APOPTOTIC AND BIOENERGETIC CHANGES IN PANCREATIC CELLS AFTER EXPOSURE TO INDIVIDUAL TOXICANTS AND THEIR MIXTURES

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Abstract: Pancreatic cancer has one of the worst fatality rates in oncology. Despite advances in treatment and recovery rates for other cancers, pancreatic cancer's lack of early symptomology culminates in a dismal five-year survival rate of 3-9% that has remained unchanged for decades. Cadmium has been implicated in the initiation of pancreatic cancer. Its prevalence in the environment make it a candidate for interactions with pesticides that have gone relatively unexplored. In this study, we examined the role of cadmium, pesticides, and their mixtures on the p53 apoptotic pathway and in the adaptive bioenergetics that foretell transition to a cancer state. We used 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) to establish a No Observable Adverse Effect Limit (NOAEL) which allowed us to identify sub-toxic chronic exposure concentrations to emulate realistic combination exposures for molecular evaluation of biological endpoints. These concentrations were used to examine p53 recruitment and downstream apoptotic markers phosphatidylserine (PS) and caspase 3/7 activity. Bioenergetic shifts were assessed by mitochondrial toxicity assays in the presence of glucose or galactose and evaluated in conjunction with mitochondrial membrane potential (MMP), oxygen consumption rates, and reactive oxygen species (ROS) production. Non-linear regression analysis led to the employment of 500nM Cd and 1µM Atrazine and Glyphosate test concentrations. Cadmium drove p53 response in HPNE cells while glyphosate and both mixtures increased p53 expression in tumor cells. Interestingly, only cadmium exhibited increased PS by annexin labeling, but mixtures showed significantly less PS, indicating a potential reinforcement of membrane integrity. No treatment groups in either cell line responded to treatment by increases in caspase activity. Taken together, we can surmise that the p53 apoptotic pathway is not initiated by exposure to these toxicants. Although ROS production was elevated in all groups and glutathione response was unaffected, it is unlikely the mild increase is responsible for differences in mitochondrial health. Summation of mitochondrial health using ATP production with cell membrane analysis is the lone parameter where mixtures behaved differently from their parts. Both mixtures distinctly presented as mitochondrial toxins and remodeled metabolism in a manner similar to that found in tumorigenic cells, indicating a potential pathway to carcinogenicity.

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CHAPTER I

INTRODUCTION

Project Summary and Background

Pancreatic cancer, PC, has one of the worst fatality rates in oncology with 57,600 new cases and 45,050 deaths estimated for 2020 (American Cancer Society 2020). PC is rarely symptomatic until late stages, and the majority of tumors are not diagnosed until stage IV and have metastasized (Sener et al. 1999; American Cancer Society 2020), making treatment difficult. According to the American Cancer Society, the five-year survival rates for a patient with a stage four diagnosis is only 3% (American Cancer Society 2020). PC is the third leading cause of cancer death, surpassing breast cancer, and will likely exceed colorectal cancer for the number two spot in the near future (Reynolds 2019). There are few risk factors defined for PC, but smokers nearly double their risk from their non-tobacco using counterparts. It has recently been found that pancreatic tumors contain regionally-dependent concentrations of cadmium. Within the tumor itself, cadmium concentrations are nearly 8-fold higher than non-cancerous tissue in the same pancreas. Areas surrounding the tumor core contain an intermediate concentration of cadmium (Buha et al. 2017; Djordjevic et al. 2019).

Cadmium is one of a family of heavy metals believed to cause cellular damage through various mechanisms, including the production of reactive oxygen species (Buha et al. 2017; Matović et al. 2015), interruption of DNA repair (Waisberg et al. 2003; Hartwig 2001), and toxic response as a result of binding with other compounds to form complexes that are able to modulate critical enzyme systems (Singh et al. 2017). Exposure to cadmium can occur through several routes, with the primary route of non-occupational exposure via the gastrointestinal system (Satarug et al. 2010). Inhalation and dermal adsorption of waste emissions from commercial or industrial processes, including mining and battery manufacturing, are other sources of cadmium (Singh et al. 2017; Waalkes 2003). With a half-life of thirty years in the body, its persistence in the environment can lead to bioacculmulation of this non-essential metal (Waalkes 2003; Schwartz and Reis 2000; Joseph 2009). Cadmium does not switch valence states as readily as other heavy metals, so it may exhibit decreased ability to produce reactive oxygen species and produce oxidative damage (El Muayed et al. 2012; Chang et al. 2013). Cadmium, like lead, can replace zinc, thereby affecting DNA, RNA, and protein synthesis, and by extension, cell division (Richter et al. 2017).

Glyphosate is the most widely applied pesticide in the world. Supposedly harmless to humans, its known mechanism of action is postulated to have effects on the gut microbiome, potentially altering the digestive dynamic that may involve pancreatic function (Nielsen et al. 2018). Atrazine, a triazine pesticide used in food crops, is also persistent in the environment, second only to glyphosate in use (Naidenko 2018). Comparing cadmium mixtures against both pesticides will give us a complete picture of pancreatic insults. Examining protein expression and bioenergetic response to cadmium-pesticide mixtures allows for the analysis

of mechanistic pathways. There are multiple potential targets for cadmium and cadmium-pesticide mixtures; p53 is a known tumor suppressor protein that often exhibits aberrant regulation in tumor formation of many types of cancers (Duffy et al. 2017). Inactivation or repression of p53 allows for the unchecked division of cells. Mutated or disrupted p53 is tightly tied to the cell's apoptotic machinery, leading to apoptotic dysregulation and uncontrolled cell growth (Al-Assaf et al. 2013). Other targets may include DNA repair mechanisms and bioenergetics. Examining systemic responses to cadmium/pesticide mixtures in pancreatic cells may give us insight into pancreatic carcinogenesis. While cadmium is identified as a known carcinogen, controversy exisits regarding glyphosate and atrazine toxicity. Early studies of glyphosate were primarily funded and performed by Monsanto itself, a clear conflict of interest. Atrazine toxicity, while shown to promote cancer in rats, has shown inconclusive results in human studies.

Due to their persistence in the environment, popular pesticides are a likely target for heavy metal interaction. Cadmium has been shown to interact synergistically with the organophosphate, Dimethoate, to effect relative body weight gain and liver weight increases of up to 25% when combined with one component at their NOAEL (Institóris et al. 1999; Singh et al. 2017). In mixtures, a response is considered additive when experimental results are the combined sum of the responses of its components. Additivity is considered the baseline for mixture toxicity. Synergism occurs when the combine response exceeds the additive response and antagonism occurs when the experimental response is less than additive. Other metal pesticide combinations have been shown to affect molecular fingerprints and altered immunity (Singh et al. 2017). Additionally, cadmium and glyphosate have been shown to affect acetylcholinesterase activity, potentially exhibiting combined

toxicity (Gupta et al. 2015). The literature on the carcinogenicity of organophosphates, such as Roundup™, is contradictory and dependent on the chemical in question. Complexes formed following the chemical interaction between pesticides and heavy metals like cadmium may be responsible for initiating mechanisms that lead to tumorigenesis. The EPA has stressed the importance of mixture toxicity studies, as toxicity in the environment is interactive rather than singular (Vanderslice et al. 1989). There are a small number of studies examing the effects of cadmium and pesticide co-exposure, but none have discussed the potential cellular impact. Identifying the molecular mechanisms underlying cadmium and cadmium/pesticide mixtures as they relate to pancreatic cancer can elucidate novel treatment options and potential early diagnosis.

Research Question

"Does cadmium metal, pesticides, and mixtures of cadmium and pesticides lead to the promotion of tumor growth and are these effects specific to a particular pesticide family."

Hypothesis

"Mixtures of cadmium and glyphosate or atrazine cause dysregulation in apoptotic pathways and/or bioenergetics leading to increased tumor development over cadmium or pesticide alone."

Research Aims

Aim #1: *Establishment of the toxicity threshold for cadmium, glyphosate, atrazine, and their mixtures on cell cultures of HPNE and ASPC1 pancreatic cell lines.*

Aim #2: *Effect of toxicant/mixture exposure on apoptosis in pancreatic cells.*

Aim #3: *Does toxicant/mixture exposure result in mitochondrial effects?*

Research Design and Methodology

Using current literature as a guide, we established biologically relevant toxicity thresholds, or NOAEL (No Observable Adverse Effect Level) for cadmium, pesticides, and the metal-pesticide mixtures in pancreatic cell lines HPNE and ASPC1 using nonlinear regression. Experiments used concentrations below our calculated NOAEL to determine if unexamined molecular pathways and protein interactions may be disrupted at this concentration. Viability testing was done using LDH and MTT assays to measure total cell number and viability. In the absence of cell cycle arrest, the effects of toxicity treatments with cadmium and its mixtures on apoptosis and bioenergetics were examined. Changes in p53, caspase, glutathione, annexin, ROS, ATP, and mitochondrial health were evaluated.

CHAPTER II

LITERATURE REVIEW

I. Environmental Pollutants and Prevalence

Pollutants are defined as atypical chemical substances within an organism or chemical substances over tolerated limits (Mathew et al. 2017). Exposure to pollutants has been implicated in a wide-range of health concerns from asthma to diabetes and heart disease (Kollmer 1991; El Muayed et al. 2012; Chang et al. 2013; Kumar et al. 2014; Kim et al. 2017). Environmental pollutants also play a role in cancer development (Parsa 2012; Lewandowska et al. 2019). Many of these pollutants have the ability to remain in the environment for prolonged periods, either as the parent compound, or as a potentially toxic metabolite such as glyphosate's metabolite, aminomethylphosphonic acid AMPA (Martínez et al. 2020; Kwiatkowska et al. 2020), making interactions with biological systems likely (Wade et al. 2002). AMPA was shown to induce oxidative stress and increase caspase activity in human neuroblastoma cells (Martínez et al. 2020). Additionally, many are lipophilic and can bioaccumulate in the body (Mathew et al. 2017).

Studies on environmental pollutants began as early as the 1900s, prompting the first water safety guidelines (Shifrin 2020). However, it was not until 1948 when the government responded to an environmental catastrophe. Extreme smog in Donora, Pennsylvania, killed 20 people and affected nearly half of the city's population, sparking the first environmental legislation (Jacobs et al. 2018). Still, it was not until the 1970s that the environmental movement became mainstream, due to the concern about factory and automobile emissions, particularly lead (Shifrin 2020). Lead, an additive to gasoline beginning in the 1920s, had known deleterious effects on human health and was finally phased out in the United States in the 1980s (Shifrin 2020). The Clean Air Act of 1996 effectively banned the use of leaded gasoline for automobiles in the United States. Allowances were made for its continued use in airplanes, race cars, farm equipment and marine engines (Bridbord and Hanson 2009). Some countries, like China, continued to use leaded gasoline well into the 2000s and beyond, making lead a continued global menace (Wang et al. 2019). While levels of lead have declined with legislative controls, this metal and others in its family do not decompose in the environment, making potential exposure a constant threat (Singh et al. 2017). Additionally, the transition to unleaded fuel involved the addition of a new additive to the environment, methylcyclopentadienyl manganese tricarbonyl, MMT, highlighting the inevitability of metal exposure (Lynam et al. 1990). The rise of industry and the expansion of chemical use to treat agricultural lands have also contributed significantly to the variety of chemicals humans are exposed to every day. With the increased exposure, there is little definitive evidence of how these chemicals affect human health, or how long they persist in the environment.

Today, the list of known pollutants is overwhelming, yet growing in number daily, with the EPA listing over 85,000 chemicals on its Toxic Substances Inventory (ATSDR 2008). It includes agricultural pesticides, industrial chemicals, heavy metals, health care products, and pharmaceuticals (El Helou et al. 2019). Due to their pervasiveness in the environment, contact with xenobiotics is inevitable. Humans are exposed to these chemicals through inhalation, dermal absorption, or consumption of the contaminated food and water. Airborne pollutants that reach the ground and can enter the ground water or remain in the soil, with uptake in the food chain as an ultimate end-point (Järup 2003; Jaishankar et al. 2014). Once inside the body, pollutants are absorbed, where they disrupt homeostasis through multiple mechanisms including damage to lipids,

IARC classifications of carcinogenic agents	
Group 1	Carcinogenic to humans
Group 2A	Probably carcinogenic to humans
Group 2B	Possibly carcinogenic to humans
Group 3	Not classifiable as to its carcinogenicity to humans
Group 4	Probably not carcinogenic to humans

EPA Cancer Classification	
Group A	Human carcinogen
Group B1	Probable human carcinogen
Group B2	Probable human carcinogen
Group C	Possible human carcinogen
Group D	Not classifiable as to human carcinogenicity
Group E	Evidence of non-carcinogenicity for humans

Table 1: A comparison between IARC and EPA cancer classification designations.

proteins, and DNA or by the dysregulation of bioenergetics and production of free radicals (Jan et al. 2015). All organ systems are affected, and clear associations between some toxicants and their target organs exist (Zona et al. 2014). For example, many heavy metals have multiple target organs. Lead is known to be both neurotoxic, causing

inhibition of acetylcholinesterase, AchE, in human erythrocytes , and nephrotoxic by inhibition of uric acid secretion (Järup 2003; Gonick 2008; Gupta et al. 2015). Mercury is associated with breast cancer through dysregulation of apoptotic mechanisms (R Wallace 2015). Some metals and insecticides, like chlorpyrifos, target acetylcholinesterase, an enzyme responsible for the hydrolysis of acetylcholine, affecting the nervous system (Gupta et al. 2015; Sandoval et al. 2019). Research into the unforeseen molecular consequences of toxicant exposure remains an area of significant interest. In addition, there is a heightened awareness that we are not exposed to singular chemicals, but mixtures of many chemicals, and the toxicological effects of those mixtures are largely undetermined.

Environmental pollutants may operate by various mechanisms to produce cellular damage. Some of the mechanisms include the production of reactive oxygen species (ROS), such as superoxide, hydroxyl, hydrogen peroxide, and oxygen radical. These radicals can damage normal cellular functioning and are often implicated in DNA damage (Hartwig 2013). Pollutants can be directly genotoxic or mutagenic, damaging DNA, or can indirectly alter epigenetic regulation of gene expression (Đukić-Ćosić et al. 2019). Interference in apoptotic machinery or autophagy has been studied in response to many toxicants (Kim et al. 2008; Đukić-Ćosić et al. 2019). Cadmium may block autophagy by interference in the fusion of autophagosomes and lysosomes and is known to induce activity of apoptotic caspases in a concentration dependent manner (Kim et al. 2008; Đukić-Ćosić et al. 2019). Alteration of protein structure or interference in protein-protein interactions may underlie this disruption (Koedrith and Seo 2011; Huang et al. 2014; Chen et al. 2015). Inorganic metals in particular can have a high affinity for

estrogen receptors and affect gene expression in cells (Wallace 2015). Some inorganic metals like mercury and cadmium act as metalloestrogens, which exert estrogenic effects due to their affinity for estrogen receptors (Wallace 2015). They can disrupt the endocrine system and dysregulate cellular signaling mechanisms (Mumtaz et al. 2002; Wade et al. 2002). Effects can be dependent on several factors, including exposure dose, exposure duration, genotype and nutritional status (Wang and Fowler 2008). Although there have been multiple attempts to categorize exposures, the United States Department of Occupational Safety and Health Administration says exposures are considered short-term, or acute, when the duration is brief, and chronic when contact is continuous. In toxicology, an acute exposure can also refer to a high dose finite exposure (Connor 2019). Cellular response to low-dose chronic exposure can be vastly different from more concentrated acute exposures (Dorian et al. 1992; Roberts et al. 2012; Jaishankar et al. 2014; Van Bruggen et al. 2018).

II. Metals and Pesticides as Pollutants

There is pervasive environmental exposure to pesticides and heavy metals, inducing stress both individually, and potentially by toxicant combinations. Heavy metals are naturally occurring inorganic elements with high densities (Tchounwou et al. 2012). The term heavy metal has been debated between different scientific disciplines (Duffus 2002), but has become a generalized term for metals exhibiting toxicty. Heavy metals have diverse applications in industry, health care, and technology, leading to broad global distribution. While some are essential nutrients, like zinc, magnesium, or iron, others have no known biological function. Essential metals play a role in multiple

cellular processes. Zinc plays a role in DNA repair machinery and is prevalent in proteins associated with the response to oxidative stress (Pieper et al. 2015). Copper is a cofactor for the free radical scavengers catalase, peroxidase, and superoxide dismutase (Tchounwou et al. 2012). Other essential elements may make up key enzymes or play roles in signaling or physiologic redox reactions (Hartwig 2001; Schröder et al. 2009; Mulware 2013; Sears 2013). Arsenic, chromium, lead, cadmium, and mercury are five non-essential metals that have been specifically studied for their detrimental effects on human health at low exposure doses (Tchounwou et al. 2012). The Environmental Protection Agency (EPA) and the International Agency for Research on Cancer (IARC) have identified these pollutants as either known or probable human carcinogens. These classifications can be found in table 1. While toxicity is routinely dependent on dose, exposure time, and other factors like genetics or nutrition, it is thought that their ability to bioaccumulate increases human risk for disease and affects multiple organ systems in the body. Yousafzai et al. in 2017 showed heavy metal accumulation in tissue with high metabolic rates, particularly the liver (Yousafzai et al. 2017) . The elevation of methylmercury concentrations in marine organisms is several orders of magnitude higher than the surrounding water. Its biomagnification ability has been established in several studies (Glasson and Tuesday 1970; Harding et al. 2018). Accumulated metals then enter the food chain and are ultimately consumed by humans, where they become systemic toxicants affecting multiple organ systems at trace concentrations < 10 ppm (Tchounwou et al. 2012). Once inside the body, heavy metals are eliminated slowly increasing their capacity to interact with cellular organelles, membranes, and proteins. Cellular responses to metal exposure can initiate apoptosis or cellular death, although the exact mechanisms

are unclear (Tchounwou et al. 2012; Wu et al. 2016). It is known that the affinity of these metals for redox cycling makes them prolific producers of free radicals (Chen et al. 2018). These radicals then go on to interact with biological molecules, including DNA. Although radicals play a role in signaling and adaptation to nutrient and oxidative changes in the environment (Schieber and Chandel 2014) , when ROS production outpaces scavenging mechanisms, free radicals may cause DNA damage, lipid peroxidation, mitochondrial dysfunction, and inflammation, all associated cancer phenotypes (Belyaeva et al. 2006; Chang et al. 2014; Chen et al. 2018). Glutathione (GSH), the body's primary responder to oxidative stress, is responsible for mediating the damage (Singhal et al. 1987; Sobrino-Plata et al. 2014). It has been noted in multiple studies that glutathione levels may be reduced by chronic metal exposure (Duruibe et al. 1989; Ivanina et al. 2008; Schröder et al. 2009; Li et al. 2016), further exacerbating toxic effects and providing a pathway to disease.

