Comparison of Cell Lines and Culture Models to Evaluate Toxicity of Pesticides and Pesticide Mixtures

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By

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ABSTRACT

There is a current shift in toxicology toward using *in vitro* cell-based assessments to screen environmental chemicals, such as pesticides, for their potential organ-specific toxicity as well as explore potential mechanisms of action. Many mammalian-derived cells lines are used to evaluate the organ-specific toxicity of chemicals; however, the ultimate use of the data obtained from these models in terms of translation to human toxicity remains challenging. Cell-based assays present distinct advantages and limitations, necessitating careful consideration by toxicologists when designing tests to address specific questions or interpret results. The primary goal of this thesis was to deepen our comprehension of cell-specific toxicity and *in vitro* assays. It is anticipated that understanding how individual factors impact variability in toxicity responses will help improve the accuracy and applicability of toxicity data derived from cell-based studies.

First, we assessed the cytotoxic effects of pesticide chlorpyrifos (CPF), and its active metabolite (chlorpyrifos oxon, CPFO), across two human cell lines representative of liver (HepG2) and kidney (HK-2). The cytotoxicity to CPF and CPFO differed between cell lines, which was attributed to lower basal expression and inducibility of metabolizing enzymes, efflux transporters, and nuclear receptors in HK-2 cells. Co-exposure of CPF with specific inhibitors of efflux transporters enhanced CPF and CPFO cytotoxicity in HepG2 cells, indicating the role of these transporters in eliminating either CPF or CPFO. Co-incubation with transporter inhibitors also increased CPF accumulation in HepG2 cells, supporting the role of efflux transporters in elimination of CPF. These results underscore the crucial role of efflux transporter expression levels in selected cell lines for assessing the potential toxicity of environmental pollutants, such as pesticides.

Understanding the role of efflux transporters in cell-specific toxicity responses to individual pesticides in cell lines prompted questions about their potential influence on mixture toxicity *in vitro*. Many pesticides act as substrates or inhibitors of efflux transporters; thus, their inhibitory effects could impede the elimination of other pesticides in a mixture, resulting in chemosensitization and increased cellular toxicity. We examined the combined toxicity of CPF with two other known P-glycoprotein (P-gp) inhibitor pesticides, endosulfan-α and heptachlor, in HepG2 cells. Binary mixtures of CPF with either endosulfan-α or heptachlor at concentrations causing less than 20% cytotoxicity ($<IC_{20}$) were tested to assess interactive effects on P-gp-mediated toxicity mechanisms. CPF with endosulfan exhibited additive cytotoxicity, while CPF

and heptachlor resulted in synergistic cytotoxicity in HepG2 cells. Our results show that pesticide mixtures had a greater ability to inhibit the efflux capacity than individual chemicals, and that pesticide interactions at efflux transporters may influence toxicokinetic and toxicity thresholds. The key finding of this chapter underscores the importance of considering the efflux transporters-mediated interaction of pesticides, particularly P-gp, in cell-based toxicity studies, given the prevalence of such interactions and exposure occurrence of pesticide mixtures.

Different cell models not only show distinct toxicity responses to chemicals due to variations in toxicokinetic factors, but they may also differ in cellular proliferation and molecular pathways, leading to varying sensitivities. We then examined the responses of porcine-origin (IPEC-J2) and human colorectal adenocarcinoma origin (Caco-2) intestinal epithelial cells to the pesticide CPF. At a functional level, CPF disrupted the epithelial monolayer barrier in differentiated IPEC-J2 cells but not Caco-2 cells. Our results suggest that disrupted barrier function in IPEC-J2 cells may involve the transcellular pathway, indicated by decreased transepithelial electrical resistance (TEER) values and increased dextran permeability at cytotoxic concentrations of chlorpyrifos, with no changes in tight junction protein expressions. However, CPF did not affect the epithelial barrier function in Caco-2 cells, possibly due to the cancerous nature of the Caco-2 cell line. Our main conclusion was that CPF disrupts the intestinal barrier function in IPEC-J2 cells by affecting transcellular permeability pathway instead of through targeting tight junction proteins.

In conclusion, the results this research highlight the different responses of cells to certain pesticides. The differences in response were observed between cell line types, as well in single cell lines when exposed to pesticides individually or in combination. Such differences in response may arise from differences of cells in expression of transporters affecting pesticide toxicokinetics and other intrinsic molecular features. Differing expression levels of enzymes, transporters, and nuclear receptors across cell lines can influence the detoxification, cellular concentration, and toxicity of individual pesticides and their interactions. Enhanced understanding of the biochemical and molecular characteristics of commonly used cell lines will increase their applicability in assessing the toxicity of environmental pollutants and improve their correlation with real-life scenarios. The findings of this thesis research directly impact the choice of cell lines for evaluating pesticides and other environmental chemicals, highlighting concerns about interpreting cell-based assays in environmental hazard assessment.

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AhR	aryl hydrocarbon receptor
ABC	ATP-binding cassette transporters
BCRP/ABCG2	breast cancer receptor protein
cDNA	complementary DNA
CES1	carboxylesterase 1
CES2	carboxylesterase 2
CLDN1	claudin-1
CPF	chlorpyrifos
CPFO	chlorpyrifos oxon
DMEM	Dulbecco's modified eagle's medium
DON	deoxynivalenol
END	endosulfan-α
FBS	fetal bovine serum
FITC–dextran	fluorescein isothiocyanate–dextran
HEP	heptachlor
HQC	high quality control
IC_{50}	inhibitory concentration affecting 50% of the population
LC-MS/MS	liquid chromatography tandem mass spectrometry
LDH	lactate dehydrogenase
LLOQ	lower limit of quantification
LQC	low quality control
MeOH	methanol
MOA	mechanism of action
MQC	medium quality control
MRPs/ABCCs	multi-drug resistant protein
NOEL	non observable effect level
Nrf2	nuclear factor erythroid 2-related factor 2
OCLN	occludin
PBS	phosphate buffer saline

P-gp	P-glycoprotein
PON-1	paraoxonase-1
PXR	pregnane X receptor
qPCR	quantitative polymerase chain reaction
Rho123	rhodamine 123
TBHP	tert-butyl hydroperoxide
TEER	trans-epithelial electrical resistance
VRP	verapamil
ZO-1	zonula occludens-1

NOTE TO READERS

This thesis is organized and formatted by following the University of Saskatchewan College of Graduate and Postdoctoral Studies guidelines for a manuscript-style thesis. Therefore, there is some repetition in content among chapters. Chapter 1 is a general literature review to support the presentation of the hypothesis and specific objectives. Chapters 2, 3, and 4 are organized as manuscripts that have either been submitted or will be submitted for publication in peer-reviewed scientific journals. Chapter 5 is a general discussion and overall conclusion. References cited in each chapter were combined and listed in the List of References section at the end of the thesis.

CHAPTER 1 – Literature Review

1.1 Approaches in Human Health Toxicity Assessment

The widespread contamination of various environmental matrices, including soil and water, has created a demand for evaluating the potential adverse impacts of these chemicals on human health. Traditionally, human systemic toxicity assessment of chemicals has relied on using animal studies, together with human exposure estimates, and conservative assessment (uncertainty) factors or linear extrapolations to define whether a chemical exposure is safe or not. However, animal tests for toxicity evaluation of chemicals can be impractical due to the high expense and uncertainties associated with extrapolation across species (Adeleye et al., 2015).

Regarding the interspecies differences between animals and humans, Olson et al. (2000) compared drug toxicities of 150 compounds in humans to various animal species including dog, primate, rat, mouse and guinea pig. Their analysis showed a general concordance rate between human and animal toxicity of 71%, which was further reduced to 63% and 43% for non-rodent and rodent alone studies, respectively. For the liver as a specific target organ, the toxicity data showed a concordance rate between human and animal of approximately 50%. Animal studies often show higher toxicity than in humans due to species differences in toxicokinetics, toxicodynamics, and higher exposure concentrations, suggesting that animal studies may not always accurately predict the disposition of chemicals and potential adverse effects in humans. Moreover, the classical animal testing approach is based on apical endpoints, which usually contribute limited information on the mechanism or mode of action.

There are also significant ethical and regulatory pressures to seek alternative methods for traditional toxicity testing strategies. Russell and Burch (1959) first defined the 3Rs (replacement, reduction, and refinement) as principles to guide more human and appropriate animal use in research. The principle of replacement is typically considered as the use of less sentient animal species (such as insects) or non-animal systems (including *in vitro* and *in silico* models) as alternative methods that can be used in biomedical research. In 2007, the US National Research Council reported a new paradigm shift in toxicity testing from a system based on high-dose studies in laboratory animals to one that incorporates new approach methods including classical and new modelling, and *in vitro* methods in order to assess key toxicity pathways (mechanisms) that contribute to biologically important effects. In the context of human health,

the disruption in regular cellular signalling pathways (also referred to as toxicity pathways) is identified using a comprehensive suite of high-throughput *in vitro* assays in human cells and cell lines following chemical exposure. These data could contribute to hazard characterization in risk assessments by filling existing gaps in our understanding of toxicity mechanisms. The ultimate aim is to ensure that exposures to chemicals remain below the threshold where significant disruptions of these biological pathways could occur (Krewski et al., 2009).

Over the past decades, significant efforts have been made to establish various predictive *in vitro* cell-based toxicity models, such as complex three-dimensional (3D) *in vitro* models and two-dimensional (2D) cell cultures (Astashkina et al., 2012). The goal of developing 3D tissue culture (including organ explants, organoids, organ-on-a-chip, and their hybrid version) was to improve cell structure and replicate complex organ systems with higher cell density, organization, diversified cell signaling, and the integration of organ architecture with its circulatory system (Maschmeyer et al., 2015). Despite their advantages, 3D models have drawbacks such as limitations in high-throughput screening, labor-intensive preparation, and challenges in standardization of protocols across laboratories (Wang and Jeon, 2022). As a result, toxicologists still routinely use monolayer adherent (also known as 2D culture models) cell cultures, which are created from dissociated cells cultured on plastic surfaces. Monolayer models are less complicated and allow higher throughput screening for the detection of toxicants and their mechanism of actions. The 2D culture models include primary cell cultures produced directly from collected tissue, stem cell-derived models, genetically engineered cells, human immortalized cell lines, and cancer cells. While 2D culture models are considered valuable as mechanistically-based predictive tools for human health risk assessments, it is equally important to consider their functional limitations in predicting *in vivo* responses to chemicals.

Exposure to pesticides is a major contributor to human health issues and can cause a wide range of adverse health effects through various mechanisms of action (MOA) and target organ toxicity (Damalas and Eleftherohorinos, 2011). Epidemiological research explores correlations between pesticide exposure and adverse outcomes such as cancer, reproductive problems, neurological impairments, and respiratory concerns. Ingestion of pesticide residues through food, skin contact when farming, and inhalation in work environments can result in both acute and chronic exposures. Although there are other elements that affect human health as well, such as air pollution and lifestyle choices, pesticides are particularly noteworthy because of their widespread

environmental presence and severe toxicological consequences (Damalas and Eleftherohorinos, 2011). Pesticide-induced toxicity has been the focus of many published toxicology studies, with the aim of characterising toxicity thresholds and understanding specific MOA to inform safety risks for the general population as well as occupational hazard. There have been considerable efforts in developing alternative toxicity testing methods using cell lines to represent various human organs to understand and predict toxicity of pesticides (Eskes et al., 2017; Oleaga et al., 2016). However, use of cell lines for toxicity assessment of pesticides or pesticide mixtures requires an understanding of factors which influence the outcome of toxicity tests, such as cellular toxicokinetic and toxicodynamic components (Kyffin et al., 2018). The following sections will describe the type of endpoints that can be assessed and considerations for cell-specific characteristics that can influence outcomes when using cell-based assays with specific reference to toxicity assessments of pesticide and mixtures.

1.2 Endpoints for Cell-Based *In Vitro* Toxicity Assessment

1.2.1 Mechanistic-based assessment

Three primary categories can be used to classify outcomes of toxicity assessments based on MOA: non-specific, specific, and reactive toxicity. Frequently, a combination of non-specific, specific, and reactive toxicity contributes to chemical-induced responses in cell-based bioassays (Neale et al., 2021). Non-specific cytotoxicity encompasses a range of toxic effects that ultimately lead to cell death. Nonpolar narcosis is often the result of exposure of cells to high concentrations of lipophilic chemicals, partitioning into and disrupting cellular membrane integrity and function (Escher et al., 2002). Determining the concentration at which 50% of the cells are inhibited/affected (i.e. IC_{50}) across different cell-based assays can be used to establish the toxicity potency of a single pesticide. Conversely, many pesticides can be quantitatively compared for their potential to induced cytotoxicity by comparing IC_{50} results from a specific *in vitro* assay. Cell viability assays are based on various cell functions such as cell membrane permeability, enzyme activity, cell adherence, replication and mitochondrial activity (Poteser, 2017).

Reactive toxicity involves energy transfer or reactive oxidative species (ROS, electrophiles) generation upon exposure to a compound, resulting in the formation of alternating covalent bonds within biomolecules like proteins, DNA, and lipids (Freyre et al., 2021). These

bonds disrupt the normal function of biomolecules, impeding downstream processes and potentially causing toxicity. For instance, endpoints related to the induction of DNA damage or genotoxicity by reactive compounds are often assessed using the Comet assay and micronucleus test (Bull et al., 2006). Chemicals, including pesticides, can also induce toxicity through oxidant-mediated responses such as membrane lipid peroxidation, metabolic perturbation and deregulation of signaling pathways and protein synthesis (Shah et al., 2020).

While non-specific toxicity arises due to the chemical interactions with biological receptors or structures in a non-selective manner, specific toxicity results from explicit binding of the compound to a selective receptor or interference with a target molecule. Most frequently employed bioassays for monitoring specific toxicity of pesticides include those that can identify their interactions with enzyme and nuclear receptors, such as thyroid, estrogen, androgen, pregnane X receptor (PXR), and aryl-hydrocarbon receptors (AhR; Kojima et al., 2011). For instance, various *in vitro* assays have been utilized in diverse toxicological investigations to detect the endocrine-disrupting effects of pesticides, manifested through interactions (either agonist or antagonist) with estrogen receptor, androgen receptor, PXR or their neurotoxic effects mediated by interactions with acetylcholinesterase (AChE), and dopamine active transporter (DAT; Silva, et al., 2015; Kojima et al., 2011). Some other endpoints include the effect of pesticides on the RNA transcription (mRNAs) and translation (proteins) of various target molecules. In past decades, 'omics' technologies provided the opportunity to assess the changes in mRNA expression (transcriptomics) and proteins (proteomics) of almost any target molecule after exposure to pesticides (Manfei et al., 2019).

1.2.2 Toxicokinetic endpoints

In vitro assays are becoming widely used as alternative methodologies to understand how toxicokinetic processes lead to a biologically effective dose and subsequent toxicity at the organ level (Silva et al., 2022). A variety of cell-based models of toxicokinetic processes are available, such as absorption across epithelial barriers (e.g. intestinal, skin, and lung), liver metabolism, blood-tissue partitioning, and translocation across endothelial barriers like the blood-brain, blood-testis, and placenta.

The movement of a chemical, including pesticides, through an epithelial barrier is referred to as absorption. The primary function of the barriers is to modulate and restrict absorption of xenobiotics, such as pesticides through skin contact, inhalation, and oral exposure

(Adler et al., 2011). These barriers include both physical aspects, such as tight junction proteins between neighboring cells, and biochemical components, such as metabolizing enzymes and transporters. The skin, intestinal, and pulmonary barriers (skin-/gut-/lung-vascular barriers) limit the absorption of pesticides into the bloodstream. Pesticides pass through these barriers via active transport and passive absorption (transcellular route), as well as tight junction absorption (paracellular pathway), suggesting compromised barrier function. Absorption is influenced not only by the physiological and pathological characteristics of the epithelial tissue and the presence of specific biochemical components but also by the physicochemical properties specific to the compound (Deepika et al., 2022).

While reproducing the complexity of *in vivo* biological barriers may be not feasible, cell-based toxicity models have been created that incorporate major essential components. Recent research uses 2D and 3D approaches to replicate skin, gut, and lung barriers, assessing xenobiotic absorption and toxicity (Wang et al., 2023). The 2D monolayer models are frequently employed because they are easy to manipulate and often utilize immortalized cell lines. In 2D monolayer models, cells are seeded on a microporous permeable membrane, thereby establishing apical and basolateral compartments similar to *in vivo* conditions. Cells cultured on transwell inserts also acquire characteristics resembling the mature epithelial cells *in vivo*, including well-defined tight junctions, polarized monolayer of epithelial cells, metabolic and active transport properties - features that are frequently absent in undifferentiated cells (Gordon et al., 2015). *In vitro* cell based assays are also used to assess how pesticides distribute from the bloodstream to various organs. Certain vital organs, like the brain, testes, and placenta, have active mechanisms (such as efflux transporters and tight junction proteins) to restrict the entry of pesticides, which are mimicked by *in vitro* cell-based models like the blood-brain barrier (BBB), and blood-testis barrier (BTB), and blood-placental barrier (BPB; Adler et al., 2011; Deepika et al., 2022).

In the last few decades, the 2D monoculture system has been widely used to study the effects of pesticides on barrier function, particularly through measures such as transepithelial electrical resistance (TEER) and nonradioactive fluorescence-labeled marker polysaccharide such as fluorescein isothiocyanate (FITC)-labeled dextran (Srinivasan et al., 2015). The toxicological evaluation of various pesticides showed significant decreases in cell viability alongside significant increases in epithelial and endothelial permeability on transwell inserts; this was evidenced by decreased TEER values and increased dextran permeability (Vidau et al.,

2009; Zhao et al., 2021; Tirelli et al., 2007; Deepika et al., 2022). Including other cellular components into *in vitro* cell-based toxicity models, especially for the intestine—such as mucus-producing Goblet cells, M-cells in the Peyer's patches, and the presence of gut microbiota—enhances the complexity of the gut culture and improves the accuracy of absorption determination (Azevedoa and Macedoa, 2020). As an example, human-derived colon cells (Caco-2) in culture exhibit tighter cell connections than found in the *in vivo* condition (Kohl, 2008). Variations between Caco-2 cells and *in vivo* human intestines have inspired researchers to incorporate HT29-MTX goblet cells, known for their mucus secretion, into *in vitro* models. This co-culture model aims to create more physiologically relevant models of the intestine, enhancing the ability to predict mechanisms of intestinal absorption. Several studies have reported improved prediction of chemical permeability across intestinal epithelial cells using mucus producing goblet cells as reviewed in Fedi et al. (2021).

The clearance of pesticides in various organs is frequently studied through different cell-based toxicity models, such as primary cells and immortalized cell cultures (Gouliarmou et al., 2018). Lipophilic pesticides commonly undergo enzymatic biotransformation, converting them into more hydrophilic metabolites. These metabolites are then eliminated directly or after conjugation with endogenous cofactors, through renal or biliary excretion. Pesticides and their metabolites are also pumped out of the cells by active efflux transporters. Enzymes and efflux transporters are more highly expressed in the intestine, liver, and kidney, but they are also expressed in other tissues to a lower degree. Bioanalytical methods like liquid chromatography coupled to tandem mass spectrometry (LC–MS/MS) are often used to measure pesticide metabolism and elimination, quantifying both the parent pesticide and its metabolic profiles over time in cells and culture media (Valdiviezo et al., 2022). Another commonly utilized method in cell-based assays for assessing transporter function in elimination of chemicals is fluorescent dye-based uptake assays, due to their feasibility and cost-effectiveness (Jouan et al., 2016). These assays use fluorescent dyes that are substrates for these transporters. When a tested chemical inhibits the activity of efflux transporter, the fluorescent dye accumulates inside the cells (Jouan et al., 2016). In addition, a wide range of endpoints ranging from activation of nuclear receptors to induction or inhibition of xenobiotic metabolizing enzymes and efflux transporters are currently available to predict the potential toxicity of pesticides and their interactions within mixtures (Janošek et al., 2006). The following sections will further elaborate on the role of

metabolizing enzymes, efflux transporters, and their nuclear regulators in pesticide elimination and their implications for cell-based toxicity results.

1.3 Cell-Specific Characteristics and Considerations for Toxicity Assessment

The human body consists of a large repertoire of cell types with different characteristics and thus appropriate selection of cell lines for *in vitro* toxicity testing is of critical importance. Most studies have focused specifically on liver-based toxicity models because the liver is a major target organ for toxicity and plays a vital role in xenobiotic biotransformation (Guguen-Guillouzo et al., 2010). However, other organs, including intestine and kidney, are also major organs involved in uptake, metabolism, and elimination of most orally absorbed xenobiotic using their specific cellular and molecular properties (Vernetti et al., 2017). Cell-specific characteristics can influence their cytotoxicity responses to chemical exposure; therefore, the predictive capacity of a cell-based toxicity model will largely depend on what human cell line is used in toxicity assessment. Various cell lines, even when derived from the same organ or tissues, can exhibit differences in apparent sensitivity to chemicals – this can be partially caused by differences in intrinsic sensitivity, such as presence of a specific biotransformation enzymes, receptor, signalling pathways, or drug transporters (Wilk-Zasadna et al., 2015; Le Fol et al., 2015; Bell et al., 2017). As an example, Bell et al. (2017) compared three cell systems - induced pluripotent stem cell-derived hepatocytes, HepaRG cells, and primary human hepatocyte spheroids. Transcriptomic analysis revealed significant changes in gene expression profiles across these cells, with primary human hepatocyte spheroids expressing higher levels of genes involved in the metabolism of endogenous and xenobiotic substances compared to the two other cell types.

1.3.1 Differences in metabolizing enzymes

The biotransformation capacity of specific cell lines is often a concern when they are used as a tool for toxicity screening and hazard assessment of chemicals. It has been shown that different cell lines have low or even absent basal expression/activity of cytochrome P450 (CYP) enzymes as compared to the *in vivo* condition (Coecke et al., 2006; Silva and Nicoll-Griffith, 2019). This is frequently the case for phase I (generally oxidation, reduction, or hydrolysis reactions) capabilities, depending on the presence of major CYP enzymes, but moreover for

phase II (generally conjugation or hydrolysis reactions) activities (Audebert et al., 2010). Several studies have shown dissimilar sensitivity of cell lines in assessing the toxicity of chemicals due to their capability or deficiency for certain metabolic functions (Theumer et al., 2018; Khoury et al., 2015; Khoury et al., 2016). Primary human cells are considered ideal for modeling biotransformation but are less commonly used due to inter-individual differences and limited viability in culture (Atienzar and Nicolas, 2018; Han et al., 2019). At present, the main alternative for use of primary human cells in toxicity testing is an immortalized organ-derived cell line (e.g., human hepatocellular carcinoma cell line, HepG2). Immortalized cell lines, with their indefinite growth *in vitro*, are appropriate models for studying tissue functions in pharmacological and toxicological research (Vinken and Blaauboer, 2017; Mueller et al., 2015). However, challenges with immortalized cells include difficulty in maintaining characteristics close to primary cell models due to genetic modifications. In addition, immortalized cell lines may undergo phenotypic and genotypic changes throughout culture passages, leading to potentially variable results in assay outcomes across studies (Kaur and Dufour, 2012).

1.3.2 Differences in drug transporters

In recent years, there has been increasing interest in and research on efflux drug transporters (phase III) – in particular how they influence intracellular accumulation of toxicants as well as diminish the toxicity of some xenobiotics (Rigalli et al., 2019; Sison-Young et al., 2015). Major phase III transporters include multidrug resistance-associated proteins (MRPs/ABCCs), P-glycoprotein (P-gp/ABCB1) gene, as well as the breast cancer resistance protein (BCRP/ABCG2) and these are expressed in many tissues such as the liver, intestine, kidney, and brain. Generally, P-gp and BCRP have very wide substrate selectivity and share many substrates. Both P-gp and BCRP play an important role in the efflux of potential harmful xenobiotics and their metabolites from the apical membrane of epithelial secretory tissues (Mottino and Catania, 2008; Gameiro et al., 2017). Environmental chemicals can also alter activity and expression of drug transporters via contaminant–transporter interactions. Given that, efflux drug transporters not only impact toxicokinetics of some chemicals (in terms of intestinal absorption, drug disposition across organ barriers and renal and hepatic elimination) but also their toxicodynamics at target sites (Fardel et al., 2012; Sawant-Basak and Obach, 2018). The specific localization and variable expression of drug transporters in different cell lines lead to variable xenobiotic access to biotransformation enzymes and dissimilar sensitivity (Dobson and

Kell, 2008; Shugarts and Benet, 2009). Wang et al. (2011) showed different endogenous expression of various transporter proteins, including P-gp, in four human lung cancer cell lines. They also determined this variance correlated positively with variation in chemosensitivity to anticancer drugs among the four cell lines. In another study, differences in the expression of several transport proteins (including ABCB1 and MRP1–MRP5) and as well as the enzyme CYP3A4 in different human colon carcinoma-derived cell lines were attributed to differences in the sensitivity of cell lines in predicting toxicity of chemicals (Pfrunder et al., 2003). Therefore, cytotoxicity as a result of chemical exposure is mainly related to both metabolic pathways and transporters in cell models, determining a chemical accumulation and biotransformation.

1.3.3 Differences in nuclear receptors

Nuclear receptors are transcription factors that regulate proliferation, differentiation, cell cycle, cell death, and metabolism. The transcriptional and protein activities of nuclear receptors are sensitive and regulated by a wide range of lipophilic compounds such as endogenous compounds, drugs, and environmental pollutants. Activated nuclear receptors regulate the expression levels of the biotransformation enzyme and efflux transporter proteins (Gronemeyer et al. 2004). Variations in the intensity of expression or functions of these proteins in various cells obviously have an influence on pesticide toxicokinetics, possibly resulting in considerably dissimilar toxicity responses to chemicals. As an example, a variety of nuclear receptors, such as the PXR and AhR, are expressed in different mammalian organs and cells (Cuperus et al., 2014; Jigorel et al., 2006). After binding to chemicals, nuclear receptors alter transcriptional expression of biotransformation enzymes and efflux transporters, enhancing the ability of the cells to dispose of the chemicals. This mechanism also serves as early warning signals of exposure to chemicals at their sub-cytotoxic concentrations and the detection of xenobiotics in the environmental samples (Leusch et al., 2014). On the other hand, chemicals that cause significant or long-term changes in homeostasis can lead to adverse effects or toxicity. This frequently includes chemicals interacting with cellular macromolecules or indirectly creating toxic compounds, such as reactive oxygen species, resulting in cellular malfunction and cytotoxicity (Williams and Iatropoulos, 2002). Increased reactive oxygen species is also capable of activating a transcription factor, named nuclear factor erythroid-derived 2-related factor (Nrf2). The activation of Nrf2 can also lead to increased gene expression of phase II metabolizing enzymes

like glutathione s-transferase, as well as efflux transporters such as P-gp, BCRP, and MRPs which are responsible for detoxifying the exogeneous chemicals (Gameiro et al., 2017).

Cell-specific toxicity responses are highly dependent on the capacity of cell models to express nuclear receptors and transcriptional factors. Since the expression and regulatory mechanism of nuclear receptors and transcriptional factors are different across the different cell lines, dissimilar molecular mechanisms and responses may be observed for the same toxicant in different cell lines. Therefore, to apply cell-based toxicity models to evaluate the expression and inducibility of enzymes and transporters by chemicals, it is important to consider both the basal expression and inducibility of these nuclear factors or regulators in the *in vitro* systems.

1.4 Evaluating Mixture Toxicity Through Cell-Based Assays

Humans are exposed to mixtures of chemicals simultaneously and studying the exposure effects of real-life mixtures on human health is complicated. Cell-based assays and other *in vitro* approaches are especially valuable for evaluating the potential toxicity of chemical mixtures on human health due to the capacity to screen a large number of mixtures and incorporate assays that inform on multiple MOA. The toxicity of a chemical mixture can be determined in one of two ways: either by evaluating the contributions of individual components to the overall toxicity of chemicals, or by considering the mixture as a whole or a single entity (Rider et al., 2018).

Hazard assessment of chemical mixtures (or environmental samples with known compound mixtures) focus on toxicity from the individual component chemicals to predict toxicity of the overall mixture. In this approach, the behaviour of chemicals in a mixture can be predicted via the concepts of dose or concentration addition for components with similar MOA or response addition for dissimilarly acting chemicals (Hernández et al., 2019). As an example, when two compounds have the same MOA, their combined effect is usually governed by the idea of concentration addition. Thus, if we have a binary mixture in which chemical A has an IC_{50} of 10 μM and chemical B has an IC_{50} of 6 μM, a combination of half the IC_{50} of A (CA = 5 μM) and half the IC_{50} of B (CB = 3 μM) will result in a 50% effect. Because they exert their effects independently, the combination effect of substances with various MOA is more challenging than simple addition (Lasch et al., 2020).

A component-based model, however, cannot always be used to predict mixture toxicity. Exceptions to concentration addition and independent action occur when the compounds within a

combination interact with each other. Chemical interactions can result in the overall toxicity being greater (synergism and potentiation) or less (inhibition, antagonism, and masking) than predicted. The interactions between compounds can influence toxicokinetic (such as absorption, transport, biotransformation, and excretion), and/or toxicodynamic (binding at the target site) processes (Cedergreen et al., 2012). For example, if one component activates an efflux transporter or detoxifying enzyme, it might result in the faster detoxification of another component in the combination, causing the resulting mixture effect to be less than the predicted toxicity (antagonistic effect). Toxicokinetic interactions can also occur in synergy, where the effects are far more intense than what the concentration addition or dosage addition models would suggest. For example, this may occur if one chemical increases the absorption of another or inhibits detoxifying enzymes or efflux transporters (Heys et al., 2016). Such interactions can occur even when each chemical in the mixture is present at their sub-cytotoxic and no-observed-adverse effect level (NOAEL; Kortenkamp and Faust, 2018; Thrupp et al., 2018). Combinations may also show different toxicity effects and interactions when the same compounds are present in different concentrations, ratios, and target cells (Mumtaz et al., 2010). The classical isobologram combination index equation (CI) values which is independent of the mechanism of action of mixture components, is widely used to describe the toxicological interactions in mixtures (Chou, 2006). This technique quantifies synergism ($CI < 1$), additive effects ($CI = 1$), and antagonism ($CI > 1$) in chemical interactions (Chou 2006, Chou and Talalay 1984).