Once heavy metals have sequestered in the body, very few can be eliminated easily by metabolic processes (Jaishankar et al. 2014). While low dose chronic exposures are likely to go untreated, chelation therapy is the standard treatment for acute metal exposure. Chelators are organic molecules with high affinity for metal ions (Kontoghiorghes 2020). Chelators bind to metals forming a soluble ring structure that can be easily excreted through the kidneys (Sears 2013). These complexes can have varying solubility, with some having greater hydrophilicity, and others having increased lipophilicity resulting in greater penetration of membranes (Sears 2013). Most chelators are non-selective rather than metal-specific and tend to have variable efficacy as a treatment regimen. Care must be taken in their administration not to disrupt the balance

of essential metals necessary for physiological function (Flora and Pachauri 2010). Calcium has been found to bind to chelators to induce hypocalcemia, resulting in dangerously low circulating calcium concentrations (Flora and Pachauri 2010; Sears 2013). EDTA is the most prevalent chelating agent, and was initially used as a chelator for lead (Flora and Pachauri 2010). EDTA is less efficacious for iron and may even increase its toxicity, instigating the use of deferoxamine, DFOA, to treat iron toxicity, eliminating its ability to participate in oxidative reactions while bound (Flora and Pachauri 2010). Synthetic chelation agents must also compete for binding with endogenous chelators like metallothionein. Some animal studies have determined that metals bound to metallothionein will exhibit an increased toxicity when they dissociate from metallothionein (Nordberg et al. 1975). Scant literature exists testing other chelators against this premise. Cadmium specifically has been found to upregulate and bind with metallothionein (Nordberg 1977; Cai and Stillman 1988; Dorian et al. 1992; Sears 2013), accounting for much of the cadmium deposited in the tissues. There are other health concerns as well. Dimercaprol is the mainstream treatment for arsenic poisoning. Originally produced as an antidote to mustard gas (Sears 2013), it induces dangerous side effects, like high blood pressure and fever (Flora and Pachauri 2010). These complications have led researchers to replace it with alternatives like DMSA and DMPS (Sears 2013). Ideally, chelating agents bind to toxic metals to form complexes that can be more easily excreted (Kontoghiorghes 2020; Flora and Pachauri 2010). However, the metal-chelator binding may also work to increase tissue deposition of complexes, or metals may be reabsorbed into the hepatic or renal circulations during excretion (Sears 2013). Considerations associated with chelation therapy include; pH,

bioavailability, and affinity of the chelator for the metal, require the continued search for treatments and combination therapies. One novel chelator, introduced in 1964, was glyphosate. Able to bind micro and macromolecules alike, glyphosate found use as a chelating agent long before its herbicidal properties were discovered (Mertens et al. 2018).

Concomitant with heavy metals, pesticides represent another environmental hazard. The term pesticide is a general term that includes fungicides, rodenticides, insecticides, and herbicides. The first recorded evidence of pesticide use dates over 4500 years ago to the Sumerian culture, which used sulfur as an insecticide (Unsworth 2010). Heavy metals themselves were used as pesticides in China, employing arsenic and mercury as a treatment for lice (Unsworth 2010; Shaban et al. 2016). Along with population growth, there was a concurrent increased demand for the development of chemical-based pesticides. There are multiple families of these pesticides, including organochlorines, organophosphates, triazines, and carbamates, each with different mechanisms of toxicity (Andreotti et al. 2009; Hernandez et al. 2019). Figure 1 contains a sampling of the most common pesticide families. Additionally, it is apparent that many pesticides within the same family work through differing mechanisms. The Pope lab in 1999 postulated that while organophosphate pesticides may share a common mechanism, variability in their targets allows for differences in their toxicological effects (Pope 1999). Some organophosphates act as direct inhibitors of the acetylcholinesterase enzyme and are not only used agriculturally, but are the active component of some nerve gases, such as sarin and VX gas (Wu et al. 2018). Others are involved with the uncoupling of oxidative phosphorylation (Karami-Mohajeri and Abdollahi 2013).

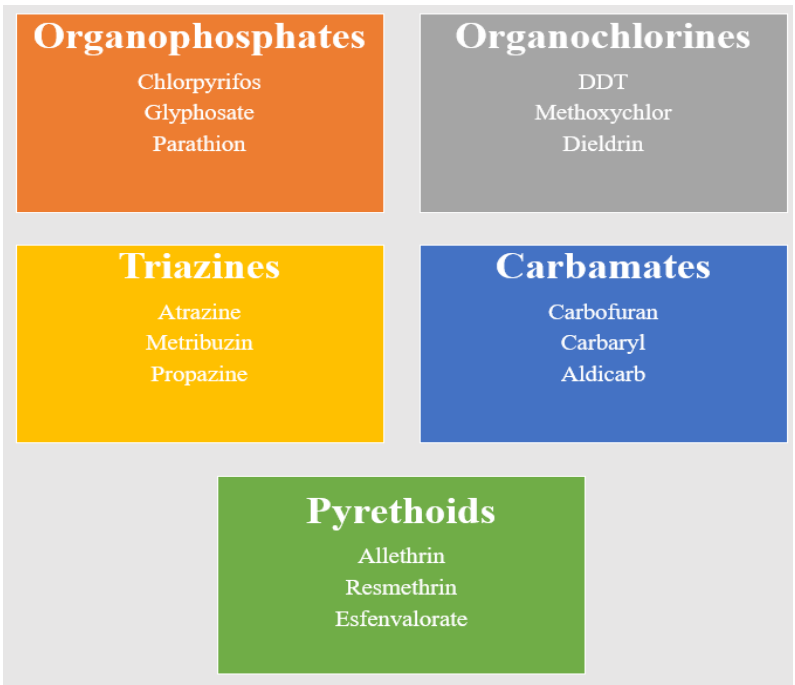

Figure 1: Pesticides are often grouped into families based on functional groups and similar modes of action.

Members of the triazine family are classified as endocrine disruptors and can interfere with or mimic the body's hormones to cause deleterious health effects (Kabir et al. 2015). Multiple pesticides across all families are suspected carcinogens (George et al. 2010; Jowa and Howd 2011; Albanito et al. 2015; Mathew et al. 2017; Andreotti et al. 2018). The ubiquity of pesticides in the environment make human exposure to them assured. Some modern food crops have been genetically altered to tolerate the mass application of these toxins (Bradberry et al. 2004). After World War II, new compounds were synthesized rapidly and applied without much oversight, including dichlorodiphenyltrichloroethane, an organochlorine known as DDT. Discovered to be a potent insecticide by Dr. Paul Muller, who won a Nobel Prize for his work, DDT was a broad-spectrum insecticide, killing a wide variety of species (IUPAC 2009). DDT was

cheap to produce and had the additional benefit of combating insect-borne illnesses like malaria and typhus (Epa and OCSPP 2014; Conis 2017). The utility of DDT quickly prompted its widespread use with an insufficient examination of its safety. It was not until 1972, after the establishment of the EPA, and decades of accumulated evidence of toxicity, that a cancellation order for DDT was given by lawmakers (OCSPP 2014). It is classified as a probable human carcinogen, with both liver and reproductive dysfunction being correlated with exposure (OCSPP 2014). DDT is still used today in developing countries where the risks insect-borne disease, such as malaria, are great and the general concern of DDT toxicity in the population are ignored. Similar to DDT, 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD; Agent Orange) is a powerful herbicide that was used by the military to clear trees and brush (Chamie et al. 2008). It is comprised of equal parts of 2,4 D, which is still in use, and 2,4 T, which is a contaminant from the manufacturing process (Chang et al. 2014). In 1997, TCDD was classified as a class 1 carcinogen by the IARC, citing positive correlations with several cancers including prostate cancer and lung cancer (Chang et al. 2014).

Outside of direct contact and absorption through the skin, pesticides can infiltrate the human body by ingestion of contaminated food or water (Roberts et al. 2012; Kim et al. 2017). Surveys of streams in the United States indicate up to 90% of the water supply may be contaminated with at least one pesticide (Norman et al. 2020). Upon deposition in the soil, pesticides can sequester for weeks to months without degradation dependent on the soil chemistry and climate conditions. From there, they may be taken up the food chain and biomagnified. Many lipophilic compounds, like DDT, can be stored within adipose cells for extended periods prior to degradation or inactivation to inert or toxic

metabolites (Katagi 2010). Many pesticides rely on powerful combinations of 'inert' ingredients and surfactants to facilitate cellular entry. Each of the inactive components can biodegrade into metabolites that may have toxicity of their own, or can amplify the toxic response initiated by the active ingredient (Benachour and Séralini 2009). Glyphosate's primary metabolite AMPA works synergistically with the POEA surfactant in Roundup™ formulations to damage cell membranes in vitro (Benachour and Séralini 2009). There is evidence indicating polyethoxylated tallow amine (POEA), the surfactant used in the formulation Roundup™, may be more toxic than glyphosate itself (Bradberry et al. 2004; Benachour and Séralini 2009; Mesnage et al. 2013; Defarge et al. 2018).

. The metabolism and elimination of pesticides may be complicated. While some appear to be excreted primarily in the parent form, others can form toxic metabolites, and others do both (Katagi 2010; Myers et al. 2016; Xu et al. 2017; Kwiatkowska et al. 2020). There appear to be vast metabolic differences between animal studies and human exposure studies where this is concerned. For example, the half-life of glyphosate in humans was estimated to be over 33 hours based on studies in rats (Järup and Akesson 2009; Buha et al. 2017), but in 2019, Connolly et al. determined glyphosate's half life to be as little as seven hours using human urine samples. Half-life values both biological and environmental are highly dependent on interactions between glyphosate and other molecules.

III. Mechanisms Underlying Cancer Development

The first recorded cancer evidence came from the Egyptian physician, Imhotep, in 2500 BC (Mukherjee 2010). Alongside it was the notation, "**no cure**." It was not until Hippocrates in ancient Greece that the condition was given a name, and the search for treatment is first documented. The term cancer comes from the Greek meaning crab in reference to the projections that extend from the tumor core. The accepted definition now refers to a group of diseases involving uncontrolled division of abnormal cells capable of metastasis (Skuse 2015). The biochemical fingerprint of specific cells, even within the same tumor, may be vastly different (Pedraza-Fariña 2006). Tumorigenic mechanisms are complex and varied, but science has identified several pathways that many cancers have in common, and they can be initiated both internally and environmentally.

Cancer cells are an extension of normal cells that have proliferated without the constraints of normal biological checkpoints (Pedraza-Fariña 2006). There can be a disruption of apoptotic mechanisms, a dysregulation of cell proliferation pathways, or both. The increased bioenergetic demand of cancer cells implies the potential involvement of mitochondrial dysfunction. Genetic factors also appear to play a role in tumorigenesis, and while some cancers seem to run in families, through the transmission of one or more genes, other cancers are still unclear. Evidence also indicates that genetic mutations, epigenetic changes, and errors in DNA repair mechanisms, may play a role in the development of cancer.

Apoptosis, or programmed cell death, is highly conserved in the animal kingdom and found in both primitive and advanced organisms. This pathway gives cells the ability to rapidly eliminate defective cells before harming the organism. Interference in apoptotic pathways is established as a property of most cancers (Eneman et al. 2000; Matsumoto et al. 2002; Waisberg et al. 2003; Waalkes 2003; Osada et al. 2010; Wu and Bratton 2013; Kolodecik et al. 2013; Hajrezaie et al. 2015).

Tumorigenesis likely occurs through the double failure of proto-oncogene activation concurrent with the silencing of tumor suppressor proteins (Mukherjee 2010), and the ratio of pro-apoptotic and anti-apoptotic proteins may be crucial in the development of the cancer state (Pistritto et al. 2016). Proto-oncogenes, such as KRAS or MYC, are generally involved in cellular proliferation and their protein products are considered to be anti apoptotic or pro proliferative (Teng 2000). Mutations in these genes can lead to unchecked proliferation. The Bcl-2 family of proteins have been found to regulate apoptosis and are associated with the outer mitochondrial membrane (Li et al. 1998; Abarikwu et al. 2011; Pistritto et al. 2016). These proteins regulate the release of cytochrome c, activating caspase 9 (Chatterjee et al. 2008; Abarikwu et al. 2011). Members of this protein family include both pro-apoptotic isoforms like Bax, as well as anti-apoptotic forms like Bcl-2. The ratio between these proteins is mediated by tumor suppressors such as p53, which has been shown to inhibit Bcl2 and upregulate Bax (Hemann and Lowe 2006; Pistritto et al. 2016). Tumor suppressor proteins like p53 and p21 help initiate apoptosis and are considered to be pro apoptotic. Though caspase independent apoptosis pathways do exist, they are not well studied and appear to be rare (Elmore 2007). Apoptosis is carried out by the caspase family of proteins with caspase 3

and caspase 7 being the main executioners Caspase 8 is the primary initiator caspase of the external or death-ligand pathway, while caspase 9 recruits the executioners in internal pathways caused by cellular stressors that converge at the mitochondria (Pistritto et al. 2016). While it is generally thought that there is little crosstalk between these pathways, Li et al. suggested cells with insufficient caspase 8 compensated via caspase 9 initiation (Li et al. 1998). Importantly to cancer development, insufficient or decreased levels of caspase 9 was found to contribute to apoptotic resistance, leaving an organism unable to clear defective cells (Kuida et al. 1998; Hakem et al. 1998; Green 1998), and its activation may occur with sustained normal mitochondrial permeability and membrane potential values (Green and Reed 1998; Hakem et al. 1998). Figure 2 diagrams the p53 apoptotic pathway.

Tumor suppressor protein p53 has been called the guardian of the genome due to its anti-tumor functionality and dysregulation in nearly 50% of all cancers (Hemann and Lowe 2006). Pro-apoptotic proteins like p53 are either inactive or under-expressed in most human cancers, allowing for uncontrolled growth (Herrero et al. 2016). This dysregulation can directly alter the p53 protein, or can stimulate a negative regulator of p53, like MDM2 or AKT (Abraham and O'Neill 2014; Hamilton et al. 2014; Herrero et al. 2016; Wang et al. 2016). Some cancers, such as lung adenocarcinomas, exhibit alterations in multiple targets of the p53 pathway (Wasylishen and Lozano 2016). Mutations of p53 are primarily missense mutations (Yue et al. 2017) and promote loss of

its ability to control proliferation or initiate apoptosis. However, oncogenic gain of function attributes have also been identified, suggesting a dual action of p53 in the regulation of tumor formation. Hanel et al. determined in mice that mutant p53 not only

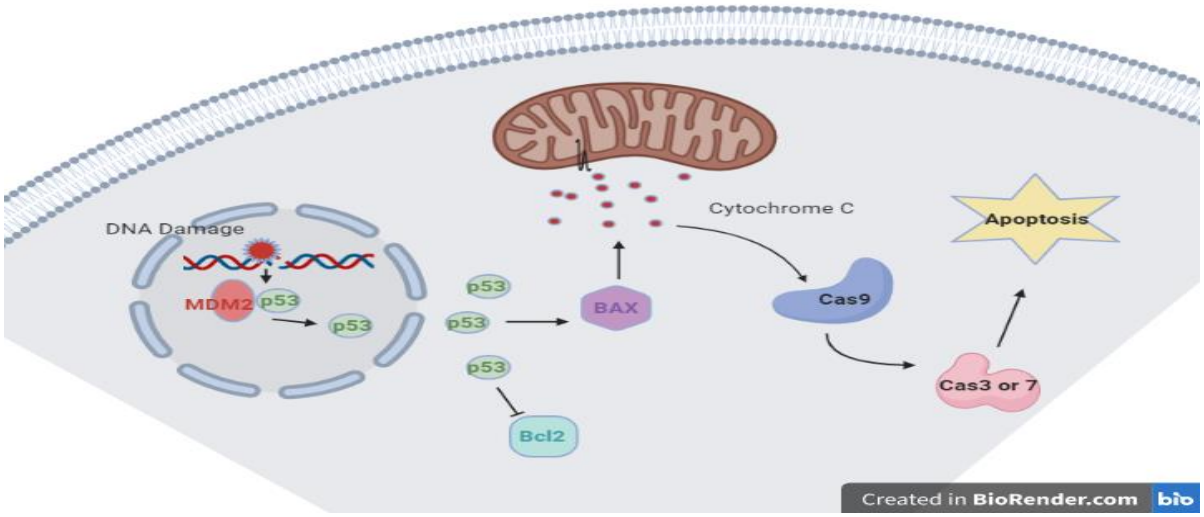

Figure 2: p53 can be activated via DNA damage and cellular stress pathways to mediate apoptosis. Phosphorylation of the p53 active site removes MDM2's association with p53, enabling signaling to pro apoptotic protein BAX. BAX initiates the release of cytochrome c by reducing mitochondrial membrane potential. Cytochrome c release activates caspase 9, which then mobilizes executioner caspases 3 and 7.

lost its suppressive properties, but had a significantly increased spectrum of tumor development as well as greater metastases and shortened survival (Hanel et al. 2013). Different mutants express different gain of function phenotypes that can include both changes in nuclear and cystolic functionality. Zhang et al. determined that mutant p53 translocated the GLUT1 transporter to the plasma membrane initiating the Warburg Effect, causing cells to transition to aerobic glycolysis for energy (Zhang et al. 2013).

The increased demand for energy required for sustained growth has implicated mitochondrial dysfunction as key in tumorigenesis. In 1920, Otto Warburg discovered

that cancer cells may have an increased glucose uptake and produce lactate via fermentation pathways even in the presence of oxygen and functional mitochondria (Zong et al. 2016; Liberti and Locasale 2016). Aerobic glycolysis is preferable to cells that require increased ATP production without the concurrent production of free radicals (Milkovic et al. 2019). This suggests the cell's ability to rewire itself to promote proliferation, leading some to suggest this may be indicative of a cell's transition to a cancer state. Mot et al. developed methodology to induce oxidative phosphorylation *in vitro*, postulating the switch from glycolysis may transition these cells back to a normal healthy metabolism (Mot et al. 2016).

Free radicals are a byproduct of oxidative phosphorylation and normal cellular metabolism, but as free radical content increases the development of cancer increases (Dally and Hartwig 1997; Liu et al. 2009; de Sá Junior et al. 2017; Djordjevic et al. 2019). Reactive oxygen species are produced by reducing oxygen with the addition of electrons and include superoxide, hydrogen peroxide, hydroxyl radical, and nitric oxide radicals (Sharma et al. 2012). They can accumulate through overproduction, failures of scavenging mechanisms, or insufficient antioxidant production (de Sá Junior et al. 2017). Their bipartite effects suggest they play have a concentration-dependent role in modifying carcinogenesis. Damage due to excessive free radical formation include; DNA fragmentation, lipid peroxidation, and protein oxidation. However, there is evidence that free radicals may have an essential role in cell signaling. Holmstrom and Finkel indicated an association between ROS and immune system functioning, stem cell self-renewal, tumorigenesis, and aging (Holmström and Finkel 2014). Studies indicate excessive amounts of ROS production trigger anti-tumor apoptotic machinery, but more

moderate concentrations induce genomic mutations that can stimulate unchecked invasive growth as shown in figure 3 (Holmström and Finkel 2014; Milkovic et al. 2019). This ability, coupled with dysregulation of DNA repair machinery, are associated with enhanced tumor development.

At its foundation, cancer can be considered a genetic disease, with somatic cells expressing the ability to transition into tumor cells with varying karyotypes (Knudson 2002). While some genetic risk factors have been identified for various cancers, the American Cancer Society reports that only 5 to 10 percent of cancers occur through inherited mutations (American Cancer Society 2020). Genomic libraries of several cancers have been constructed, and thousands of different genes have been identified as upregulated or downregulated, influencing the cancer state (Pedraza-Fariña 2006; Filbin

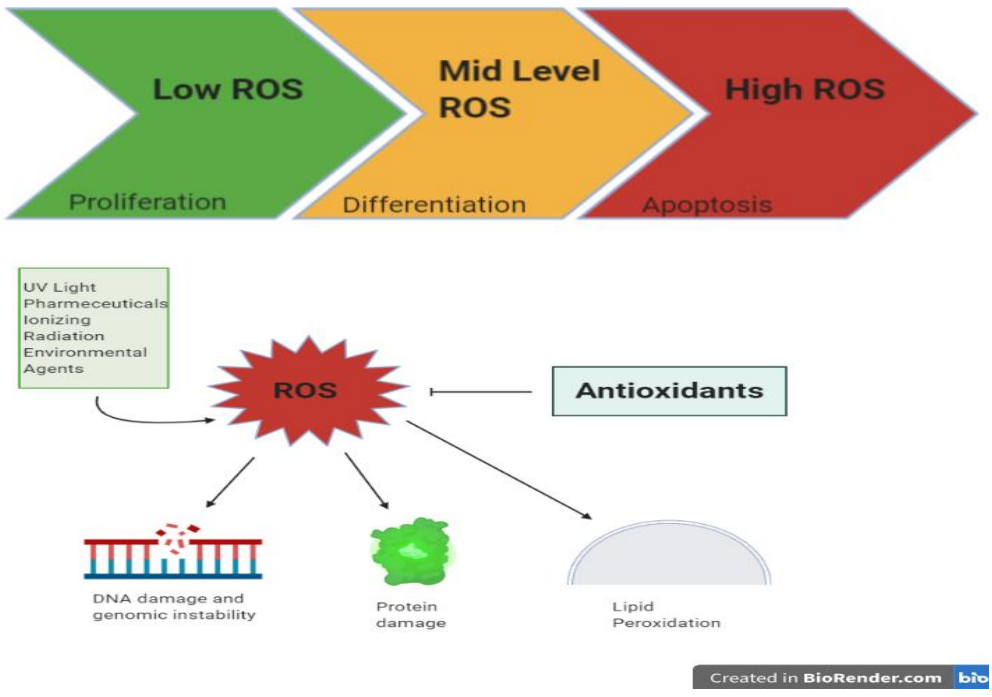

Figure 3: Reactive oxygen species can be produced by normal metabolism or numerous external insults. These free radicals have multiple targets for effect including proteins, cell membranes, and nucleic acids. Heavy metals and pesticides may produce ROS concurrent with a reduction in antioxidants like glutathione.

and Monje 2019). Carcinogenesis is often characterized by ineffective DNA repair mechanisms, allowing mutations and DNA lesions to proliferate unchecked (Knudson 2002; Parsa 2012; Osterman et al. 2014; El Helou et al. 2019). DNA fragmented by cellular injury or errors in mitosis cannot be correctly remedied due to impaired functioning of the repair machinery. Outside of nuclear DNA, Tan et al. determined that depleting mitochondrial DNA compromised tumorigenesis and transfers of mtDNA from a host cell could affect mitochondrial function (Tan et al. 2015). Epigenetic changes that specifically alter gene expression are common in many cancers. Aberrant hypomethylation is a hallmark of gliomas (Filbin and Monje 2019), and many cancers exhibit global hypomethylation with coincident hypermethylation at specific loci (Knudson 2002; Huang et al. 2008; Doi et al. 2011; Buha et al. 2017; Liu and Pilarsky 2018; Hirao-Suzuki et al. 2018).

Interference of various biochemical processes by pollutants is critical for their role in carcinogenesis. The toxicant itself may have the ability to replace essential elements necessary for normal function, or even alter the structure of particular proteins, rendering them inactive (Hartwig 2001; Bertin and Averbeck 2006; Joseph 2009; Koedrith and Seo 2011; Guilherme et al. 2012; Meza-Joya et al. 2013; Buha et al. 2017). Alternatively, toxicants such as glyphosate can interfere with essential minerals. Glyphosate's ability to chelate metals, specifically iron and zinc, make these nutrients unavailable for their essential functions and may allow for the mobilization of more toxic metals such as cadmium instead (Mertens et al. 2018). Interference in antioxidant response systems has been detected, notably a decrease in glutathione expression in response to both pesticides and metals, particularly glyphosate and cadmium (Ivanina et

al. 2008; Sobrino-Plata et al. 2014). Many heavy metals have been found to replace essential metals in biomolecules. Cadmium replacement of zinc in DNA repair mechanisms promotes genomic instability, and (Jin et al. 2003) found it prohibited mismatch repair in yeast.

Many pollutants initiate the production of free radicals, leading to DNA lesions or interference in cellular respiration. Many metals such as mercury, arsenic, lead and cadmium are known free radical generators and have been reported to decrease the activity of free radical scangers like catalase, glutathione, and superoxide dismutase (Ercal et al. 2001; Schröder et al. 2009; Jan et al. 2015). The effects of pesticides like glyphosate on antioxidant activity in animal studies is more conflicting and may be dependent on both species and organ system. A study in goldfish showed whole formulation Roundup^{TM} could decrease GSH activity in the liver while increasing catalase activity in the liver and kidney (Lushchak et al. 2009). In zebrafish, antioxidant systems were reported to be largely unresponsive to low glyphosate exposures, but decreased superoxide dismutase activity was recorded in the liver in response to a 58 µM exposure (Guilherme et al. 2012), and in piglets, glyphosate significantly increased catalase and superoxide dismutase in the duodenum across all treatment groups starting at a 10 ppm concentration, but had no effect in the jejunum at low doses (Qiu et al. 2020). There are specific mutagenic and epigenetic alterations in DNA methylation and histone modifications associated with different pollutants (Ray et al. 2014). Lead can dysregulate methyltransferase expression in rats, indicating epigenetic activity (Schneider et al. 2013; Nye et al. 2015). Micro RNA regulation of cellular proliferation has been reported (Basu et al. 2010; Rawat et al. 2019). The function of miRNA can be altered by exposure to

pollutants and have a role in carcinogenesis by regulating cellular proliferation (Stahlhut Espinosa and Slack 2006; Basu et al. 2010; Rawat et al. 2019). miRNA16 expression is increased in many tumor cells and protects the cell through proliferative Bcl2 protein, protecting the tumor cell from apoptosis and increasing its metastatic potential (Basu et al. 2010). There are indications that pesticides may also have the ability to regulate gene expression through activation of enzymatic activity of deacetyltransferase or methyltransferases (Kim et al. 2016).

IV. Pancreatic Cancer – Causes and Mechanisms

Pancreatic cancer (PC) is one of the deadliest documented malignancies. Despite improvements in the treatment and prognosis of other cancers, the five-year survival rate of pancreatic adenocarcinoma is 9% for all stages (American Cancer Society 2018; Rosenzweig 2019). Because pancreatic cancer is asymptomatic until late in the progression of the disease, more than half of diagnoses do not occur until the cancer has spread outside the pancreas, dropping the survival rate to 3%, a value relatively unchanged since the 1960s (Ansari et al. 2016). Contributing to this poor prognosis is the lack of early diagnostics and lack of effective treatments for late-state PC (Wood et al. 2019). The 2018 American Cancer Report identifies PC as the eleventh most commonly diagnosed cancer, estimating 55,440 new diagnoses and 44,340 deaths for the upcoming year. Projections predict it to become the second deadliest cancer following lung cancer by 2030 (Rahib et al. 2014). The risk of a person developing PC is about 1.4%, a relatively small number, but a number that hasn't declined in the last couple decades like

many other cancers (Ansari et al. 2016). Although the pancreas has both endocrine and exocrine function, PC is derived largely from cells in the ductal epithelia, with only 5% occurring in insulin-producing endocrine cells (Ansari et al. 2016; Amundadottir 2016).

Identifying the risk factors for PC has been difficult and the reports are mixed and equivocal. Among environmental toxicants, the only risk factor consistently identified is smoking (Nitsche et al. 2011; Yuan et al. 2017; Nimmakayala et al. 2018). Smokers and tobacco users appear to run an increased risk of not only developing PC but also exhibit higher mortality rates (Yuan et al. 2017). Cigarette smoke is a noxious mixture of pollutants that include hydrogen cyanide, formaldehyde, carbon monoxide, benzene, radioactive elements such as uranium, and several damaging heavy metals like arsenic, lead and cadmium (American Cancer Society 2017). Second hand smoke can increase lung cancer risks 20-30% in non-smokers (CDC 2018) with increased risk also associated with breast cancer and leukemia (Office on Smoking and Health (US) 2010). Although bystanders incur decreased risk of pollutant exposure with e cigarettes, vaping aerosols contain many of the pollutants found in traditional cigarettes, including heavy metals (Office on Smoking and Health (US) 2010). Genetics accounts for roughly 10% of pancreatic adenocarcinoma diagnoses (Ansari et al. 2016; Barone et al. 2016), which translates into a moderately elevated risk approximately 1.8 times normal (Jacobs et al. 2010).

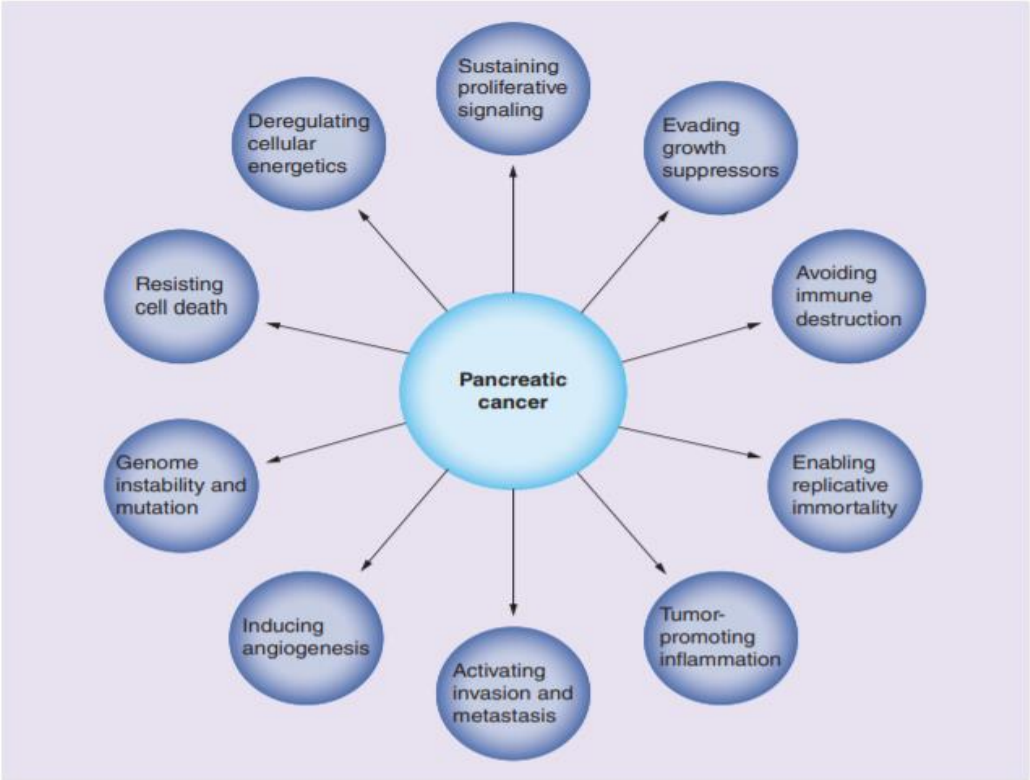

Figure 4: Mechanisms involved in pancreatic cancer initiation and promotion. (Ansari et al. 2016; Hanahan and Weinberg 2011)

There is a discussion of whether these correlations are attributed wholly to the genome or in part to the similarity of environmental conditions and exposures present in a particular shared space (Amundadottir et al. 2004). Familial studies have identified several cancer driver genes, with KRAS and p53 being two of the most prevalent in PC (Lucas et al. 2013; Kahlert et al. 2014; Holst et al. 2017). KRAS oncogene mutations appear in more than 90% of cases, (Grigor'eva et al. 2014; Mann et al. 2016; Wood et al. 2019), affecting all cellular processes including cell proliferation. Mutations in tumor suppressor protein p53 are also commonplace, and testing has shown PC tumors likely to show co-occurrence of these two alterations with an odds ratio of 1.56 meaning that you are 1.56-

times more likely to develop PC with the two mutations than if you had no mutations (Lu and Zeng 2017). In a study by Kahlert et al., both patient serum and tumor cell exosomes were used to isolate DNA fragments found to contain both KRAS and p53 mutations concurrently (Kahlert et al. 2014). In addition to mutation, epigenetic restructuring has also been implicated in PC initiation. Many studies have assessed methylation of central promoter sites to identify an epigenetic biomarker profile (Kisiel et al. 2015; Liu and Pilarsky 2018; Eissa et al. 2019). Figure 4 summarizes the pancreatic cancer profile.

Although the specific pathways are unclear, the utilization of several mechanisms for initiation and promotion are involved in PC development. KRAS oncogene signaling plays an important role in multiple pathways, including cellular metabolism, autophagy, cellular proliferation, DNA repair, and apoptosis (Deer et al. 2010; Rachagani et al. 2011; Kolodecik et al. 2013; Grigor'eva et al. 2014; Mann et al. 2016; Kamisawa et al. 2016; Chuang et al. 2017; Doiron and DeFronzo 2018).

Evidence shows that KRAS mutations may also affect the response to environmental toxicants, potentially increasing the risk for PC development, (Kolodecik et al. 2013), and highlighting the multiple modalities of action. Prevailing theories include dysregulation of DNA repair mechanisms and disruption of apoptotic pathways (Osterman et al. 2014; Piciucchi et al. 2015). DNA damage is an intermittent consequence of multiple factors, including environmental toxicant exposure as well as normal metabolism. The body has mechanisms and systems to prevent DNA lesion accumulation, which can lead to genomic instability and tumorigenesis (Osterman et al. 2014). Using primary pancreatic tissue samples, Osterman et al. identified elevated levels of activated DNA damage recognition proteins in malignant samples compared to

controls, indicating an elevated repair response in tumor cells. Once activated, the damage is repaired, or the apoptotic machinery is initiated (Osterman et al. 2014). Evidence also exists of disruption of apoptotic pathways in PC (Perugini et al. 2000; Matsumoto et al. 2002). Increased genomic instability produced by DNA damage coupled with reduced apoptosis can generate conditions favorable to tumor development.

Pancreatic adenocarcinoma was first documented in 1761 by Giovanni Battista Morgagni, an Italian anatomist and the father of anatomic pathology (Ansari et al. 2016). A more definitive description came in 1858 with the advent of the microscope, but effective surgical techniques did not follow until 1937 (Ansari et al. 2016). Despite subsequent surgical advances in pancreatic resection procedures and technology, the five-year survival rate has remained unchanged. The lack of survival rate change can be attributed in some part to the limited response to chemotherapy and radiation protocols in pancreatic adenocarcinoma (De La Cruz et al. 2014; Piciucchi et al. 2015; Ansari et al. 2016).