The widespread use of pesticides and their potential mixture toxicity effects, combined with constraints associated with animal studies, has necessitated the use of *in vitro* cell-based methods for assessing human toxicity. The fundamental objectives of *in vitro* toxicity testing are to predict biological responses to chemicals at different concentrations and understand the mechanisms underlying toxic effects when they come into contact with target cells or tissues in the body. To achieve this, it is important that the biological properties of applied *in vitro* cell-based toxicity model mirror, to some extent, those of target cells or tissues *in vivo*, encompassing both toxicodynamic and toxicokinetic aspects. A significant challenge of incorporating cell-based approaches arises from overlooking intrinsic features, such as their biotransformation, elimination capacity, and the expression of organ specific molecular targets which are unique to the organ models being used. This oversight can cast doubts on the applicability of these models in addressing hypothesis questions, especially in routine toxicological studies like determining

the potency of pesticides to induce cytotoxicity (IC_{50}) or their interactions when present in a mixture. Efflux transporters, such as P-gp, play a crucial role in influencing the potency of pesticides to induce cytotoxicity and their interactions with each other. Transporters can modulate the intracellular concentration of pesticides by actively extruding them from cells, thereby potentially reducing their cytotoxic effects. Additionally, competition for transport by a particular transporter among multiple pesticides can affect their intracellular concentrations and subsequent toxicity. Understanding the role of transporters is essential for assessing the toxicological effects and interactions of pesticides in biological systems. The applicability of *in vitro* cell-based toxicity models for understanding mechanisms of organ toxicity via their interaction with particular target molecules has also been demonstrated in many toxicological studies as reviewed by Knudsen et al. (2015). However, not all *in vitro* cell-based toxicity models are capable of predicting specific organ toxicity. Cell models, including *in vitro* immortalized cell lines, typically specialize in addressing a narrow set of potential mechanisms. The suitability of cell-based models to predict toxicity of xenobiotics largely depends on the specific cell line employed, highlighting the importance of careful consideration in their selection. In this context, we explored and discussed the challenges and opportunities of integrating *in vitro* data into human toxicity assessment of pesticides and their mixtures.

1.5 Exposure, Disposition and Toxicity of Specific Pesticides to Humans

Pesticides are comprised of many different categories of chemicals and are designed to be deliberately spread into the environment to kill off pests. However, they can also cause adverse effects on non-target species through water, soil, and contaminated plants, either by similar or different mechanisms (Lushchak et al., 2018). In this research, we focus on three insecticides: chlorpyrifos (CPF), endosulfan (END), and heptachlor (HEP). The following paragraphs provide a brief overview of their exposure sources, pathophysiological effects, mechanisms of toxicity, and how human cells absorb, biotransform, and eliminate these pesticides.

1.5.1 Chlorpyrifos

Chlorpyrifos (CPF) is one of the most widely used organophosphates insecticides worldwide used for the control of different insects in fruits, vegetables, crops, and households (Solomon et al., 2014). Despite improving crop yields by effectively controlling pests, CPF has

been shown to pose risks to human and animal health. The World Health Organization (WHO, 2019) classified CPF as a moderately hazardous pesticide (Class II). Both the U.S. Environmental Protection Agency (EPA) in 2021 and the European Commission in 2020 imposed usage restrictions for CPF (EPA, 2021; EU, 2020). Nevertheless, CPF remains widely used in many developing countries, with monitoring reports indicating its presence in various environmental matrices (Andersen et al., 2022).

Humans and animals can be exposed to CPF through oral, dermal, and inhalation routes (ur Rahman et al., 2021). Farm workers and pesticide applicators are at risk of exposure to higher concentrations due to frequent contact with CPF and pesticide mixtures. However, exposure levels vary based on duration, pesticide concentration, and use of personal protective equipment (PPE). Non-occupational exposure to trace levels of CPF in contaminated food or water is also a significant concern for the general public, particularly children (ur Rahman et al., 2021). CPF toxicity has been associated with different neurological issues, developmental and behavioral abnormalities, endocrine disruption, genotoxicity, hematological cancers, cardiovascular diseases, immunotoxicity, histopathological changes, and oxidative stress (ur Rahman et al., 2021). The main mechanism of CPF is inhibiting acetylcholinesterase (AChE) in both target insects and non-target organisms, leading to harmful nervous system effects. Besides inhibiting AChE, CPF can induce toxicity by interacting with enzymes, affecting neurotransmitter receptors, impairing neuron differentiation, affecting macromolecules synthesis, disrupting signal transduction, and generating oxidative stress (Eaton et al., 2008; ur Rahman et al., 2021).

Due to its lipophilic nature, CPF can be readily absorbed via the skin, lungs, and gastrointestinal tract. It has been determined that CPF undergoes first-pass metabolism resulting in either CPF-oxon (CPFO) through desulphuration reaction or a dearylation reaction that detoxifies CPF to hydrolysis products, 3,5,6-trichloro-2- pyridinol (TCP) (Sams et al., 2004) (Fig. 1.1). CPFO, like other active metabolites of many organophosphate insecticides, exerts acute toxicity by inhibiting AChE through the phosphorylation of a serine residue in catalytic site of the enzyme. CPFO interact with different hepatic and extrahepatic esterases, such as carboxylesterases (CES1 and CES2) and paraoxonase-1 (PON-1) to form TCP and diethylphosphate (DEP), which are involved in the detoxification and removal of CPFO (Smith et al., 2009). The human isoforms that metabolize CPF are different CYPs, such as CYP1 A2, 2B6, 2C9, 2C19, and 3A4 (Sams et al., 2004). CYP 2B6, 3A4, and CYP 1A2 are the most

important in respect to formation of the CPF-oxon (Eaton et al., 2008; Leoni et al., 2012), whereas CYP3A4 and CYP2C19 have the highest catalytic activity for diarylation of CPF, producing TCP, DEP, and diethyl thiophosphate (DETP). Thus, cell lines with high CYP2B6 and CYP1A2 activity and low CYP2C19, PON1, CES1, and CES2 activity might be particularly susceptible to the toxic effects of CPF, because a comparatively high concentration of CPFO would be predicted.

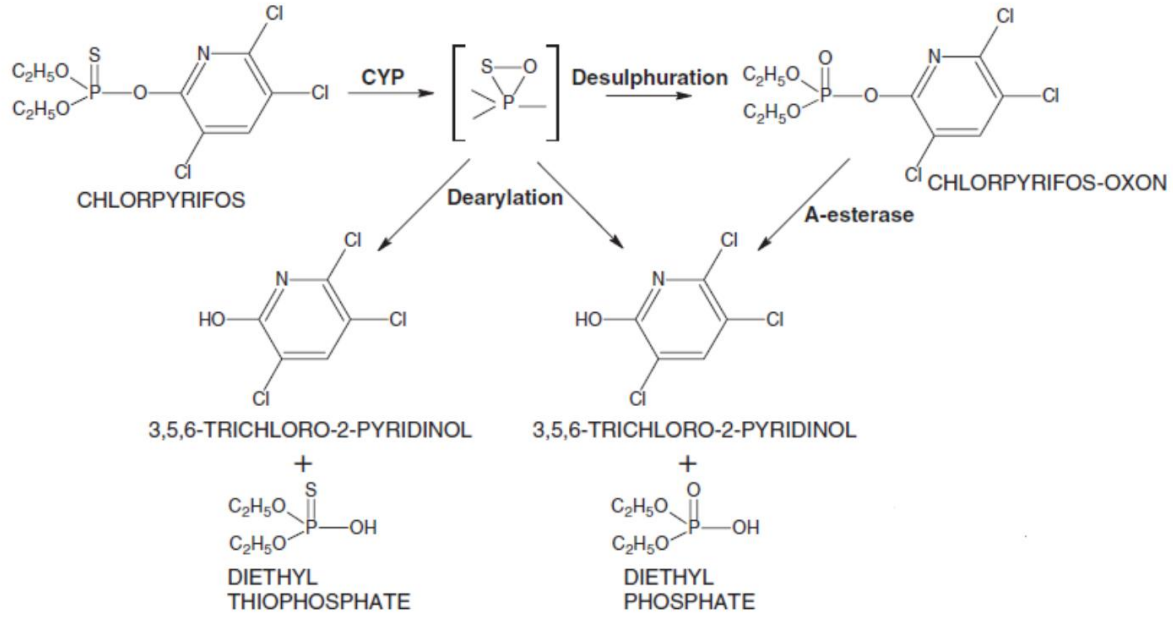

Figure 1.1: Biotransformation of chlorpyrifos (Sams et al., 2004).

Two efflux transporters, BCRP and P-gp, were shown to interact with CPF and/or its metabolite that are known to might be human BCRP and P-gp substrates indicating their protective and defense mechanisms for detoxification. The previous studies also have reported increased expression and transport activity of the drug efflux transporter, BCRP, in human villous trophoblasts cells, human placental cells, and enterocytes exposed to CPF in vitro (Agarwala et al., 2004; Ridano et al., 2012; Ridano et al., 2017; Halwachs et al., 2016). Furthermore, it was demonstrated that rats treated with the CPF had increased P-gp in kidney, adrenal, liver, jejunum, and stomach (tissues associated with elimination of xenobiotics) compared to control rats (Lanning, 1996). Despite the importance of MRPs in the elimination of many pesticides and the interaction of organochloride pesticides with MRPs as their inhibitors,

there is no evidence supporting the potential interaction of CPF as either a substrate or an inhibitor of MRPs in mammals (Chedik et al., 2018).

1.5.2 Endosulfan

Endosulfan (END) is an organochlorine insecticide with two isomeric forms, α-END and β-END. END is classified as a persistent organic pollutant and remains in the environment for extended periods (Silva, and Beauvais, 2010). END can be absorbed through ingestion, inhalation, skin contact, or transplacental transfer. Similar to CPF, the main exposure route for the general population is exposure to residues or trace levels, while occupational exposure can result in higher exposure rates and concentrations. END is a bioaccumulative and can cause histopathological damage to major organs, often detectable within the initial hours of exposure (reviewed by Menezes et al., 2017). Exposure to END in humans has been linked to hyperstimulation of the central nervous system, gastrointestinal discomfort (including nausea and vomiting), respiratory difficulties, and developmental abnormalities (Menezes et al., 2017). The primary mechanism of END toxicity is its action as a non-competitive GABA antagonist. END disrupts neurotransmission by blocking the chloride channel associated with the GABA receptor, resulting in overstimulation in pests and neurological symptoms in humans (Menezes et al., 2017). The severe neurotoxic effects of END have resulted in its ban in many countries. Despite restrictions and bans, END remains detectable in various environmental media in some countries due to its extensive production, application, and environmental persistence (Guo et al., 2022).

In mammals, END is readily metabolized into END sulfate and END diol, which can further be converted into END lactone, hydroxyether, and ether. Studies using human liver microsomes have shown that cytochromes CYP2B6 and CYP3A4 primarily catalyze the formation of END sulfate from α-END (Casabar et al., 2006), while CYP3A4 and CYP3A5 mediate this process from β-END (Lee et al., 2006). The metabolism studies also indicate that a significant amount of the parent compound remains present in tissues and excreta. Biliary excretion of END has been shown to be significant in mammals. Evidence also suggests that END is a substrate for P-gp efflux transporter, and a potential inhibitor of various transporters, including human P-gp, BCRP, and MRP (Bain et al., 1996; Chedik et al., 2018).

1.5.3 Heptachlor

Heptachlor (HEP) is an organochlorine pesticide widely used for insect control in many countries, and it has been banned by the U.S. and other developed nations due to its toxicity to

humans and animals (ATSDR, 2007). Despite regulations, HEP remains detectable in soils and in human and wildlife tissues due to its persistence (Khuman et al., 2020). Similar to CPF and END, the general population is exposed to HEP in trace amounts, primarily through residual contamination in food and water (Reed and Koshlukova, 2014). Due to its lipophilicity, heptachlor epoxide is readily absorbed through the skin, lungs, and gastrointestinal tract, and tends to bioaccumulate in body fat. Once absorbed, HEP heptachlor is metabolized by CYP2B and CYP3A enzymes into its more toxic and persistent form, HEP epoxide (Kania-Korwel et al., 2013). Other metabolites found in rats and humans include 1-hydroxychlordene, 1-hydroxychlordene epoxide, and 1,2-dihydroxydihydrochlordene. HEP epoxide has been reported to bioaccumulate in various organs, especially the liver, causing chronic effects including neurotoxicity, reproductive and developmental issues, liver toxicity, and hepatic tumor promotion (Williams and Numoto, 1984; Arisekar et al., 2021). HEP is a chloride channel blocker insecticide with significant mammalian toxicity. Its toxicological effects are attributed to both the parent compound and its metabolite, HEP epoxide (Reed and Koshlukova, 2014). Similar to END, HEP has been shown in several studies to inhibit various efflux transporters, including MRP1, MRP2, BCRP, and P-gp (Chedik et al., 2018), increasing the potential for interactions with other chemicals.

1.6 Research Objectives and Hypotheses

1.6.1 Overall research goal

The overall goal of this thesis research is to enhance our understanding of cell-specific toxicity and *in vitro* assays to better inform the use of cell lines and interpretation of *in vitro* toxicity data related to pesticides and human hazard assessment. To achieve this goal, I conducted three different studies with the following main objectives:

1) Determine how intrinsic cellular traits of human cell lines (specifically efflux transporters) contribute to their cell-specific cytotoxicity responses to pesticide exposure (Chapter 2).
2) Identify the putative role of efflux transporters on cytotoxicity responses to individual pesticides and their binary mixtures (Chapters 2 and 3).
3) Establish the relative sensitivity of two mammalian intestinal epithelial cell lines to assess impacts of a pesticide on intestinal barrier function (Chapter 4).

1.6.2 Specific research objectives

CHAPTER 2: The Role of Efflux Transporters in Cytotoxicity and Intracellular Concentration of Chlorpyrifos and Chlorpyrifos Oxon in Human Cell Lines

Summary: Various human cell lines derived from target organs are frequently employed in toxicological research to assess potential organ-specific toxicity resulting from exposure to individual pesticides (reviewed in Allen et al., 2005). However, these studies often overlook critical factors such as the distinct baseline levels of cellular components affecting toxicokinetics (such as inducibility of transporters), cell line-specific toxicity responses, and regulations.

Objectives:

- Investigate whether the cytotoxicity of pesticide chlorpyrifos (CPF) and its metabolite chlorpyrifos oxon (CPFO) differ in human cell lines.
- Assess whether variations in basal expression and inducibility of transporters and their regulators (nuclear receptors) in human cell lines contribute to cell-specific toxicity
- Examine if transporters P-gp and BCRP play functional roles in preventing cellular accumulation and cytotoxicity.

Hypothesis:

- Different cell lines will exhibit dissimilar cytotoxicity responses to the model compounds tested (CPF and CPFO).
- The cell-specific cytotoxicity responses to CPF and CPFO can be attributed to basal expression or inducibility of P-gp and BCRP in human cells.
- The cellular concentrations and cytotoxicity of CPF and CPFO in human cells will be related to the functional role of P-gp and BCRP.

CHAPTER 3: Efflux Transporter Inhibition Influences the Combined Toxicity of Pesticides in a Human Liver Cell Line

Summary: Individual pesticides have been shown to interact with cellular efflux drug transporters as their substrates or inhibitors (Chedik et al., 2022; Hernández et al., 2017). However, because humans are frequently exposed to pesticide mixtures (Ottenbros et al., 2023), there is concern that interactions between pesticides in combination with efflux transporters may affect the elimination of them in the mixture, resulting in chemosensitization and increased cellular toxicity. We investigated the potential interactive effects of CPF as a P-gp substrate with

either endosulfan-α (END) or heptachlor (HEP) in HepG2 cells, and we evaluated the role of P-gp in binary mixture cytotoxicity responses.

Objectives:

- Determine the cytotoxicity effects of pesticides individually and their binary combinations at their sublethal concentrations on HepG2 cells.
- To investigate the impact of individual pesticides and their combinations in binary mixtures on P-gp expression and activity in HepG2 cells.
- Examine how the expression and activity of P-gp in HepG2 cells relate to the observed cytotoxicity responses to binary pesticide mixtures.

Hypothesis:

- Exposure to binary mixtures of pesticides will result in higher cytotoxicity effect than individual pesticides.
- Individual pesticides and their binary mixtures can modulate the expression and activity of P-gp.
- The cytotoxicity results of binary pesticides mixtures will be attributed to P-gp expression and activity.

CHAPTER 4: Effects of Chlorpyrifos on Epithelial Barrier Properties in Two Mammalian Intestinal Cell Culture Models

Summary: Mammals are typically exposed to pesticides via the oral pathway, with the intestine serving as the primary exposure site. Intestinal epithelial cells obtained from porcine (IPEC-J2) or human (Caco-2) sources are frequently used to study the effects of pesticides on intestinal barrier function. Despite their extensive use, there is a scarcity of knowledge on cell line-specific toxicity responses and regulations.

Objectives:

- Assess and compare the cellular responses of two commonly used intestinal epithelial cells, IPEC-J2 and Caco-2, to intestinal barrier dysfunctions of a model compound pesticide CPF.
- To determine the potential underlying mechanism responsible for the CPF effects on the intestinal barrier function in Caco-2 and IPEC-J2 cells.

Hypothesis:

- The effect of the pesticide CPF on intestinal barrier function in differentiated IPEC-J2 and Caco-2 will be cell-specific.
- The barrier function in intestinal epithelial cell models will correspond to cell-specific intrinsic features and the expression of tight junction proteins.

CHAPTER 2 – The Role of Efflux Transporters in Cytotoxicity and Intracellular Concentration of Chlorpyrifos and Chlorpyrifos Oxon in Human Cell Lines

Preface

The role of xenobiotic transporters in pesticide distribution and clearance, and their impact on toxicity outcomes is known for certain pesticides. In recent decades, *in vitro* assays have emerged as alternative approaches to toxicity testing to support hazard characterization. However, there is still a lack of attention on how the expression of xenobiotic transporters in cell models may influence the cytotoxicity responses. In this chapter, we optimized a method to assess the cytotoxicity of a model compound across two cell lines, with the ultimate goal of identifying the role of efflux transporters in the accumulation and cytotoxicity results.

The content of Chapter 2 was adapted from Goldar, S, Gachumi, G, Siciliano, S.D, Hogan, N.S. 2024. The role of efflux transporters in cytotoxicity and intracellular concentration of chlorpyrifos and chlorpyrifos oxon in human cell lines. *Toxicology In Vitro*. 105942 (https://doi.org/10.1016/j.tiv.2024.105942). Copyright (2024) with permission from ScienceDirect.

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Steven Siciliano: conceptualization, funding acquisition, writing - review and editing, resources.

Natacha Hogan: conceptualization, supervision, funding acquisition, writing - review and editing, resources.

2.1 Abstract

In this study, we investigated the role of two efflux transporters, p-glycoprotein (P-gp) and breast cancer resistance protein (BCRP), in the cytotoxicity and intracellular accumulation of the organophosphate pesticide chlorpyrifos (CPF) and its active metabolite, CPF-oxon (CPFO), in a human-derived hepatoma cell line (HepG2) and kidney epithelial cell line (HK-2). The cytotoxicity to CPF and CPFO differed between cell lines. For both compounds, IC_{50} values were lower in HK-2 compared with HepG2 cell lines, which could be attributed to lower basal expression and inducibility of metabolizing enzymes, transporters, and nuclear receptors in HK-2 cells. In HepG2 cells, co-exposure of CPF with a specific inhibitor of either P-gp (verapamil or VRP) or BCRP (Ko143) enhanced the cytotoxicity of CPF while co-exposure of CPFO with VRP enhanced the cytotoxicity of CPFO, suggesting the role of these transporters in the export of CPF and CPFO. Inhibition of efflux transporters did not affect the cytotoxicity of CPF and CPFO in HK-2 cells. Co-incubation of CPF with P-gp and BCRP inhibitors increased the intracellular concentration of CPF in HepG2 cells suggesting that both transporters play a role in limiting the cellular accumulation of CPF in HepG2 cells. Our results provide evidence that inhibition of efflux transporters can enhance CPF-induced toxicity in HepG2 cells through enhanced cellular accumulation and raises additional questions regarding how pesticide-transporter interactions may influence toxicity of mixtures containing pesticides and other environmental chemicals.

2.2 Introduction

Pesticide exposure can pose a significant risk to human health. Exposure routes can be through contact with the skin, ingestion, or inhalation and adverse effects can arise from acute high-dose exposures as well as chronic low-level exposures (Damalas and Eleftherohorinos, 2011). The type of pesticide, the duration and route of exposure are among the many factors that influence the type of injury and organ system affected. Therefore, evaluating the potential toxicity of pesticides to target organs is an essential component to human health risk assessment (Reffstrup et al., 2010). The conventional approaches to hazard characterization of chemicals rely on *in vivo* experimental models and encounter significant challenges including high costs, time-intensive procedures, low throughput, and ethical concerns. In recent decades, *in vitro*

assays have emerged as alternative approaches to toxicity testing, using human cell-based models and incorporating mechanistic endpoints to support hazard characterization (Zhang et al., 2018). Although *in vitro* assays are well suited to high-throughput analysis, they also have limitations; in fact, the relevance and reliability of *in vitro* toxicity data is often questioned due to the metabolic capacity and toxicokinetic limitations of cell lines (Coecke et al., 2013). Xenobiotic-metabolizing enzymes and membrane transporters influence pesticide toxicokinetics by regulating the concentration of pesticides in various tissues and cells (Chedik et al., 2018). Metabolizing enzymes and transporters also facilitate the access of chemicals to target molecules, thereby impacting toxicodynamics (Chedik et al., 2018, Guéniche et al., 2020). While the role of xenobiotic-metabolizing enzymes is recognised and well-studied in terms of pesticide toxicity (Ginsberg et al., 2009), transporters are less characterized, although they can contribute significantly to cell-specific toxicity.

Xenobiotic transporters are classified into two superfamilies: ATP-binding cassette (ABC) transporters, which act mainly as efflux pumps, and solute carrier (SLC) transporters, which function primarily as drug uptake transporters (Clerbaux et al., 2019). Among the ABC group, p-glycoprotein (P-gp; encoded by the ABCB1 gene), and breast cancer resistance protein (BCRP; encoded by the ABCG2 gene) are two major contributors to xenobiotic efflux in key cellular sites of xenobiotic elimination, including hepatocytes and renal tubular cells. Numerous pesticides from various classes can interact with these efflux transporters as either substrates, inhibitors, or inducers, which had an impact on the toxicokinetic and toxicity of pesticides (reviewed in Chedik et al., 2018, Guéniche et al., 2020). In liver and kidney, P-gp and BCRP are located in the apical membrane of cells, which mediate the secretion of xenobiotics and their metabolites out of cells (into the bile and renal tubule, respectively), limiting their intracellular accumulation and impeding the delivery of compounds to the systemic circulation (Gameiro et al., 2017). Xenobiotics can also modulate transporter expression levels by either suppressing or inducing transcriptional activation in a cell-specific manner (Czuba et al., 2018). For instance, organochlorine and organophosphate pesticides have been reported to alter transporter mRNA expression *in vitro* (Bucher et al., 2014; Chedik et al., 2018). This sort of transporter modulation is primarily regulated by the nuclear receptor superfamily of transcription factors, including the pregnane X receptor (PXR), aryl hydrocarbon receptor (AhR), and nuclear factor erythroid 2–related factor 2 (Nrf2) (Amacher, 2016). Nonetheless, the expression and inducibility of these

efflux transporters and their regulators in cell lines are often disregarded in the assessment of *in vitro* assay methods.

Chlorpyrifos (O-O-diethyl-O- {3, 5, 6 trichloro-2-pyridyl}-phosphorothioate; CPF) is one of the most used organophosphate pesticides worldwide (Foong et al., 2020; Kaushal et al., 2021). Biotransformation of CPF occurs via cytochrome P450 (CYP) enzymes to form the major toxic metabolite CPF-oxon (CPFO) (Dar et al., 2019), while inactivation of CPFO occurs through B-esterases carboxylesterases (CES) and A-esterase paraoxonase1 (PON-1; Moser and Padilla, 2016; Nandi et al., 2022). Aside from the enzymatic detoxification of CPFO, *in vitro* and *in vivo* studies provide evidence that the efflux transporter P-gp protects against CPF-induced acute toxicity through active efflux of CPF and CPFO (Bain and LeBlanc, 1996; Alharbi et al., 2017; Lanning et al., 1996). Other studies have reported CPF as a potential BCRP substrate *in vitro* (Halwachs et al., 2016; Kuhnert et al., 2020) and capable of upregulating the BCRP expression in human placental explants (Ridano et al., 2017; Ridano et al., 2012) and human placental trophoblast cell lines (Halwachs et al., 2016). While CPF and its metabolite, CPFO, appear to interact with critical efflux transporters in studies using different cell lines, there is limited data on how these transporters influence intracellular concentrations of CPF and CPFO and toxicity thresholds across organ-specific cell lines. Understanding the function of transporters and their role in chemical toxicity is critical since these transporters can be altered by other mixture components via inhibition or activation. Such interactions can account for non-linear dose-response scenarios and potentially explain unexpected mixture toxicity, emphasizing the importance of understanding pesticide-transporter interactions for *in vitro* hazard characterisation and *in vivo* extrapolation.

In the present study, we assessed the putative role of P-gp and BCRP transporters in cell-specific toxicity and intracellular concentrations of CPF and CPFO in human hepatoblastoma cells (HepG2) and human kidney proximal tubule epithelial cells (HK-2). We also compared the capacity of these two cell lines to express biotransformation enzymes responsible for CPFO detoxification to highlight their role in cytotoxicity responses. These cell lines exhibit differential expressions of metabolizing enzymes and drug transporters (Guguen-Guillouzo and Guillouzo, 2010). Specifically, HepG2 cells are reported to have relatively high expression of the efflux transporters P-gp and BCRP (Brandon et al., 2006; Rigalli et al., 2012) while HK-2 cells exhibit no or low-level expression of these transporters (Jenkinson et al., 2012). Therefore, the combined

use of two *in vitro* models with different capacities in transporter expression, specific inhibitors of efflux transporters, as well as application of a selective and sensitive analytical method, could yield clear advantages in the study of CPF interaction with efflux transporters and cytotoxicity response.

2.3 Materials and Methods

2.3.1 Chemicals and reagents

Dulbecco's Modified Eagle's Medium (DMEM), DMEM/F12, fetal Bovine Serum (FBS), penicillin-streptomycin (10,000 U/mL), phosphate buffer saline (PBS), and 0.25% Trypsin-EDTA were purchased from Gibco; Thermo Fisher Scientific, Inc., Canada. CPF (purity >99.67%), CPFO (purity >99%) and CPFO-d10 (purity >99%) were of standard analytical grade and supplied by Toronto Research Chemicals (TRC, Toronto, Ontario, Ca). CPF-d10 (99.0%) was purchased from Cambridge Isotope Laboratories, Inc (MA, USA). Verapamil (VRP; purity ≥ 99%), Ko143 (purity ≥ 98%), and neutral red powder were purchased from Sigma Aldrich (Oakville, ON, Ca). TRIzol reagent was purchased from Invitrogen (Burlington, ON, Ca). The Pierce™ BCA Protein Assay Kit, Applied Biosystems™ High-Capacity cDNA Reverse Transcription Kit, and UltraPure™ DNase/RNase-Free Distilled Water were purchased from ThermoFisher. The SsoAdvanced Universal SYBR Green Supermix was purchased from BioRad (Mississauga, Ontario, Ca). Methanol (MeOH), formic acid, and water were of LC/MS grade and purchased from Fisher Scientific (Ottawa, Ontario, Ca).

2.3.2 Cell culture

HepG2 (human hepatocellular carcinoma; HB-8065) and HK-2 (human kidney proximal tubule; CRL-2190) cell lines were obtained from the American Type Culture Collection (ATCC). Cells were initially grown as monolayer cultures in 75 cm^2 flasks and maintained with the appropriate medium that is recommended by the supplier. HepG2 cells were cultured in high glucose DMEM with pyruvate, and supplemented with 10% FBS, and 1% penicillin-streptomycin. HK-2 cells were grown in DMEM-F12 containing 5% FBS and 1% penicillin-streptomycin. Cells were cultured in an atmosphere at 95% relative humidity and 5% CO_2 at

37°C. The cells were sub-cultured after trypsinization once or twice per week and resuspended in a complete medium using a 1:4 split ratio.

2.3.3 Cell viability assay

To establish the cytotoxicity of CPF and CPFO for each cell type, HepG2 and HK-2 cells were seeded at densities of 2×10^4 and 1.2×10^4 cells/well, respectively, in 96-well cell culture plates and were allowed to attach for 24 hours (h). Cells were then treated with varying concentrations of CPF or CPFO by making a two-fold serial dilution based on solubility limits for CPF (0, 12, 24, 47, 94, 188 µM) and CPFO (0, 2, 4, 8, 16, 32, 63, 125, 250 µM). We excluded concentrations higher than 188 µM CPF due to its limited solubility, as the low solubility of CPF could affect the IC50 and dose-response curve. The CPF and CPFO stock solutions were prepared in 100% MeOH and kept in a freezer at −20°C protected from ambient light. Their working solutions were prepared daily in FBS-free cell culture media. The final MeOH concentrations did not exceed 1% (v/v) and vehicle-treated cells were used as controls. The neutral red uptake assay was carried out following a 24 h exposure period as described by Repetto et al. (2008). Briefly, after 24 h, exposure media was removed, cells were washed with PBS, and then treated with 40 $\mu g mL^{-1}$ neutral red. After 2 h incubation at 37°C, cells were washed with PBS again and a de-staining solution containing glacial acetic acid, ethanol, and water (1:50:49, respectively) was added to each well. After 10 min agitation at room temperature, the absorbance was read at 540 nm. Cell-free wells with medium were also included for determining background absorbance. The mean background absorbance was subtracted from the absorbance reading of all solvent control and treated wells. Cell viability was calculated relative to the solvent control (MeOH) wells using equation 2.1.

$$\left[\frac{\text{(Absorbance value of treated samples)}}{\text{(Absorbance value of MeOH control samples)}}\right] \times 100 \qquad (2.1)$$

Cell viability was also evaluated in cells exposed to CPF or CPFO when co-incubated with verapamil (VRP) or Ko143, as known inhibitors of efflux transporters P-gp and BCRP, respectively. Exposure conditions and cytotoxicity assay methodology for the co-exposures were similar to the single compound exposures described above. Cells were exposed to CPF, CPFO, VRP and Ko143 at concentrations $<IC_{20}$ previously established for each compound and cell line. Briefly, cells were exposed to either CPF (50 µM for HepG2 cells and 20 µM for HK-2 cells) or

CPFO (20 μM for HepG2 cells and 2 μM for HK-2 cells) with or without each of VRP (20 μM for HepG2 cells and 10 μM for HK-2 cells) or Ko143 (10 μM for HepG2 cells and 5 μM for HK-2 cells) for 24 h. The final MeOH concentration in all wells was 0.5 % (v/v), and vehicle-treated cells were used as controls.