V. Cadmium and Cancer

Early identification is imperative in increasing the survival of PC, and several external risk factors have been identified. Although most environmental factors are considered controversial, smoking is the exception. Smoking may double the risk of developing PC and is involved in 20-30% of cases (Nitsche et al. 2011; Yuan et al. 2017; Nimmakayala et al. 2018). Additionally, smokers and tobacco users show a 40% increased risk of death from pancreatic cancer than their non-smoking counterparts (Yuan et al. 2017). In a 2017 analysis of 1,037 PC patients, Yuan et al. assessed tobacco usage

and mortality data, finding that mortality rates were significantly higher for smokers than for both former and non-smokers. Using cell culture techniques, research by Nimmakayala et al. showed an increase in stem cell markers from pancreatic cells after a 20-week exposure to cigarette smoke extract, alluding to the potential of the toxicant to de-differentiate pancreatic cells. The compound mixture's ability to induce these changes could not be replicated in vivo (Nimmakayala et al. 2018). Cell culture and animal studies have also identified cadmium, a component of cigarette smoke, as a potential risk factor for PC (Buha et al. 2017; Wallace et al. 2019), finding concentrations of cadmium of up to 14 ppm at the tumor's core, a value up to 7 times higher than non-tumor tissue. Other risk factors generally considered include chronic pancreatitis and obesity (Piciucchi et al. 2015), although these findings are less consistent. Studies on other factors, including obesity, and pollutant interactions, hope to further elucidate the etiology of pancreatic cancer initiation and are done using both animal models and cell culture (De La Cruz et al. 2014; Amundadottir 2016). The heavy metal, cadmium, is a ubiquitous naturally occurring element involved in the manufacturing process leading to environmental contamination in food and water supplies, paints, metal coatings, fuel and industrial emissions, batteries, and tobacco smoke (Waalkes 2003). Cadmium has no known biological function and can have robust effects on an organism's physiology. Discovered in 1817 by a German chemist, it has been designated a human carcinogen by the International Agency for Research on Cancer, the World Health Organization, and the United States Toxicology Program (Waalkes 2003; liza et al. 2012). The mechanisms of cadmium carcinogenicity are summarized in figure 5.

Previous studies by Djordjevic et al. discovered significantly increased concentrations of cadmium in tumor tissue with respect to healthy pancreatic tissue (Djordjevic et al. 2019). Cadmium concentrations declined as samples were taken distally from the tumor core, and tissue immediately near the tumor exhibited significantly higher cadmium concentrations compared to 'normal' tissue at the most distal sampling locations (Djordjevic et al. 2016; Djordjevic et al. 2017; Buha et al. 2017). Human exposure to cadmium in the general population primarily occurs through the gastrointestinal system or via inhalation. It is estimated that the average person ingests approximately 30 μg per day (Schwartz and Reis 2000). Smokers inhale an additional 2 μg per cigarette, potentially doubling the lifetime burden (Schwartz and Reis 2000; Waalkes 2003). An analysis from the National Statistics for Health and Center for Disease Control and Prevention database by Mannino et al. indicated that urinary levels of cadmium increased with age in all populations, however, levels in smokers were significantly higher than in non-smokers (Mannino et al. 2004). The physiological response to cadmium exposure is weak and the clearance of cadmium is long, leading to significant bioaccumulation in humans, which adds to potential adverse outcomes (Waalkes 2003). Cadmium accumulates in several different organs in the body, but has been linked specifically to the kidneys, liver, and pancreas, and is associated with malignancies in those organs (Abel and DiGiovanni 2008). Cadmium is eliminated very slowly and has a biological half-life of up to 30 years, which significantly increases the risk for adverse effects (Schwartz and Reis 2000; Waalkes 2003; Joseph 2009). A likely explanation for the long half life of cadmium in the body is its attachment to metallothionein, which is almost completely reabsorbed by the kidneys (ATSDR 2008). Experiments done by Singhal et

al. identified the antioxidant glutathione as the first potential cellular defense against cadmium toxicity. Once conjugated to glutathione, cellular damage following cadmium exposure is attenuated (Singhal et al. 1987; Sobrino-Plata et al. 2014). Another protein known to chelate cadmium metal is metallothionein (MT), a metal-binding protein that is important in the cellular response following metal exposure (Suzuki et al. 1983; Waalkes et al. 1992; Bae et al. 2003; Lei et al. 2005). MT contains seven binding sites for either essential metal homeostasis or non-essential metal cytotoxic response, making it an effective defense against cadmium toxicity, especially following acute exposure. Glutathione and metallothionein function to scavenge free radicals and bind of metal ions for storage or detoxification respectively (Ivanina et al. 2008), but this initial defense system is quickly saturated (Ivanina et al. 2008). Once conjugated to these protective proteins, cadmium's toxicity appears to be mitigated, but the complex is sequestered rather than eliminated, allowing for its bioaccumulation (Baron and Schweinsberg 1988; Dorian et al. 1992). Interestingly, although metallothionein binds cadmium ions and protects against reactive oxygen species, it has been reported that 'remobilized' cadmium, which is released from MT, was five times more toxic than cadmium chloride when delivered to rats (Nordberg et al. 1975). Conjugated 1.1mg/kg Cd-MT injections were fatal within seven days, which were attributed to the release of cadmium from MT. Much larger doses of 2.5 mg/kg cadmium chloride were tolerated without mortality for 30 days (Nordberg et al. 1975). Absorption of cadmium in the body may occur with dietary deficiencies of essential metals such as zinc, calcium, or iron. (Klaassen et al. 1999; Okazaki et al. 2000; Chmielowska-Bąk et al. 2013). There are conflicting results regarding cadmium's ability to induce glutathione production. One study suggests a dose

dependency of the cadmium-glutathione relationship, with GSH production decreasing with higher concentrations of cadmium in the hepatopancreatic cells of oysters, while Zheng et al. reported increased GSH production and activity with higher concentrations of cadmium in the bacteria, *A. Ferroxidans* (Ivanina et al. 2008; Zheng et al. 2018). Li et al. determined that glutathione activity in rats may be decreased in the presence of cadmium (Li et al. 2016). Conversely, substantial evidence exists that metallothionein is upregulated upon exposure to metals (Waalkes et al. 1992; Eneman et al. 2000; Lei et al. 2005). In 1996, Oshisho et al. linked the expression of MT to poor prognosis in pancreatic tumors. While it is known that chronic low-dose exposure to cadmium has greater biological impact than a single acute dose, (ATSDR), it has been shown that only one injection of its complex with MT can cause renal damage in rats (Dorian et al. 1992), highlighting the potential role of MT complexing in the tissue deposition of cadmium. However, the cadmium-MT complexes role in tumor development is still unclear.

Cadmium toxicity may be elicited via several different mechanisms, including reactive oxygen species generation, interference in DNA repair mechanisms, and epigenetic and protein modifications Like other carcinogenic metals such as lead, mercury, and arsenic, cadmium can switch valence states, resulting in the production of free radicals (Belyaeva et al. 2006). The action of cadmium is similar to the action of zinc, in its structure, reactivity and the ease at which it replaces other metals in biological molecules (Suzuki et al. 1983; Hartwig 2001; Hamann et al. 2012; Ugwuja et al. 2015).

It has also been postulated that cadmium has a role in the dysregulation of the methylome epigenetic architecture (Huang et al. 2008). Figure 7, devised by Waisberg et al., illustrates the many mechanisms for cadmium toxicity as they are related to tumor

development (Waisberg et al. 2003).

The production of reactive oxygen species has several deleterious effects on cellular processes, with DNA damage one of the most significant (Bhatti et al. 2011; Liu et al. 2009; Wu et al. 2016). Electron spin resonance spectra have shown that cadmium can induce the formation of superoxide anions, hydroxyl radicals, and hydrogen peroxide *in vivo* (Liu et al. 2009), and increased ROS fluorescence has been reported *in vitro* (Yang et al. 1997; Chang et al. 2013; Zhang et al. 2015). Several studies have determined this

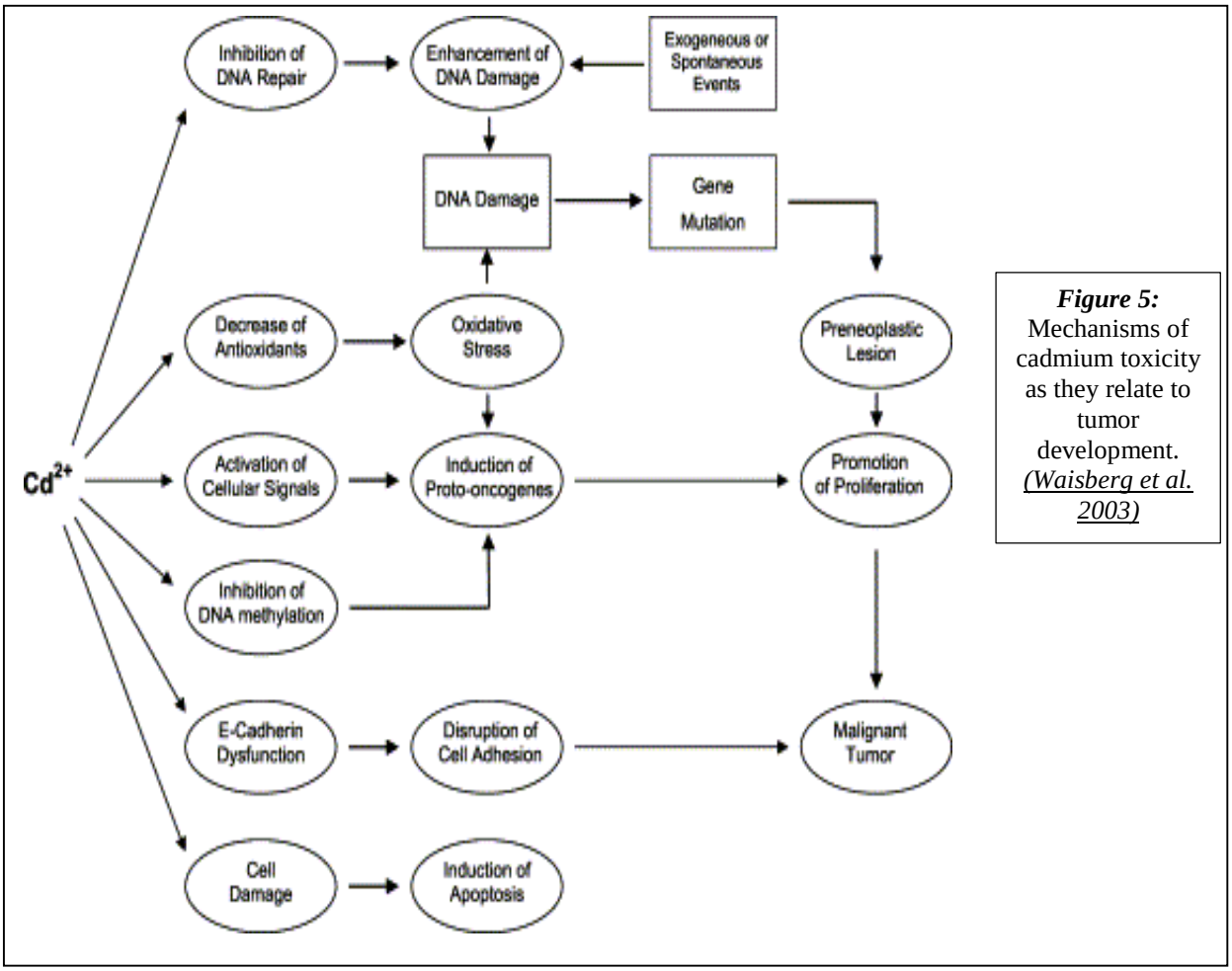

Figure 5: Mechanisms of cadmium toxicity as they relate to tumor development. *(Waisberg et al. 2003)*

increased signal may happen indirectly, by reducing the transcription of antioxidant genes like glutathione (Waisberg et al. 2003; Shukla and Singhal 1984). Rather than producing more free radicals, they may not be scavenged as efficiently after cadmium exposure.

While it has been noted that cadmium-induced ROS production appears to decrease following low-dose chronic exposures compared to acute high dose controls (Liu et al. 2009). It is the steady accumulation of DNA lesions caused by these radicals that is thought to be responsible for the progression of apoptotic tolerance, an important implication for tumorigenesis (Liu et al. 2009; Osterman et al. 2014). Compounding this problem is the influence of cadmium in DNA repair mechanisms. There are three biological mechanisms used by the body to repair DNA damage: excision repair for either bases or whole nucleotides, mismatch repair, and recombination repair (Waisberg et al. 2003). While cadmium has not been determined to be mutagenic itself, research indicates a cadmium-mediated interference that occurs early in the process of damage recognition and repair protein binding (Waisberg et al. 2003; Hartwig 2001), making the cell more susceptible to toxic effects. Cadmium's semblance to zinc is instrumental in this dysregulation. Zinc finger domains are closely associated with DNA protein interactions and DNA repair protein structures (Hartwig 2001). Replacement of zinc by cadmium in these domains leads to incomplete, incorrect, or loss of protein function by improper folding resulting in decreased DNA binding capacity (Hartwig 2001; Cai and Stillman 1988). This is illustrated in figure 6. Hartwig et al. reported that DNA binding was inhibited at only nanomolar concentrations of cadmium (Hartwig et al. 1996). Changes in epigenetic profiles, particularly methylation, have been associated with

cadmium exposure (Huang et al. 2008; Doi et al. 2011; Castillo et al. 2012; Hirao-Suzuki et al. 2018).

The term epigenetics refers to molecular genetic alterations that impact gene

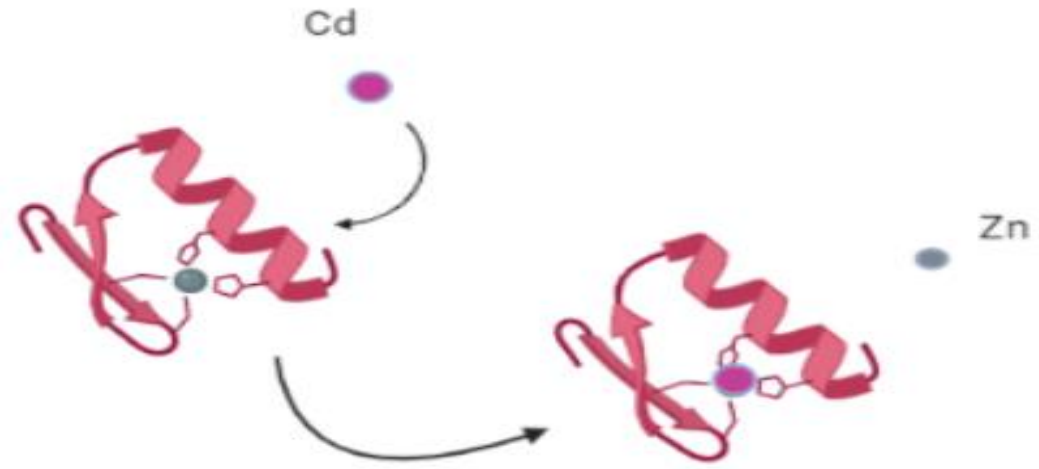

Figure 6: Zinc finger domains are frequently found in DNA repair pathways and transcription factors. Cadmium's similarity to zinc allows for its substitution into these proteins associated

expression (Buha et al. 2017). The current thought is that cadmium's role in these processes may stem from interruption of DNA methyltransferase (DNMT) activity, specifically *de novo* methylation by DNMT3, while more prevalent DNMT1 appeared unaffected. (Vilahur et al. 2015). Of the known DNMTs in humans, DNMT3a and DNMT3b are primarily responsible for laying new epigenetic marks and DNMT1 maintains existing modifications. In a 2012 study, Castillo et al. reported a change in both DNA methylation and DNMT protein expression in rat livers when exposed to cadmium, and that these effects could be sex-dependent (Castillo et al. 2012). Supporting the Castillo study, Takashi et al. found a significant decrease in DNMT expression correlated to global de novo hypomethylation with a four-hour cadmium exposure in

chicks (Doi et al. 2011). Other studies have shown that cadmium can reprogram pancreatic epithelial cells, transforming them into cancer stem cells (Yu et al. 2016). The mechanism behind this process is poorly understood. However, it is believed that low-level chronic cadmium exposure induces the expression of transcription factors responsible, at least in part, for the transition (Yu et al. 2016). There has been little work done examining the epigenetic implications of cadmium. Therefore, more investigation is needed to better understand cadmium's influence on genomic machinery.

VI. Glyphosate and Atrazine in Cancer

Glyphosate (*N*-(phosphonomethyl)glycine) was first synthesized by Swiss chemist Dr. Henri Martin in 1950 (Benbrook 2016). Lack of pharmaceutical usefulness diminished the utility of glyphosate, so it was sold to outside companies and first marketed as a chelating agent in 1964 (Toy and Uhing 1964). Dr. John Franz of the Monsanto Company identified its herbicidal properties in the 1970s, and the first formulations of Roundup™ hit the shelves in 1974, with glyphosate as its active ingredient (Benbrook 2016). Roundup™ is now the most commonly used herbicide in the world, both commercially and residentially, accounting for the 1.6 billion kilograms of glyphosate applied since its debut (Benbrook 2016). Due to the development of genetically engineered Roundup™ tolerant crops in 1996, the global use of glyphosate has risen almost 15%. It has been determined by Benbrook et al., that two-thirds of the total volume of this herbicide has been applied in the last ten years while regulatory agencies are continuing to increase acceptable tolerance limits (Benbrook 2016). An analysis by Myers et al. in 2016 determined the presence of glyphosate and its

metabolites in several crops, including soybeans, and corn and suggested recommended daily intake limits were likely based on outdated science (Myers et al. 2016). Over 8.6 billion kilograms of the pesticide glyphosate have been applied globally since its inception, and use has increased 15-fold since the inception of resistant crops in 1996 (Benbrook 2016). While acute toxic effects appear to be minimal, evidence exists for health effects stemming from ultra-low chronic exposure from the environment (Van Bruggen et al. 2018).

Glyphosate is a broad-spectrum herbicide belonging to the vast organophosphate (OP) family of pesticides (see figure 7), representing many different mechanisms of action (Pope 1999). Some OPs have been used as nerve agents like Sarin of VX gas, which function by inhibiting acetylcholinesterase, leaving the body no way to break down acetylcholine and subject to uncontrolled muscle contraction. This same principle is used in organophosphate insecticides such as chlorpyrifos and malathion. Unlike organophosphate insecticides or nerve gasses, glyphosate cannot enter the central nervous system and effect acetylcholinesterase activity (Casida 2017; Isenring 1996). Glyphosate's herbicidal usefulness is derived from its ability to inhibit the 5-enolpyruvalshikimate-3-phosphate synthase pathway (ESPS) in plants, although the exact mechanism is still under debate (Mertens et al. 2018). This pathway was thought to be only present in plants, rendering glyphosate non-toxic for humans.

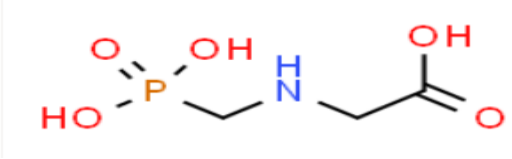

Figure 7: Structure of glyphosate http://www.chemspider.com

Multiple studies have demonstrated that glyphosate is less toxic alone than in formulation with surfactants and adjuvants added in a proprietary formulation (Myers et al. 2016; Druart et al. 2010; Benachour et al. 2007; Lin and Garry 2000). Benchour et al. determined in 2008 that glyphosate alone exhibited toxicity once dilutions reached 1% and higher, roughly 59 µM. The World Health Organization and the IARC declared glyphosate a probable carcinogen in 2015 (Tarazona et al. 2017; Myers et al. 2016), and it has been linked to several different cancers, including pancreatic islet cell adenoma, non-Hodgkin's lymphoma (Guyton et al. 2015), and acute myeloid leukemia (Andreotti et al. 2018). In 2018, groundskeeper Dwayne Johnson was awarded $289 million concerning his terminal cancer diagnosis, initiating a cascade of legal action against Monsanto (Telford 2019).

Conflicting data about glyphosate's toxicity muddles exposure and disease prevention strategies. It is recognized that a large body of early work may have been tainted by undisclosed conflicts of interest stemming from funding supplied by Monsanto (Samet 2019; Myers et al. 2016). Still, a 2018 study by Panzacchi et al. exposed rats to glyphosate at the United States Acceptable Daily Intake (US ADI) dose of 1.75mg/kg/day and found the dose did not affect mortality or body weight during chronic exposure over 125 days and measured no metabolic or cellular changes (Panzacchi et al. 2018). The authors also noted small sample sizes and large deviations limit their conclusions (Panzacchi et al. 2018). It is traditionally accepted that glyphosate breaks down quickly in the body and does not bioaccumulate, but it is constantly present in the food and water supply and is therefore consumed (Contardo-Jara et al. 2009). A 2018 study conducted by Connolly et al. analyzed urine samples of seven individuals exposed

to glyphosate and determined a biological half-life ranging between 5 and 10 hours using regression analysis (Connolly et al. 2019). These values are lower than those recorded in the IARC, which suggests total clearance of glyphosate occurs within 24 hours (Connolly et al. 2019; Tarazona et al. 2017; Van Bruggen et al. 2018). Glyphosate has the capacity to remain in the soil and water for much longer periods, contaminating the food supply, and this indeed is the main route of exposure (Pope 1999). Reported values for half-life in the soil tend to average about 47 days (Isenring 1996). It has also been determined that glyphosate and its primary metabolite, aminomethylphosphonic acid (AMPA), are present in over 90% of some food crops (Myers et al. 2016) ensuring consumption. While accidental ingestion typically leads to only mild, temporary effects, symptoms such as gastrointestinal erosion have been reported in larger, acute exposures (Bradberry et al. 2004; Isenring 1996). The limited pharmacokinetic date on glyphosate in vertebrates are not sufficient to predict its potential consequences in various tissues (Panzacchi et al. 2018).

Epidemiological studies have linked a higher incidence of cancer development to people exposed to low concentrations of organophosphates, like glyphosate, for extended periods (Elersek and Filipic 2011; Pope 1999). Research is currently directed at the molecular effects of low dose/chronic glyphosate exposure. Studies have associated the production of ROS with dysregulation of cellular processes, particularly DNA damage (Guilherme et al. 2012; Soares et al. 2019). DNA lesions can profoundly affect the heterogeneity of tumors, complicating diagnosis and treatment (de Sá Junior et al. 2017). De Sa Junior et al. (2017) also speculate that increased ROS may alter cell signaling mechanisms in ways that favor tumor formation. Studies have shown that glyphosate

may also interfere in aromatase production, affecting estrogen and the reproductive system (Benachour et al. 2007). In the same study, Benchour et al. determined an inhibition in aromatase production in only 24 hours with a 210 µM dose. Additional research indicated that low-dose glyphosate exposure can induce apoptotic machinery, specifically executioner caspases 3 and 7, and can promote mitochondrial toxicity without membrane damage (Benachour and Séralini 2009). It has been hypothesized that chronic low-dose exposure through the diet can induce cells to respond to toxicity by establishing a reduced metabolic steady–state as an attempt to maintain homeostatic control (Malatesta et al. 2008). Examination of cellular organelles by Malatesta et al. in 2008, showed no structural differences in mitochondrial volume or number but detected a decrease in mitochondrial membrane potential and increased lysosomes on the cytoplasm. Animal studies examining the glyphosate-mediated genotoxicity did not produce a change in measurable genotoxicity at doses in excess of 200 µM (Casida 2017). Continual low dose exposures to glyphosate are not affecting viability, but are initiating molecular changes that impact homeostasis, including energy production and mitochondrial health.

Atrazine, a predecessor to glyphosate, is now believed to be decreasing in the environment while levels of its metabolite, DEA, continues to rise (American Society of Agronomy 2020). DEA is classified as Group 2B, a probable human carcinogen by the IARC (PubChem). Globally, atrazine is the second most widely used herbicide; its structure is illustrated in figure 8. Due to its limited solubility and its lack of soil binding, it is the most prevalent herbicide found in surface and drinking waters (Naidenko 2018). It is a synthetic compound belonging to the triazine family of pesticides, identified by

their nitrogen ring. Attempting to identify novel classes of pesticides for agricultural use, atrazine was first registered in 1957 (CDC 2019) and works as a broad spectrum herbicide by inhibiting photosynthesis (Bara et al. 2014). Like glyphosate, atrazine appears to be cleared from the body relatively rapidly, with a proposed biological half-life of about 11 hours, but can exist in the soil for several months (LeBlanc and Sleno 2011; ATSDR 2003). It is not thought to bioaccumulate, despite its ability to sequester in fat cells, and is thought to be neutralized by glutathione conjugation (Santos and Martinez 2012; LeBlanc and Sleno 2011; Abarikwu et al. 2011).

The majority of reports regarding atrazine's toxicity has centered on its role as an estrogen disruptor and its potential role in reproductive dysregulation (Cooper et al. 2000; Hayes et al. 2006; Albanito et al. 2015). While rat studies show differing mechanisms from humans, both seem to revolve around dysfunction in the estrus cycle. Atrazine does not appear to interact directly with estrogen receptors, but exerts its effects either by

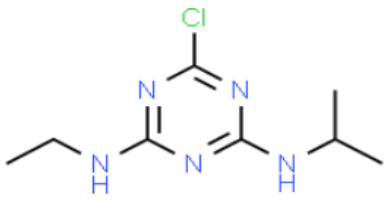

Figure 8: Atrazine structure http://www.chemspider.com/

altering the release of luteinizing hormone or upregulating aromatase activity (Albanito et al. 2015; Cooper et al. 2000). In amphibians, atrazine can chemically castrate and feminize exposed males by depleting androgens at concentrations of 0.1ppb (Hayes et al. 2006). Atrazine is not considered to be mutagenic or genotoxic (Tennant et al. 2001), and there is conflicting evidence of its carcinogenicity (Neuberger et al. 2004; Tsuda et

al. 2005; Sathiakumar and Delzell 1997; Jowa and Howd 2011). Thorpe et al. suggest an association between atrazine in the water supply and childhood cancer. However, those assumptions were not supported by Neuberger et al. Their investigation suggested no significant relationship between atrazine and elevated kidney and pancreatic cancer cases in the community, though this case cluster study involved a review of records and relied heavily on interviews to establish exposure history. A study using rats showed increased tumor formation of various types in response to atrazine (Pintér et al. 1990), initiating controversy about its carcinogenicity. Though the IARC recognizes there is ample evidence it is carcinogenic in animals, they designated atrazine as not classifiable regarding human cancers.

VII. The Importance of Studying Chemical Mixtures

The ubiquity and persistence of pollutants in the environment demands that the interactions of pollutants in mixtures be explored for combined effects, additivity or synergism. Our food and water safety is threatened with both agrichemicals and other environmental pollutants known to contaminate both food and water. Heavy metals and pesticides are present in the food supply, and multiple studies have confirmed combinations of contaminants in variable concentrations (Akoto et al. 2013; Roychowdhury et al. 2003; Clarke et al. 2015). Environmental concentrations of xenobiotics are dependent on several factors, including soil geochemistry, rainfall, and production methodology (Wallace and Buha Djordjevic 2020; Clarke et al. 2015). The detrimental health effects initiated by these compounds is varied, affecting multiple

systems, and is dependent on the nature of the exposure. Analysis of disease burden becomes enormously difficult when accounting for the effects of chemical mixtures and their physiological implications. Surprisingly, very little work has been done to further our understanding of the toxicity associated with chemical mixtures (Prüss-Ustün et al. 2011; Clarke et al. 2015).

Mixture analysis is complicated by existing analytical methods designed to test across a single broad-spectrum group, rather than across multiple classes of xenobiotics (Clarke et al. 2015). Current legal permissible intake levels are assessed only for individual pollutants, due to the complexity of mixture analysis and identification in any given area. While several regulatory agencies use the no observable adverse effect limit (NOAEL) to establish toxicological thresholds for xenobiotics, no effort has been made with respect to mixtures (Wade et al. 2002). How these contaminants work together to interrupt cellular machinery remains to be determined. The Agency of Toxic Substances and Disease Registry, ATSDR, has developed a chemical mixtures program, mandated to develop interaction profiles similar to their profiles of individual toxicants, and has identified mixture analysis as one if the six priority goals for the agency (de Rosa et al. 2004).

Combinations of mixtures present an interesting challenge to research scientists. Not only is determining likely combinations of toxicants essential, but the sequential order of exposure could be highly relevant in toxic response (Hernandez et al. 2019). Health impacts can be easily over- or underestimated depending on the experimental approach. Whole mixture studies often neglect to identify which component of the mixture is primarily responsible for the toxic response or potential interaction between components, while component-based strategies typically underestimate risk (Bopp et al.

2019; Hernández et al. 2017; Hernandez et al. 2019). The concentration/dose is of utmost importance since much of the existing body of work relies on a single large dose that does not accurately reflect real-life situations. It is now believed that low concentrations at or below the NOAEL are essential to determine the toxicity of mixtures and that experimentation must include data on the individual components of the mixture as well (Tsatsakis et al. 2018; Kostoff et al. 2018; Hernandez et al. 2019). Many compounds can interact chemically, potentially affecting the magnitude or even the mechanism of the toxic response (Bopp et al. 2019). Most experimental models presume an additive response, where the sum effect of the combined toxicants is predicted to reflect the additive sum of the respective toxicities (Bopp et al. 2019). Additivity is the preferred assumption for mixtures with undetermined mechanisms of action (Hernandez et al. 2019). Other types of interaction include potentiation, antagonism, and synergism. Potentiation can be seen when a chemical does not exert toxicity in a given system without another chemical, suggesting that the second chemical 'potentiates' the action of the first chemical. Two separate teams determined potentiating effects with triazine herbicides. Atrazine was found to increase the cytotoxicity of arsenic in human liver carcinoma cells (Tchounwou et al. 2001), while similar findings in insects where reported following exposure to a triazine/organophosphate mixture (Schuler et al. 2005). Synergism results from the concurrent action on different molecular targets, resulting in a greater than additive toxicity. Synergism is predicted to be the most common combined effect at biologically relevant concentrations (Wang et al. 2015; Hernández et al. 2017; Wallace and Buha Djordjevic 2020), though Hernandez et al. concluded these interactions are difficult to quantify at daily intake levels. Chemical interactions between

pollutants in a mixture is one explanation for increased toxicity of the mixture over the individual chemicals. Mixtures of cadmium and chlorpyrifos were found to increase cadmium transport across cell membranes 20%, resulting in accumulation in liver cells (Chen et al. 2013). Cadmium mixtures with atrazine had synergistic effects in earthworms, while cadmium mixtures with butachlor and λ Cyhalothrin behaved differently, expressing additivity and antagonism respectively (Wang et al. 2012; Wang et al. 2015). Triazines, in general, have been identified as overrepresented in synergistic interactions along with organophosphate and pyrethroid insecticides (Hernández et al. 2017). Antagonism is an interaction of chemicals resulting in a less than additive toxic response. The reduction in toxicity is due to one chemical blocking the actions of another chemical, or two compounds that interact on a chemical level to negate each compounds effect. Many studies have identified antagonistic responses between similar metal species like cadmium and zinc and different families of toxicants (Ugwuja et al. 2015; Vellinger et al. 2012). Metals and pesticides, like glyphosate, are frequently found to show a less than additive response in their toxicities (Zbigniew and Wojciech 2006; Xu et al. 2017; Zhou et al. 2014). There is evidence that synergism may increase directly with the complexity of the mixture (Wade et al. 2002), and work continues to effectively translate exposure doses into relevant test concentrations (Hernandez et al. 2019). Wallace et al. proposed a differential toxicity model in which a mixture has the ability to elevate toxic responses at lower concentrations and exhibit mixture-specific unique toxicity at higher concentrations as shown in figure 9, underscoring the importance of this determination (Wallace and Buha Djordjevic 2020).

Advancing technologies in spectrophotometry have allowed researchers to identify mixtures in several food commodities, such as rice, maize, spices, and vegetables (Akoto et al. 2013; Roychowdhury et al. 2003; Clarke et al. 2015). In response to the identification of both heavy metals and pesticides in food, examination of mixture

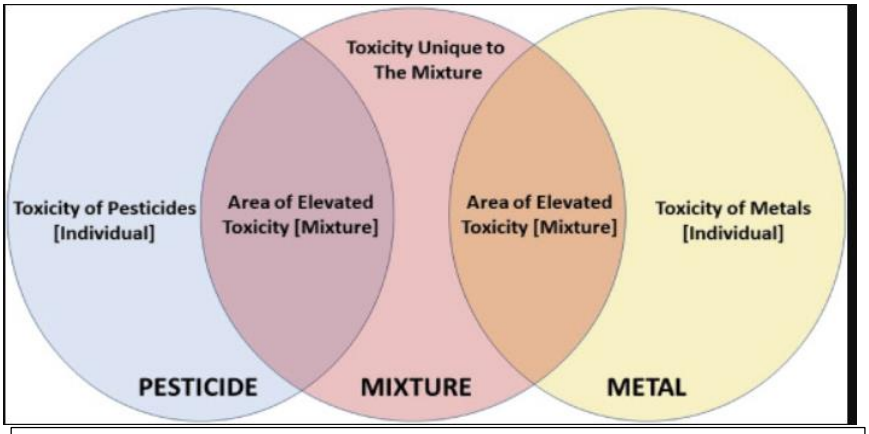

Figure 9: A schematic representing potential differences in toxicity in metal/pesticide mixtures from (Wallace and Buha Djordjevic 2020)

toxicity at the individual pollutant safe exposure levels has established a number of novel effects in both animals humans. In the honey bee, there was a disruption of both redox systems and vitamin A metabolism in response metal-pesticide mixtures (Jumarie et al. 2017). In a 2014 study by Zhou et al., a mixture of atrazine and cadmium significantly increased DNA damage in earthworms, though not additively (Zhou et al. 2014). A study in chicken embryos found that exposure to a combination of cadmium and glyphosate at environmentally permitted concentrations resulted in 100% mortality compared to 85.7 % for cadmium and 40.6% for glyphosate alone (Szabó et al. 2018). In rat liver, low-dose combinations of cadmium and organochlorines can differentially affect amino acid metabolism, and produce widespread changes in metabolic biomarkers expression compared to either toxicant alone (Xu et al. 2015). However, higher dose combinations of

cadmium and organochlorines were directly neurotoxic. Cadmium and chlorpyrifos were shown to decrease mitochondrial membrane potential and increase oxidative stress (Xu et al. 2017). In the thyroid, exposure to mixtures at the daily reference dose elicited a significant increase in circulating thyroid-stimulating hormone levels (TSH) (Wade et al. 2002). From *in vitro* studies with HeLa cells, exposure to mixture profiles of various organochlorines resulted in an upregulation of gene transcription for CYP1A1, GST, and p53, but only at high concentrations (Mumtaz et al. 2002; Tully et al. 2000). The exact mechanism for these effects is unknown, but may be through an increase in ROS. Additionally, the presence of heavy metals with organic xenobiotics tends to affect antioxidant response systems differentially than in individual testing, by decreasing glutathione and altering xenobiotic metabolism (Schröder et al. 2009).