2.3.4 Quantitative real-time RT-PCR (qPCR)

To assess whether cytotoxicity responses of cell lines could be attributed to cell-specific expression of metabolizing enzymes, drug transporters, and nuclear receptors, basal transcript expression of target genes were assessed via qPCR. The expression of transporters and nuclear receptors was also evaluated following exposure to CPF and CPFO. Information on target genes of interest and reference genes, including primer sequences for qPCR, are listed in Table 2.1. Briefly, HepG2 cells (6×10^5 cells/well) and HK-2 cells (3.5×10^5 cells/well) were seeded in 6-well plates and incubated. Cells were then cultured for 24 h in cell culture medium (for the basal expression comparison) or were exposed to concentrations of CPF ($<IC_{20}$: 20 and 50 μM for HepG2 and $<IC_{20}$: 8 and 20 μM for HK-2), CPFO ($\leq IC_{20}$: 2 and 20 μM for HepG2 cells and $<IC_{20}$: 0.2 and 2 μM for HK-2), or solvent control (0.02% MeOH). Total RNA was extracted using TRIzol™ Reagent (Life Technologies, Carlsbad, CA) following the manufacturers protocol. RNA concentration was determined using a Nanodrop spectrophotometer and quality was verified on a 1% agarose gel. Complementary DNA (cDNA) was synthesized from 1 μg of RNA using the High-Capacity cDNA Reverse Transcription kit (Applied Biosystems) according to manufactures instructions.

The cDNA was diluted 10-fold to 5 ng/μL for qPCR. Each 20 μL reaction contained 2 μL of cDNA, 0.8 μL of both forward and reverse primer (0.4 mM), and 10 μL of SSoAdvanced Universal SYBR Green Supermix (BioRad, Hercules, CA) and reaction were run using a CFX96 Real-time C1000 Thermal Cycler (BioRad, Hercules, CA). PCR conditions were as follows: 95°C for 3 min; 40 cycles of 95°C for 20 s, and 60°C for 30 s. After 40 cycles, the PCR products from each reaction were subjected to melt curve analysis to ensure that only a single product was amplified.

Samples were run in duplicate, and assays included no reverse transcriptase controls (no RT control) and samples that did not receive any cDNA template. All primer sets were tested for efficiency using a 5-point standard curve generated by a serial dilution of cDNA. The standard

curve method was used to interpolate the relative mRNA abundance of target and reference genes for each sample based on RNA input. To compare basal expression of target genes between cell lines, the reference genes *GAPDH* and *HMBS2* were first determined to be stably expressed and then following normalization, differences in transcript expression for genes of interest were expressed relative to HK-2 as the calibrator group. For the CPF/CPFO exposures, three reference genes (*GAPDH, HMBS2,* and *GUSB*) were used for assay normalization and data were expressed as fold-change relative to the MeOH control.

Table 2.1. Primer sets used for real-time PCR.

Category	Gene	Primer Sequence (5′-3′)	Source
Efflux Transporter	*ABAB1*	Fwd: CACCCGACTTACAGATGATG Rev: GTTGCCATTGACTGAAAGAA	(Dauchy et al., 2009)
	ABCG2	Fwd: CAGGTGGAGGCAAATCTTCGT Rev: ACACACCACGGATAAACTGA	(Tsunoda et al., 2007)
Metabolizing Enzyme	*PON-1*	Fwd: TTCACCCGATGGCAAGTATG Rev: ACGAGGGTATTAAAGTCAAGGG	(Aboufarrag, 2019)
	CES1	Fwd: AGAGGAGCTCTTGGAGACGACAT Rev: ACTCCTGCTTGTTAATTCCGACC	(Chiorean et al., 2012)
	CES2	Fwd: GTAGCACATTTTCAGTGTTCC Rev: GTAGTTGCCCCCAAAGAA	(Cecchin et al., 2005)
Nuclear Receptor	*PXR*	Fwd: CCCAGCCTGCTCATAGGTTC Rev: CTGTGATGCCGAACAACTCC	(Liu et al., 2011)
	AhR	Fwd: GGCTCAGGTTATCAGTTTATT Rev: ACTATCATGCCACTTTCTCC	(Gramatzki et al., 2009)
	Nrf2	Fwd: CGGTATGCAACAGGACATTG Rev: ACTGGTTGGGGTCTTCTGTG	(Zhang et al., 2014)
Reference Gene	*GAPDH*	Fwd: TCGGAGTCAACGGATTTGGT Rev: TTCCCGTTCTCAGCCTTGAC	(Pecorelli et al., 2020)
	HMBS2	Fwd: TGCTATCTGGGGAGTGATTACC Rev: GGCTGTTGCTTGGACTTCTC	(Rowland et al., 2019)
	GUSB	Fwd: AGCCAGTTCCTCATCAATGG Rev: GGTAGTGGCTGGTACGGAAA	(Li et al., 2009)

PON-1 (Paraoxonase-1); *CES1* (Carboxylesterase 1); *CES2* (Carboxylesterase 2); *PXR* (Pregnane X receptor); *AhR* (Aryl hydrocarbon receptor); *Nrf2* (Nuclear factor erythroid 2-related factor 2); *GAPDH (*Glyceraldehyde 3-phosphate dehydrogenase); *HMBS2 (*Hydroxymethylbilane synthase); *GUSB (*Glucuronidase beta).

2.3.5 LC-MS/MS conditions

An Agilent 1260 Infinity system (Agilent Technologies, Mississauga, Ontario, Ca) and Agilent Eclipse plus C18 column (4.6 x 100 mm, 3.5 μm) were used for the chromatographic

separation. Isocratic elution was employed using methanol/water (95:5 v/v) with 0.1% acetic acid at a 600 µL/min flow rate. Column temperature was maintained at 30°C and injection volume was 10 µL. Analyte detection and quantification were achieved using TSQ Altis mass spectrometer (Thermo Scientific, Mississauga, ON) that has an ion source with heated electrospray ionization (HESI) probe, and MS was operated in positive ionization using multiple reaction monitoring (MRM) mode. Two transitions were monitored, one as the quantifier ion and the other as qualifier ion. Both ion source and compound-dependent parameters were optimized via direct infusion. The following ion source parameters were employed: spray voltage 3500 V, sheath gas 60 (arb), auxiliary gas 23 (arb), sweep gas 2 (arb), vaporizer temperature 400°C, ion transfer tube temperature 380°C, and default calibrated RF lens value. CPF and CPFO ionized as $[M + H]^+$, however, due to isotopic contribution by chloride, $[M + 2 + H]^+$ was also observed at an almost similar abundance. Depending on the signal intensity and/or stability, either $[M + H]^+$ or $[M + 2 + H]^+$ was chosen as precursor ion. Table 2.5.S shows MRM transitions monitored and the corresponding retention time and collision energies for both analyte and internal standards. Chromeleon software v.7.03 (Thermo Scientific, Mississauga, ON) was used for data acquisition and processing.

2.3.6 Intracellular concentration of CPF and CPFO by LC-MS/MS

HepG2 cells were cultured in 6-well plates at a density of 6×10^5 cells/well in 2 mL of media. Cell culture media was replaced after 24 h with fresh media containing CPF (50 µM), either alone or in combination with pharmacologic efflux pump inhibitors (10 µM Ko143 or 20 µM VRP), and the solvent control (MeOH at 0.5%) as described above. Following 2 and 6 h of CPF exposure, the cell culture medium was collected and stored at -80°C overnight for later analysis. The initial concentration of CPF in the cell culture media (0 h) was also quantified by collecting media samples from a set of wells immediately after addition. After removing the culture medium, HepG2 cells were washed two times with ice-cold PBS. Each sample received 1 mL of methanol containing internal standards, with CPF-D10 at 80 µM and CPFO-D10 at 40 nM. Samples were frozen at –80°C overnight and then cells were vortexed and centrifuged at 10,000 × *g* for 10 min at 4°C, pellet discarded, and supernatant retained. In preparation for LC-MS/MS analysis, 5 µL and 10 µL aliquot of extract were diluted to a final volume of 200 µL with MeOH for CPF and CPFO quantification, respectively. Before initiating the experiments, BCA

protein assays and trypan blue cell counting were performed to ensure uniform cell counts in all treated wells. To quantify residual CPF and CPFO in the media, the samples were thawed and briefly vortexed. Then, 5 µl of the culture media was diluted with 195 µl of MeOH containing internal standards. The final concentration of the internal standards was 2 µM for CPF-D10 and 2 nM for CPFO-D10 in calibration standards and samples. For each analyte, a calibration curve was made ranging from 0.5-1024 nM for CPF and 0.05-12.8 nM for CPFO. The preparation of stock solutions, calibration standards, and quality control samples are detailed in 2.6 Supplementary Information - Methods with concentrations details in Table 2.1.S. The concentration [C] of CPF and CPFO in HepG2 cells and culture media are defined using equation 2.2.

$$[\mathbf{C}] = \frac{\mathbf{V}\,\text{fraction}}{\mathbf{V}\,\text{cell}} \times [\mathbf{C}]\,\text{fraction} \qquad (2.2)$$

where [C] fraction represents the concentration of a compound in the culture media or cells fraction, V fraction is the total volume of the cellular or culture media fraction (200 µL), and V cell represents the volume of cells or culture media within the fraction (e.g., 5 µL of culture media; Gordon et al., 2016). LC-MS/MS method validation for CPF and CPFO quantification are described in supplementary information Tables 2.1.S to 2.4.S.

2.3.7 Statistical analyses

Data were analyzed with GraphPad Prism (v.8, GraphPad Software, San Diego, CA, USA). The inhibitory concentration causing 50% cytotoxicity (IC_{50}) was derived from a non-linear regression curve calculated in GraphPad Prism ([Inhibitor] vs. normalized response, variable slope, and least squares fit). Differences in basal expression of target genes were compared between cell lines using Student's t-test for unpaired data. For data other than the basal comparisons, one-way analysis of variance (ANOVA) was performed with Dunnett's multiple comparison test to determine differences between exposure groups. A value of $p \leq 0.05$ was considered statistically significant. Three independent assays were performed for assessment of cell viability and gene expression with five replicates and three replicates in each assay, respectively. All results are expressed as mean ± standard deviation (SD).

2.4 Results

2.4.1 Cytotoxicity of CPF and CPFO to HepG2 and HK-2 cells

A concentration-dependent decrease in the viability of HepG2 and HK-2 cells was observed after 24 h exposure of CPF and CPFO (Fig. 2.1). The determined IC_{50} values of CPF were 180.9 ± 8.3 μM for HepG2 cells and 73.6 ± 7.6 μM for HK-2 cells (Fig. 2.1A). The IC_{50} values for HepG2 and HK-2 cells exposed to CPFO were 73.5 ± 5.8 and 10.6 ± 1.5 μM, respectively (Fig. 2.1B).

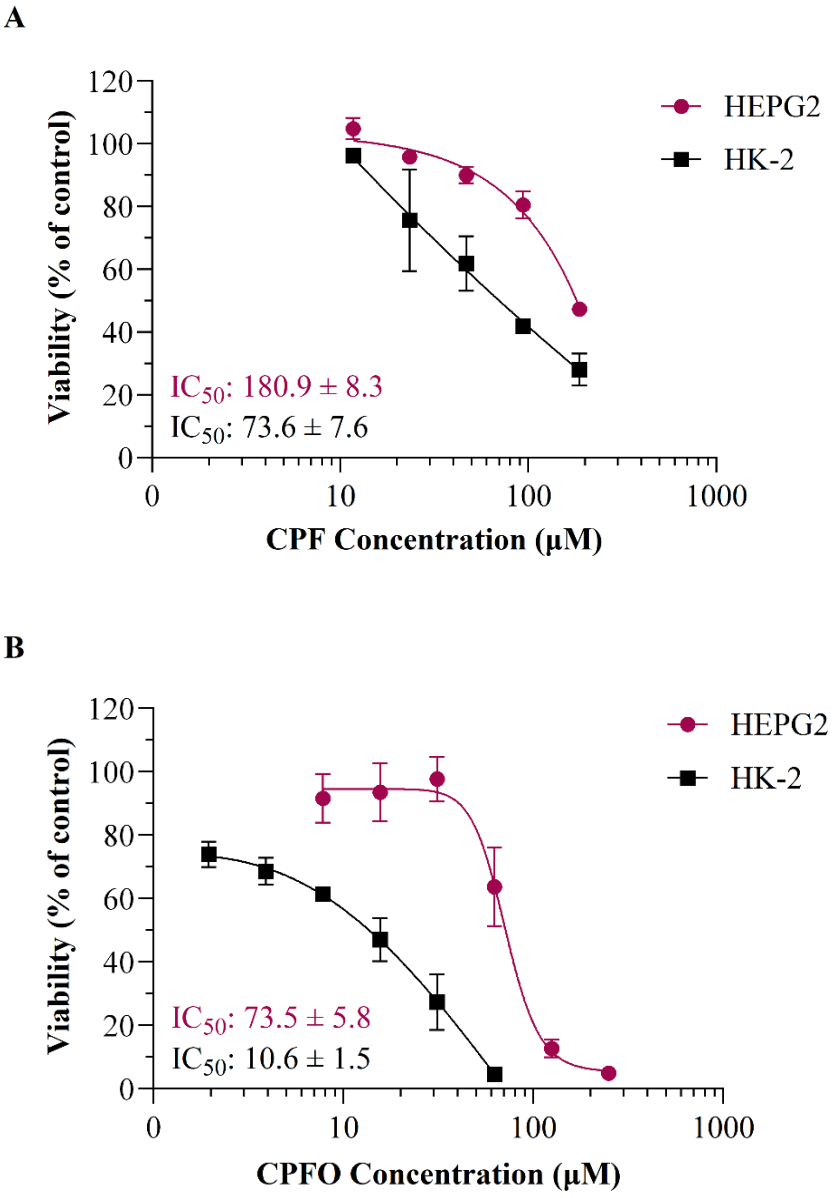

Figure 2.1. Viability of HepG2 and HK-2 cells after 24 h exposure to CPF (A) and CPFO (B) as determined by neutral red assay. The percentage of viable cells normalized to solvent treated control (≤ 1% MeOH) were plotted after background subtraction. Data are means ± SD and represent three independent assays (*n*=3).

2.4.2 Cell line-specific expression of enzymes, transporters, and nuclear receptors

Basal expression of all target genes was significantly higher in HepG2 cells compared to HK-2 cells (Fig. 2.2). Expression of the efflux transporters *ABCB1* and *ABCG2* were 2.5-fold and 71-fold higher, respectively, in HepG2 cells relative to HK-2 cells. HepG2 cells also had higher basal expression of the metabolic enzyme *CES2* (5-fold), the nuclear receptors *AhR* (1.6-fold) and transcription factor *Nrf2* (4.6-fold) when compared to expression HK-2 cells. There was no detectable amplification of *PXR*, *CES1*, and *PON-1* in HK-2 cells while HepG2 cells had measurable transcript levels for these target genes.

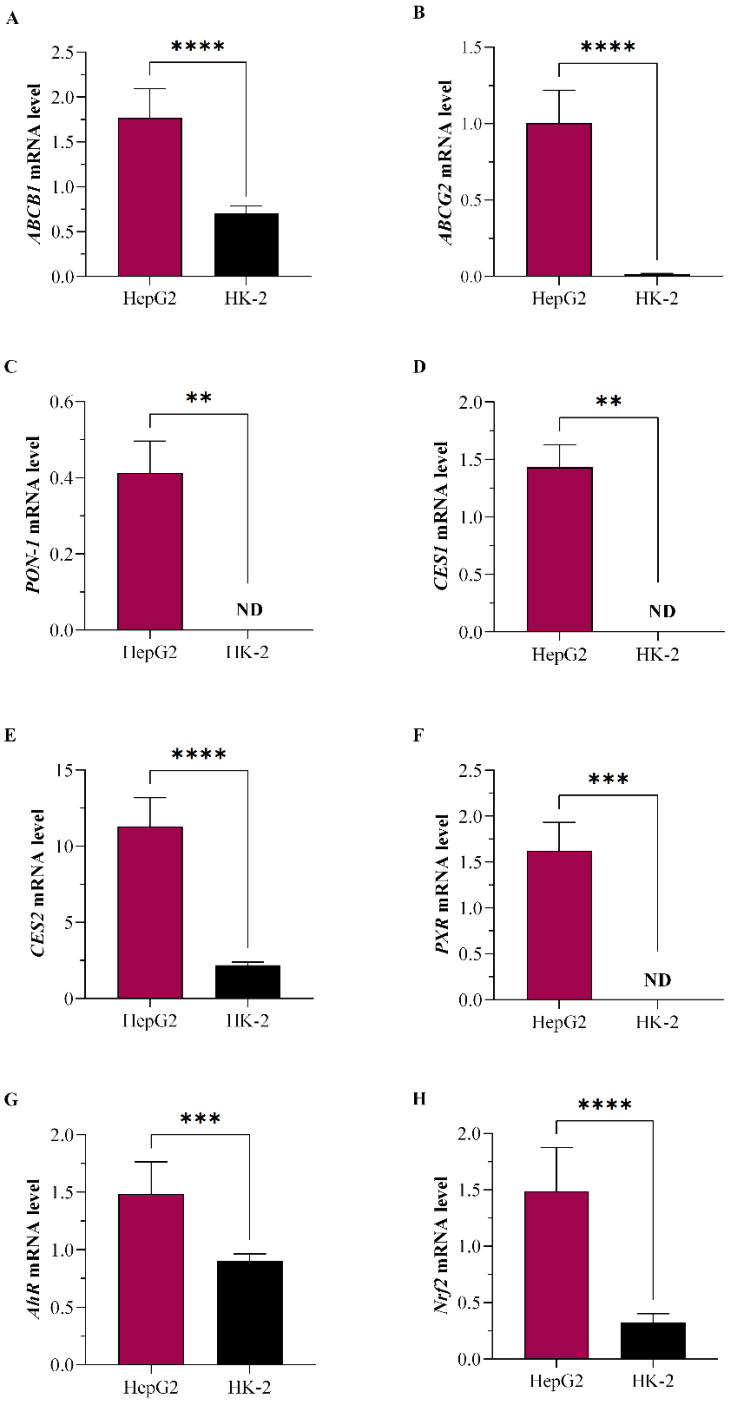

Figure 2.2. Basal mRNA levels of drug efflux transporters *ABCB1* (A) and *ABCG2* (B), metabolizing enzymes *PON-1* (C), *CES1* (D), and *CES2* (E), nuclear receptors *PXR* (F), *AhR* (G), and *Nrf2* (H) in HepG2 and HK-2 cells. Data are means ± SD (n = 3). ** p<0.01, *** p<0.001 **** p<0.0001. ND: Not detected. *P-gp* (P-glycoprotein); *BCRP* (Breast Cancer

Receptor Protein); *PON-1* (Paraoxonase-1); *CES1* (Carboxylesterase 1); *CES2* (Carboxylesterase 2); *PXR* (Pregnane X receptor); *AhR* (Aryl hydrocarbon receptor); *Nrf2* (Nuclear factor erythroid 2-related factor 2).

2.4.3 Modulation of efflux transporter and nuclear receptor expression by CPF and CPFO

In HepG2 cells, there was no effect of CPF exposure (at 20 or 50 µM) on expression of ABCB1 when compared to the solvent control (0.02% MeOH). In contrast, HepG2 cells exhibited significant increase in *ABCB1* expression upon exposure to both 2 and 20 µM concentrations of CPFO (Fig. 2.3A). In HK-2 cells, however, 8 and 20 µM of CPF as well as 0.2 and 2 µM of CPFO showed no effect on the mRNA expression of *ABCB1* (Fig. 2.3A). Likewise, CPF did not change the mRNA level of *ABCG2* in HepG2 cells. However, exposure to 20 µM CPFO significantly increased *ABCG2* expression in this cell line as compared to the solvent control. CPF and CPFO did not induce any changes in *ABCG2* mRNA expression in HK-2 cells (Fig. 2.3B). Similarly, CPF did not alter expression of nuclear receptor *PXR* mRNA in HepG2 cells. Conversely, the mRNA expression of *PXR* in HepG2 cells was significantly increased to 2-fold greater than control by 20 µM CPFO. The basal expression of *PXR* mRNA was not detected in HK-2 cells (Fig. 2.4A). The treatment of HepG2 cells with 20 µM CPF decreased mRNA expression of *AhR*, whereas CPFO did not exhibit any alteration in *AhR* mRNA expression. Neither CPF nor CPFO exposure altered the mRNA expression of *AhR* in HK-2 cells (Fig. 2.4B). No statistically significant changes in *Nrf2* mRNA expression were observed after exposure to CPF and CPFO in HepG2 and HK-2 cells (Fig. 2.4C).

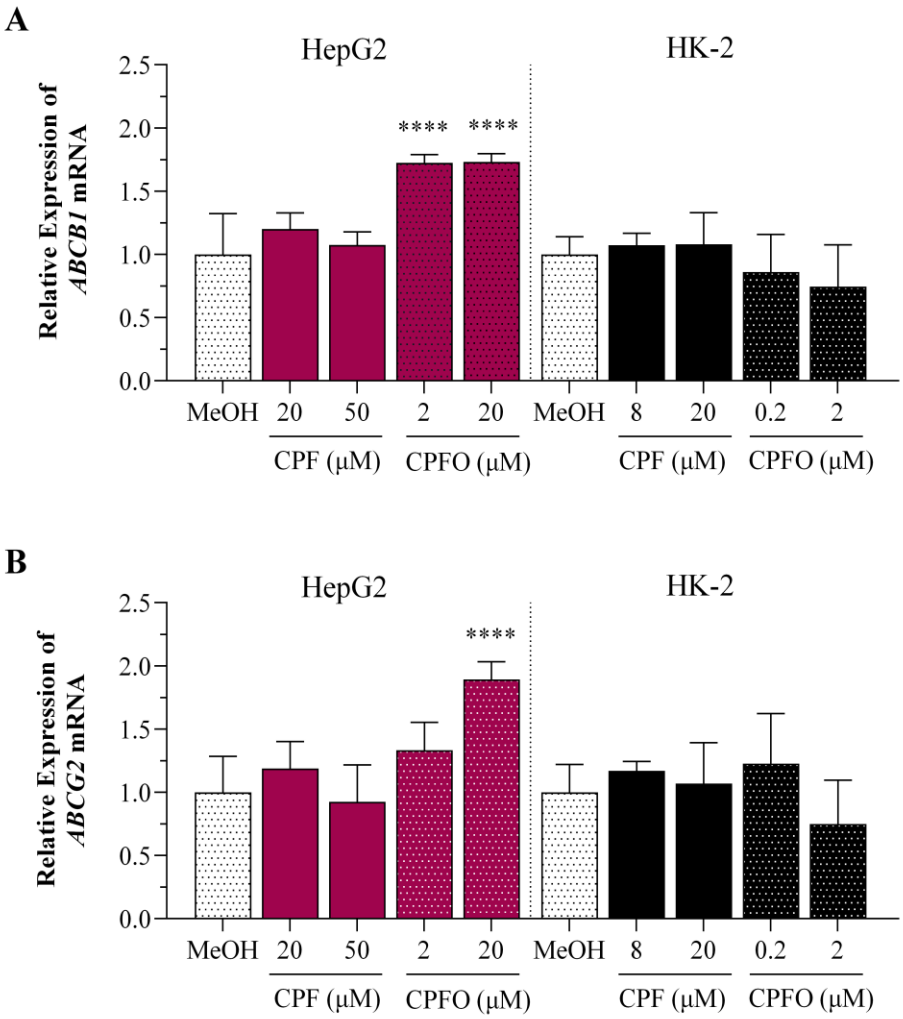

Figure 2.3. Effects of 24 h exposure to concentrations (<IC_{20}) of CPF and CPFO on mRNA expression of *ABCB1* (A) and *ABCG2* (B) in HepG2 and HK-2 cells. Data are presented as mean ± SD (*n*=3) and expressed relative to MeOH (0.02%) as vehicle control within cell type. Asterisks represent statistically significant differences between exposed and MeOH control (one way ANOVA, Dunnett's multiple comparisons test). **** p<0.0001.

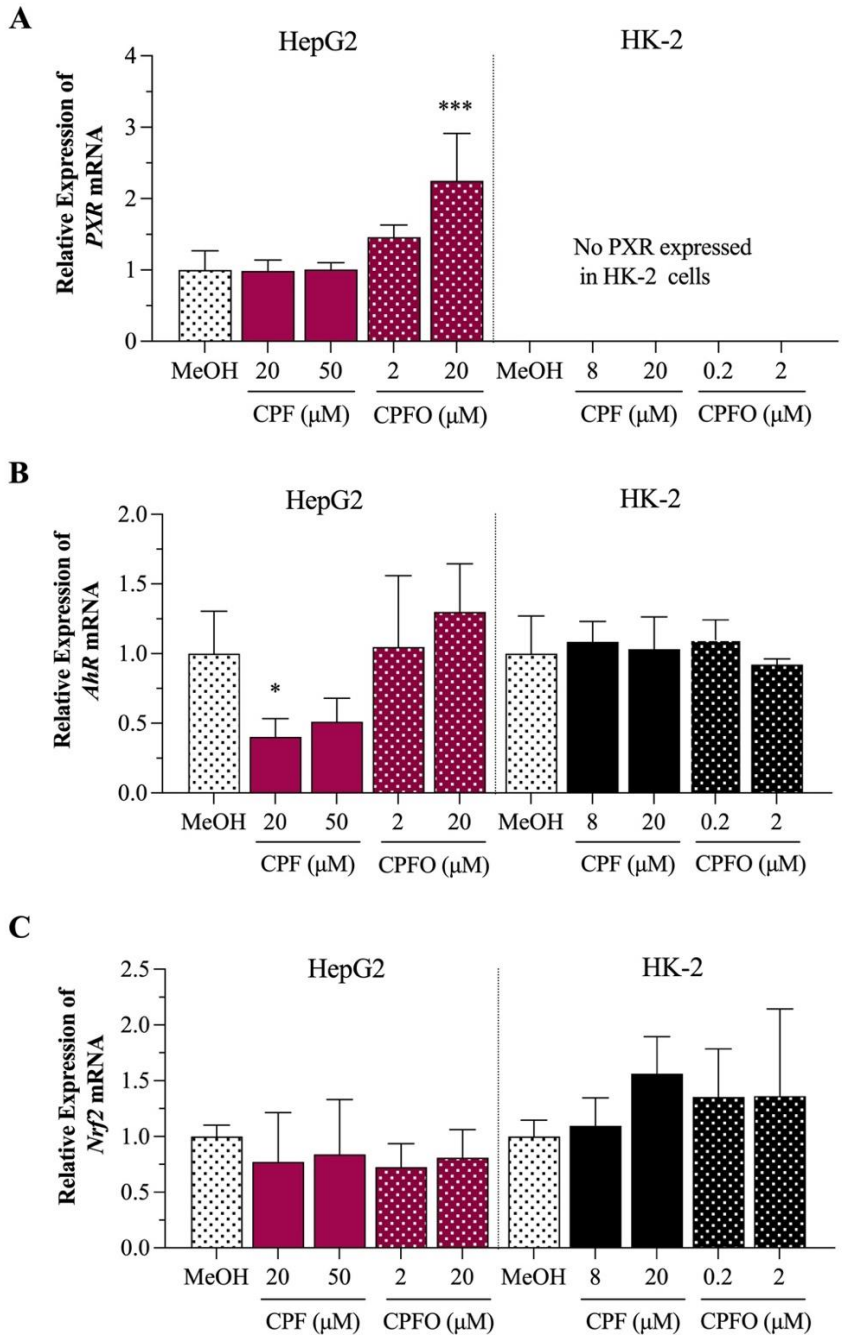

Figure 2.4. Effects of 24 h exposure to concentrations (<IC_{20}) of CPF and CPFO on mRNA expression of *PXR* (A), *AhR* (B), and *Nrf2* (C) in HepG2 and HK-2 cells. Data are presented as mean ± SD of 3 independent assays (n=3) and are expressed relative to MeOH (0.02%) as vehicle control within cell type. Asterisks represent statistically significant differences between exposed and MeOH control (one way ANOVA, Dunnett's multiple comparisons test). * p<0.05 and ***p<0.001.

2.4.4 Effects of inhibitors on the cytotoxicity of CPF and CPFO

In both HepG2 and HK-2 cells, the selected concentrations of VRP and Ko143 resulted in less than 10% cytotoxicity compared to the solvent control alone (MeOH at 0.5%, Fig. 2.5A and B). In HepG2 cells exposed to 50 μM CPF, co-exposure with either VRP (20 μM) or Ko143 (10 μM) resulted in 24% and 60% cytotoxicity, which was significantly higher than CPF alone (Fig. 2.5A). In HepG2 cells exposed to 20 μM CPFO, co-exposure with VRP increased cytotoxicity compared to CPFO alone but there was no effect of co-incubation with Ko143 (Fig. 2.5A). co-incubation of CPF (20 μM) and CPFO (2 μM) with either VRP or Ko143 had no effect on cytotoxicity in HK-2 cells (Fig. 2.5B).

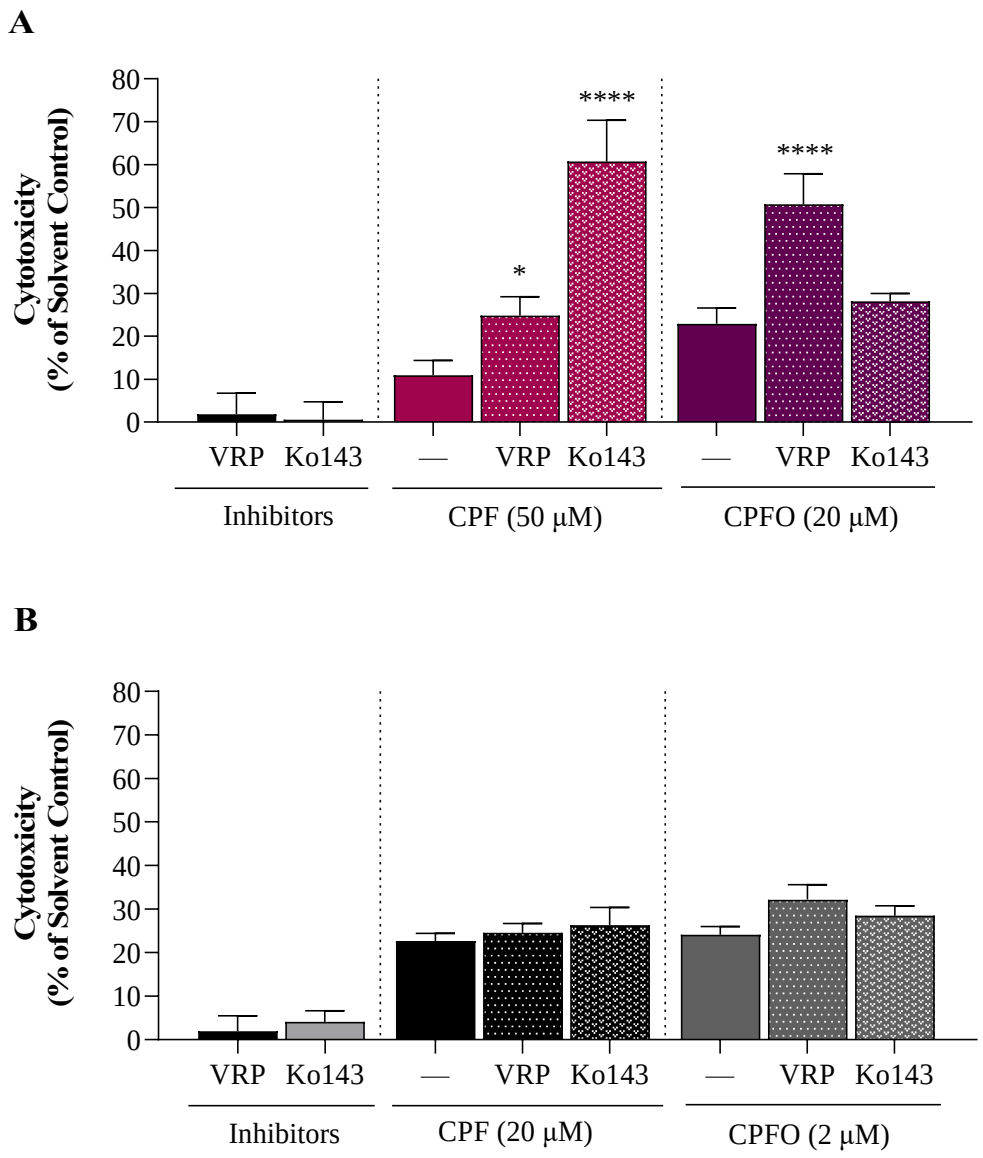

Figure 2.5. Cytotoxicity in HepG2 cells (A) and HK-2 cells (B) treated with concentrations (<IC_{20}) of CPF and CPFO in the presence of transporter inhibitors, verapamil (VRP) at 20 µM for HepG2 cells or 10 µM for HK-2 cells, and Ko143 at 10 µM for HepG2 cells or 5 µM for HK-2 cells. Data are expressed as mean ± SD of three independent assays (n=3). Asterisks represent significant differences between the pesticides alone and their co-incubation with VRP or Ko143 (one-way ANOVA with Dunnett's multiple comparison test). * p<0.05, **** p<0.0001.

2.4.5 Intracellular and media concentrations of CPF and CPFO in HepG2 cells

Measured concentrations of CPF and CPFO in HepG2 cells and in culture media are shown in Table 2.2. In cells exposed to the solvent control (0.5% MeOH), there was no detection of CPF or CPFO at both 2 and 6 h timepoints. After 2 h of co-exposure to CPF (50 μM) with VRP (20 μM) or Ko143(10 μM), there were no differences in intracellular concentration of CPF or CPFO compared to cells exposed to CPF alone. However, after 6 h of exposure, HepG2 cells exposed to CPF with either VRP or Ko143 had significantly higher intracellular concentration of CPF when compared to CPF alone. After 6 h exposure of CPF with Ko143, the cellular concentration of CPFO was below the LLOQ (lower limit of quantification).

The concentration of CPF in the culture media was also measured after 2 and 6 h of exposure to CPF or in combination with transporter inhibitors. There was no measurable level of CPF or CPFO in media containing the solvent only (0.5% MeOH) taken from cells after 2 and 6 h of exposure. The initial CPF concentration in the cell culture media, measured immediately after adding CPF alone or with transporter inhibitors, was 3.07 ± 0.4 μM/well (CPF alone), 3.27 ± 0.2 μM/well (CPF + VRP), and 3 ± 0.3 μM/well (CPF + Ko143) and were not significantly different between treatment groups. CPF concentrations in CPF + VRP and CPF + Ko143 media taken from cells after 2 h or 6 h of exposure were not significantly different from CPF alone. However, the concentrations of CPFO were significantly lower in CPF + Ko143 media taken from cells at both the 2 h and 6 h time points when compared to CPF alone. The conversion of CPF and CPFO in culture media (in the absence of cells) was also measured to assess the potential abiotic transformation of CPF to CPFO in media and it was found that CPFO concentrations were below the LLOQ at after both 2 and 6 h of incubation.

Table 2.2. Concentration of CPF and CPFO in HepG2 cells and culture media after 2 or 6 h exposure to 50 µM CPF with and without efflux transporter inhibitors, VRP and Ko143.

	CPF (µM/well)		CPFO (nM/well)	
	2 h	**6 h**	**2 h**	**6 h**
Cells				
MeOH (0.5%)	ND	ND	ND	ND
CPF	40.06 ± 1.67	31.47 ± 2.23	1.17 ± 0.06	1.52 ± 0.36
CPF + VRP	43.58 ± 0.69	37.03 ± 0.27*	1.14 ± 0.15	1.07 ± 0.24
CPF + Ko143	42.22 ± 0.9	36.75 ± 1.29*	1.10 ± 0.04	< LLOQ*
Culture Media				
MeOH (0.5%)	ND	ND	ND	ND
CPF	1.70 ± 0.18	1.28 ± 0.13	2.43 ± 0.05	4.01 ± 0.36
CPF + VRP	1.72 ± 0.25	1.38 ± 0.41	2.31 ± 0.19	3.74 ± 0.91
CPF + Ko143	1.90 ± 0.26	1.55 ± 0.24	1.58 ± 0.1*	1.96 ± 0.19*

Data are presented as the mean of three exposure replicates ± SD (n=3). Asterisk (*) represent significant differences ($p \leq 0.05$) versus CPF alone, within each sample matrix (cells and media) and each time point (one-way ANOVA, Dunnett's multiple comparison test). LLOQ: lower limit of quantification. ND: not detected.

2.5 Discussion

The effectiveness of cell lines in assessing pesticide toxicity relies on their functional characteristics, such as the expression of xenobiotic metabolizing enzymes and efflux transporters (Gómez-Lechón et al., 2014). A recent meta-analysis by Alehashem et al. (2022) highlighted both biotranformation capacity and membrane transporters as important characteristics contributing to the suitability of cell lines in predicting *in vivo* pesticide toxicity. In this study, we aimed to compare the toxicity and responsiveness of two human cell lines, HepG2 and HK-2, to the organophosphate pesticide CPF and metabolite CPFO while examining their specific expression of efflux transporters, metabolising enzymes, and nuclear receptors. We also explored the functional and preventive roles of efflux pumps P-gp and BCRP in the cytotoxicity of CPF and CPFO in these cell lines as well as how inhibition of these transporters influences intracellular accumulation in HepG2 cells specifically.

The cytotoxicity of CPF and its active metabolite, CPFO, to HepG2 and HK-2 cells was first assessed and compared between cell lines. For HepG2, the 24 h IC_{50} values for CPF and CPFO were 180.9 ± 8.3 μM and 73.5 ± 5.8 μM, respectively, which are lower than what is reported in other studies. Zhou and Li (2018) reported an IC_{50} of 886.53 μM in HepG2 cells using MTT assay while Tadee et al. (2020) found that 100 μg/mL (280 μM) CPF decreased HepG2 cell viability to approximately 50% of the solvent control as measured by the sulforhodamine B (SRB) cytotoxicity assay. To the best of our knowledge, our 24 h IC_{50} values for CPF (73.6 ± 7.6 μM) and CPFO (10.6 ± 1.5 μM) in HK-2 cells are the first reported for this cell type. The kidney cells exposed to CPF and CPFO displayed greater cytotoxicity when compared to liver cells. Cell-specific sensitivity to CPF was previously reported in a comparative study of responses across cell types (Kovalkovičová et al., 2015). Polláková et al. (2012) found that cytotoxicity to CPF, assessed by microscopic indications of cellular damage and LDH leakage into the medium, was greatest in rabbit kidney (RK13) when compared to rat and murine liver cells. Dellai et al. (2016) also found dissimilar cytotoxic responses to CPF among three human tumour cell lines with IC_{50} values rank order of A549<HT29<MCF7. The cytotoxicity of both CPF and CPFO was also reportedly higher in mouse embryonic stem cells compared to differentiated fibroblasts cells (Estevan et al., 2013). We also observed that CPFO was more cytotoxic than CPF in both HepG2 and HK-2 cells. Other studies have also reported CPFO to have a greater potency than CPF in decreasing cell viability in cultured neuronal HT22 cell line

(Naime et al., 2020) and D3 mouse embryonic stem cells (Estevan et al., 2013). Naime et al. (2020) suggested this may be due to the higher pro-oxidant activity of CPFO in comparison to CPF, highlighting the importance of CPF metabolism (particularly, bioactivation to CPFO) in developing non-cholinergic toxicity.

Cell-specific cytotoxicity can be attributed to differences in expression of enzymes, transporters, and their transcriptional regulators (Le Fol et al., 2015, Bell et al., 2017). We found that basal levels of *ABCB1* and *ABCG2* mRNA were approximately 2.5-fold and 70-fold higher, respectively, in HepG2 cells compared to HK-2 cells. Several other studies have characterised basal expression of efflux transporters in HepG2 cells (Brandon et al., 2006; Rigalli et al., 2012; Kim et al., 1993). Notably, Sukowati et al., (2012) reported relatively high mRNA and protein expression of both ABCB1 and ABCG2 in HepG2 cells and suggested that this protects HepG2 cells from doxorubicin-induced cytotoxicity. Conversely, there are limited studies examining basal expression of transporters in HK-2 cells. Jenkinson et al., (2012) found that although the expression pattern of ABC transporters in HK-2 cells is closer to kidney tissue as compared to the SLC family, the overall low or lack of drug transporter expression is a major limitation to using HK-2 cells in studies of xenobiotic-induced nephrotoxicity. We also found that mRNA expression of metabolising enzymes *PON-1, CES1,* and *CES2* were markedly lower or absent in HK-2 cells compared to HepG2 cells. These enzymes are important in modulating the toxicity of specific organophosphate pesticides, including CPF, in humans (Gupta et al., 2019; Matthews et al., 2011). Similar to AChE, CES enzymes have serine, histidine, and glutamic acid at their active site and are capable of irreversible binding to CPFO (Eaton et al., 2008). Human PON-1, which is synthesized in the liver, hydrolyzes the oxon group formed after oxidative desulfurization of the organophosphate parent compound by the CYP enzymes in the liver (Choi et al., 2006; Matthews et al., 2011). Therefore, it is possible that higher expression of the enzymes PON-1, CES1, and CES2 provides the ability of HepG2 to metabolize CPFO, resulting in lower cytotoxicity in HepG2 compared to HK-2.

Ligand-activated transcription factors also play an important role in regulating expression and activity of metabolizing enzymes and efflux transporters, thus, influencing detoxification capacity and toxicity response (Amacher, 2016). As with enzymes and transporters, we found a lack or lower expression of nuclear receptors in HK-2 cells compared to HepG2 cells. Overall, the higher basal expression of the enzymes, efflux transporters, and their regulators observed in

HepG2 cells compared to HK-2 cells in this study suggests greater biotransformation and transporter competence in HepG2 cells and may infer greater sensitivity of HK-2 cells to CPF and CPFO. However, cell-specific proliferative, DNA-repair, and antioxidant capacities are also crucial factors influencing chemical toxicity. The basal glutathione (GSH) content is a good measure of antioxidant defense capacity in different cells and consequently the cytotoxicity responses. Rau et al. (2004) found higher basal GSH levels in HII4E (rat) cells compared to PLHC-1 (fish) cells, leading to lower cytotoxicity in HII4E cells upon exposure to prooxidants. In addition, oxidative stress is a contributing factor to CPF-induced toxicity in diverse cell lines (Ki et al., 2013; Ventura et al., 2015). In HepG2 cells, the basal GSH level is similar to that of human primary hepatocytes (Dvořák et al., 2003), while the intracellular GSH levels in HK-2 cells is lower than other renal proximal tubular cell lines (Racusen et al., 1997; Gunness et al., 2010). Therefore, other defense mechanisms and enzymes, such as GSH, could play a potential role in protecting HepG2 cells from CPF- and CPFO-induced oxidative stress and cytotoxicity. Finally, protein-level quantification of the transporters, enzymes and nuclear receptors would provide a more functional characterisation and comparison of HepG2 and HK-2 cells, although there is reportedly good correlation between mRNA and protein expression for transporters in HepG2 cells and liver tissue (Shirasaka et al., 2009; Hayeshi et al., 2008).

Based on the cytotoxicity results obtained for each cell line, we then exposed cells to concentrations ($\leq IC_{20}$ 24 h) of CPF and CPFO to examine whether these chemicals modulate expression of transporters and nuclear receptors. Exposure to CPFO induced *ABCB1* and *ABCG2* mRNA levels in HepG2 cells. Other studies report that exposure to CPFO induced *ABCB1* expression in Caco2 cells (Agarwala et al., 2004) and human placental cells (Ridano et al., 2017) as well as *ABCG2* expression in MCF-7 cells (Herriage et al. 2022). Lanning et al. (1996) observed an increased *ABCB1* expression in various organs (kidney, adrenal, liver, jejunum, and stomach) in rats with exposure to CPF and hypothesized that this induction was due to CPFO, as CPF is rapidly biotransformed to CPFO. Similar to *ABCB1* and *ABCG2*, we found that CPFO exposure resulted in an increase in *PXR* mRNA levels in HepG2 cell, but there was no such response in HK-2 cells. These findings suggest a relationship between basal *PXR* expression and its inducibility, as well as induction of *ABCB1* and *ABCG2*, following CPFO exposure. The correlations between the mRNA expression levels of efflux transporters and *PXR* have been reported in other *in vitro* models. For example, both *PXR* and *ABCG2* expression were induced

by proinflammatory cytokines in MCF7 breast carcinoma cells (Malekshah et al. 2011). Ott et al. (2009) reported that exposure to the PXR ligands, rifampicin and hyperforin, induced *PXR* and *ABCB1* mRNA expression as well as increased P-gp activity in porcine brain capillary endothelial cells. We hypothesise that the increase in *PXR* mRNA expression observed in HepG2 cells following CPFO exposure suggests a potential involvement of *PXR* in the expression and activity of P-gp and BCRP transporters by CPFO. Conversely, the absence of *ABCB1* and *ABCG2* induction by CPF and CPFO in HK-2 cells, coupled with the lack of *PXR* expression in HK-2 cells, implies that PXR may play a role in upregulating *ABCB1* and *ABCG2* specifically in response to CPFO. The higher cytotoxicity of CPF and CPFO in HK-2 cells compared to HepG2 cells could also be attributed to the absence of *PXR* expression in HK-2 cells. The AhR is another nuclear receptor that plays a major role in xenobiotic metabolism by regulating the expression of diverse enzymes involved in detoxification and metabolic processes. We also observed a decrease in expression of *AhR* with CPF exposure, but this effect was not observed in HK-2 cells. CPF is an AhR agonist (Long et al., 2003; Takeuchi et al., 2008). There are limited studies examining effect of CPF on expression of *AhR*. In a study by Moyano et al. (2020), exposure to CPF or CPFO (24 h) had no effect on *AhR* expression in MDA-MB-231 and MCF7 cells; however, longer exposure (14 days) resulted in a dose-dependent increase in *AhR* expression. There are likely cell-specific and time-dependent factors involved in the modulation of *AhR* expression by CPF and other pesticides.

The functional role of P-gp and BCRP in the cytotoxicity of CPF and CPFO in these cell lines was also explored using binary combinations of pesticides with the pharmacologic efflux pump inhibitors, VRP and Ko143. Several studies have demonstrated that these compounds can enhance the cytotoxicity of xenobiotics that are substrates for P-gp and BCRP, respectively, through inhibition of these efflux transporters (Lee et al., 2022; Xia et al., 2007; Liu et al. 2014). We observed that co-exposure of CPF with either of the transporter inhibitors significantly increased cytotoxicity in HepG2 cells compared to CPF alone. One possible explanation for this increased cytotoxicity is that inhibition of efflux transport of CPF (as a substrate for P-gp and BCRP) results in an accumulation of CPF inside the cell leading to increased toxicity. Similarly, Cavret et al. (2005) demonstrated that inhibition of P-gp transporters by VRP enhanced accumulation of diazinon within the Caco-2 cells, increasing diazinon-induced cytotoxicity. We also saw that the cytotoxicity of CPF in HepG2 cells was more enhanced with Ko143 compared

to VRP, suggesting that BCRP plays a major role in the efflux of CPF. It is also possible that Ko143 could inhibit P-gp-mediated efflux as seen in human embryonic kidney cell lines at concentrations >1 μM (Weidner et al. 2015). For CPFO, cytotoxicity in HepG2 cells appeared to be primarily P-gp-dependent as co-exposure with VRP resulted in increased cytotoxicity compared to CPFO alone and there was no change in cytotoxicity with co-exposure with Ko143. This is supported by Lanning et al., (1996) who proposed that P-gp plays a role in the efflux of CPFO and provided evidence of an interaction between CPFO and P-gp in Sf9 cells expressing the human MRD1 gene. The co-exposure of HK-2 cells to CPF or CPFO with transporter inhibitors did not affect cytotoxicity response, which is supported by the lack or limited expression and activity of P-gp and BCRP this specific cell line. Although HepG2 cells have limited phase I CYP450 enzyme capacity (Steinbrecht et al., 2019), they appear a more suitable cellular system (at least compared to HK-2 cells) for examining the function role of efflux transporters in CPF- and CPFO-induced cellular toxicity. Overall, our results from the co-exposures indicate that both efflux transporters may be involved in movement and their inhibition results in accumulation of CPF and/or CPFO, influencing cell-specific cytotoxicity responses to these compounds. However, due to the potential non-selective inhibition of other efflux transporters by VRP and Ko143, further studies are required to elucidate the specific roles of these transporters in the transport of CPF and CPFO.

To further examine the relationship between enhanced cytotoxicity and cellular accumulation of CPF and CPFO, we measured the cellular and media concentrations of CPF and CPFO when HepG2 cells were exposed to CPF alone as well as in the presence of efflux inhibitors. We focused on CPF exposure to specifically address cellular toxicity to the parent compound, whether it be through accumulation of CPF or through potential metabolism and increase in intracellular CPFO. Co-exposure to CPF with VRP or Ko143 increased intracellular CPF concentration after 6 h of exposure as measured using LC-MS/MS, supporting the hypothesis that increased cytotoxicity is due to higher intracellular concentration of CPF. These inhibitors have demonstrated effectiveness in reducing efflux capacity in other cell lines, resulting in measurable increase in cellular concentrations of pesticides and leading to enhanced cytotoxicity (Pivčević and Žaja, 2006; Chedik et al., 2018). The effect of efflux inhibition on intracellular CPF also appears time dependent with no change at the early time-point of 2 h; however, CPF concentration does appear to be trending upwards at this time-point. Intracellular

concentration of CPFO did not increase in the presence of efflux inhibitors and decreased, which was somewhat surprising as it would be expected that, with increased CPF inside the cell, there would be greater potential for cellular metabolism to the more cytotoxic CPFO. In fact, we measured a decrease in CPFO in cells and in culture media, specifically with co-exposure to Ko143. One possibility is that HepG2 cells may further metabolize CPFO into other metabolites along with potential for formation of CPFO-protein adducts, which together could explain the lower levels of CPFO and greater cytotoxicity through induction of oxidative stress (Yang and Bartlett, 2016).

In the culture media, CPF concentrations were very low (1-2 μM/well) when measured after 2 and 6 h of CPF exposure, whether alone or co-exposure with inhibitors. This can be attributed to the highly lipophilic nature of CPF (log Kow = 4.7), which allows for rapid, passive diffusion of CPF into cells (Yang et al., 2017). There is also potential for CPF loss via water-air transfer and adherence to plastic (Georgieva et al., 2021; Kramer et al., 2015), resulting in decreased concentration in culture medium; however, our measured intracellular CPF concentrations are approximately 84% of dosed concentration at 2 h and 70% at 6 h, indicating that the majority of CPF was taken up by the cells. Yang et al (2017) exposed human neurons *in vitro* to 10 μM CPF for 48 h and measured very low levels of CPF in cell culture media with most CPF distributing into cells. For CPFO, they reported <1 nM in cellular media and <1 ng/million cells, which are in the range of concentrations measured in this study. Yang et al. (2017) further suggested that a decrease in CPFO media in the presence of neurons (compared to media alone) may be attributed to the formation of protein adducts, as mentioned previously. In our study, inhibition of efflux transporters could have prevented CPF efflux out of cell and subsequently more CPFO formation through metabolic conversion, although this was not reflected in an increase in CPFO in the cells. We cannot discount cellular metabolism of the efflux inhibitors themselves having in indirect effects on the concentration and efflux capacity of CPF and CPFO in HepG2 cells, especially given that CES1 (an enzyme responsible for the detoxification of CPFO) can hydrolyse the potent and selective Ko143 inhibitor in mammalian cells (Liu et al. 2017).

Although CPF and its metabolite, CPFO, were the specific compounds used for this study, our results support that cell-specific expression of transporters, metabolic enzymes, and nuclear receptor can influence toxicity thresholds (Hafey et al., 2022; Chedik et al., 2018). We

also demonstrate that inhibiting efflux transporters enhance pesticide-induced toxicity through increased cellular accumulation or possibly other mechanisms of action, such as altered biotransformation mechanisms or increased reactive oxygen species production. Characterizing transporter expression and activity is important when using *in vitro* models to assess cytotoxicity or sub-cytotoxic responses to pesticides, especially if extrapolating *in vitro* data for the purpose of chemical risk assessment. Pesticide-transporter interactions are also a relevant scenario for pesticide mixtures, where modulation of efflux transporter activity or expression can greatly influence interactive cell-specific toxicity responses.

2.6 Supplementary Information – Methods

Preparation of stock solutions, calibration standards, and quality control samples for LC-MS/MS assay

A stock solution of a reference standard for CPF (50 μM), and CPFO (5 μM) were prepared in MeOH. A mixed standard working solution, containing both CPF and CPFO at 1280 nM and 128 nM, respectively, was prepared by pipetting the required volume from the reference stock solutions and diluting with MeOH. The stock solutions of internal standards (IS), CPF-D10, and CPFO-D10 were prepared in methanol at 1mM and 10 μM for CPF and CPFO respectively. Similarly, a mixed internal standard working solution of CPF-D10 and CPFO-D10 was prepared by pipetting the required volume of each analyte and diluting it with MeOH for a final concentration of 40 μM for CPF-D10 and 40 nM for CPFO-D10. The calibration standards were prepared by spiking the blank matrix (lysed cells) with known concentrations of the analyte. Quality control (QC) samples were prepared at four levels, the lower limit of quantification control (LLOQC), low quality control (LQC), medium quality control (MQC), and high-quality control (HQC). The concentrations for all standard and quality controls working solutions are listed in Supplementary Table 2.1.S. Internal standards were spiked into each calibrant at a final concentration of 20 μM for CPF-D10 and 2 μM for CPFO-D10.

Table 2.1.S. Analyte concentrations (nM) in calibration standard solution and quality control solutions

Calibration level	CPF	CPFO
1	0.5	0.05
2	2	0.2
3	8	0.8
4	16	1.6
5	32	3.2
6	64	6.4
7	128	12.8
8	256	-
9	384	-
10	512	-
11	704	-
12	1024	-
Lower limit of quantification (LLOQ)	0.5	0.05
Low-quality control (LQC)	1.5	0.15
Medium-quality control (MQC)	448	4.48
High-quality control (HQC)	960	9.60

Validation of the analytical method for quantification of CPF and CPFO

The developed LC-MS/MS method was validated for selectivity, linearity, accuracy, and precision, matrix effect, recovery, dilution integrity, and stability following the European Medicines Agency (EMA, 2011). Calibration curve was generated by plotting peak area ratios of analyte to internal standard vs analyte concentration. Linearity was established in concentration ranges 0.5-1024 nM and 0.05–12.86 nM for CPF and CPFO, respectively, and a weighing of 1/x was applied. The calibration curves for both CPF and CPFO yielded a mean correlation coefficient ≥ 0.998. Assay selectivity using the specific mass transitions was performed by analysis of blank cell lysed samples/matrix obtained from liver and kidney cells (n = 6). No interference from extracted matrix could be detected in CPF, CPFO, and internal standards MS channels. Inter-day and intraday precision and accuracy were found to be within ± 15% for both

CPF and CPFO (Supplementary Table 2.2.S). The LLOQ was established at 0.05 nM for CPFO and 0.5 nM for CPF with a precision of ≤ 20% for both.

Table 2.2.S: Inter-day and intra-day precision and accuracy (%) for CPF and CPFO.

		Nominal concentration (nM) for CPF			
CPF		0.5	1.5	448	960
Intra-assay	Precision (% RSD)	7.2	2.3	3.2	1.0
	Accuracy (%)	108.0	99.3	92.8	99.9
Inter-assay	Precision (% RSD)	12.5	1.8	2.8	1.4
	Accuracy (%)	106.6	100.2	93.3	99.5
		Nominal concentration (nM) for CPFO			
CPFO		0.05	0.15	4.48	9.60
Intra-assay	Precision (% RSD)	4.6	2.4	1.3	1.2
	Accuracy (%)	100.0	100.0	100.6	103.3
Inter-assay	Precision (% RSD)	14.1	1.7	1.4	1.6
	Accuracy (%)	106.6	100.0	102.0	103.4

Recovery, matrix effects, and carryover: Extraction recoveries of all analytes from cell lysates were calculated by comparing pre- spiked samples and post-spiked samples at LQC, MQC, and HQC (n: 3). The mean extraction recoveries for CPF were 88.5% ± 7.9 (LQC), 94.7% ± 3.2 (MQC), and 102.5% ± 8.1 (HQC). For CPFO, the mean extraction recoveries were 108.5% ± 9.1 (LQC), 104.1% ± 3.6 (MQC), and 116.4% ± 9.2 (HQC). These results provide a comprehensive overview of the extraction efficiency, emphasizing both mean values and the associated variability represented by the RSD at different concentration levels. No significant matrix effect for CPF and CPFO was observed in the post-spiked extracted cells compared to standards prepared in neat solvent (methanol). The matrix effect (%) for CPF in lysed cells determined at LQC, MQC, and HQC levels were 106.2 ±5.2, 106.3 ±3.7, and 100 ±9.8, respectively. The results for CPFO show values of 103.4 ±5, 104 ±2.4, and 109 ±5.7 for LQC, MQC, and HQC respectively. An injection of a solvent blank following injection of the high concentration standard did not show carry-over at the retention times corresponding to analytes and IS.

Stability and dilution integrity: Various stability studies were evaluated at both LQC and HQC and included benchtop (4 h), autosampler (12 h and 24 h), -20°C, and freeze and thaw cycles. For freeze and thaw cycles, analytes were frozen at -80°C, allowed to thaw at room temperature and refrozen again for at least 12 h for each cycle. Dilution integrity was evaluated at 50-fold and 20-fold, and this criterion was chosen to match the dilution factors applied to the samples. Quantitative results for stability (only freeze thaw cycles shown) and dilution tests are shown in Supplementary Table 2.3.S and 2.4.S, respectively. The precision (RSD) and accuracy values for each analyte met the acceptable criteria of within ± 15%.

Table 2.3.S. Results of post-preparative stability tests of CPF and CPFO.

Analyte		Concentration Value (%)					
		One Cycle		Two Cycle		Three Cycle	
		Accuracy (%)	RSD (%)	Accuracy (%)	% RSD	Accuracy (%)	% RSD
CPF	LQC	88.9	7.3	87.3	9.2	91.4	5.3
	HQC	85.9	8.8	88.5	7.4	99.4	2.3
CPFO	LQC	110.2	5.6	111.3	7.3	114.1	9.5
	HQC	104.0	2.3	107.4	4.1	106.4	3.7

Table 2.4.S. Precision (% RSD) and accuracy (% DFN) of spiked samples (n = 3) at higher concentrations of the upper limit of quantification (ULOQ) and diluted 20- and 50-fold.

Analyte	Stock Concentration (nM)	Dilution factor	Nominal level (nM)	Measured level (nM)	RSD (%)	DFN (%)
CPF	1600	50x	32	22.7 ± 0.2	1.9	71
	2000	20x	100	87.9 ± 3.5	4.9	87
CPFO	160	50x	3.2	2.9 ± 0.03	1.3	90
	200	20x	10	10.3 ± 0.3	3.4	104

Table 2.5.S. Multiple reaction monitoring (MRM) transitions, the corresponding retention time and collision energies for both analyte and internal standards. Bolded values represent quantifier ion.

Compounds	Precursor (*m*/*z*)	Quantifier/Qualifier (*m*/*z*)	Retention Time (min)	Collision Energy (V)	RE Lens (V)
CPF	351.91	**199.96**/96.97	2.88	19.91/31.96	56
CPFO	333.95	**277.90**/197.96	2.11	17.51/30.02	62
CPF-D10	360.01	**98.89**/199.04	2.84	31.33/20.75	57
CPFO-D10	346.04	**200.97**/282.03	2.09	32.63/19.66	74

CHAPTER 3 – Efflux Transporter Inhibition Influences the Combined Toxicity of Pesticides in a Human Liver Cell Line

Preface

In the previous study described in Chapter 2, we showed that cell-specific expression of efflux transporters can influence a pesticide intracellular concentration and toxicity threshold *in vitro*. The findings of Chapter 2 raise the question of how efflux transporters may influence mixture toxicity. In this chapter, we examined the binary combination of CPF with two organochlorine pesticides, endosulfan-α (END) and heptachlor (HEP), considering previous reports suggesting the potential inhibition of P-gp activity by HEP and END *in vitro* and *in vivo*. This chapter delves further into the complexities of chemical interactions and their potential implications for *in vitro* toxicological outcomes.

The material described within this chapter will be submitted for publication to *Toxicology Letters.* This chapter has been reformatted from the original submitted version for inclusion in the thesis.