Many of the mechanisms altered by mixture exposure are also identified as necessary mechanisms in tumorigenesis, prompting the theory of co-carcinogenicity (Wallace and Buha Djordjevic 2020). A co-carcinogen is defined as not carcinogenic alone, but able to facilitate the cancer state when combined with another chemical. In a study examining pancreatic cancer risk, an increased presence of heavy metals in subjects exposed to pesticides was reported (Camargo et al. 2019) . The exposure to undetermined contaminant cocktails has the potential to increase our risk of multiple pathologies, and much more work remains to be done to bridge the knowledge gap (Cedergreen 2014).

VIII. Potential role for Cadmium, Glyphosate and Atrazine in the Development of Pancreatic Cancer

Pancreatic cancer's dismal survival rates make it imperative to identify potential causative agents. It is one of the few cancers on the rise for both incidence and death due difficulty in diagnosis and misdiagnoses (Rosenzweig 2019). The heavy metal cadmium has been identified as a potential contributing factor in pancreatic carcinogenesis (Camargo et al. 2019; Buha et al. 2017; Djordjevic et al. 2019; Luckett et al. 2012; Yu et al. 2016; Schwartz and Reis 2000; Chen et al. 2015; Djordjevic et al. 2017; Ishihara et al. 1987; Qu et al. 2012; Wallace et al. 2019; García-Esquinas et al. 2014). Areas of high cadmium concentration has been identified in pancreatic tumors, with decreasing cadmium concentrations radiating out from the tumor focus into the unaffected surrounding normal tissue (Djordjevic et al. 2019; Buha et al. 2017). Known risk factors associated with pancreatic cancer can also be associated with cadmium exposure, particularly smoking, mandating we look at cadmium metal as a risk factor to pancreatic tumorigenesis (Schwartz and Reis 2000).

The ability of cadmium to bioaccumulate has been well established. A 1996 study highlighted this using a crayfish model where crayfish were fed plants allowed to bioaccumulate cadmiumfor two weeks (Devi et al. 1996). Accumulation of cadmium in the hepatopancreas of the crayfish rose 2,634% and went from an initial concentration of 176.8 ppb to 4657.6 ppb on day 14, greater than a 26-fold increase (Devi et al. 1996). A pilot study in Italy measured the metal content in several fish species, finding cadmium present in levels much higher than established regulatory levels (Pastorelli et al. 2012).

In Thailand, a 90% of rice crops tested positive for cadmium (Chunhabundit 2016). Even though the levels were considered within safety guidelines, it was calculated that the average Thai diet exceeded the acceptable monthly intake by 168% over the values set the WHO (Chunhabundit 2016). Consumption of seafood from rural Louisiana, where cadmium accumulation due to industrial waste is elevated, resulted in much higher incidence of pancreatic cancer cases associated with the elevated levels of cadmium (Luckett et al. 2012). Once consumed, most cadmium is bound to metallothionein and is distributed throughout the body with little excretion (Nordberg 1977; Cai and Stillman 1988; Dorian et al. 1992; Sears 2013). The half-life of cadmium in the body is known to be 10-30 years, and continuous exposure with age increases body burden relatively without any substantial mechanism for removal.

Smoking is the sole modifiable risk factor for pancreatic cancer identified in epidemiological studies (Schwartz and Reis 2000). One cigarette contains 1.5-2 µg of cadmium, which is present in both mainstream and second-hand smoke at concentrations of 1000-3000ppb (Martin 2008). Concentrations of cadmium in the adipose tissue of smokers were determined to be four times that of non-smokers (Mussalo-Rauhamaa et al. 1986). Cadmium is not added as part of the manufacturing process but preferentially accumulates in broadleaf plants like tobacco from the air (Kim et al. 2010), where its gains association with pesticide use. Proposed mechanisms of cadmium-induced tumorigenesis include transdifferentiation, ROS production, and interference in DNA repair mechanisms. Cadmium's similarity to zinc allows it to replace this essential metal in multiple cell systems. The pancreas contains high levels of zinc, and it has been shown that dietary insufficiencies of zinc can mobilize stored cadmium (Pieper et al.

2015; Kim et al. 2019). Since zinc is involved in DNA repair mechanisms, its replacement by cadmium may render that protective machinery ineffective (Padjasek et al. 2020; Buha et al. 2017). Once damaged, replication of damaged DNA can lead to uncontrolled growth and proliferation resulting in the heterogeneous fingerprint seen in cancers. There is also evidence that cadmium may be involved in the upregulation of proto-oncogenes and inactivation of p53 tumor suppressor mechanisms. Dysregulation of p53 has been observed in multiple studies of pancreatic adenocarcinoma (Jing et al. 2018; Deer et al. 2010; Kolodecik et al. 2013; Perugini et al. 2000; Weissmueller et al. 2014; Chien et al. 2017; Mello et al. 2017).

The ability of a tumor cell to de-differentiate from its original state is considered a hallmark of cancer. Cadmium has been shown to induce the formation of fully functioning hepatocytes from pancreatic cell tissue at concentrations as low as 360 µM (Konishi et al. 1990). These transdifferentiated cells stained for increased metallothionein production (Waalkes et al. 1992), which was found to be associated with worse histological grade and shorter survival (Ohshio et al. 1996; Buha et al. 2017).

Although cadmium is considered to be a "redox inactive" metal (Buha et al. 2017), it is capable of generating increased levels of ROS through an unspecified mechanism. Cadmium has also recently been identified as a potential mitochondrial toxin (Wallace et al. 2019), indicating bioenergetics may be altered in the presence of excess free radical. In pancreatic β-cells, Chang et al. showed that cadmium induces apoptosis through mitochondrial-dependent pathways, further implicating cadmium in its role in cell cycle dysfunction (Chang et al. 2013).

Pesticide exposure has also been identified as a risk factor for pancreatic cancer (Schwartz and Reis 2000). While DDT and some organochlorines have been linked to an increased cancer risk, atrazine exposure has not been associated with pancreatic cancer. However, one study did mention an increasing trend in the incidence of pancreatic carcinogenesis in atrazine mixtures with acetochlor (Lerro et al. 2015). Similarly, few studies exist of the association of glyphosate and pancreatic cancer specifically, though cohort studies with multiple cancer endpoints were examined (Mink et al. 2012). Glyphosate was determined to produce a nonsignificant trend towards increased pancreatic cancer incidence (Andreotti et al. 2018). Correlations between glyphosate exposure and the incidence of PC are complicated by concurrent risk factors such as smoking among study participants. All available cohort studies were based on occupational exposures, where glyphosate tends to be in higher concentrations. Correlations between glyphosate exposure and pancreatic effects in rats have been reported following application doses of glyphosate, an effect that was mitigated by zinc supplementation (Tizhe et al. 2014). Glyphosate and cadmium exposures can have similar biological endpoints, and given their colocalization in the digestive system and association with pancreatic cancer, analysis of their combined toxicity will illuminate molecular changes contributing to the onset of this disease.

CHAPTER III

METHODOLOGY

***Cell Lines and Cell Culture Maintenance*:** All cell lines were obtained from American Type Culture Collection (ATCC, Manassas, VA). Pancreas hTERT-HPNE ("human pancreatic Nestin-expressing" cells; ATCC® CRL- 4023™, immortalized pancreatic control cells) and AsPC-1 (ATCC® CRL-1682™, pancreatic tumor cells) were grown and maintained as described in the ATCC-suggested protocols. A photo of each can be found in figure 10. Briefly, hTERT-HPNE cells were maintained in Dulbecco's Modified Eagle's Medium (DMEM; 75% - Sigma-Aldrich, St. Louis MO) and a medium supplement, M3 Base (25%; Incell Corp. – Austin, TX). This combined base media was then sterile filtered and supplemented with: sterile L-glutamine (2mM), sodium bicarbonate (1.5 g/L), fetal bovine serum (5%), human epidermal growth factor (hEGF, 10 ng/mL), D-glucose (1 g/L) and Puromycin (750 ng/mL). Media was exchanged every 2-3 days and cells were subcultured weekly with a subculture ratio of approximately 1:8. AsPC-1 cells were maintained in RPMI-1640 medium (Sigma-Aldrich, St. Louis, MO) with the addition of fetal bovine serum (10%). Media was exchanged every 2-3 days and cells were subcultured weekly with a subculture ratio of approximately 1:6. Cells were

maintained in a 37° incubator supplemented with humidified 95% air/5% CO_2. All media supplements were obtained through Sigma- Aldrich or Incell (St. Louis, MO or San Antonio, TX, respectively). HTERT-HPNE pancreatic control cells are healthy human pancreatic ductal epithelial cells used for adherent cell cultures. This cell line was isolated from a 52-year-old male, and transfected with human telomerase reverse transcriptase (HTERT) for induced immortalization (Feldmann et al. 2009). HPNE pancreatic ductal cells express the neuronal stem cell marker Nestin. The protein Nestin is considered as a marker for exocrine progenitor cells and is first expressed during embryonic development of the pancreas and maintained in the adult pancreas (Carrière et al. 2007). Testing has indicated a role for Nestin producing cells in the regeneration of the pancreas in response to disease states such as pancreatitis (Carrière et al. 2007; Ishiwata et al. 2006). It has been determined that this cell line has wild type KRAS and TP53, making it useful as a control cell line for investigations on pancreatic cancer initiation (Carrière et al. 2007), and is the only normal pancreatic cell line currently available from the American Type Culture Collection, ATCC.

AsPC1 tumor cells were collected from a 62 year old woman with metastatic adenocarcinoma in the head of the pancreas and transfected with SV40 virus for immortality (Chen et al. 1982). AsPC1 cells are of ascinar epithelial cell origin and are an adherent cell line that produces abundant mucin (Deer et al. 2010). One of many tumor lines available through ATCC, multiple studies have suggested AsPC1 cells form tumors readily when injected into the pancreas of mice, but these tumors tend to be smaller than those of other cancer cell lines (Deer et al. 2010; Katayama et al. 2003. KRAS appears to be activated in the AsPC1 cell line, as it is with most pancreatic tumors,

while studies show it inconsistently exhibits mutation in tumor suppressor genes TP53 and SMAD4 (Deer et al. 2010). Both cell lines exhibit adherent, monolayer growth on

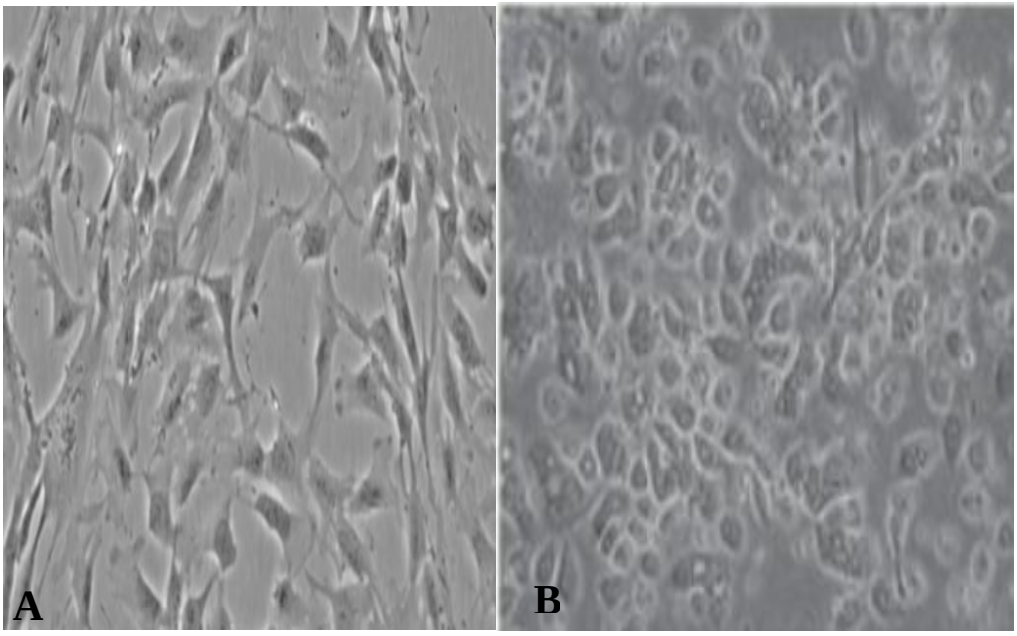

Figure 10: Control pancreatic HPNE cells (A) and AsPC1 pancreatic tumor cells (B)

glass and plastic surfaces, allowing for easy visual examination. HTERT-HPNE and AsPC1 cells used in this study are modified for immortality, meaning they can grow nearly indefinitely in culture.

***Cell Culture Treatments*:** Before assay initiation, the adherent cells were detached by the addition of warmed (37°C) 0.25% trypsin. After detachment, cell suspensions were centrifuged for 3-5 minutes to pellet the cells. Pellets were washed with base growth media (no supplements), and the pellets were re-suspended in 11 mL of growth media (refer to above) which yields a cell density of approximately 2 x 10^5 cells/mL to 5 x 10^5 cells/mL. Aliquots (100 µL) of cell suspension were added to each well of a black/clear

96-well plate for a final density of 2-5 x 10^4 cells/well. Cells were allowed to adhere for at least 24 hours before assay. All assays were run in low-serum assay media composed of MEM without phenol red (Fisher #MT90009PB) with the addition of 2 mM glutamine, 1.5 g/L sodium bicarbonate, and 1% FBS.

Experimental Methods

Aim 1

LDH Cell Viability Assay: LDH assays were performed to determine 1) the number of viable cells within the culture and 2) the percentage of the total cell number that were viable. Lactate Dehydrogenase (LDH) is released from dead/dying cells and is indicative of necrosis due to membrane damage or rupture. A schematic of the assay is found in figure 11. LDH activity was measured using the Cytotox-ONE™ Homogeneous Membrane Integrity Assay kit (G7891 Promega; Madison, WI). Assay procedures were performed following the kit instructions for use. Determination of cellular proliferation/growth was completed using the following formula:

Live cell # (RFU) = [Total LDH]-[Media LDH]

*% Viability = ([Live cell RFU]/[Total LDH])*100*

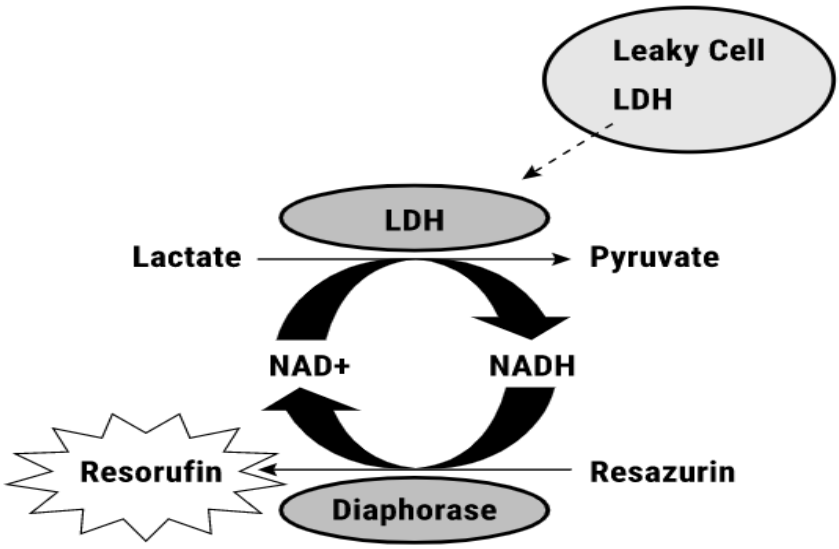

Figure 11: LDH leakage by damaged cells is detectable by the conversion of resazurin to a fluorescent resorufin product. https://www.promega.com/resources/guides/cell-biology/cell-viability

Briefly, 20,000-50,000 cells per well were plated and allowed to adhere as per general methods. First, both HPNE and AsPC1 cell lines were exposed to 50 µM concentrations of each individual toxicant: cadmium, atrazine, glyphosate, and Roundup^{TM}. The preliminary mixture concentrations contained identical concentrations of pesticide with the addition on 1 µM Cadmium. This concentration had been verified in our lab to be sub-toxic. The second LDH experiment included only cadmium, atrazine, and glyphosate in serial dilutions from 1 mM to 1 nM. Experiments for this test were performed using n=6. Stock solutions of each toxicant (10 mM) were used as the starting point of all dilutions. The test concentrations along with a control were diluted in cytotox media. Each well received 50 µL of toxicant and plates were returned to the incubator at 37°C with 5% CO_2 for 48 hours. Post incubation, half of the cells were lysed using 3 µL 0.9% Triton X and returned to the incubator for 1 hour. 50 µL of LDH substrate was then

added to all wells in a 1:1 ratio with treatments, and the reaction was protected from light at room temperature for ten minutes. The reaction was then terminated with 50 µL of stop solution, and the plates were read on a Bio-Tek plate reader at 530/25nm excitation and 590/25 emission.

Statistics: Data was expressed as mean ± SEM of 3 analyses (n = 3) in duplicate. Data was treated as parametric and analyzed with GraphPad Prism (v 8.00, GraphPad Software, La Jolla, CA). Analyses were performed using a two-way ANOVA (treatment x cell line) followed by Tukey's post hoc tests to compare the differences in means between groups.

MTT Viability Assay: MTT is taken up into live cells and converted to formazan (see figure 12). Experiments examined the effects of cadmium and the pesticides glyphosate and atrazine. The assays were designed to evaluate cell viability in response to serial dilutions of toxicants following a 48 h exposure. Cells were exposed to eight concentrations ranging from 1 mM to 1 nM. The eight treatment groups were as follows: 1. $CdCl_2$ dilutions with and without 500 nM Atrazine, 2. $CdCl_2$ dilutions with and without 500 nM Glyphosate, 3. Atrazine dilutions with and without 500 nM $CdCl_2$, and 4. Glyphosate dilutions with and without 500 nM $CdCl_2$. Before beginning the assay, the adherent cells were detached by the addition of warmed (37°C) 0.25% trypsin. Cells were then plated into clear 96 well plates at a density of 2-5 x 10^4 cells per well and allowed to adhere for 24 hours. 12 mM MTT stock solution was made in PBS and stored at 4°C until use. After seeding and cell attachment, growth media was removed and cells were exposed to 100µL $CdCl_2$, pesticide, or mixture in assay media ranging from 1 nM to 1 mM for 48 hours. Treatments were made by serial dilutions of a 10 mM stock solution of

each toxicant, in a 1:10 ratio. After exposure for 48 hours, 10 µL of 12mM MTT was added to each well. Plates were then returned to the incubator at 37°C and 5% CO_2 for four hours. Post incubation, 75 µL of treatment was removed and replaced by 50 µL of

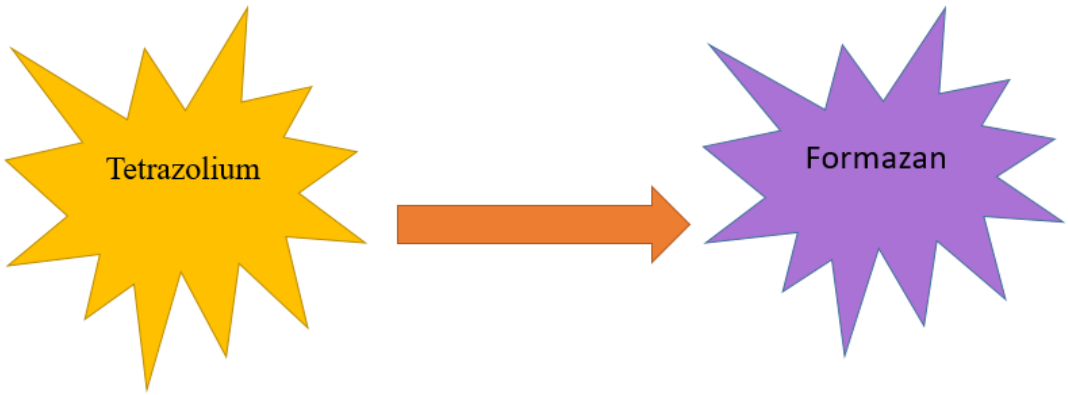

Figure 12: MTT viability assays depend on the uptake of tetrazolium into metabolically active cells where it is converted to a formazan product.

DMSO and mixed thoroughly to solubilize formazan crystals. Plates were incubated an additional ten minutes before being mixed again to ensure maximum formazan solubility, then placed in a Biotek plate reader set to 540nm to measure absorbance.

Statistics: LC50 values were determined using GraphPad Prism statistical software (v 8.00, GraphPad Software, La Jolla, CA), and data was expressed as mean ±SEM of 6 analyses (n = 6) tested in duplicate. Absorbance data was analyzed by non-linear regression analysis using a single site inhibition curve to determine LC50 values. Two-way ANOVA and Tukey's post-hoc tests were used to compare the differences in means between groups. One-way ANOVA was used to compare LC50 values between individual toxicants and mixtures. All viability measures were expressed as percent of control.

Aim 2

p53 ELISA: Experiments measured the expression of p53 protein present in response to toxicant exposures versus untreated controls. Cell lines were plated in a black walled 96 well plate at a concentration of 1-2 x 10^4 cells per well and allowed to adhere for 24 hours. Media was removed and cells were treated with toxicants or mixtures as outlined in general cell culture methods using an n of 4 in duplicate. Treatments were removed after 48 hours and cells were fixed with 100 µL of 4% paraformaldehyde for 15 minutes at room temperature. After fixing, cells were washed twice with 100 µL of tris buffered saline (TBS) for 5 minutes on a plate shaker at room temperature and permeabilized according to the Thermo Scientific kit protocol (product #62216, Waltham, MA). Permeabilization buffer was removed and cells washed as above followed by a 20 minute incubation with a quenching solution. After quenching and an additional wash step, samples were blocked using the kit supplied blocking buffer for 20 minutes before the addition of 1:1000 anti p53 antibody and incubated at 4°C overnight. Following the incubation, cells were washed three times for five minutes on the plate shaker using a 1X wash buffer consisting of 5% 20X TBS, 1% Surfact-Amps 20, and Molecular Biology grade ultrapure water. 100 µL horseradish peroxidase (HRP) conjugate was added to each well and incubated at room temperature for 30 minutes, followed by an additional three wash steps using supplied wash buffer. TMB substrate was added to initiate the reaction and stopped with stop solution after 13 minutes. The absorbance was read immediately at 450nm. After the plate was read, contents were removed, and the plate washed twice with ultrapure water followed by the addition of Janus green dye (100 µL) and incubation on a plate shaker for 5 minutes. Four wash steps using 200µL ultrapure

water followed to ensure all excess stain was removed. An elution buffer was added to each well and the plate was read immediately at 615 nm. Data from the A_{450} read was divided by data from the A_{615} read to account for inconsistencies in cell number during plating.

Statistics: Data were expressed as mean ± SEM of 6 analyses (n = 6) in duplicate. Untreated control data was run through Shapiro-Wilks test for normality in small data sets (n<50). Data was treated as parametric and analyzed with GraphPad Prism (v 8.00, GraphPad Software, La Jolla, CA). Analyses were performed using a two-way ANOVA (treatment x cell line) followed by Tukey's post hoc tests to compare the differences in means between groups.

Annexin: The presence of early apoptotic membrane changes were identified using the RealTime-Glo Annexin V Apoptosis Assay (Promega, Madison, Wisconsin). Cells were plated in white clear bottomed culture plates at a density of 2-5 x 10^4 cells per well using an n = 3 in duplicate. Four wells were kept as no-cell blanks to assess background signal. Treatments were applied according to general methods and incubated at 37°C and 5% CO_2 for 48 hours. A 2X detection reagent was prepared by adding 24 µL of 1000X Annexin NanoBiT substrate, 1000X calcium chloride, Necrosis Detetion Reagent, 1000X Annexin V-SmBiT, and 1000X Annexin V-LgBiT to 12 mL of prewarmed assay media as per the included kit protocol. Post incubation, treatment was removed and replaced with 100 µL pre-warmed assay media and an equal 100 µL volume of 2X detection reagent was added to each well. The plate was immediately read on a Synergy Biotek

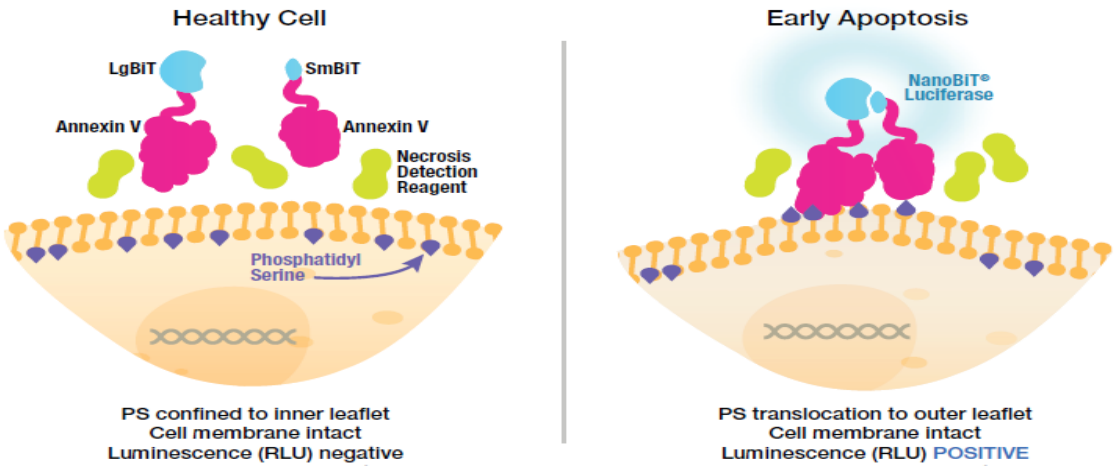

Figure 13: Annexin protein subunits bind to phosphatidylserine that has flipped to the outer side of the cell membrane in the initial phases of apoptosis and provide a luminescent signal. *www.promega.com*

plate reader set to measure luminescence. A schematic of the assay can be found in figure 13.

Statistics: Data were expressed as mean ± SEM of 3 analyses (n = 3) in duplicate. Data was treated as parametric and analyzed with GraphPad Prism (v 8.00, GraphPad Software, La Jolla, CA). Analyses were performed using a two-way ANOVA (treatment x cell line) followed by Tukey's post hoc tests to compare the differences in means between groups.

Caspase 3/7 Activity Assay: Caspase 3/7 activity was measured using Caspase-Glo 3-7 assay (Promega, Madison, Wisconsin). In short, a kit supplied substrate containing tetrapeptide DEVD from the caspase active site was mixed with assay buffer to form the caspase reagent. The addition of the reagent to the well results in cell lysis and cleavage of the substrate by caspase. The cleaved substrate then produces a luminescent signal in the presence of luciferase. The signal is proportional to

the amount of caspase present. Cells were seeded at 1-5 x 10^4 cells per well in a black walled clear bottom plate and allowed to adhere for 24 hours. After incubation, media was removed and replaced by 50 µL of Caspase reagent and returned to the incubator for 30 minutes. Without removal of the reagent, cells were then treated with 50 µL of toxicants or mixtures of 500 nM cadmium and 1 µM pesticide determined in aim 1. The plates were then covered in foil to protect them from light and placed on a plate shaker for 30 seconds at 350 x rpm and incubated at room temperature for one hour. Post incubation, the plate were read in a Biotek plate reader at 485/20 nm excitation and 528/20 nm emission sto detect fluorescence.

Statistics: Data were expressed as mean ± SEM of 6 analyses (n = 6) in duplicate. Data was treated as parametric and analyzed with GraphPad Prism (v 8.00, GraphPad Software, La Jolla, CA). Analyses were performed using a two-way ANOVA (treatment x cell line) followed by Tukey's post hoc tests to compare the differences in means between groups.

Aim 3

DCFH Measurement of Oxidative Stress: Formation of free radicals after toxicant exposure was determined by measuring the fluorescence emitted by 6-carboxy-2',7'-dichlorofluorescin (DCFH). After the cells were incubated with toxicant, the amount of fluorescence (proportional to free radical formation) was measured using a Biotek plate reader set to an excitation wavelength of 485 nm and an emission wavelength of 530 nm. Cells were plated at 10^4 cells per well in a black 96 well plate and allowed to adhere for

24 hours. Before assay, growth media was removed from each well and replaced with 50µL DCFH and returned to the incubator for 30 minutes. Upon completion of incubation, excess DCFH was removed and treatments were added to wells. Treatment groups were as outlined in the above general methods cell treatment section. Plates were returned to the incubator and read after one hour on a Biotek plate reader set at 485/528 nm for excitation/emission to identify increases in production of reactive oxygen species (ROS).

Statistics: Data were expressed as mean ± SEM of 6 analyses (n = 6) in duplicate. Data was treated as parametric and analyzed with GraphPad Prism (v 8.00, GraphPad Software, La Jolla, CA). Analyses were performed using a two-way ANOVA (treatment x cell line) followed by Tukey's post hoc tests to compare the differences in means between groups.

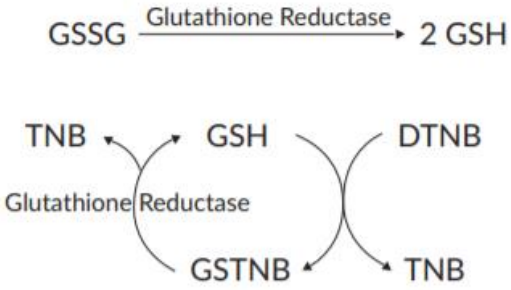

Figure 14: Enzymatic recycling is used to identify both oxidized and reduced forms of glutathione. www.caymanchem.com

Total Glutathione: Glutathione (GSH) is widely distributed in plants and animals, and works to detoxify toxicants. The Glutathione Assay kit (Cayman Chemical, Ann Arbor, MI) uses enzymatic recycling as seen in figure 14, to identify both reduced and oxidized glutathione to measure total glutathione present in the cell. Cells were harvested at a concentration of 1-4 x 10^6 and diluted by a factor of ten in 10 mL growth media. These

cells were then replated into 24 well plates at a density of 1-4 $x10^5$ using 0.5mL per well. We used an n = 3, requiring 18 wells for five treatments and one control per cell line. Cells were seeded for 24 hours and grown an extra day to ensure adequate growth for assay signal. Growth media was then removed and replaced with 1mL toxicant treatments in cytotox media. Cells were then incubated for 48 hours at 37°C with 5% CO_2. Post incubation, cells were again harvested with 0.3 mL trypsin per well and reincubated for 15 minutes. The trypsin and cells were collected from each well, placed in separate microfuge tubes, and then counted and diluted to a final density of 1-5 $x10^6$ cells per mL. Samples were centrifuged at 500 rpm for 5 minutes at 4°C, then washed with 1 mL cold 1X PBS. After a second centrifugation, the supernatant was discarded, and the wash step was repeated. The cell pellet was then resuspended in 500 µL 5% MPA, mixed thoroughly and sonicated. Cells were sonicated for one minute with on/off intervals of ten seconds to prevent sample overheating. Immediately following sonication, samples were centrifuged at 12,000 rpm for 10 minutes at 4°C. Post incubation, 25 µL of 1X glutathione reductase solution and 25 µL of kit supplied 1X NADPH solution were added to each well. Treated samples were added to the plate in 100 µL quantities and mixed. 50 µL supplied 1X Chromogen substrate was added and gently mixed. Plates were read immediately at 405 nm.

Statistics: Data were expressed as mean ± SEM of 3 analyses (n of 3) in duplicate. Data was treated as parametric and analyzed with GraphPad Prism (v 8.00, GraphPad Software, La Jolla, CA). Analyses were performed using a two-way ANOVA (treatment x cell line) followed by Tukey's post hoc tests to compare the differences in means between groups.

Glutathione Adducts: Cell lines were grown to confluence as described in general methods, then trypsinized, counted, and resuspended in 10mL growth media. Suspended cells were then plated on a 24 well plate, and allowed to adhere for 24 hours as above. Cells were then exposed to treatments as outlined in general methods, using an n = 3 and incubated for a 48h exposure. Post exposure, treatments were aspirated, and wells were washed three times with 1X PBS on an orbital shaker at 350 rpm for 1 minute per wash cycle. Immediately after washing, wells were lysed using 200 µL RIPA lysis buffer with added protease and phosphatase inhibitors and incubated on ice for 20 minutes. After lysing, a rubber spatula was used to scrape wells, and the contents were collected and transferred to a labeled microfuge tube. Tubes were sonicated for one minute, 10 s on and 10 s on ice alternately, before being centrifuged for 10 minutes at 12,000 rpm and 4°C. The supernatants were collected and transferred to fresh microfuge tubes and stored overnight at -80°C. Reagents were prepared as per kit manufacturer (Cayman Chemical, Ann Arbor, MI) instruction, and conjugate diluent was diluted to a concentration of 100ng/mL in PBS, and 100µL was added to each well of the supplied 96 well plate. The plate was then incubated at 4°C overnight. Post incubation, lysate samples were warmed to room temperature, and the diluted conjugate was removed from wells. Each well was washed twice with 1X PBS as described above and blotted dry. 200µL of assay diluent was added to each well and incubated at room temperature for 1h to block. Once assay diluent was removed, 50µL of lysate was added to each well with n=3 in duplicate. A 1:500 primary antibody dilution was added to each sample and incubated at room temperature on an orbital shaker for 1h, followed by a ternate of washings with 250 µL of wash buffer. Immediately after washing, a 1:1000 dilution of secondary antibody was

applied with an additional 1h incubation and identical wash step. 100µL reaction substrate was then added to each well and placed on the orbital shaker for 20 minutes followed by the addition of 100µL stop solution. Plate was immediately read on a Biotek plate reader set at 450nm.

Statistics: Data were expressed as mean ± SEM of 3 analyses (n = 3) in duplicate. Data was treated as parametric and analyzed with GraphPad Prism (v 8.00, GraphPad Software, La Jolla, CA). Analyses were performed using a two-way ANOVA (treatment x cell line) followed by Tukey's post hoc tests to compare the differences in means between groups.

Mitochondrial Membrane Potential: Changes in mitochondrial membrane potential (MMP) can be an early indicator of cell death, as it can coincide with the opening of membrane transition pores and increased membrane permeability (Kwiatkowska et al. 2020; Wu and Bratton 2013). This increased permeability can signal the apoptotic cascade (Kim et al. 2013; Kwiatkowska et al. 2020). A cationic, lipophilic dye, JC10, selectively enters the mitochondria where it concentrates and forms aggregates in the matrix. In healthy cells, these aggregates fluoresce red. In apoptotic cells, JC10 leaks out of the mitochondria into the cytosol and remains in monomeric form, where it fluoresces green (Abcam 2020). The ratio between healthy (red) and unhealthy (green) gives us a picture of cellular mitochondrial health dependent on JC10's ability to permeate the mitochondrial membrane and is reflective of the mitochondrial membrane potential. Cells were grown to confluence as described in the general cell culture methods section, trypsinized, and counted on a Corning cell counter. 2-5 $x10^{-4}$ cells per well were plated onto a black walled, clear bottom 96 well plate. Cells were allowed to

adhere for 24 hours before growth media was removed and treatments administered. Toxicant treatments were 100 µL per well as follows: 500 nM cadmium, 1 µM atrazine, 1 µM glyphosate, and mixtures of cadmium/atrazine and cadmium/glyphosate as outlined in general methods. The plate was laid out with an n of 4 in duplicate. Treated cells were incubated at 37°C and 5% CO_2 for 48 hours. Post incubation, 50 µL per well of JC-10 dye solution was added, prepared following Abcam JC-10 kit protocol (Abcam, Cambridge, UK). Cells were then returned to the incubator at 37°C and 5% CO_2 for 1 hour. After incubation, 50 µL of kit provided assay buffer B was added to each well prior to reading fluorescence. Samples were run through a Biotek plate reader both at 540ex/590em for the aggregate form and at $490_{ex}/525_{em}$ for the monomeric form. Analysis is done ratiometrically, dividing the RFU for aggregates by the RFU for monomers.