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Author Contributions:

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Natacha Hogan: conceptualization, supervision, funding acquisition, writing - review and editing, resources.

3.1 Abstract

Individual pesticides are known to interact with cellular efflux drug transporters. However, humans are often exposed to mixtures of pesticides and there is concern that inhibitory effects by some pesticides can reduce the elimination of others in a mixture, resulting in chemosensitization and increased cellular toxicity. Here, we investigated the combined toxicity of the organophosphorous pesticide, chlorpyrifos (CPF) with two other pesticides that are known P-glycoprotein (P-gp) inhibitors, endosulfan-α (END) and heptachlor (HEP) in HepG2 cells. The cytotoxicity of single pesticides (24 h) followed a rank order of potency of END > HEP > CPF. Binary mixtures of these pesticides were tested at concentrations of $<IC_{20}$ to determine if there were interactive effects and whether toxicity was through P-gp mediated mechanisms. The mixture of CPF + END exhibited additive effects. By contrast, the binary mixture of CPF + HEP resulted in a more than additive or synergistic cytotoxicity response. Intracellular rhodamine 123 (Rho123) accumulation assay showed that HEP could inhibit P-gp activity . In contrast, CPF and END did not exhibit any inhibitory effects on P-gp activity. There was no effect of these pesticides or their binary mixtures on the mRNA expression of *P-gp* and nuclear pregnane X receptor (*PXR*), suggesting that the inhibitory effects of these pesticides on P-gp may not be attributed to changes in its transcriptional regulation. Induced expression of *Nrf2*, a transcriptional regulator of antioxidant proteins and P-gp, by the binary mixtures suggest a cytoprotective response to oxidative stress. Our results show that pesticide mixtures have a greater ability to inhibit the efflux capacity of HepG2 cells than individual chemicals and that pesticide interactions at efflux transporters may influence toxicokinetic and toxicity thresholds.

3.2 Introduction

Humans are frequently exposed to mixtures of multiple different pesticides through nutritional sources and occupational settings (Kim et al., 2017). Pesticides in a mixture can influence the toxicity of one another at the cellular level by interacting at the biological target site (i.e., toxicodynamics) and/or modulating the cellular concentration of parent or their metabolites (i.e., toxicokinetics; Chedik et al., 2022; Hernández et al., 2017). Such interactions can increase toxicity (synergism or potentiation) or decrease toxicity (antagonism) in target organs and cells, making mixture toxicity difficult to predict based on toxicological data of

individual pesticides. Toxicokinetic interactions of mixtures can change the concentration of a pesticide at its target site via biotransformation or transporter-mediated uptake or efflux, causing a shift in the threshold for effects (Hernández et al., 2013). Interactions of pesticides on biotransformation processes are often a mechanism for enhanced or attenuated toxicity of mixtures; however, interactions with drug transporters are equally important to consider owing to the major contribution of transporters to cellular accumulation and elimination of pesticides (Guéniche et al., 2020).

Xenobiotic (drug) transporters are a class of proteins that regulate the movement of different xenobiotics across cell membranes in multiple organ systems (Nigam, 2015). P-glycoprotein (P-gp), encoded by ATP-binding cassette sub-family B member 1 (ABCB1), is an efflux transporter found on apical membranes of various cells with excretory functions, such as enterocytes, the renal proximal tubular cells, and the canalicular pole of hepatocytes (Thiebaut et al. 1987). In these organs, P-gp acts as an ATP-dependent efflux pump that facilitates the elimination of xenobiotics, reducing their cellular concentration and toxicological effects; thus, the fundamental role of P-gp is to regulate the systemic exposure of its substrates (Elmeliegy et al., 2020). Many pesticides have been identified to interact with P-gp as either substrates or inhibitors (Chedik et al., 2022). For example, organochlorine pesticides (e.g., dichlorodiphenyltrichloroethane known as DDT and endosulfan), organophosphate insecticides (e.g., chlorpyrifos and parathion) and pyrethroid pesticides (e.g., permethrin and fluvalinate) are all reported to inhibit the efflux activity of P-gp in mammalian cells *in vitro* (Bain and Leblanc, 1996; Bircsak et al., 2013; Chedik et al., 2018). While characterization of pesticide-transporter interactions is often limited to toxicokinetic studies of single compounds, it is becoming increasingly well-established that inhibition of transporter activity by environmental chemicals, including pesticides, may impact the toxicokinetics of other chemical, resulting in chemosensitization and increased cellular toxicity (Chedik et al., 2018; Pivčević and Žaja, 2006; Kurth et al., 2015). Consequently, co-exposure to pesticides that inhibit P-gp may increase the systemic bioavailability of other pesticides that are P-gp substrates (Guéniche et al., 2020).

The regulation of the ABCB1 gene is highly complex and controlled by various transcription factors such as pregnane X receptor (PXR) and nuclear factor erythroid-derived 2-related factor 2 (Nrf2; Abalenikhina et al., 2022; Jigorel et al., 2006). The interaction of various pesticide groups with different nuclear receptors was reviewed by Kojima et al. (2010).

Activation of PXR by pesticides can alter transcriptional expression of the *ABCB1* and consequently transport of P-gp substrates (Guéniche et al., 2020). For example, Bucher et al., (2014) showed that several organochlorine pesticides could induce mRNA expression of the *ABCB1* in cultured human hepatoma HepaRG cells via activation of PXR. CPF has also been reported to induce mRNA expression and activate *PXR*, consequently upregulating *ABCB1* expression in the liver and intestine of the rainbow trout (De Anna et al., 2021). Exposure to xenobiotics can lead to the development of oxidative stress which can trigger the induction of *Nrf2* expression and activity (Shchulkin et al., 2021; Ghanem et al., 2015). This, in turn, may result in protecting cells from oxidative damage by up-regulating the expression of Nrf2-related antioxidant genes, such as phase II detoxifying/ antioxidant enzymes (Shaw and Chattopadhyay, 2020; Aleksunes and Klaassen, 2012) and *P-gp* (Wu et al., 2019). As another example, overexpression of *Nrf2* in alveolar A549 cells resulted in reduced intracellular paraquat concentration and toxicity, potentially via the upregulation of P-gp expression and activity (Wu et al., 2019). Similarly, Lu et al., (2014) found that paraquat exposure induced *Nrf2* expression in both A549 cells and mice, reducing paraquat toxicity. Given the role of P-gp in governing the intracellular accumulation and concentration of pesticides, it is imperative to understand how pesticides, both individually and in mixtures, influence expression of *ABCB1* and its transcriptional regulators.

The aim of this study was to determine and compare cytotoxic effects of individual pesticides and their binary mixtures, and to investigate potential associations between cytotoxicity and the activity and expression of the P-gp/*ABCB1* transporter *in vitro*, using the human hepatocyte cell line, HepG2. As HepG2 cells have little to no expression of major P450s (including CYP 2B6 and 3A4), this makes the cell line an appropriate model to identify the role of efflux transporters in potential pesticide interactions (Westerink and Schoonen, 2007). Our previous research demonstrated that inhibition of efflux transporters can enhance cytotoxicity of the organophosphorus pesticide, chlorpyrifos (CPF) in HepG2 cells, likely though reduced efflux and increased intracellular concentrations (Goldar et al. submitted). Thus, in the present study, we furthered the scope of our study of pesticide-transporter interactions by examining the combined toxicity of CPF with two other pesticides that are known P-gp inhibitors, endosulfan-α (END) and heptachlor (HEP) (Sreeramulu et al., 2007; Bircsak et al., 2013; Bain and Leblanc,1996). Understanding the *in vitro* interactions of pesticides with P-gp will provide

guidance on how efflux transporters may contribute to the overall disposition and toxicity of pesticides and their mixtures. Identifying the potential interactive toxicity of these particular pesticides is also relevant given that they have been identified together in many environment matrices (Wołejko et al., 2022; Keswani et al., 2022; Navarrete et al., 2018; Adeyi et al., 2021).

3.3 Materials and Methods

3.3.1 Chemicals

Chlorpyrifos (CPF) was of analytical standard grade (purity >99.67%) and supplied by Toronto Research Chemicals (TRC, Toronto, Ontario, Ca). Endosulfan-α (END; purity ≥ 98.0 %), Heptachlor (HEP; purity ≥ 98.0 %), verapamil (VRP; purity ≥99%), and rhodamine 123 (Rho123) were purchased from Sigma Aldrich (Oakville, Ontario, Ca). Stock solutions of CPF, HEP, END, VRP and Rho123 were prepared in 100% Methanol (MeOH) and stored at −20°C, protected from ambient light. Working solutions were prepared daily in FBS-free cell culture media. The concentration of MeOH did not exceed 0.25% v/v and was included in all assays as the solvent control.

3.3.2 Culture of HepG2 cells

HepG2 cell line (human hepatocellular carcinoma; HB-8065) was obtained from the American Type Culture Collection (ATCC) and grown as monolayer cultures in Dulbecco's Modified Eagle's Medium (DMEM) with high glucose and pyruvate, and supplemented with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin (Thermo Fisher Scientific, Inc., Ca) in an atmosphere with 95% relative humidity and 5% CO2 at 37°C, as recommended by the supplier. Upon reaching confluency in 75 cm2 flasks, cells were detached using 0.25% Trypsin-EDTA (Thermo Fisher Scientific, Inc., Ca), counted, and then sub-cultured (1-2× per week) at a 1:4 split ratio in complete medium. Before proceeding with assays, cell viability was assessed microscopically using trypan blue exclusion to ensure >95% viable cells.

3.3.3 Cytotoxicity of individual pesticides and binary mixtures

The cytotoxicity of individual pesticides and their binary mixtures was assessed using the neutral red assay. HepG2 cells were initially seeded at densities of 2×10^4 cells/well in 96-well

cell culture plates cells were then allowed to adhere for 24 h at 37°C and 5% CO2 in a humidified atmosphere. Then cells were exposed to five concentrations of HEP (0-100 µM) or END (0-50 µM) for 24 hours (h). The cytotoxicity of CPF in HepG2 cells was determined in our previous study (Goldar et al., submitted). After 24 h exposure, cell viability was assessed by neutral red assay using methods previously described by Repetto et al (2008). Briefly, the media was removed after the 24 h exposure, and the cells were washed with phosphate buffer saline (PBS, Thermo Fisher Scientific, Inc., Ca). Subsequently, the cells were treated with 40 µg/ml neutral red (Sigma Aldrich, Oakville, Ontario, Ca) and incubated for 2 h. Cells were then washed again with PBS, and a de-staining solution (50% ethanol, 49% deionized water and 1% glacial acetic acid) was added to each well. The plate was agitated at room temperature for 10 min and absorbance measured at 540 nm in Epoch 2 Microplate Spectrophotometer (Bio-Tek Instruments Inc., Winooski, VT, USA) using blank wells (media only, without cells) as a reference. Background absorbance from these blank wells was subtracted from absorbance readings of both solvent control and treated wells. The percentage of live cells was calculated relative to the solvent control wells (MeOH-treated cells) using Equation 3.1:

$$\left[\frac{(\text{Absorbance value of treated samples})}{(\text{Absorbance value of MeOH control samples})}\right] \times 100 \qquad (3.1)$$

Cell viability was also assessed in HepG2 cells exposed to binary combinations of pesticides (CPF + END and CPF + HEP). Concentrations causing less than 20% cytotoxicity (< IC_{20}) were determined from the scaled dose-response curves. The 50 µM concentration of CPF used in the binary mixture was determined from dose-response data presented in Chapter 2. This concentration was then combined in binary mixtures with END at 3 and 6 µM or HEP at 6 and 12 µM for 24 h exposures in HepG2 cells. The neutral red assay was performed as previously described for individual compounds to determine cytotoxicity of binary mixtures. The type of interaction (synergistic, additive, or antagonistic effect) was evaluated by the combination index analysis using the Chou and Talalay (1984) method. The quantitative measurement of the interaction was defined using Equation 3.2.

$$CI = \frac{a}{A} + \frac{b}{B} \qquad (3.2)$$

Where *A* and *B* are concentrations of individual pesticide *A* and *B* that resulted in a specified effect (e.g., IC_{20}, IC_{50}), and *a* and *b* are the combination of concentrations that produced the same effect level as the mixture. The type of interaction is classified based on combination index as follows: CI < 0.8: synergism; 0.8 < CI < 1.2: additive effect; and CI > 1.2: antagonism (Balázs et al., 2021).

3.3.4 Lactate dehydrogenase (LDH) assay

Cell leakage induced by individual pesticides and their binary combinations was evaluated by the LDH assay (Abcam Inc., BioVision, Toronto, Ontario, Ca). HepG2 cells were seeded and grown under identical conditions as described above for neutral red uptake assay. Briefly, cells were exposed to each of the following compounds and combinations: CPF (50 µM), END (6 µM), HEP (12 µM), CPF (50 µM) + END (6 µM), and CPF (50 µM) + HEP (12 µM). HepG2 cells were also exposed to tert-butyl hydroperoxide (TBHP; 100 µM) and 0.25% v/v MeOH as positive and negative controls, respectively. After 24 h exposure, 10 µL supernatant samples were collected and processed following the manufacturer's protocol. Absorbance was detected at 450 nm using Epoch 2 Microplate Spectrophotometer. Equation 3.3 was used for data analyses:

$$LDH\ release\ (\%) = \frac{(Test\ Sample - Negative\ Control)}{(positive\ Control - Negative\ control)} \times 100 \qquad (3.3)$$

3.3.5 Rhodamine-123 accumulation assay

The inhibitory effect of individual and binary pesticide mixtures on P-gp-mediated Rho123 transport was evaluated according to previously published methods (Dogra et al., 2018). Rho123, a cationic fluorescent dye, is a well-established substrate for P-gp and is frequently used as an indicator of P-gp activity (Jouan et al., 2016). HepG2 cells were exposed to individual pesticides (at concentrations $<IC_{20}$) or their binary combinations, as previously mentioned, for 6 h. The solvent control (0.25% v/v MeOH) and a standard P-gp inhibitor VRP (40 µM) were used as negative and positive controls, respectively. After 6 h of exposure, media was replaced with Hank's Balanced Salt Solution (HBSS, Sigma Aldrich, Oakville, Ontario, Ca) solution containing 10 µM Rho123 (Sigma Aldrich, Oakville, Ontario, Ca) and incubated at 37°C for 30 min (37°C, 5% CO_2). Cells were then washed with ice-cold PBS while kept on ice and lysed with 0.1%

Triton X-100 to solubilize the probe completely. Subsequently, fluorescence intensity was measured at the 485 nm excitation and 535 nm emission wavelengths using a SPARK multimode microplate reader (Tecan, Austria GmbH). Rho123 cellular uptake was normalized to the protein levels in each sample using the Pierce™ bicinchoninic acid (BCA) Protein Assay Kit (Thermo Fisher Scientific, Inc., Ca).

3.3.6 Quantitative real-time RT-PCR (qPCR)

Briefly, HepG2 cells were seeded in 6-well plates to a final density of 6×10^5 viable cells/well in 2 mL of medium and cells were allowed to adhere for 24 h. The medium was removed, and fresh FBS-free medium was added containing either the solvent control (0.025% v/v MeOH), the concentrations $<IC_{20}$ of individual pesticides (CPF: 50 µM, END: 6 µM, and HEP: 12 µM), or their binary combinations (50 µM CPF + 6 µM END or 50 µM CPF + 12 µM HEP) for 6 h. Total RNA from samples was isolated using the RNeasy Mini Kit with an on-column DNase I treatment, according to the manufacturer's protocol (Qiagen, Ontario, Ca). RNA concentration was assessed using NanoDrop 2000 (NanoDrop Technologies, Wilmington, DE, USA), and its quality was confirmed through analysis on a 1% agarose gel. The Applied Biosystems™ High-Capacity cDNA Reverse Transcription Kit (Thermo Fisher Scientific, Inc., Ca) was utilized to synthesize cDNA from 1 µg of RNA according to the manufacturer's recommendation and cDNA was stored at −20°C.

The qPCR primers used to measure mRNA expression of *ABCB1*, *PXR*, and *Nrf2* are listed in Table 3.1 and were previously used for targeted gene expression analyses in HepG2 cells (Goldar et al., submitted). The cDNA was diluted tenfold to achieve a concentration of 5 ng/µL for qPCR. For each qPCR reaction, 2 µL of cDNA, 0.8 µL of both forward and reverse primers (0.4 µM each), and 10 µL of SsoAdvanced Universal SYBR Green Supermix (BioRad, Ontario, Ca) were combined to form a final reaction volume of 20 µL. The qPCR analysis was performed using a CFX96 Real-time C1000 Thermal Cycler (BioRad, Ontario, Ca). After an initial 3 min hot start, a total of 40 cycles were run, involving denaturation at 95°C for 20 s, followed by an annealing step at 60°C for 30 s, and an elongation step at 72°C for 30 s. Following 40 cycles, the PCR products from each reaction underwent melt curve analysis to verify the amplification of only a single product. Each cDNA was analyzed in duplicate, and assays encompassed no reverse transcriptase controls (no RT control) and samples devoid of

cDNA template. The efficiency of all primer sets was evaluated using a five-point standard curve generated through a five-fold serial dilution of cDNA. Relative gene expression was based on the $2^{-\Delta\Delta Ct}$ method (Pfaffl, 2001) using *GAPDH* and *HMBS2* as housekeeping genes. Data were expressed as fold-change relative to the MeOH control.

Table 3.1. Primer sets used for real-time PCR

Gene	Primer Sequence (5′-3′)	Product (bp)	Source
ABCB1	Fwd: CACCCGACTTACAGATGATG	81	Dauchy et al. (2009)
	Rev: GTTGCCATTGACTGAAAGAA		
PXR	Fwd: CCCAGCCTGCTCATAGGTTC	153	Liu et al. (2011)
	Rev: CTGTGATGCCGAACAACTCC		
Nrf2	Fwd: CGGTATGCAACAGGACATTG	263	Zhang et al. (2014)
	Rev: ACTGGTTGGGGTCTTCTGTG		
GAPDH	Fwd: TCGGAGTCAACGGATTTGGT	181	Pecorelli et al. (2020)
	Rev: TTCCCGTTCTCAGCCTTGAC		
HMBS-2	Fwd: TGCTATCTGGGGAGTGATTACC	146	Rowland et al. (2019)
	Rev: GGCTGTTGCTTGGACTTCTC		

PXR (pregnane X receptor); *Nrf2* (nuclear factor erythroid 2-related factor 2); *GAPDH* (glyceraldehyde 3-phosphate dehydrogenase); *HMBS2* (hydroxymethylbilane synthase).

3.3.7 Statistical analyses

Data were analyzed by GraphPad Prism (v.8, GraphPad Software, San Diego, Ca). The inhibitory concentration causing 50% (IC_{50}) cytotoxicity and inhibitory concentrations at other levels (i.e., IC_{20}, IC_{40}) for each pesticide were calculated in GraphPad Prism using a four-parameter logistic regression to interpolate IC values. Data are presented as means ± standard deviation (SD) from at least three technical replicates of three independent assays for cytotoxicity, LDH, and Rho123 accumulation assays. For qPCR, two independent assays were conducted, with two wells analyzed per assay, resulting in two technical replicates for statistical analysis. All data were analyzed using a one-way analysis of variance (ANOVA), followed by either Tukey's multiple comparisons test to assess each group in the binary cytotoxicity assay or

Dunnett's multiple comparisons test to compare with the control group in the LDH, Rho123, and qPCR assays. A value of $p \leq 0.05$ was considered statistically significant.

3.4 Results

3.4.1 Cytotoxicity of individual pesticides and binary mixtures

Both END and HEP caused a concentration-dependent decrease in viability of HepG2 cells after 24 h exposure (Fig. 3.1). After 24 h of exposure, END exhibited higher cytotoxicity compared to HEP, with IC_{50} values of 23.6 ± 2.3 µM for END and 32.1 ± 2.7 µM for HEP. The dose-response curve and IC_{50} value for CPF (180.9 ± 8.3 µM) in HepG2 cells were obtained and reported in a previous chapter (Chapter 2).

The selected concentrations of pesticides for the binary mixtures produced less than 20% cytotoxicity effects in HepG2 cells after 24 h exposure (Fig. 3.2). HepG2 cells exhibited cytotoxicity of about 11.5% for 50 µM CPF, 11% for 6 µM END, and 7% for 3 µM END when exposed individually. The co-exposure to 50 µM CPF with 3 µM of END resulted in a significantly enhanced cytotoxicity of 18% compared to 3 µM END alone. Combinations of 50 µM CPF with 6 µM END also significantly increased cytotoxicity to 25.4% compared to all individual pesticides (Fig. 3.2A). After 24 h of exposure, HepG2 cells exhibited 5% cytotoxicity for 6 µM HEP and 11% cytotoxicity for 12 µM HEP. Upon combination exposure to 50 µM CPF with either 6 µM or 12 µM of HEP, the cytotoxicity of cells significantly increased compared to individual pesticides, leading to 38.6% and 52.5% cytotoxicity, respectively (Fig. 3.2B).

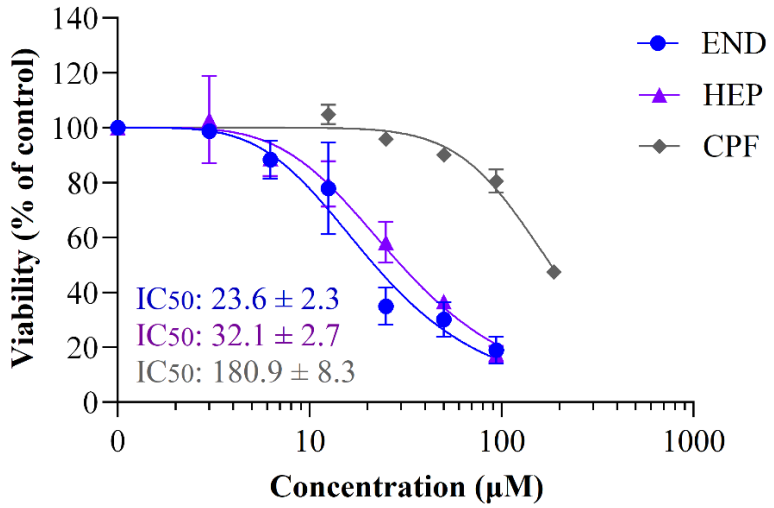

Figure 3.1. Viability of HepG2 cells after 24 h exposure to END, HEP, and CPF as determined by neutral red uptake assay. The dose-response curve and IC50 value for CPF derived from an exposure and analysis conducted as part of Chapter 2. The percentage of viable cells normalized to solvent treated control (≤ 1% MeOH for CPF and ≤0.25% for END and HEP) were plotted after background subtraction. Data are means ± SD and represent three independent assays (*n*=3).

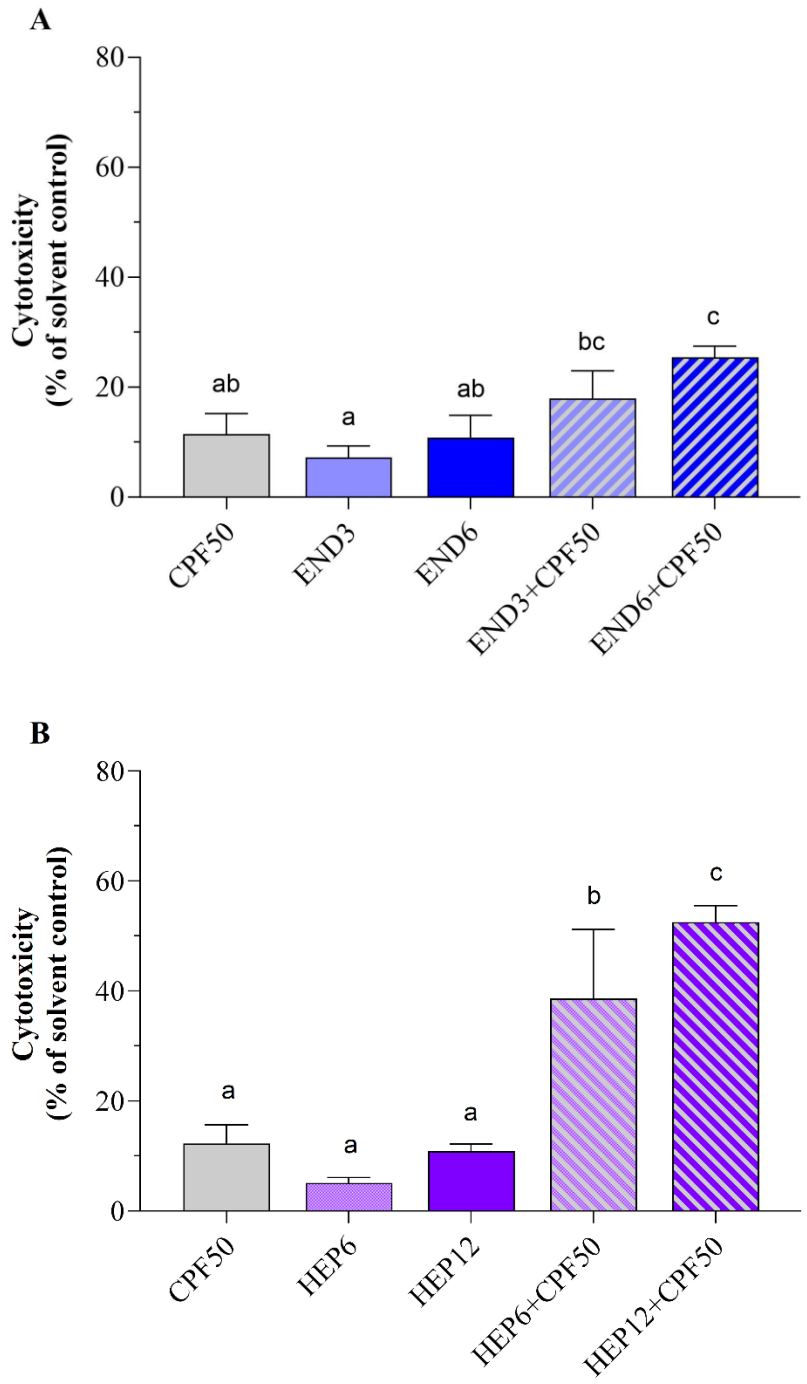

Figure 3.2. Cytotoxicity in HepG2 cells treated for 24 h with concentrations <IC_{20} of individual pesticides and their binary mixture: (A) endosulfan (END) and chlorpyrifos (CPF) and (B) heptachlor (HEP) and chlorpyrifos (CPF). Data are expressed as mean ± SD of three independent assays (n=3). Different superscript letters indicate significant difference (P<0.05) across the exposures as assessed by one-way ANOVA with Tukey's post hoc test.

Values of combination index, achieved after 24 h treatment of HepG2 cells with binary mixture of pesticides, are summarized in Table 3.2. Under our experimental conditions, qualitative evaluation of the CPF + END combination presented additive effects with CI values of 1.07 ± 0.17 and 1.14 ± 0.15 in the cells exposed to 50 μM CPF + 3 μM END and 50 μM CPF + 6 μM END for 24 h, respectively. In contrast, a synergistic effect was observed after treatment with the combination of 50 μM CPF + 6 μM HEP and 50 μM CPF + 12 μM HEP with CI values of 0.65 ± 0.24 and 0.63 ± 0.35 (Table 3.2).

Table 3.2. The combination index values (CI) for the binary mixtures of pesticides.

Pesticide Concentration (μM)	**Measured Cytotoxicity %**	**Estimated Inhibitory Concentration (IC, μM) of Individual Pesticides**		**Combination Index (CI)**
CPF 50 + END 3	18.0 ± 4.9	CPF IC_{18}: 72.9 ± 13.2	END IC_{18}: 7.6 ± 0.7	1.07 ± 0.17 (Additive)
CPF 50 + END 6	25.4 ± 2.0	CPF IC_{25}: 96.7 ± 17.9	END IC_{25}: 9.5 ± 0.7	1.14 ± 0.15 (Additive)
CPF 50 + HEP 6	38.6 ± 12.5	CPF IC_{39}: 136.8 ± 35.1	HEP IC_{39}: 20.7 ± 8.4	0.65 ± 0.24 (Synergism)
CPF 50 + HEP 12	52.5 ± 2.9	CPF IC_{52}: 188.8 ± 64.0	HEP IC_{52}: 33.6 ± 17.0	0.63 ± 0.35 (Synergism)

Data are expressed as mean ± SD of three independent assays. CPF (chlorpyrifos); END (endosulfan-α); HEP (heptachlor). CI < 0.8: synergism; 0.8 < CI < 1.2: additive effect; and CI > 1.2: antagonism.

3.4.2 Effect of pesticides and their combinations on LDH release

The LDH assay was used to assess cell membrane damage and subsequent enzyme leakage induced by these pesticides and their combinations. After 24 h of exposure, the LDH contents did not increase in groups treated with individual pesticides compared to the control (Fig. 3.3). The binary mixture of CPF + END and CPF + HEP also did not show any significant increase in LDH release compared with the solvent control. For the positive control, the LDH content in the TBHP exposed cells increased significantly (P<0.0001) compared with the control (Fig. 3.3).

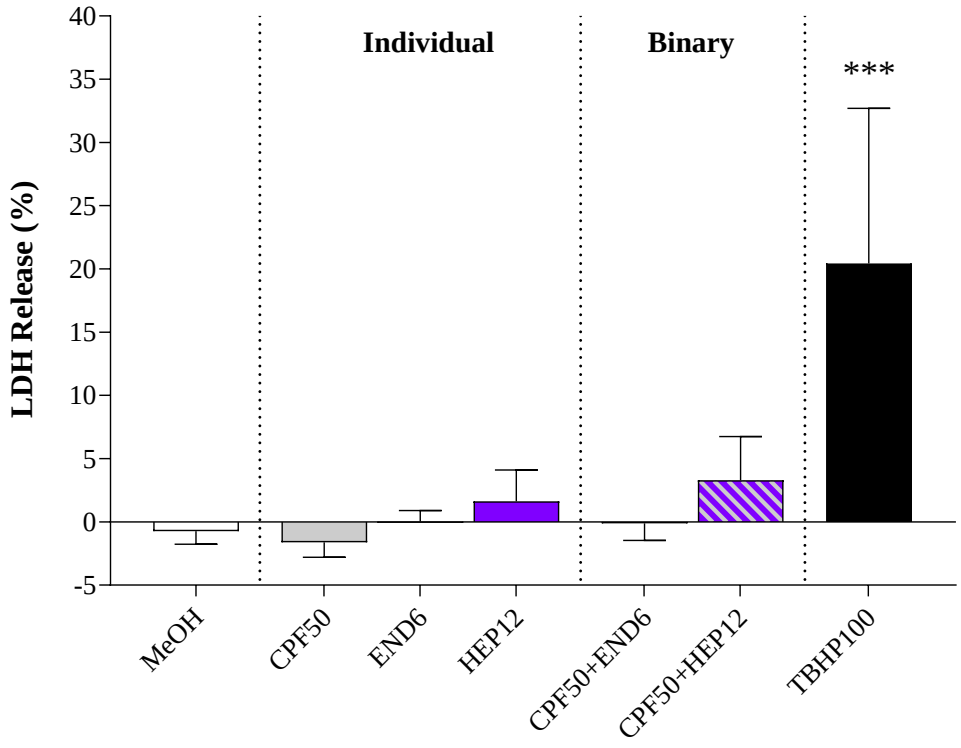

Figure 3.3. Effects of individual pesticide concentrations and their binary mixtures on LDH release in HepG2 cells after 24 h exposure. MeOH (0.25% v/v) and TBHP100 (100 µM) were negative and positive controls, respectively. All values are expressed as mean ± SD and represent three independent assays (n=3) in which each treatment had a minimum of three technical replicates. Asterisks represent significant difference from MeOH solvent control (one-way ANOVA with Dunnett's post-hoc test). ***$p < 0.001$.

3.4.3 Effect of pesticides and their combinations on the accumulation of Rho123

We examined the effect of individual pesticides and their binary combinations on P-gp transport activity by measuring the intracellular accumulation of Rho123. At concentrations causing less than 20% cytotoxicity, CPF and END individually did not induce any significant alteration in the amount of Rho123 within HepG2 cells (Fig. 3.4). Treatment with 12 µM HEP significantly increased the fluorescence intensity in HepG2 cells, inducing a 30% increase in Rho123 levels compared to the control (Fig. 3.4). The combined treatment of cells with CPF + END resulted in a 37% increase, and CPF + HEP caused a 52% increase in Rho123 intracellular amount in HepG2 cells compared to the solvent control (Fig. 3.4). Upon exposure to 40 µM of VRP, a well-characterized P-gp inhibitor, HepG2 cells demonstrated an approximately 56% increase in Rho123 fluorescence compared to the control (Fig. 3.4).

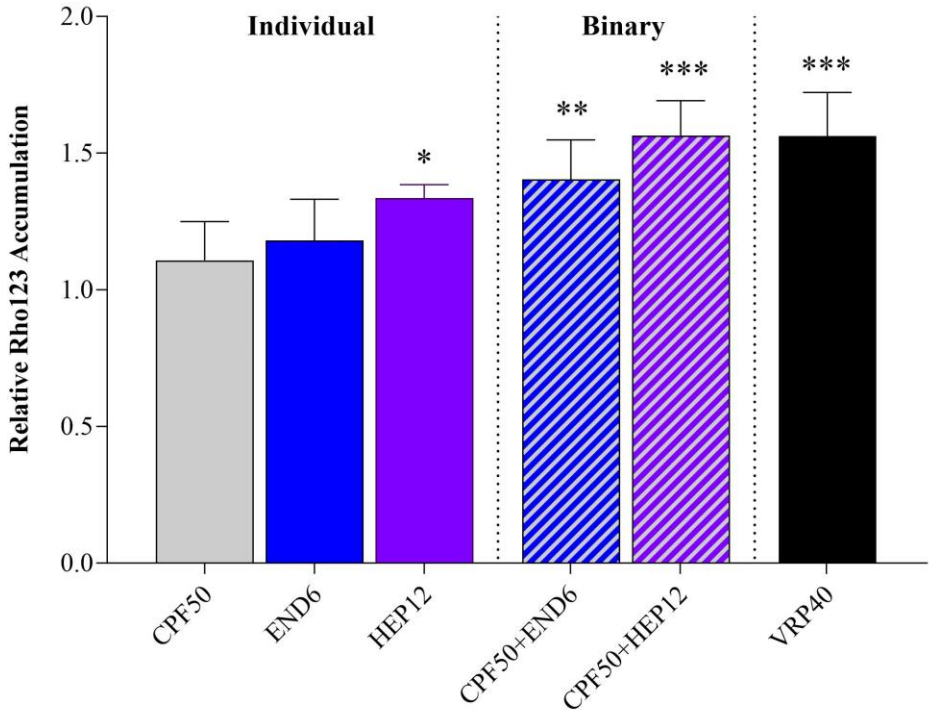

Figure 3.4. Relative Rho123 intracellular accumulation in HepG2 cells after 6 h exposure to individual pesticides or their binary combinations. Data are presented as mean ± SD of three independent assays (*n*=3), and expressed the fluorescent dye accumulated in exposed cells cells relative to MeOH (0.25%). Asterisks represent significant differences between exposed and

MeOH control (one-way ANOVA with Dunnett's multiple comparisons test). * $p<0.05$, ** $p < 0.01$, *** $p < 0.001$.

3.4.4 Effects of individual pesticides and binary mixtures on *P-gp*, *PXR*, and *Nrf2* expression

Since the alteration of *P-gp* transporter expression might also contribute to the reversal of its activity, the effect of individual pesticides and their binary mixtures on the mRNA levels of *P-gp* and its regulators, *PXR* and *Nrf2*, were evaluated using qPCR. After treatment with individual pesticides (50 μM CPF, 6 μM END, and 12 μM HEP) for 6 h, there were no noticeable differences in *P-gp*, *PXR*, and *Nrf2* mRNA expression (Fig. 3.5A, B, and C). The mRNA expression of *P-gp* and *PXR* were also not significantly affected by binary combination of pesticides (Fig. 3.5A and B). However, the cells exposed to the binary mixture of CPF + END and CPF + HEP showed an increase in *Nrf2* expression compared to the control (Fig. 3.5C).

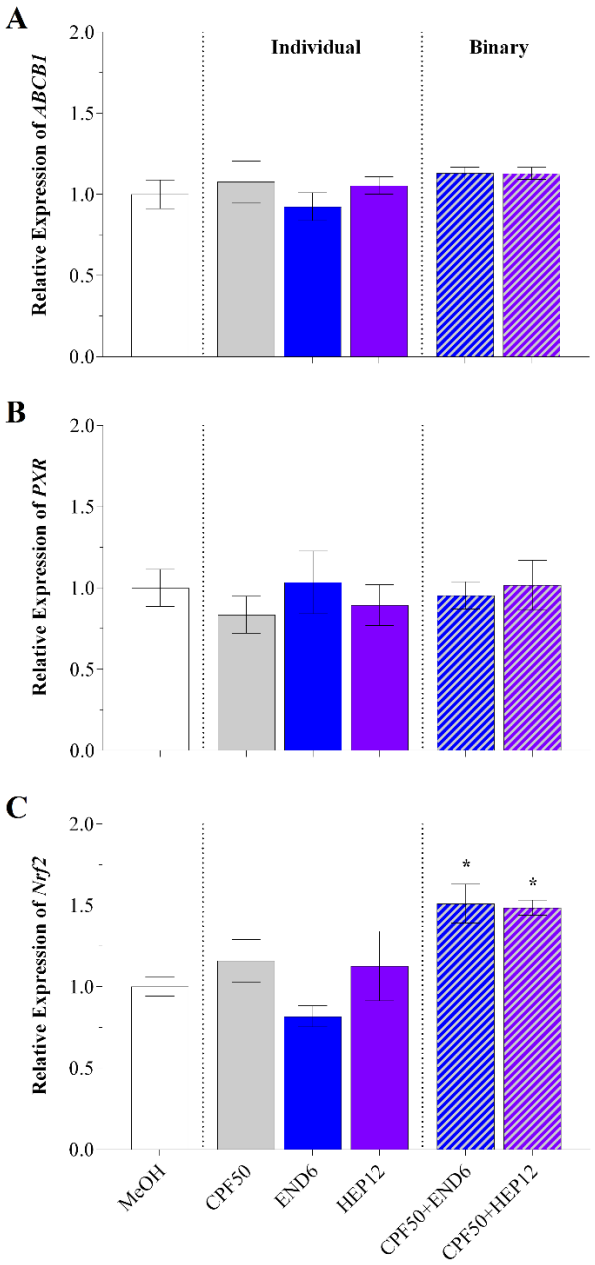

Figure 3.5. Effects of 6 h exposure to pesticides at concentrations <IC_{20} and their binary mixtures on mRNA expression of *ABCB1* (A), *PXR* (B), and *Nrf2* (C) in HepG2. The data are presented as the mean ± standard deviation (SD) of two independent assays, each with two technical replicates (n=4), and are expressed relative to MeOH (0.025%) as the vehicle control. Asterisks represent statistically significant differences between exposed and MeOH control (one way ANOVA, Dunnett's multiple comparisons test). *$p < 0.05$.

3.5 Discussion

Pesticide mixtures can change the concentration of a pesticide at its target site *via* various mechanisms, which can alter thresholds for effects/toxicity. The alteration of biotransformation processes is often a mechanism for enhanced or attenuated toxicity of mixtures. However, pesticide interactions mediated by transporters are important to consider owing to the major contribution of transporters to cellular accumulation and elimination of pesticides (Hernández et al., 2017). In our previous study, a pharmacologic P-gp inhibitor, VRP, produced chemosensitizing effects toward CPF in HepG2 cells by inhibiting the efflux of CPF by P-gp (Goldar et al., submitted). In the present study, we investigated the potential interactive effects of CPF with two other pesticides and putative inhibitors of P-gp activity, endosulfan (END) and heptachlor (HEP), in cytotoxicity of HepG2 cells, and we examined the potential role of P-gp inhibition in the cytotoxicity responses to these binary pesticide mixtures.

Concentration-response curves in HepG2 cells were generated for each pesticide to identify concentrations causing less than 20% cytotoxicity for testing binary combinations. HEP and END both exhibited a concentration-dependent decrease in the viability of HepG2 cells, with the IC_{50} values of 23.6 ± 2.3 μM for END and 32.1 ± 2.7 μM for HEP, while the CPF IC_{50} value of 180.9 ± 8.3 μM was obtained from our previous research (Goldar et al., submitted). Based on these results, the rank order cytotoxicity of these pesticides in HepG2 cells is END > HEP > CPF. Several previous studies also report higher cytotoxicity for OC pesticides (e.g., END, HEP) as compared to organophosphate pesticides (e.g., CPF; Jamil et al., 2005; Sultana Shaik et al., 2016; Andersen et al., 2002).

We further evaluated the combined cytotoxic effect of CPF + END and CPF + HEP mixtures at their $<IC_{20}$ to identify potential pesticide interactions. Exposure to these binary mixtures resulted in higher cytotoxicity when compared to the individual pesticides. The combination index values revealed an additive effect of the CPF + END mixture, while CPF + HEP exhibited a synergistic response. To the best of our knowledge, this is the first study to report mixture toxicity responses for the pesticides CPF + HEP. Søfteland et al. (2014) observed an additive response at low concentrations (1 μM in 1:1 mixture) of CPF + END but a synergistic effect at high concentrations (100 μM in 1:1 mixture) in the reduction of cell viability of fish hepatocytes. In contrast, Sultana Shaik et al. (2016) reported synergistic cytotoxic and genotoxic effects on human blood lymphocytes with exposure to CPF + END. Although we

observed additive effects CPF + END in HepG2 cells, differences between our results and those of previous studies could be attributed to different exposure durations, exposure concentrations, and cell types. The LDH assay was employed to further identify the potential mechanism of cytotoxicity. LDH release is a widely recognized marker for chemical-induced cell death via cell membrane damage (Bonfoco et al., 1995). There was no increase in LDH release with exposure to individual pesticides nor their binary mixtures, indicating maintenance of the cytoplasmic membrane of the HepG2 cells. Therefore, the enhanced cytotoxicity observed with binary mixtures, as measured *via* the neutral red assay, could occur through other mechanisms, such as apoptosis. However, other studies have also reported that the neutral red uptake assay can detect toxicity after exposure to chemicals before any leakage of LDH is observed (Fotakis and Timbrell, 2006; Repetto et al., 2008).

We assessed the role of P-gp in cytotoxicity responses by examining how individual pesticides and their combinations influence levels of Rho123, a P-gp substrate, in HepG2 cells. The Rho123 accumulation in cells serves as an indicator of the potential of chemicals to inhibit P-gp activity (Jouan et al., 2016). Among the three individual pesticides, only HEP exposure resulted in significant accumulation of Rho123, suggesting inhibition of P-gp-mediated efflux from the cells. The inhibitory effect of HEP on P-gp activity is previously reported (Bain and Leblanc, 1996). Exposure to both binary mixtures also resulted in increased Rho123 accumulation in cells compared to the negative control; therefore, P-gp inhibition by pesticide mixtures may have contributed to the enhanced cytotoxicity. Results from our previous research indicate that P-gp plays a role in the elimination of CPF in HepG2 cells and that efflux transport is a defense mechanism against the CPF cytotoxicity (Goldar et al., submitted). Given that HEP significantly inhibited P-gp activity, the synergistic cytotoxicity response observed in the CPF + HEP mixture could be attributed to the inhibition of P-gp by HEP, disrupting the P-gp-mediated transport of CPF out of HepG2 cells and synergistic cytotoxic effects. However, further investigation is required to elucidate the exact molecular mechanism. In the present study, END alone did not increase intracellular accumulation of Rho123 and did not inhibit P-gp efflux activity in HepG2 cells. Lacher et al. (2015) and Bucher et al (2014) also reported a lack of Rho123 accumulation in P-gp–expressing cells following END exposure and concluded that this pesticide is not a P-gp inhibitor. However, our results contrast with those of Bain and Leblanc (1996), where 100 μM END resulted in >80% P-gp inhibition in B16/F10 cells. Similarly, other

studies report on the P-gp inhibitory activity of END (Sreeramulu et al., 2007; Bircsak et al., 2013) but results appear to be dependent on pesticide concentration, cell lines, and the methods employed to assess P-gp inhibition.

The synergistic cytotoxicity of CPF + HEP could also be explained by mechanisms other than efflux inhibition – for example, modulation of cytochrome P450 (CYP) enzyme activity. Both organochlorine and organophosphate pesticides are recognized inducers of CYP enzymes, particularly CYP 2B6 and 3A4, and these enzymes are the primary catalysts for the production of oxon derivatives (Hernández et al. 2013; Chedik et al., 2018). In the binary combination of CPF and HEP, either or both pesticides may induce cellular enzyme activity that enhances biotransformation of CPF into its active metabolite (CPFO), contributing to a synergistic cytotoxicity response. As an example, Savary et al., (2014) reported a synergistic effect between two OC pesticides, endosulfan and methoxychlor, in the metabolically competent human HepaRG cell (another hepatocyte cell line). However, in HepG2 cells, which have little to no expression of major P450s (including CYP 2B6 and 3A4), only an additive response was observed after exposure to each pesticide at 100 μM in mixture. Savary et al., (2014) reported that both pesticides increase the activity of CYP2B6 in HepaRG cells, potentially leading to higher production of their active metabolite and the observed synergistic effect. Hernández et al. (2013) described the synergism between organophosphate pesticides and triazine herbicides *via* the induction of the CYP450 monooxygenases by triazines, which they suggested increased the rate of formation of the highly toxic oxon metabolite, thus enhancing the toxicity of the organophosphate pesticides. Utilizing metabolically competent cells, such as HepaRG cells, significantly influences the results of *in vitro* mixture toxicity studies. HepG2 cells have a limited capacity for expressing CYP enzymes (Seo et al., 2019) and so this cell line might not serve as an appropriate *in vitro* model for identifying pesticide interactions mediated by CYP450 enzyme induction.

The toxicity interaction of pesticides in a mixture may also be attributed to chemical-induced modulation of *ABCB1* transporter expression as well as expression of its transcriptional regulators (Martin et al., 2008). However, we saw no effect of pesticide exposure, alone or in binary mixture, on expression of *ABCB1* and *PXR* mRNA in HepG2 cells under the conditions tested. Similarly, Bucher et al. (2014) reported no effect of 10 μM END on *ABCB1* expression in HepaRG cells; however, they did observe induction of *ABCB1* mRNA expression after 48 h

exposure to higher concentration of HEP (100 μM; Bucher et al., 2014). Our results suggest that increased accumulation of Rho123 following exposure to HEP and HEP + CPF mixture is due to inhibition of P-gp efflux activity rather than a reduction in *ABCB1* expression at the transcriptional level.

The mRNA expression of *Nrf2* which is a cytoprotective transcription factor against oxidative stress and a transcriptional regulator of P-gp was also measured after exposure to individual pesticides and the binary mixtures. Exposure to xenobiotics can lead to the development of oxidative stress and an overproduction of reactive oxygen species, which can trigger the induction of *Nrf2* expression (Raghunath et al., 2018). While individual pesticides had no effect on *Nrf2* mRNA expression and cytotoxicity in HepG2 cells, the binary combinations induced *Nrf2* expression, perhaps as an antioxidant mechanism to deal with oxidative stress response to the binary mixtures. Measuring the activity and protein levels of Nrf2 following exposure to pesticides could provide insight into the role of Nrf2 and oxidative stress in the observed cytotoxicity of binary pesticide mixtures.

In conclusion, the findings of our study provide evidence for modulation of P-gp activity as a mechanism for synergistic cytotoxicity of pesticide mixtures in HepG2 cells. These results help further our understanding of how pesticides may interact through transporter-mediated mechanisms to amplify toxicity when present in mixtures. Considering the interaction of pesticides through efflux transporters is crucial for hazard assessment of pesticide co-exposure and the potency of pesticides in mixtures. Further research and investigations are necessary to comprehensively understand the complexity of pesticide interactions and the role of efflux transporters in cytotoxicity of pesticide mixtures.

CHAPTER 4 - Effects Of Chlorpyrifos on Epithelial Barrier Properties in Two Mammalian Intestinal Cell Culture Models

Preface

In previous chapters, we evaluated the capacity of *in vitro* cell-based models to assess cytotoxicity of individual pesticides and their binary mixtures, focusing on active efflux transporters. However, in this chapter, our focus shifts to the capability of *in vitro* cell culture models to identify a more functional, organ-specific response, rather than just general cytotoxic effects of pesticides. To accomplish this, we selected two intestinal epithelial models, Caco-2 and IPEC-J2 cells, to compare the intestinal epithelial barrier dysfunction of a model compound CPF.

The material described within this chapter will be submitted as a multi-authored manuscript to *Food and Chemical Toxicology* for publication.

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Natacha Hogan: conceptualization, supervision, funding acquisition, writing - review and editing, resources.

4.1 Abstract

Exposure to environmental contaminants, including pesticides, via ingestion is known to alter the environment, permeability, and function of the gastrointestinal tract (GIT). The toxic effects of environmental chemicals on the intestinal barrier function are often characterized by *in vitro* cellular models using various mammalian intestinal epithelial cell lines. Different cell lines exhibit distinct cellular and molecular features that impact their responses to xenobiotics. There is limited understanding and comparison of the cell line-specific toxicity responses among different intestinal epithelial cell lines, which makes it challenging to select a suitable model for studying intestinal barrier function and toxicity assessment of chemicals. Understanding the differential responses of various intestinal epithelial cell lines to xenobiotics, along with investigating the specific molecular and cellular mechanisms underlying these effects, enhances the predictive capacity of *in vitro* studies assessing chemical impacts on intestinal health and barrier function. In this study, we examined how two types of intestinal epithelial cells, one from porcine origin (IPEC-J2) and the other from humans (Caco-2), responded to chlorpyrifos (CPF), a pesticide commonly found in water and food. Both Caco-2 and IPEC-J2 cells exhibited concentration-dependent viability reduction after 24 h CPF treatment and there was no difference in calculated IC_{50} between the cell lines. In differentiated IPEC-J2 cells, exposure to CPF disrupted intestinal barrier function as measured by decreased TEER and increased permeability to dextran. Conversely, there was no effect of CPF on differentiated Caco-2 cells, likely due to the fact that these cells are derived from a cancer/tumor cell line. We also assessed the effect of CPF exposure on the expression of tight-junction-related proteins associated with barrier function. Although there was a decrease in barrier function in IPEC-J2 exposed to higher concentrations of CPF, this was not reflected in altered mRNA expression of tight junction proteins. In Caco-2 cells, CPF exposure resulted in increased expression of tight junction proteins, suggesting a protective response against CPF-induced damage. Our results provide evidence that CPF disrupts intestinal barrier function in IPEC-J2 cells by affecting the transcellular permeability pathway rather than targeting tight junction proteins. This research highlights the importance of considering the unique characteristics and cell type-specific sensitivity when assessing intestinal toxicity of environmental chemicals.

4.2 Introduction

The gastrointestinal tract (GIT) represents the major site of exposure to xenobiotics from the oral ingestion of environmental pollutants. Pesticide exposure from environmental sources, such as drinking water and food, is a particular concern and potential driver of GIT toxicity and associated adverse health outcomes. Exposure to pesticides through ingestion has been associated with acute and chronic effects on the GIT, such as inflammation, deregulation of microbiota (known as dysbiosis), increased permeability, and histological lesions (Huang et al., 2020; Lima et al., 2022). In addition, intestinal damage from pesticide exposure is suggested to be linked to neurotoxicity, metabolic and endocrine diseases, and developmental disorders in the human population (Di Tommaso et al., 2021).

Traditionally, evaluating the toxicity of environmental pollutants, such as pesticides, has involved conducting studies on whole animals to study the damage they cause to the intestines upon ingestion. However, such studies are resource-intensive, laborious, and may not provide insight into underlying mechanisms of toxicity. In addition, the use of animals in experimentation raises ethical issues, and current guidelines and legislation recommend following the "3R" principles: "Replace, Reduce, and Refine" (Russell and Burch, 1959). Thus, *in vitro* models of intestinal epithelial cells are being used to assess the impact of xenobiotics on cellular growth and proliferation, absorption, and barrier function, while reducing the expense and ethical issues associated with the use of animal experiments (Costa and Ahluwalia, 2019). Intestinal epithelial cell lines have been generated from different animal species and humans to investigate the potential hazard of chemicals on the mammalian intestinal epithelium (Creff et al., 2021).

The intestinal barrier is responsible for controlling the movement of luminal contents, such as pathogens and xenobiotics, either via the paracellular route (between cells) or transcellular route (through cells) into the underlying mucosa (Schoultz and Keita, 2020). One of the most common systems used to recreate *in vitro* the intestinal interface involves culturing epithelial cells on semipermeable membranes, often called transwell inserts. Intestinal epithelial cells, when cultured on transwell inserts, undergo differentiation into enterocytes, displaying features such as cell polarity, microvilli development, and expression of tight junction proteins and digestive enzymes (Simon-Assmann et al., 2007). Once the cells have been established on the transwell inserts, the configuration provides access to both apical and basolateral

compartments whereby the xenobiotic can be added to the apical compartment and barrier integrity measured. One can measure the integrity and tightness of the epithelial cell monolayer by measuring the trans-epithelial electrical resistance (TEER), which is the electrical, ohmic resistance of the cell layer (Schoultz and Keita, 2020, Srinivasan et al., 2015). The transwell insert configuration is also amenable to direct assessment of barrier permeability, where a paracellular tracer compound is applied to the apical compartment post-exposure and then measured in the basal compartment (Bednarek, 2022). Altered barrier function may arise from varied cellular processes including modified cell proliferation, membrane integrity disruption, or apoptotic events. Additionally, chemical influences at the molecular level, such as perturbations in the synthesis, subcellular localization, or degradation pathways of tight junction proteins, can contribute to this impairment. These proteins restrict passive substance diffusion through the paracellular spaces between epithelial cells. Important tight junction proteins in the small intestine, such as occludin (OCLN), claudins (CLDNs), are attached to the cytoskeleton by scaffolding proteins such as zonula occludens-1 (ZO-1), which is essential for controlling barrier function (Zhao et al., 2021; Lima et al., 2022). These *in vitro* toxicity tests provide the opportunity to explore cellular and molecular mechanisms, offering insights into how various pollutants, such as pesticides, can disturb normal intestinal function and potentially result in specific intestinal toxicity. Furthermore, by evaluating the ability of chemicals to disrupt intestinal barrier function or integrity, we can understand not only their local effects on the gastrointestinal tract but also how toxic substances might permeate the intestinal barrier and enter systemic circulation.

The main challenge with cell-based models frequently arises from overlooking their original and intrinsic features that regulate intracellular concentration (e.g. biotransformation and elimination capacity) and signaling pathways (e.g. cell proliferation and differentiation). The Caco-2 cell line is the most widely used *in vitro* model for pharmacological and toxicological studies on intestinal barrier function. Caco-2 cells can be grown on culture inserts to polarize and differentiate, expressing enzymes, transporters and tight junction proteins (Hidalgo et al., 1989). However, Caco-2 cells were originally derived from a human colon adenocarcinoma, which may influence their ability to represent the physiology of intestinal epithelial cells *in vivo* (Sun et al., 2008). Porcine intestinal epithelial cells (IPEC-J2), derived from the jejunum of piglets, present a non-transformed and non-tumorigenic cell line alternative for intestinal toxicity studies. IPEC-J2

cells maintain their differentiated features and closely resemble primary intestinal epithelial cells (Geens and Niewold, 2011; Vergauwen, 2015). IPEC-J2 cells have been predominantly utilized in nutritional toxicology to evaluate the effect of naturally occurring toxins, such as mycotoxins (Akbari et al., 2017; Springler et al., 2016), but have been under-utilized in intestinal toxicity studies of pesticides and other anthropogenic compounds.

The purpose of this study was to evaluate and compare the permeability responses of Caco-2 and IPEC-J2 cells to different CPF concentrations. For this comparison, we used the organophosphate insecticide, chlorpyrifos (CPF), which is contributing to frequent human exposure through contaminated food and drinking water sources (ur Rahman et al., 2021). Chronic exposure to CPF increases paracellular intestinal permeability and can alter expression of tight junction proteins (Condette et al., 2014; Liang et al., 2019) and similar responses are reported *in vitro* across studies using intestinal cells lines (Tirelli et al., 2007; Réquilé et al., 2018). We measured response of Caco-2 and IPEC-J2 cells to CPF using TEER, fluorescein isothiocyanate (FITC)-dextran (4 kDa) permeability assay and mRNA expression of select tight junction proteins (specifically OCLN, CLDNS, and ZO-1). We also used the mycotoxin deoxynivalenol (DON) as a positive control for these assessments as the effects of DON on the intestine are well described *in vivo* and *in vitro* (e.g. altered intestinal structure, reduced expression of junctional proteins, reduced barrier function, altered nutrient absorption, etc.; reviewed in Pinton and Oswald, 2014). It is anticipated that this research will aid in the understanding of cell-specific differences in response to CPF exposure and the identification of the mechanisms underlying CPF-induced intestinal barrier dysfunction.

4.3 Materials and Methods

4.3.1 Chemicals

Chlorpyrifos (CPF; purity >99.67%) and deoxynivalenol (DON; purity >99%) were of standard analytical grade and supplied by Toronto Research Chemicals (TRC, Toronto, ON) and Sigma Aldrich (Oakville, ON), respectively.

4.3.2 Culture of IPEC-J2 and Caco-2 cells

The IPEC-J2 cell line, which was obtained from the jejunum of a neonatal pig, was kindly provided by Professor Dr. Anthony Blikslager (North Carolina State University, USA). The Caco-2 cell line (human colorectal adenocarcinoma; HTB-37) was obtained from the American Type Culture Collection (ATCC, Manassas, VA). IPEC-J2 cells were cultured in a mixture of Gibco Dulbecco's modified eagle medium (DMEM)/Ham's F-12 medium (1:1) (Thermo Fisher Scientific, MA), supplemented with 5% fetal bovine serum (FBS; Thermo Fisher Scientific), 1% penicillin-streptomycin (Thermo Fisher Scientific), 10 μg/ml insulin, 5.5 μg/ml transferrin, and 5 ng/ml selenium (known as ITS; Thermo Fisher Scientific), and 10 ng/ml epithermal growth factor (EGF; Sigma Aldrich). Caco-2 cells were cultivated in DMEM with a composition of 1.0 mmol/l sodium pyruvate, 0.1 mmol/l nonessential amino acids, and 1.5 g/l sodium bicarbonate (Thermo Fisher Scientific). The medium was supplemented with 10% FBS and 1% penicillin-streptomycin. Both cell lines were cultured in an atmosphere at 95% relative humidity and 5% CO_2 at 37°C. The cells were sub-cultured by trypsinization using 0.25% Trypsin-EDTA (Thermo Fisher Scientific) once or twice per week and reseeded in a complete medium at a 1:4 split ratio.

4.3.3 Cell viability assay

The neutral red uptake assay was performed to verify the cytotoxic potential of CPF in Caco-2 and IPEC-J2 cells, following the protocol reported by Repetto et al. (2008). Caco-2 and IPEC-J2 cells were seeded at densities of 2×10^4 cells/cm^2 in 96-well cell culture plates and were allowed to attach for 24 hours (h) at 37°C and 5% CO_2. Cells were then exposed to CPF at one of five concentrations (12, 24, 47, 94, and 188 μM). After 24 h, the exposure media was removed, and the cells were washed with phosphate buffer saline (PBS, Thermo Fisher Scientific). Then, the cells were treated and incubated with 40 μg/ml neutral red (Sigma Aldrich) for 2 h. After washing the cells with PBS again, each well received a 1:50:49 mixture of glacial acetic acid, ethanol, and water. Following a gentle 10 min mixing of the plates at room temperature, the absorbance at 540 nm was recorded using the Epoch 2 Microplate Spectrophotometer (Bio-Tek Instruments Inc., Winooski, VT, USA), with blank wells containing only medium (without cells) used as a reference. The ultimate concentrations of MeOH did not surpass 1% (v/v), and cells treated with the solvent control served as controls. The absorbance from these blank wells was

subtracted from the absorbance readings of both the solvent control and treated wells. The percentage of live cells was calculated relative to the solvent control wells using equation 4.1:

$$\frac{(\text{Absorbance value of treated samples})}{(\text{Absorbance value of MeOH control samples})}] \times 100 \qquad (4.1)$$

4.3.4 Monolayer integrity and permeability assays

The barrier function and integrity in Caco-2 and IPEC-J2 cell monolayers after CPF exposure was measured by TEER. Caco-2 and IPEC-J2 cells were seeded on transparent polyethylene terephthalate (PET) 24-well ThinCert™ transwell inserts (6.5 mm diameter, 0.33 cm^2 area, 0.4 µm pore size; VWR, ON) at a density of 2×10^4 cells/cm^2. Growth media was renewed every second day by replacing 0.5 mL and 1 mL of complete medium into the apical and basolateral chambers, respectively. Caco-2 cells were cultured for 18–21 days, while IPEC-J2 cells were allowed to differentiate for 8–12 days. TEER values were measured before the medium was changed to monitor the progression of confluence using a Millicell ERS-2 epithelial cell volt-ohm meter (Millipore Corporation, Billerica, MA). When the TEER readings reached a plateau, differentiated Caco-2 and IPEC-J2 cells were treated with either vehicle control (0.1% MeOH), various concentrations of CPF (2, 6, 19, 57, and 170 µM), or a positive control DON (10 µM for IPEC-J2 cells or 20 µM for Caco-2 cells). After 24 and 48 h exposure, TEER values were measured and the change in TEER was reported as a percentage relative to the baseline or initial value before treatment.

Paracellular permeability was determined by the flux of FITC-Dextran (Sigma Aldrich) through differentiated Caco-2 and IPEC-J2 monolayers after 48 h CPF exposure (2, 6, 19, 57, and 170 µM). After 48 h exposure, medium was removed from both apical and basolateral chamber, and cell monolayers were washed two times with HBSS (Sigma Aldrich). Then, 200 µl of 1 mg/ml FITC–dextran prepared in HBSS was added to the apical compartment of each insert, while 600 µl of HBSS was added to the basolateral well. The plate was covered with foil to prevent light inactivation of the fluorescent marker and placed in the incubator at 37°C. After 2 h, 120 µl aliquots were taken from the basolateral chamber and placed into a black, clear bottom 96 well plate. The fluorescence intensity of the basolateral HBSS aliquots was measured immediately after sampling at the 485 nm excitation and 528 nm emission wavelengths using a

SPARK multimode microplate reader (Tecan, Austria GmbH). The permeability results are expressed relative to the solvent control.

4.3.5 Quantitative real-time RT-PCR (qPCR)

The mRNA expression of tight junction proteins was evaluated in Caco-2 and IPEC-J2 cells following exposure to CPF. Briefly, Caco-2 and IPEC-J2 were seeded in the transparent PET membrane insert (12 wells, 0.4 μm pore size) at a density of 2×10^4 per cm^2 and were allowed to differentiate as previously described. Differentiated Caco-2 and IPEC-J2 cells were treated with CPF (2, 6, 19, 57, and 170 μM), solvent control (0.1% v/v MeOH), or 10 μM DON (as a positive control) in fresh FBS-free medium for 6 h. Total RNA of the cells was isolated using the RNeasy Mini Kit, including a DNase I treatment, according to the protocol provided by the manufacturer (Qiagen, ON). The concentration of RNA was measured using NanoDrop 2000 (NanoDrop Technologies, Wilmington, DE, USA), and its quality was verified by visualising on a 1% agarose gel. Extracted RNA samples were stored at −80°C until cDNA synthesis. The Applied Biosystems™ High-Capacity cDNA Reverse Transcription Kit (Thermo Fisher Scientific) was employed to generate complementary DNA (cDNA) from 1 μg of RNA, with the reaction conducted in a final volume of 20 μl. The resulting cDNA was stored at -20°C.

Information on target genes of interest and reference genes, including primer sequences for qPCR, are listed in Table 4.1. Primers were tested for efficiency using a dilution series of cDNA. The efficiency of the primers was between 95% and 110%, and only one product was formed, as assessed by melting curve analysis. The cDNA samples were diluted 10-fold to obtain working concentrations of 5 ng/μl before a quantitative polymerase chain reaction (qPCR). Then, 2 μl input of sample cDNA at working concentrations, 0.8 μl of both forward and reverse primer (0.4 mM), and 10 μl of SsoAdvanced Universal SYBR Green Supermix (Bio-Rad, Ontario) in a final 20 μl reaction volume was analyzed by quantitative PCR in a BioRad CFX96 Real-time C1000 Thermal Cycler. Following an initial hot start for 3 min, 40 cycles were run with a denaturation step at 95°C for 20 second, an annealing step at 60°C for 30 second, and an elongation step at 72°C for 30 second. After completing 40 cycles, melt curve analysis was performed on the PCR products from each reaction to confirm the amplification of a single product. Duplicate analyses were conducted for each cDNA sample and assays included controls without reverse transcriptase (no RT control) and samples lacking a cDNA template. Relative

gene expression was based on the $^{2-\Delta\Delta Ct}$ method (Pfaffl, 2001) using B-actin (ACTB), Glyceraldehyde 3-phosphate dehydrogenase (GAPDH), and hypoxanthine phosphoribosyltransferase 1 (HPRT1) as housekeeping genes. Data were expressed as fold-change relative to the solvent control.

Table 4.1. Primer sets used for real-time PCR.

Cell line	Gene	Primer Sequence (5′-3′)	References
Caco-2	*ZO-1*	Fwd: TCACGCAGTTACGAGCAAGT Rev: TGAAGGTATCAGCGGAGGGA	Gong, S., et al. (2022)
	OCLN	Fwd: TCCTATAAATCCACGCCGGTTC Rev: CTCAAAGTTACCACCGCTGCTG	Park, H. Y et al. (2015)
	CLDN1	Fwd: CTGGGAGGTGCCCTACTTTG Rev: ACACGTAGTCTTTCCCGCTG	Yang, M., et al. (2019)
	ACTB	Fwd: CTGGCACCCAGCACAATG Rev: GCCGATCCACACGGAGTACT	Westerink and Schoonen, (2007)
	GAPDH	Fwd: TCGGAGTCAACGGATTTGGT Rev: TTCCCGTTCTCAGCCTTGAC	Pecorelli et al. (2020)
	HPRT1	Fwd: AGACTTTGCTTTCCTTGGTCAG Rev: TCAAGGGCATATCCTACAACAA	Wang, S., et al. (2016)
IPEC-J2	*ZO-1*	Fwd: CGGCGAAGGTAATTCAGTGT Rev: TCTTCTCGGTTTGGTGGTCT	Huang, Y., et al. (2022)
	OCLN	Fwd: CTACTCGTCCAACGGGAAAG Rev: ACGCCTCCAAGTTACCACTG	Liao, P., et al. (2017)
	CLDN1	Fwd: TGCCTCAGTGGAAGATTTACTCC Rev: TGGTGTTCAGATTCAGCAAGGA	Yu, C., et al. (2016)
	ACTB	Fwd: GGATGCAGAAGGAGATCACG Rev: ATCTGCTGGAAGGTGGACAG	Liao, P., et. (2017)
	GAPDH	Fwd: TCATCATCTCTGCCCCTTCT Rev: GTCATGAGTCCCTCCACGAT	Li, Y., et al. (2018)
	HPRT1	Fwd: CCGAGGATTTGGAAAAGGT Rev: CTATTTCTGTTCAGTGCTTTGATGT	Omonijo, F. A., et al. (2018)

ZO-1 (zonula occludens-1); OCLN (occludin); CLDN1 (claudin 1); ACTB (B-actin), GAPDH (Glyceraldehyde 3-phosphate dehydrogenase); and HPRT1 (hypoxanthine phosphoribosyltransferase 1)

4.3.6 Statistical analyses

Data were analyzed with GraphPad Prism (v.8, GraphPad Software, San Diego, CA, USA). The inhibitory concentration causing 50% (IC_{50}) cytotoxicity were derived from a non-linear regression curve calculated in GraphPad Prism ([Inhibitor] vs. normalized response,

variable slope, and least squares fit). For all data, one-way analysis of variance (ANOVA) was performed with Dunnett's multiple comparison test to determine differences between treatments and negative controls. The results were averaged over three different independent assays ($n = 3$) with three replicates per assay for cell viability, TEER measurement, and FITC-dextran permeability assays and two replicates per assay for measurement of gene expression. A value of $p \leq 0.05$ was considered statistically significant. All results are expressed as mean ± standard deviation (SD).

4.4 Results

4.4.1 Cytotoxicity of CPF to Caco-2 and IPEC-J2 cells

Caco-2 and IPEC-J2 cells exhibited a concentration-dependent decrease in the viability after 24 h exposure of CPF (Fig. 4.1). The IC_{50} value of CPF were 104.2 ± 12.7 µM in Caco-2 and 109.4 ± 10.5 µM in IPEC-J2 cells (Fig. 4.1).

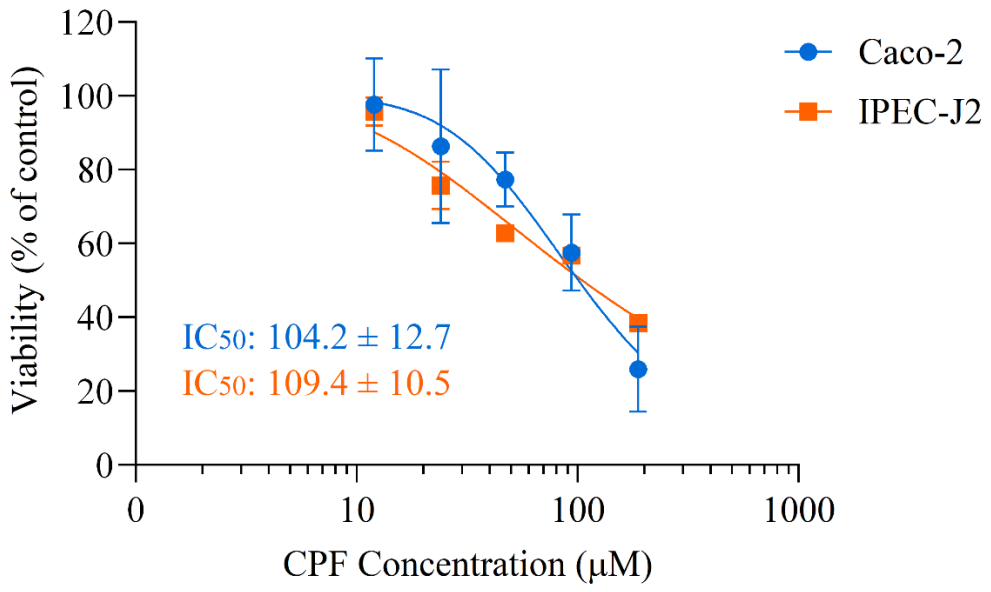

Figure 4.1. Viability of Caco-2 and IPEC-J2 after 24 h of CPF exposure as determined by neutral red assay. The percentage of viable cells normalized to solvent treated control (≤ 1% MeOH) were plotted after background subtraction. Data are means ± SD and represent three independent assays (*n*=3).

4.4.2 Effect of CPF on permeability in Caco-2 and IPEC-J2 cells

In Caco-2 cells, exposure to 19, 57, and 170 μM of CPF resulted in significantly higher TEER at 24 h compared with solvent control (0.1% MeOH; Fig. 4.2A). In contrast, TEER was not affected by incubation of cells with different concentrations of CPF after 48 h (Fig. 4.2A). At 24 h, there was a significant decrease in the electrical resistance of IPEC-J2 cells after treatment with 170 μM of CPF compared to the solvent control (Fig. 4.2B). However, upon reaching the 48 h exposure, the TEER values in IPEC-J2 cells treated with 2, 57 and 170 μM CPF decreased significantly compared to solvent control (Fig. 4.2B). A significant reduction of the TEER was also observed in both IPEC-J2 and Caco-2 cells after 24 and 48 h of exposure to DON as a positive control (Figure. 4.2A and B). TEER values (Ω× cm2) of Caco-2 and IPEC-J2 cells after exposure to CPF are shown in supplementary information Tables 4.1.S and 4.2.S, respectively.

Exposure to CPF at different concentrations did not increase the permeability of FITC-dextran in Caco-2 cells after 48 h exposure (Fig. 4.3A). However, IPEC-J2 monolayer became significantly permeable with FITC-dextran upon treatment with 170 μM CPF compared to the control group (Fig. 4.3B). Following a 48-h treatment with a positive control of 10 μM DON in IPEC-J2 and 20 μM DON in Caco-2, the permeability of FITC-dextran also significantly increased (Fig. 4.3A and B).

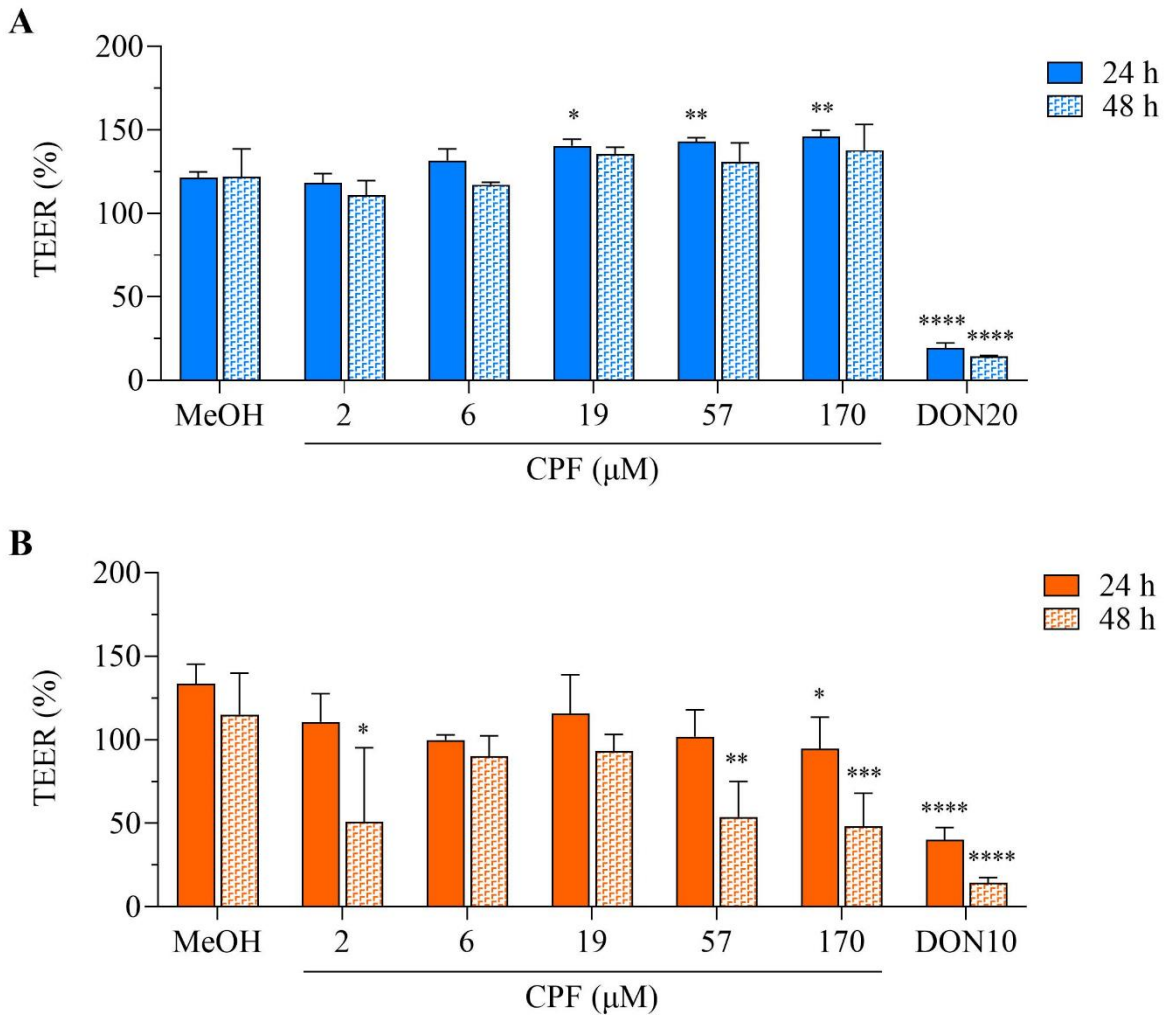

Figure 4.2. TEER of Caco-2 (A) and IPEC-J2 (B) cells monolayer treated with different concentrations (µM) of CPF at 24 and 48 h. TEER values are expressed as percentage of initial TEER value at 0 h. Data are expressed as mean ± SD of three independent experiments for IPEC-J2 (*n*=3) and two independent experiments for Caco-2 cells (n=2). Asterisk represents significant differences between cells exposed to CPF and those exposed to 0.1% MeOH, as negative control (one-way ANOVA with Dunnett's multiple comparison test). * $p<0.05$, ** $P<0.01$, *** $p<0.001$, **** $p<0.0001$. DON: deoxynivalenol (µM).

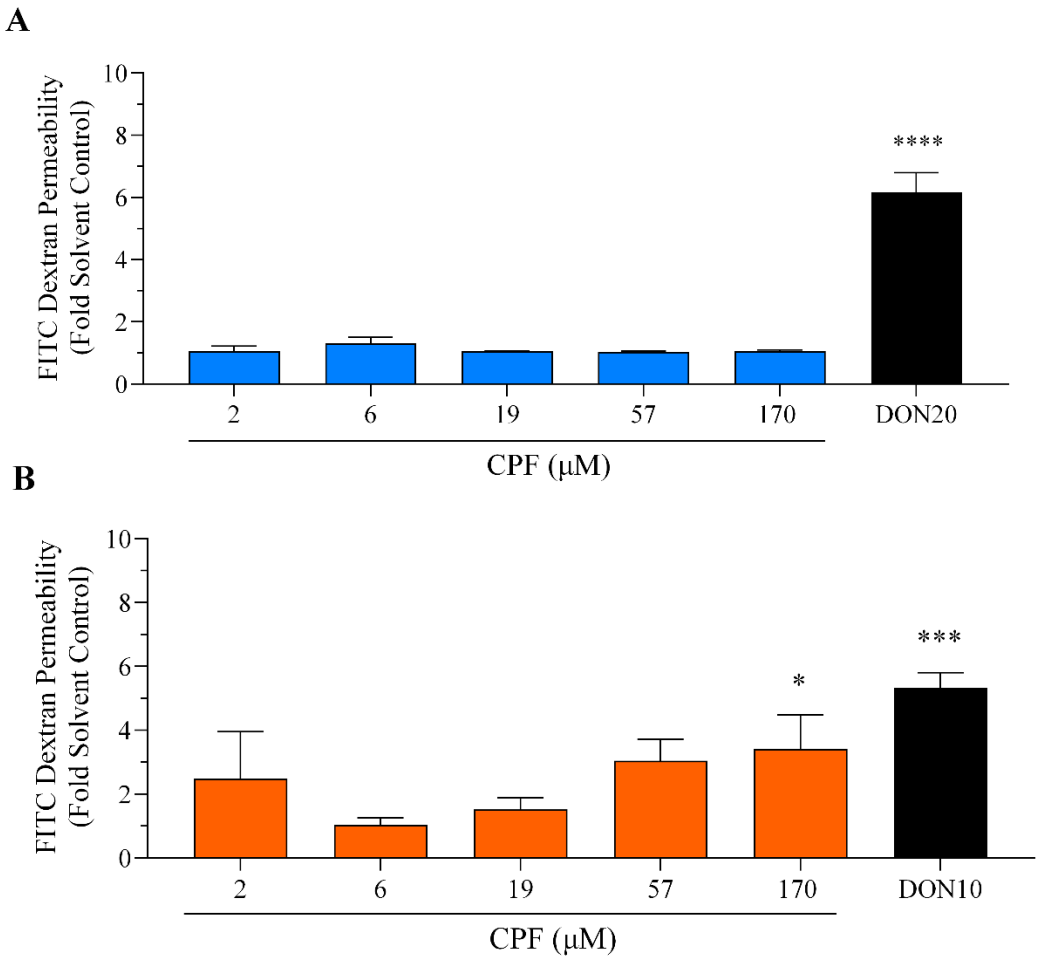

Figure 4.3. Paracellular flux of FITC-dextran in Caco-2 (A) and IPEC-J2 (B) cell monolayers exposed to different CPF concentrations at 48 h. Data are expressed as mean ± SD of three independent experiments for IPEC-J2 (*n*=3) and two independent experiments for Caco-2 cells (*n*=2) and expressed relative to MeOH (0.1%) as vehicle control within cell type. Asterisk indicates significant differences between exposed cells and MeOH using one-way ANOVA with Dunnett's multiple comparisons test, *p<0.05, *** p<0.001, **** p<0.0001. DON: deoxynivalenol (µM).

4.4.3 Comparison of gene expression in Caco-2 and IPEC-J2 cells in response to CPF

In Caco-2 cells, *ZO-1* and *OCLN* expression was significantly increased compared to the solvent control with exposure to 57 µM CPF (Fig. 4.4A and 4.4B), while *CLDN1* levels were

increased with exposure to 6, 19, 57, and 170 µM CPF (Fig. 4.4C). In IPEC-J2 cells, there was no effects of CPF exposure on expression of tight junction proteins *ZO-1*, *OCLN*, and *CLDN1* when compared to the solvent control (Fig. 4.4D to F). However, *ZO-1*, *OCLN*, and *CLDN1* was increased both Caco-2 and IPEC-J2 cells with exposure to 10 µM DON (positive control) (Fig. 4.4A to F).

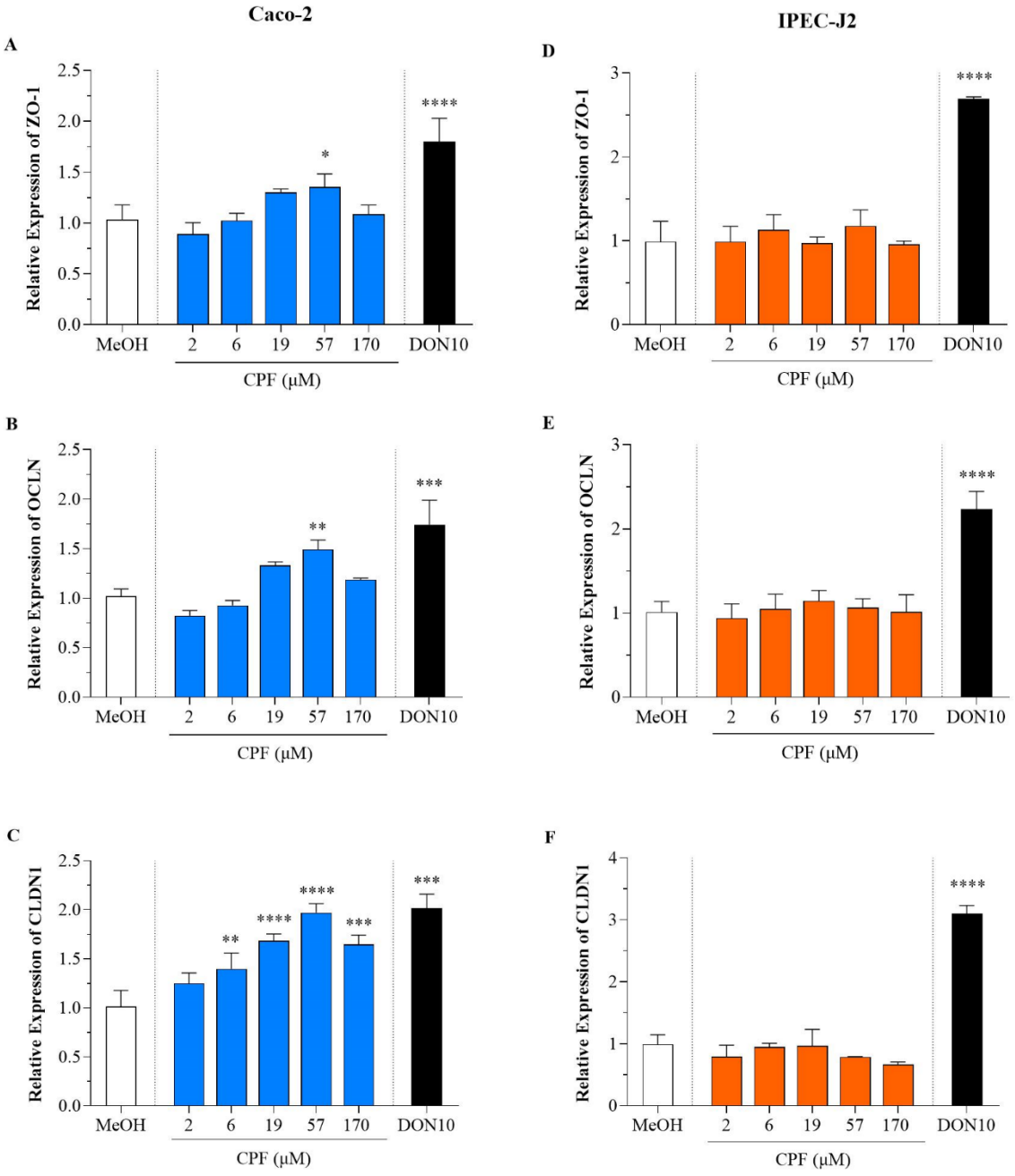

Figure 4.4. mRNA expression of *ZO-1* (A), *OCLN* (B), and *CLDN1* (C) in Caco-2, and *ZO-1* (D), *OCLN* (E), and *CLDN1* (F) in IPEC-J2 cells after 6 h exposure to different CPF concentrations (2, 6, 19, 57, 170 µM) and 10 µM of deoxynivalenol (DON10, as a positive control). Data are presented as mean ± SD (*n*=3) and expressed relative to MeOH (0.1%) as vehicle control within cell type. Asterisk represents statistically significant differences between

exposed and MeOH control (one way ANOVA, Dunnett's multiple comparisons test). * $p<0.05$, **$P<0.01$, ***$p<0.001$, ****$p<0.0001$.

4.5 Discussion

Mammalian intestinal epithelial cells of both non-human and human origins have been established in monolayer culture systems to assess the bioavailability and toxicity of pesticides. While Caco-2 cells are commonly used in studying pesticide effects on intestinal permeability, their inherent cancerous characteristics pose challenges that may affect the translation of *in vitro* results to real-life scenarios. Thus, integrating other intestinal epithelial cell lines in such studies could offer valuable insights. The *in vitro* models using porcine intestinal epithelial cells (e.g. IPEC-J2) are well-established and used extensively for toxicology studies on mycotoxins; however, they are not commonly utilised for mechanistic toxicity assessment of other xenobiotics. Comparing the behavior of different intestinal epithelial cell lines to xenobiotics, and specifically their molecular and cellular responses, will aid in the development of useful *in vitro* assessment tools to assess and predict the impact of chemicals on intestinal health and barrier function.

We initially conducted exposure with undifferentiated/non-polarized Caco-2 and IPEC-J2 cells to assess CPF cytotoxicity and identify concentrations that cause low cytotoxicity for subsequent assays to assess effects on intestinal barrier function. We found that these two cell lines exhibited comparable sensitivity to CPF exposure as indicated by their similar IC_{50} values. There is little information available on the effects of CPF on viability of intestinal epithelial cells. Fernando (2021) saw a concentration-dependent reduction in cell viability in rainbow trout intestinal (RTgutGC) cells with 24 h exposure to CPF. They found that 100 μM of CPF resulted in 55% cytotoxic response, which is consistent with the IC_{50} values obtained in our study. In contrast, Tirelli et al. (2007) found no effect of up to 250 μM CPF (24 h exposure) on cell viability in an undifferentiated and differentiated co-culture of Caco-2/TC7 cells. The discrepancy in CPF cytotoxicity results across studies using Caco-2 cells could be due to differences in experimental conditions, incubation protocols, and the heterogeneity of Caco-2 cell subpopulations. The genetic instability of Caco-2 cells can contribute to variability in cytotoxicity and permeability responses both inter- and intra-laboratory (Kus et al., 2023; Cai et

al., 2022). This study represents the first cytotoxicity assessment of CPF on IPEC-J2 cells and our findings reveal that both intestinal cell lines exhibited comparable cytotoxicity to CPF.

We used the same wide range of CPF concentrations derived from cytotoxicity results to evaluate the influence of CPF on the barrier integrity of differentiated Caco-2 and IPEC-J2 cell monolayers grown on transwell inserts using TEER and FITC-dextran permeability assays. The concentrations used in TEER and FITC-dextran permeability assays are intended to evaluate not only the integrity of tight junction proteins in differentiated IPEC-J2 and Caco-2 cells following exposure to sublethal concentrations of CPF but also to assess their permeability responses to higher and cytotoxic CPF concentrations at their differentiated forms. We saw an increase in TEER values in differentiated Caco-2 cells after 24 h CPF exposure; however, this increase was no longer seen after another 24 h of CPF exposure (48 h time point) when TEER values were again comparable to the solvent control. It is well-established that the TEER values are inversely proportional to the barrier permeability (Srinivasan et al., 2015), so a macromolecular tracer flux assay was used immediately following TEER measurements to determine whether CPF exposure directly affected paracellular permeability of the Caco-2 monolayer. CPF exposure did not impact permeability in Caco-2 cells, indeed supporting the lack of CPF effects on TEER.

Contrary to the observations in Caco-2 cells, IPEC-J2 cells were more sensitive to CPF exposure in terms of effects on intestinal barrier function. Exposure to CPF resulted in decreased TEER at both timepoints measured, with a greater effect with increasing CPF concentration and a more produced decreased at 48 h as compared to 24 h. Again, TEER values were supported by FITC-dextran permeability results, in that exposure to CPF after 48 h exposure increased the movement of dextran from apical to basal chamber. Tirelli et al. (2007) observed decreased TEER values in Caco-2/TC7 cells when exposed to the highest concentration of CPF (250 µM) while Fernando (2021) reported a concentration-dependent decrease in TEER values in RTgutGC cells following exposure to cytotoxic concentrations of CPF (50, 100, and 500 µM). The impacts of CPF on barrier function observed on IPEC-J2 cells may occur via disruption of tight junctions leading to increased paracellular permeability; however, since we observed barrier dysfunction in IPEC-J2 cells with exposure to two highest concentrations of CPF (57 and 170 µM), the decrease in TEER and increase permeability may be a result of cell death, thus movement via a transcellular pathway rather than paracellular pathway. In addition, exposure to the mycotoxin DON, as positive control, clearly affected intestinal barrier function in both Caco-

2 and IPEC-J2 cells, as evidenced by decreased TEER and increased FITC dextran permeability. DON, a well-characterized lipophobic mycotoxin, is known to disrupt the integrity of the intestinal epithelial barrier by inhibiting tight junction proteins synthesis and potentially through additional mechanisms such as decreasing protein stability and accelerating the degradation of tight junction proteins in the lysosome (Pinton et al., 2009; Li et al., 2021). Based on effects of DON on Caco-2 and IPEC-J2 cells, it appears that both cell lines are responsive to a compound that affects tight junction proteins, primarily influencing the paracellular pathway. In contrast, for lipophilic compounds like CPF that can accumulate in biological membranes, the mechanisms underlying effects in IPEC-J2 cells observed in this study may involve cell death and transcellular routes rather than targeting tight junctions in the paracellular pathway.

The differences in functional responses in Caco-2 and IPEC-J2 cells to CPF could also be attributed to the distinct cellular proliferation pathways and intrinsic features of cell models. The Caco-2 cell line is derived from colonic cancer cells. Typically, normal epithelial cells rely on a basement membrane for survival and growth signals, undergoing apoptosis when cultured in suspension. However, cancer-derived Caco-2 cells could evade apoptosis, resulting in uncontrolled proliferation after exposure to a chemical (Cerda et al., 2001). Caco-2 cells possess multiple mutations that activate cellular repair mechanisms or enable survival following damage, which would otherwise render normal non-cancerous cells like IPEC-J2 cells unviable (Nossol et al., 2015). Another explanation for the differing responses observed in Caco-2 and IPEC-J2 cells is that exposure to the same extracellular CPF concentrations may not result in similar intracellular CPF concentrations. The discrepancy could be attributed to differences in the expression of transporters responsible for CPF elimination between the cell lines. The variability in expression of efflux transporters, such as P-glycoprotein (P-gp) and breast cancer resistance protein (BCRP), has been shown to alter intracellular concentrations of pesticides (Chedik et al., 2018; Hodgson, 1999) and thus would likely influence the inherent sensitivity and differences in cell-specific responses across *in vitro* intestinal cell models. Previous studies have shown that Caco-2 cells express various drug transporters (Elsby et al., 2008, Taipalensuu et al., 2001), while IPEC-J2 cells have low expression of intestinal transport proteins, particularly P-gp and BCRP (Saaby et al., 2016; Ozgür et al., 2018). Since CPF is substrate for P-gp and BCRP, differences in basal expression of these efflux transporters could drive cell-specific toxicity responses to CPF. Our previous research indicates that P-gp expression plays a protective role in

cell-specific cytotoxicity to CPF, influencing the intracellular concentration of CPF and cytotoxicity thresholds (Goldar et al., submitted). Collectively, higher expression of the P-gp and BCRP efflux pump in Caco-2 cells, compared to levels in IPEC-J2 cells, might also contribute to the dissimilar responses observed between Caco-2 and IPEC-J2 cells.

Tight junctions are important functional proteins that regulate the paracellular pathway and maintain the strength of the epithelial barrier (González-Mariscal et al., 2014). Alterations in the expression levels or localization of ZO-1, OCLN, and CLDN-1 can disrupt the integrity of epithelial monolayer, compromising the barrier function of the intestinal epithelium. We found different effects of CPF exposure on expression genes that encode for tight junction proteins in Caco-2 and IPEC-J2 cells. In IPEC-J2 cells exposed to CPF, there was no change in expression of *ZO-1*, *OCLN*, and *CLDN-1*, further supporting the hypothesis that the decreased TEER observed at high concentrations of CPF in IPEC-J2 cells might be associated with cytotoxicity rather than paracellular damage. Conversely, in Caco-2 cells, CPF significantly increased in the mRNA expression of *ZO-1, OCLN and CLDN-1*, suggesting increased production of the encoded tight junction proteins, which is supported by TEER and permeability results in this intestinal cell line. Increased tight junction expression could serve as a compensatory and protective mechanism within Caco-2 cells, possibly acting against the damaging effects of CPF. The reported upregulation of tight junction proteins as a protective mechanism to preserve paracellular permeability has been previously documented in other cell lines (Ling et al., 2016; Balbuena et al., 2011). Contrary to our study, Réquilé et al. (2018) reported a decrease in mRNA expression of tight junction proteins *ZO-1* and *OCLN* after CPF exposure in Caco-2/TC7 cells but this may be related to the influence of co-culture conditions.

Previous studies of tight junction proteins have also revealed additional crucial functions beyond their barrier roles. These proteins actively participate in the regulation of epithelial apoptosis and proliferation, thereby exerting a significant impact on these essential cellular processes (Kuo et al., 2022). It is suggested that increased expression of tight junction proteins, such as ZO-1 and CLDN1, may result in increased proliferation in different cancer cell lines (reviewed in Kozieł et al., 2021). For example, Singh et al. (2012) have reported that CLDN1 expression induces resistance to anoikis (detachment-induced cell death) in colon cancer cells. In another study, Kuo et al. (2021) reported that ZO-1 was critical for upregulation of epithelial proliferation and successful completion of mitosis. In our study, a CPF-stimulated increase in

tight junction proteins in Caco-2 cells could be similarly activating mechanisms that contribute to the resistance of this cell line to CPF.

In conclusion, this study focused on two intestinal cell lines in order to compare the responsiveness of these *in vitro* cell models for intestinal toxicity assessment and further our understanding of CPF effects on intestinal epithelial cells. The results demonstrate a differential impact of CPF on intestinal epithelial barrier integrity and mRNA expression of tight junctions in Caco-2 and IPEC-J2 cells. The results of current study suggest that intestinal barrier disruption of IPEC-J2 cells may not be exclusively attributed to the paracellular pathway. Instead, transcellular routes and cell death might contribute to the observed responses in IPEC-J2 cells, in contrast to Caco-2 cells where such effects were not observed. The absence of a permeability effect of CPF in Caco-2 cells, even at cytotoxic concentrations, could be attributed to the cancerous nature of these cells and their ability to evade regulated cell proliferation pathways. This underscores the importance of considering the characteristics and differential responsiveness of these intestinal cell lines when using them to assess toxicity of pesticides and other xenobiotics on intestinal barrier function.

4.6 Supplementary Information

Table 4.1.S. TEER values (Ω× cm2) of Caco-2 cells after 0, 24, and 48 h exposure to different CPF concentrations.

Concentrations (µM)	Assay Replicate #1 TEER Values (Ω × cm2)						Assay Replicate #2 TEER Values (Ω × cm2)					
	0 h		24 h		48 h		0 h		24 h		48 h	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
0.1% MeOH	765.9	10.9	911.1	12.4	843.7	56.5	680.0	32.4	845.6	51.9	912.4	43.5
CPF 2	754.7	3.4	864.5	13.1	789.0	33.7	687.5	32.1	843.2	23.3	809.7	95.9
CPF 6	691.1	7.9	876.4	46.1	803.2	13.5	623.9	5.3	856.0	20.3	740.8	62.9
CPF 19	695.4	23.9	956.6	13.0	963.7	28.5	612.7	48.2	879.2	45.5	814.8	23.9
CPF 57	775.1	34.7	1096.8	28.2	1076.4	40.2	665.5	20.1	966.7	15.3	824.4	112.1
CPF 170	735.5	27.0	1093.2	47.0	1092.9	65.2	690.3	73.3	990.7	40.5	873.9	27.9
DON 20	632.4	87.0	119.7	1.0	91.5	11.1	629.1	82.4	136.2	24.3	93.2	13.5

Data are presented as the mean of three technical replicates ± SD (n=3).

Table 4.2.S. TEER values (Ω× cm2) of IPEC-J2 cells after 0, 24, and 48 h exposure to different CPF concentrations.

Concentration (µM)	Assay Replicate #1 TEER Values (Ω × cm2)						Assay Replicate #2 TEER Values (Ω × cm2)						Assay Replicate #3 TEER Values (Ω × cm2)					
	0 h		24 h		48 h		0 h		24 h		48 h		0 h		24 h		48 h	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
0.1% MeOH	1881.4	596.1	2568.9	505.4	2505.1	192.9	3563.0	306.7	4260.5	63.2	3311.6	674.4	381.0	84.3	547.1	43.3	434.0	26.8
CPF 2	2815.3	608.7	2814.9	672.7	1445.2	738.2	2426.8	252.4	3181.8	1002.1	1648.9	399.5	412.5	87.6	425.9	58.1	351.5	33.4
CPF 6	3284.8	176.1	3162.9	133.9	3264.7	274.1	3486.5	217.7	3569.1	712.7	2674.9	779.7	457.5	28.4	344.2	60.7	322.6	22.1
CPF 19	3226.2	386.7	2959.2	415.5	2802.8	774.3	2597.4	799.8	3439.5	594.9	2564.0	240.8	325.1	84.0	403.1	119.2	330.7	32.1
CPF 57	2781.5	467.4	3266.8	528.0	2903.7	330.8	2597.4	799.8	2041.8	729.8	1002.9	640.9	468.1	42.6	424.2	59.8	285.2	68.3
CPF 170	2112.6	307.6	2372.7	92.8	1393.8	422.5	2373.5	295.0	1769.6	708.1	647.6	167.4	365.0	66.0	346.6	21.7	177.3	70.0
DON 10	3625.5	152.7	1711.6	182.0	623.9	90.7	2817.8	622.1	911.4	157.4	418.1	78.2	364.4	84.6	155.8	10.2	42.7	10.2

Data are presented as the mean of three technical replicates ± SD (n=3). The TEER values for the first two assay replicates (#1 and #2) were obtained using a different device, which accounts for the higher TEER values recorded in these assays compared to the third assay replicate.

CHAPTER 5 – General Discussion and Conclusions

5.1 Primary Contributions of Research

5.1.1 Cell line-specific responses to chlorpyrifos can be attributed to distinct regulatory and signaling pathways

There is evidence indicating that different cell lines possess unique regulatory and proliferation signaling pathways and that these pathways may contribute to the diversity of cellular responses to chemicals across cell lines (Harmsen et al., 2008; Tremblay et al., 2006). The research findings presented in this thesis emphasizes the effectiveness of cell lines in elucidating toxicity mechanisms triggered by chlorpyrifos, particularly interactions with key target molecules (i.e. nuclear receptors). An increased expression of nuclear receptors is considered as the molecular initiating event for detoxification of a number of pesticides (Tebourbi et al., 2011). Induction of nuclear receptors can lead to activation of further key events, including nuclear receptor-associated gene transcription and expression of proteins (e.g. metabolizing enzymes and transporters) that subsequently modify the pesticide accumulation and toxicity in organs (Lichtenstein et al., 2020). In Chapter 2, we found differential basal expression of nuclear receptors, enzymes and transporters between liver and kidney cell lines and also observed that chlorpyrifos oxon could induce the expression of nuclear receptors and efflux transporters in the liver cell line specifically. The two cell lines also displayed varying sensitivities to pesticides, leading us to hypothesize that expression and inducibility of nuclear receptors can, at least in part, protect cells from chemical-induced cytotoxicity. As such, understanding relevant signalling pathways, metabolic capacity, and transporter kinetics of specific cell lines are important when choosing cell lines for generating toxicity data using cell-based assays.

Cell lines can also display varying sensitivities to pesticides due to differences in cellular proliferation and molecular pathways. In our study, we observed differential effects of chlorpyrifos on intestinal epithelial barrier integrity and tight junction mRNA expression in two intestinal epithelial cell lines, Caco-2 and IPEC-J2 cells. While chlorpyrifos disrupted the epithelial barrier function in IPEC-J2 cells, it had no effect on Caco-2 cells. Our results described

in Chapter 4 suggest that the increased permeability induced by chlorpyrifos in IPEC-J2 cells is more likely attributed to the transcellular pathway, supported by observed effects at the highest concentrations tested as well as unchanged tight junction gene expression at sublethal levels. The lack of permeability disruption in Caco-2 cell monolayers at the cytotoxic concentration might be due to its colorectal adenocarcinoma origin, which harbors genetic mutations like in p53 and other tumor suppressor genes. Mutations may reduce toxicity response to chlorpyrifos by evading cell death mechanisms and promoting uncontrolled cell proliferation. Our results suggest that IPEC-J2 cells are best suited for assessing the impact of chemicals on transcellular permeability through mechanisms such as cell membrane damage or cell death, due to the stable cellular proliferation pathways of IPEC-J2 cells. On the other hand, both Caco-2 and IPEC-J2 cells respond similarly to deoxynivalenol, a positive control for assessing the impact of chemicals on the paracellular pathway, in terms of both permeability and tight junction mRNA expression. The similar responses observed across these two cell lines after exposure to deoxynivalenol implies that both cell lines are sensitive to substances that affect tight junction proteins. As such, not all developed intestinal cell lines exhibit the same functional toxicity responses, such as disruption of epithelial barrier function by pesticides due to their dissimilar molecular and cellular features and signaling pathways. When using these cells in chemical exposure investigations, it is critical to examine and comprehend the main causes of these diverse reactions.

The observed discrepancy in toxicity results between HepG2 and HK-2 cells, as well as between IPEC-J2 and Caco-2 cells, following exposure to chlorpyrifos underscores how the selection of cell lines can substantially impact the interpretation of experimental outcomes. This discrepancy between results obtained from cell lines illustrate the potential for misleading data that may not accurately reflect *in vivo* responses. Therefore, the selection of appropriate cell lines based on the specific research question is crucial for enhancing the validity and relevance of the findings. This process involves considering key factors such as the physiochemical properties of the compound under investigation and the characteristics and limitation of the cell lines.

5.1.2 Efflux transporters influence the cell-specific toxicity of chlorpyrifos

Cell-specific toxicity responses to chemicals, such as pesticides, can arise from variability in the competency of specific cell lines to detoxify and eliminate them from cells

(Coecke et al., 2013). While the role of biotransformation enzymes in cell-specific pesticide toxicity has been extensively studied, the contribution of transporters has received limited attention. The efflux transporters function as important cellular defense systems against a variety of pesticides and their metabolites by limiting their concentrations in the cells (Chedik et al., 2018). While various organ-derived cell lines are used in pesticide toxicity assessments, not all exhibit the required efflux transporter functions for elimination of them. This discrepancy leads to differential toxicity responses across different cell lines.

The research presented in this thesis investigated and compared the cytotoxicity responses of different cell lines originating from various organs (kidney, liver and intestine) to a model compound chlorpyrifos. Our findings revealed that kidney cells (HK-2 cells) exhibited greater sensitivity to chlorpyrifos compared to liver (HepG2 cells) and intestinal (Caco-2 and IPEC-J2) cells, as evidenced by lower IC_{50} values in HK-2 cells. Existing *in vivo* studies indicate that chlorpyrifos (similar to many other pesticides) can induce injury in the liver, kidney, and intestine and the extent of toxicity in these organs is dependent on a variety of factors, such as the concentration, route of exposure, duration, and individual susceptibility (Lushchak et al., 2018). We hypothesized that greater toxicity of chlorpyrifos and its active metabolite to kidney cells compared to liver and intestine cells was in some part related to lower capacity of kidney cells in metabolize and eliminate them by detoxifying enzymes and efflux transporters.

The research findings presented in Chapter 4 also highlight cell-specific responses to chlorpyrifos that are relevant to ingestion and intestinal toxicity. Differentiated human colon-derived Caco-2 cells were less sensitive as compared to porcine jejunal IPEC-J2 cells. The differing response to toxicity between these two intestinal epithelial cell lines may be partly due to variations in the expression of efflux transporters across the Caco-2 and IPEC-J2 cells. The expression levels of *ABCB1/P-gp* appear comparable between the jejunum and Caco-2 cells (Taipalensuu et al., 2001), whereas *ABCB1* expression levels and P-gp function in IPEC-J2 cells are lower compared to those observed in the human jejunum *in vivo* and Caco-2 cells (Ozgür et al., 2018; Saaby et al., 2016). In this research, the higher expression of P-gp in Caco-2 cells could help this cell line to eliminate P-gp substrate chlorpyrifos and resulting higher thresholds of toxicity to chlorpyrifos. As many pesticides are substrate for efflux transporters, their elimination from cells could be limited in cell lines that express low levels and/or activities of efflux transporters, resulting in intracellular accumulation and greater sensitivity. Therefore, it is

important to recognise not only the function of metabolizing enzymes, but also the role of efflux transporters in specific cell lines to effectively assess and interpret *in vitro* cytotoxicity response. Our data supporting the role of efflux transporters in chlorpyrifos toxicity has implications for understanding the impact of environmental mixtures containing chlorpyrifos and other pesticides that interact with these transporters and is further discussed in section 5.1.3 below.

5.1.3 Efflux transporters and chemosensitisation with pesticide mixtures

Most chemical hazard assessments are predominantly based on assessments of individual substances. However, humans are exposed to a cocktail of chemicals and as such, impacts on organ systems cannot be attributed to individual compounds. Many pesticides act as substrates or inhibitors of efflux transporters, which raises concerns that inhibitory effects from some pesticides could reduce the elimination of others or increase cellular bioaccumulation when combined, resulting in chemosensitisation and increased cellular toxicity (Kurth et al., 2015). Predicting chemical interactions and associated mechanisms of action driving toxicity responses has been of great interest to the toxicological studies and regulatory agencies for the past several decades. Because of species variations in enzymes and transporters, animal models are not commonly used to predict the hazards of chemical interactions in humans. Instead, cell-based assay systems, non-cell-based models, and *in silico* tools are frequently employed to identify chemical interactions (Fardel et al., 2012).

In Chapter 3, we demonstrated that chlorpyrifos was a P-gp transporter substrate and that the pesticide heptachlor was a P-gp inhibitor. When HepG2 cells were exposed to a binary mixture of these pesticides at concentrations causing less than 20% cytotoxicity, a synergistic cytotoxic response occurred, which we hypothesized was due to heptachlor inhibiting of chlorpyrifos efflux from cells. Our results demonstrate that using cell-based assays with cell lines expressing P-gp, like HepG2 cells, can effectively detect potential pesticide interactions mediated by P-gp. The ratio of the cytotoxicity response (e.g., IC_{10}, IC_{20}) of pesticides when exposed alone compared to the cytotoxicity of their mixture (in the presence of a transporter modulator) could serve as a screening approach to predict the potential interactive nature of pesticides in a mixture. Our finding shows that low levels of chlorpyrifos and heptachlor can increase cell susceptibility to other xenobiotics when they are in a mixture. The chemosensitization processes as a relevant toxicological effect in cells in organs should be

considered in predicting the synergistic interaction of pesticides. Overall, our results demonstrate that selecting a competent cell line expressing efflux transporters enables us to effectively predict interactions between chemical mixtures. *In vitro* assays and traditional immortalized cell lines, employing basic toxicity assays like standard cytotoxicity assays, can serve as initial approaches to identify whether a compound is a substrate, or an inhibitor of a given transporter.

5.2 Limitations

In two of the studies (Chapter 2 and 3) presented in this thesis, the hepatoblastoma-derived HepG2 cell line was used as a model of liver hepatocytes. However, this cell line has the disadvantage of low basal expression levels of biotransformation enzymes, particularly in phase I cytochrome P450 enzyme, when compared to primary cultured hepatocytes (Steinbrecht et al., 2019). Therefore, while they are still considered the most widely used liver-derived cell line in toxicology studies, HepG2 cells are limited for use in chemical biotransformation research and investigating the interplay mechanisms of CYP enzymes and transporters. For instance, numerous studies have demonstrated that chlorpyrifos is a substrate for multiple CYP metabolizing enzymes (e.g. CYP3A4 and CYP2B6) and efflux transporters (P-glycoprotein and breast cancer resistance protein; Sams et al., 2004; Chedik et al., 2022). Bioassays that use cell lines with low or absent expression of these biotransformation enzymes or transporters (e.g. HepG2) may not yield cellular toxicity results reflective of adverse effects of pesticides on similar cell types *in vivo*. In addition, HepG2 cells actually express multiple efflux transporters, which makes it challenging to attribute cellular responses to a specific efflux transporter (Rigalli et al., 2012). Alternatively, cells that are manipulated to either stably or transiently express a specific drug transporter could be used to screen pesticides and other chemicals for transporter-based toxicity in chemical hazard assessment. Although cell lines that are engineered to overexpress a single transporter may yield irrelevant cellular toxicity results, they can effectively and efficiently identify chemicals that have a potential to interact with efflux transporters (Gameiro et al., 2017).

Throughout the research presented in this thesis, we evaluated mechanisms underlying cell-specific responses to pesticide exposure by assessing the mRNA expression (as a precursor to proteins) of efflux transporters, nuclear receptors, or tight junction proteins. Although mRNA and protein levels are often correlated, post-transcriptional mechanisms can also influence

protein levels irrespective of mRNA abundance (Buccitelli and Selbach, 2020). Some of these post-transcriptional processes include variable mRNA-to-protein conversion rates by microRNAs, protein degradation, and the ability of cells to maintain stable protein levels despite mRNA fluctuations. The addition of targeted measurement of protein levels or enzyme activities into the findings of our current research would help strengthen our results and further enhance our understanding of mechanisms involved in cell-specific toxicity and applicability of cell lines in the hazard assessment of pesticides.

Through this thesis research we contributed information on the toxicokinetic and toxicodynamic processes associated with toxicity of chlorpyrifos, a widely used organophosphate pesticide that continues to be of considerable interest to both researchers and regulatory bodies. However, like many hydrophobic organic chemicals, chlorpyrifos is subject to a range of loss processes when under *in vitro* bioassay conditions including sorption, volatilization, and biotransformation. Chlorpyrifos has a high octanol-water partition coefficient (K_{ow}: 4.7) and moderate Henry's law constants (H: 4.2×10^{-6} atm·m^3/mol at 25°C), which suggests a propensity for sorption to plastic and culture medium constituents, and potential volatility, characteristics that would certainly impact its distribution, bioavailability, and thus the effective concentrations driving uptake and toxicity. In the current research, we utilized methanol as the solvent and employed a solvent spike exposure by directly adding pesticides into culture medium. While solvent spiking is an accepted exposure method, it can result in non-uniform distribution and limited bioavailability of a lipophilic chemicals, such as chlorpyrifos. Addition of a solvent to increase the solubility of chlorpyrifos could have had an impact on cell proliferation, differentiation or modulation of signaling pathways (Tanneberger et al., 2010). Passive dosing is one approach that could address these challenges by continuously releasing hydrophobic organic compounds from a predominant reservoir, typically a biologically inert polymer like silicone. A passive dosing method could ensure stable and defined concentrations of freely dissolved compounds, while avoiding the need for spiking with co-solvents (Smith et al., 2010). Another benefit of passive dosing over traditional spiking techniques is that effect data from *in vitro* research can be extended to the *in vivo* condition by enhancing the relationship between the *in vitro* exposure and internal exposure in tissue or cells (Smith and Schäfer, 2017).

5.3 Future Research

The research findings presented in this thesis emphasize the inherent benefits and limits of conventional cell-based assays in characterising the toxicity of individual and pesticide mixtures. We completed three distinct studies that addressed specific research questions that are difficult to obtain through *in vivo* studies, such as the effects of pesticides at the cellular level and their specific interactions with cellular feature that influenced cell-specific toxicity responses. While the simplicity of conventional cell-based assays cannot fully replicate the intricate interactions and systemic responses of organs and tissues, there are opportunities for future research to push the complexity of cell-based culture systems and use these systems for comprehensive toxicity assessment of pesticides and their mixtures.

The monoculture of cell lines used in this research offered insights into toxicity mechanisms in hepatocytes and enterocytes. However, monocultures might not fully capture complex interactions with other cells in these organs, possibly underestimating the overall toxicity. Pesticide exposure can cause injury to liver and intestine not only by directly damaging parenchymal cells, namely hepatocytes and enterocytes, but also by interacting with other cells in these organs. Both intestine and liver have substantial populations of innate immune cells, such as macrophages in the intestine and Kupffer cells (resident macrophages) in the liver. In the liver, pesticide exposure can activate these immune cells to release pro-inflammatory cytokines as well as produce reactive oxygen species, all capable of initiating apoptosis (Lasram et al., 2014; Cataudella et al., 2012). In the intestine, pesticides can activate immune cells to secrete cytokines which influence enterocyte barrier function (Liang et al., 2019; Lima et al., 2022). Future research could employ co-cultured liver and intestinal models, incorporating Kupffer cells or human macrophage-like cells (such as differentiated monocytic THP-1 cells) to allow the assessment of additional biological responses of immune-mediated organ toxicity (Zinchenko et al., 2006). Such an approach would more accurately capture the complexity of cell interactions that contribute to the cellular toxicity of chlorpyrifos and other pesticides. By evaluating both cytotoxicity and mechanistic endpoints in monoculture and co-culture systems, we can assess whether the presence of multiple cell types alters toxicity and whether co-culture models better recapitulate the *in vivo* scenario.

Another critique of monoculture cell-based *in vitro* methods, particularly those used to study intestinal toxicity, is the fact that they lack capacity for mucus production and microbiome

communities. The mucus layer acts as both a physical and chemical barrier in the intestinal epithelium against luminal contents (Le et al., 2023). Co-culture of Caco-2 cells with mucin-secreting HT29-MTX cells effectively resembles the small intestine and provides better predictability of chemical permeability compared to Caco-2 culture alone. The tight junctions of HT29-MTX cells are less robust than those of Caco-2 cells. In Chapter 4, we observed a lower sensitivity of Caco-2 cells to chlorpyrifos compared to IPEC-J2 cells. We hypothesized that this difference could be attributed to the smaller tight junction pore size in Caco-2 cells compared to that in IPEC-J2 cells. Co-culturing Caco-2 cells with mucin-secreting HT29-MTX cells could potentially better mimic the permeability characteristics of the human intestinal barrier and consequently enhance responsiveness to chlorpyrifos. The intestinal microbiome is also now understood to play a critical role in modulating intestinal function and influencing pesticide metabolism and toxicity (Peters et al., 2020). There is significant interest in the gut microbiota due to their pivotal roles in host metabolism, absorption, immunity, intestinal health, and the maintenance of health in other organs. As an example, chlorpyrifos can disrupt gut microbiota balance, known as dysbiosis, causing adverse effects on intestine and various other organs (Yuan et al., 2019). Intestinal cell-based models alone, like Caco-2 and IPEC-J2 cells used in this thesis research, do not consider the influence of microbiota-driven metabolism of CPF in the lumen of the digestive tract. Additional research could integrate a commensal microbiome into *in vitro* intestinal epithelial cell models to explore the potential influence of microbiota on the biotransformation and elimination processes of chlorpyrifos (and other pesticides), and consequently intestinal epithelial barrier health.

This thesis research also highlighted the significance of detoxifying enzymes and efflux transporters in toxicity responses of organ-derived cells *in vitro*. Understanding the interplay between organs that occur *in vivo* is also important for better assessing toxicokinetic processes influencing toxicity of pesticides. Exposure to pesticides via ingestion occurs via uptake in the gastrointestinal tract, after which they move into the bloodstream and reach liver and other organs. Metabolism and transporter-mediated processes in enterocytes of the intestine can actively reduce the bioavailability of these absorbed pesticides to the liver via the portal vein, through first-pass effects (Paul, 2019). Experimental exposure systems, including bi-cultures of gut epithelial cells and liver cell lines using transwell membranes, have been developed to study liver and intestine interactions (Lau et al., 2004; Choi et al., 2004). Future studies could utilize

the intestine-liver bi-culture system to investigate the toxicokinetic processes in intestine epithelial cells and how the resulting metabolome, along with detoxification mechanisms (such as efflux transporters) in the enterocytes could impact liver hepatocytes. For example, we could compare two bi-culture systems: both featuring intestinal cells on the apical side of a transwell insert and hepatocytes on the basal side. One system would involve intestinal epithelial cells expressing P-gp (e.g., Caco-2 cells), while the other would lack P-gp expression on the transwell insert membrane. Subsequently, one could compare the concentrations of chlorpyrifos and its metabolites, along with the molecular hepatotoxic responses, in liver cells after exposure to chlorpyrifos in the apical compartment. This exposure approach and comparison would help better understand the contribution of P-gp activity in intestinal epithelial cells on the toxicity in liver cells.

The research presented in this thesis was part of a larger project that aimed to identify and validate approaches that could be used to assess human and ecological risk of contaminated groundwater mixtures at a legacy-contaminated industrial site. Chemical analyses of groundwater samples obtained from the site revealed elevated concentrations of various agri-chemicals, petrochemicals, and metals exceeding environmental protection guidelines (Boamah et al., 2024; Gasque-Belz et al., 2023). *In vitro* toxicity assessment with the cell lines used in this thesis research demonstrated mostly non-cytotoxic effects in liver HepG2 cells and Caco-2 intestinal epithelial cells with exposure to 100% groundwater from two locations on the industrial site (from low- and high-impacted wells) using technique described in Niss et al. (2018). In addition, we did not observe significant effects of groundwater exposure on markers of apoptosis, cell cycle, or oxidative stress in HepG2 cells. Using the same methods described in Chapter 4, we evaluated and found no effects of groundwater exposure on intestinal barrier function in Caco-2 and IPEC-J2 cell lines. In parallel research conducted by a fellow graduate student, a rodent model was used to identify the potential target organs, biological processes, and likely drivers of toxicity associated with oral exposure to the contaminated groundwater. Their results supported our *in vitro* assessment in that there were no adverse impacts on liver and intestine following *ad libitum* oral exposure to 0.05% v/v contaminated groundwater for up to 60 days (Boamah et al., 2024). However, the testes, kidneys, immune system, and lungs were found to be target organs. Although there was concordance in toxicity of groundwater samples (or lack thereof) on the liver and intestine, there are challenges relating *in vitro* cytotoxicity to *in vivo*

organ toxicity, as highlighted through the findings of this thesis research. The correspondence between *in vitro* and *in vivo* outcomes suggests that the lack of toxicity observed *in vivo* can be predicted using simple *in vitro* exposures coupled with mechanistically informative, functional, and organ-specific endpoints. Future research could focus on investigating whether *in vitro* cell-based models employing organ-specific endpoints, have the capacity to detect toxicity in organs where adverse effects were observed *in vivo*, such as the kidneys, immune system, and lungs. Further studies into the correspondence between *in vitro* and *in vivo* outcomes will help validate the predictive potential of high-throughput and low-cost *in vitro* approaches in site-specific hazard assessments and risk identification for environmental mixtures to human health.

5.4 Overall Conclusion

This thesis research demonstrated that different cell lines may produce varied toxicity responses to pesticide exposure due to cell-specific differences in toxicokinetics and other intrinsic molecular features. These results also help further our understanding of how pesticides may interact through transporter-mediated mechanisms to amplify toxicity when present in mixtures and that such chemosensitisation should be considered in hazard assessment of pesticides using cell-based assays. The results obtained from the intestinal epithelial cell models emphasize that no single model is universally appropriate for all tested assays and toxicological questions. A systematic tiered-approach incorporating multiple cell lines with known capabilities and limitations would provide an alternative, enhanced testing strategy for capturing organ-specific toxicity responses that most closely reflect real-life scenarios. Finally, the results of this thesis research have direct implications for the selection of cell lines in the assessment and screening of pesticides and other environmental chemicals and raise questions regarding the interpretation of cell-based assays in the context of environmental hazard assessment.

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