Statistics: Data were expressed as mean ± SEM of 3 analyses (n = 4) in duplicate. Data was treated as parametric and analyzed with GraphPad Prism (v 8.00, GraphPad Software, La Jolla, CA). Analyses were performed using a two-way ANOVA (treatment x cell line) followed by Tukey's post hoc tests to compare the differences in means between groups.

Oxygen Consumption: Oxygen consumption rate is a measure of normal cell function and was measured by an assay kit from Cayman Chemical (600800). In short, ATP production is essential to cellular health and oxidative phosphorylation is the most efficient means of its synthesis. Oxygen is required for oxidative phosphorylation to proceed and the rate of its consumption is an indication of mitochondrial health. Cells were seeded at 4-8 x 10^4 cells per well and exposed to toxicants for 48 hours. The

phosphorescent oxygen probe (Item #600801) was reconstituted using 1mL Molecular Biology grade distilled water prior to running experiment. Cells were removed after their incubation, and treatments were replaced with 150 µL of fresh cytotox media. The prepared phosphorescent oxygen probe was added to each well (10 µL) and overlaid with 100 µL warmed (37°C) HS mineral oil (Item No. 660910) using a repeating pipette. The protocol calls for 380ex and 650em with peaks at 360-340ex and 630-680em. Using available equipment, the plate was read kinetically on a BioTek Synergy 2 plate reader every 5 minutes for 2 hours at 340 ±30 nm excitation and 590 ±35 nm emission using a gain of 90.

Statistics: Data was expressed as mean ± SEM of 3 analyses (n = 3) in duplicate. Data was treated as parametric and analyzed with GraphPad Prism (v 8.00, GraphPad Software, La Jolla, CA). Analyses were performed using a two-way ANOVA (treatment x cell line) followed by Tukey's post hoc tests to compare the differences in means between groups.

Mitochondrial Toxicity- Membrane Integrity and ATP Production: HPNE and AsPC-1 cell lines were plated at a final density of 20,000-50,000 cells/well and allowed to adhere for at least 24 h prior to the assay. Toxicant exposure began by removing the growth media and replacing with treatment groups supplemented with either 25 mM glucose or 10 mM galactose. To establish the relationship between cellular viability and the loss of ATP, we used a luciferin-based detection system (Mitochondrial ToxGlo™; Promega). The foundation of the mitochondrial toxicity tests is that substituting 10 mM galactose for 25 mM glucose will increase susceptibility to mitochondrial toxins by eliminating aerobic glycolysis as an alternative energy source in the presence of galactose in order to

highlight toxicant effect on oxidative phosphorylation. The cells were exposed to treatment for 48 h prior to the initiation of the assay. The first step in the assay is assessment of necrotic protein presence as a marker of membrane integrity using bis-AAF-R110 substrate. This substrate cannot cross intact cell membranes of live cells and delivers a signal proportional to non-viable cells. After toxicant incubation and addition of substrate, fluorescence is measured at 485 ±20 nm excitation and 528 ±20 nm emission. After measurement, plate was allowed to equilibrate to room temperature. The second step in the multiplex was to quantify the amount of ATP present by directly adding 100 µL of luciferin-based ATP detection system into the wells and shaken on an orbital shaker for 5 minutes at 500rpm. Plate was read on a Bio Tek plate reader set for luminenesence and the luminescent signal is directly proportional to the amount of ATP present. Comparisons of the two data sets can identify whether the toxicant is a mitochondrial toxin or if cytotoxic mechanisms unrelated to mitochondrial health are present. Values were calculated as percent control and evaluated. There are several alternatives in data interpretation; *i.)* No change in membrane integrity or ATP reduction means the compound is not a mitochondrial toxin. *ii.)* If there is a reduction in ATP with commensurate changes in membrane activity, the compound is not a mitochondrial toxin, but is causing primary necrosis. *iii.)* Reduction in ATP with no change in membrane activity means the compound is a mitochondrial toxin. *iv.)* Reduction in ATP with discordant changes in membrane activity means the compound is a mitochondrial toxin.

Statistics: Data were expressed as mean ± SEM of 3 analyses (n = 3) in duplicate. Data was treated as parametric and analyzed with GraphPad Prism (v 8.00, GraphPad

Software, La Jolla, CA). Analyses were performed using a two-way ANOVA (treatment x cell line) followed by Tukey's post hoc tests to compare the differences in means between groups.

CHAPTER IV

RESULTS

Aim 1

Establishment of the toxicity threshold for cadmium, glyphosate, atrazine, and their mixtures on cultured of HPNE and ASPC1 pancreatic cells.

Experiment 1.1: LDH

Initial experiments used the LDH assay to examine the effects of four toxicants in HPNE and AsPC1 cell lines; cadmium, atrazine, glyphosate, and whole Roundup^{TM}, by exposing cells to 50 µM of each toxicant or 50 µM pesticide with 1µM cadmium for mixtures. Two way ANOVA revealed significant viability effects of treatment ($F_{7,32}$= 65.08; $p<0.0001$), cell line ($F_{1,32}$ = 4.675; p=0.0382), and significant interaction between treatment X cell line ($F_{7,32}$37.59; $p<0.0001$). Analysis of cell number showed significant effects of treatments ($F_{7,32}$=11.79; $p<0.0001$) and the interaction of treatments and cell line ($F_{7,32}$=6.980; $p<0.0001$). Cell line alone did not have significant effects on cell numbers ($F_{1,32}$=0.2174; p=0.6442). In HPNE cells, cadmium was the only treatment group to show a significant (p=0.0359) decrease in viability (10%) compared to control values. The only treatment group to show a decline in cell numbers from controls at this

concentration was the cadmium/atrazine mixture ($p=0.005$). No mixture treatment differed from either component alone in either viability or cell number, and glyphosate was not found to produce results that differed from Roundup™.

AsPC1 cells showed both decreased viability and cell number following cadmium treatment at the 50 µM concentration ($p<0.0001$) with viability and cell number only averaging 50% of controls. No other treatment groups had a significant viability response compared to untreated controls. The cadmium/Roundup™ mixture also significantly decreased cell number ($p=0.0002$). Cadmium alone was more effective at reducing cell viability compared to its mixture with atrazine ($p<0.0001$) and glyphosate ($p=0.0004$), but as part of a mixture with Roundup™ ($p=0.779$). None of the pesticides tested exhibited any effects on cell viability at the concentration tested. Figure 15 shows the LDH results for both cell lines.

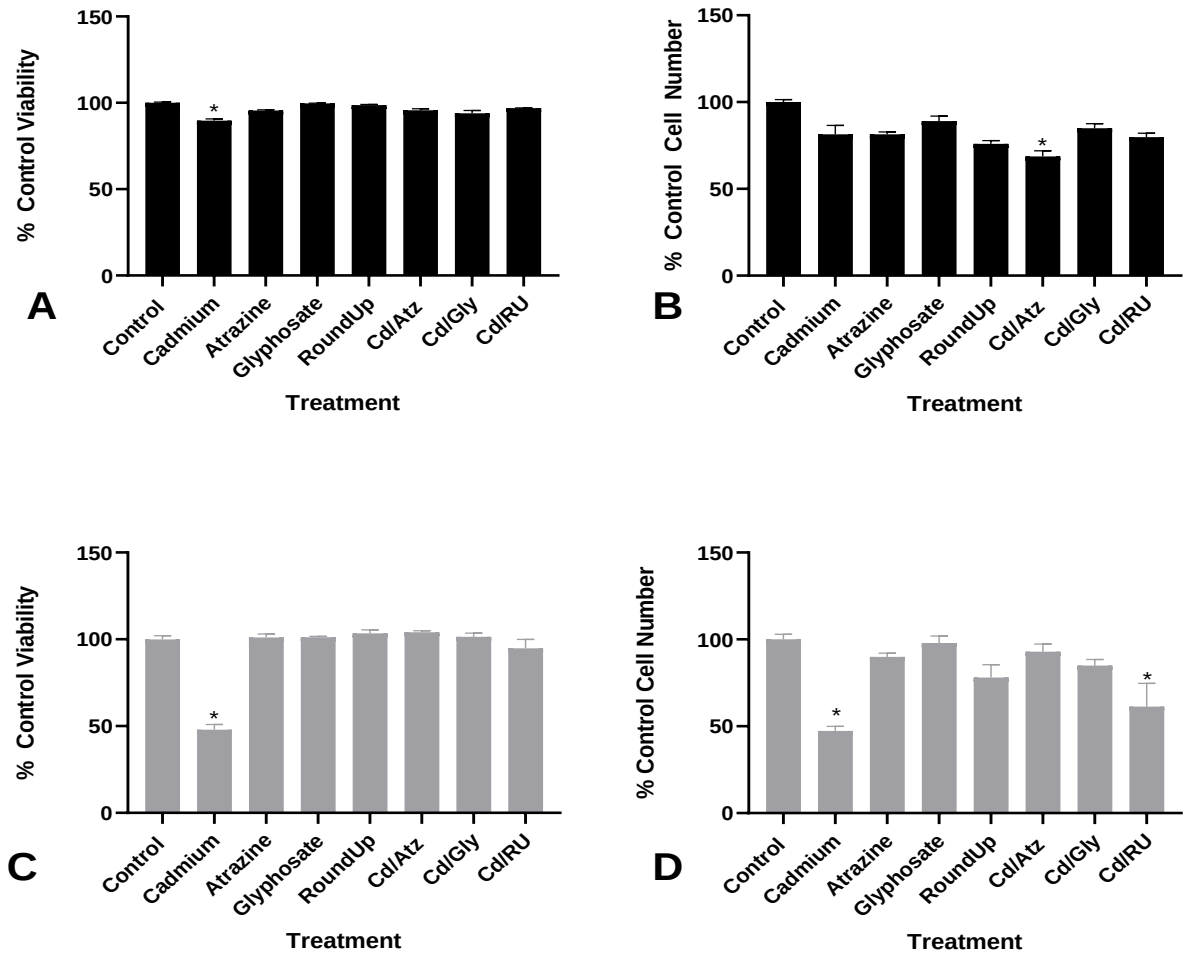

Figure 15: LDH testing used a concentration of 50 µM for individual toxicants. Mixtures were 50 µM pesticide with 1 µM cadmium (Cd). Cadmium resulted in a significant loss of viability in HPNE cells (A), while the cadmium/atrazine mixture had an effect on HPNE cell number (B). In AsPC1 cells cadmium decreased viability about 50% (C) and had a similar effect on cell number along with its mixture with glyphosate (Cd/Gly) (D). All data was analyzed with one way ANOVA and Dunnet's post hoc test. Abbreviations: Cadmium (Cd), Atrazine (Atz), Glyphosate (Gly)

Experiment 1.2: MTT Cytotoxicity and LC50 Determination

Experiments to determine the LC50 and identify sub-toxic test concentrations were performed using MTT viability testing as outlined in the methods chapter. Cadmium LC50 values were determined in two separate experiments, once with glyphosate mixtures and once with atrazine mixtures. All toxicant groups were assessed using non-linear regression best fit curves in a single-site model. In the glyphosate studies, cadmium alone exhibited a LC50 value of 36.5±4.51 µM in HPNE cells, whereas exposure to the mixture of cadmium and glyphosate resulted in a LC50 of 26.1±4.55 µM Cadmium exposure yielded an LC50 value of 46.5±9.36 µM alone and 38.9±4.56 µM when combined with 500 nM atrazine. Atrazine alone exhibited an LC50 value of 127.8±45.86 µM and with the addition of 500nM Cd, the LC50 value was 119.2±20.02 µM in HPNE cells. No test concentration had effects different from control after exposure to glyphosate or its mixture with 500 nM Cd, so non linear regression analysis could not be completed.

In cadmium chloride trials with 500nM glyphosate, AsPC1 cells exhibited an LC50 value of 4.4±0.295 µM for cadmium alone and 2.57±0.195 µM with the addition of 500nM glyphosate. In trials with atrazine, AsPC1 cells exhibited an LC50 value of 4.9±0.783 µM for cadmium and 3.91±0.542 µM when combined with

500 nM atrazine. These values indicate a ten fold greater sensitivity to cadmium for AsPC1 cells than HPNE cells. AsPC1 cells appeared to be less sensitive to atrazine exposure than HPNE cells, and LC50 values for atrazine alone was calculated to be 520.5±32.67 µM and 468.1±260.16 µM when combined with 500nM cadmium in the

tumor cell line. Again, no concentration of glyphosate resulted in viability different from controls, and LC50 values could not be determined.

Comparisons between LC50 values were assessed by one way ANOVA and determined there was a significant difference between cell lines for cadmium response in both its trials ($p=0.0190$ and $p<0.0001$), with AsPC1 LC50 values having an 8-9 fold shift to the left of HPNE values, suggesting greater sensitivity. In the cadmium chloride LC50 trials, no mixture with 500nM of either pesticide exhibited differences from cadmium alone, indicating cadmium was the driver of the response. Neither atrazine nor glyphosate exhibited differences in LC50 values between cell lines at the concentrations tested, and their mixtures with 500nM Cd also did not show differences from the parent pesticide in these trials. Figures 16 (HPNE) and 17 (AsPC1) show the non-linear regression curves for each toxicant.

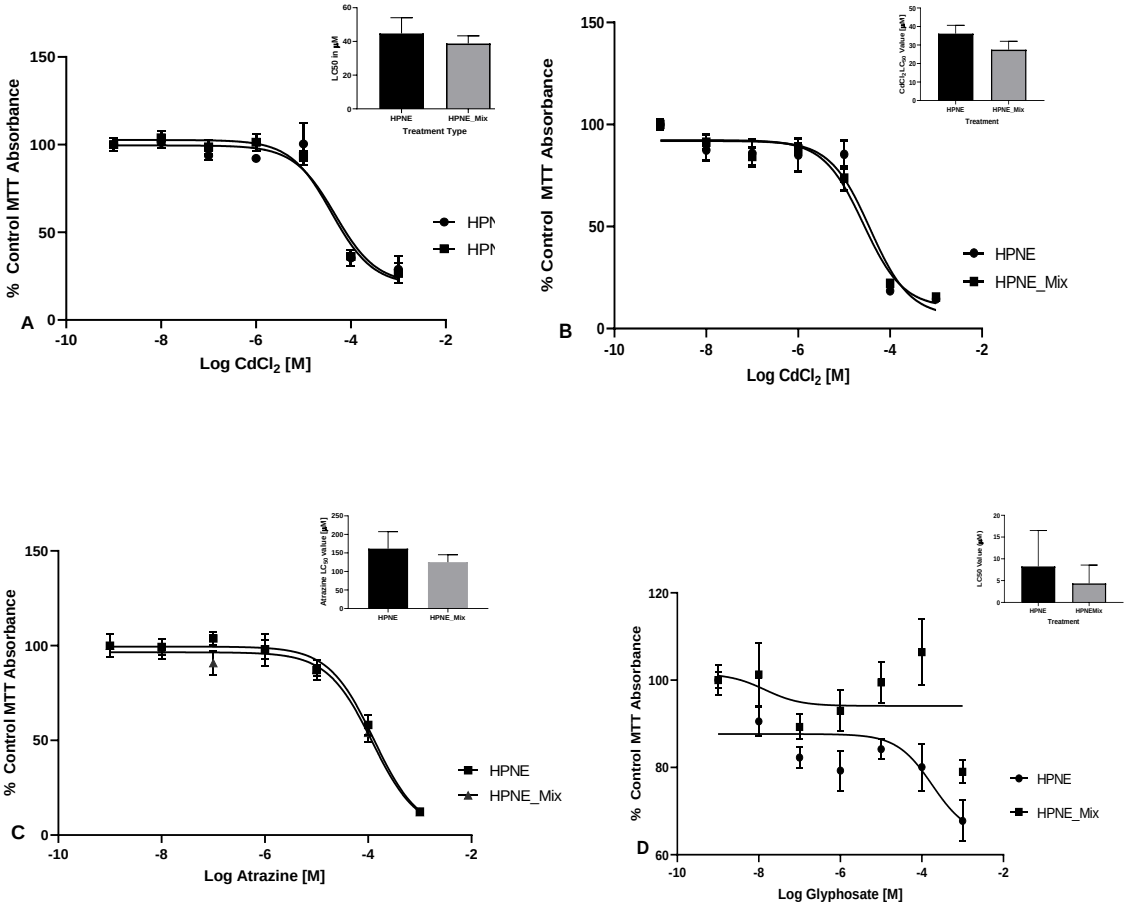

Figure 16: Non-linear regression analysis of LC50 values for HPNE cells in response to (A) cadmium chloride and its mixture with 500 nM atrazine, (B) cadmium chloride and its mixture with 500 nM glyphosate, (C) atrazine and its mixture with 500 nM Cd, and (D) glyphosate and its mixture with 500 nM Cd. While cytotoxicity threshold values appear to be around 10 µM for both cadmium groups, neither group seems affected by the addition of pesticide. While glyphosate had no discernable curve at the tested concentrations, atrazine and its mixture exhibited a threshold around 10 µM. N=6 and results are presented ±SEM.

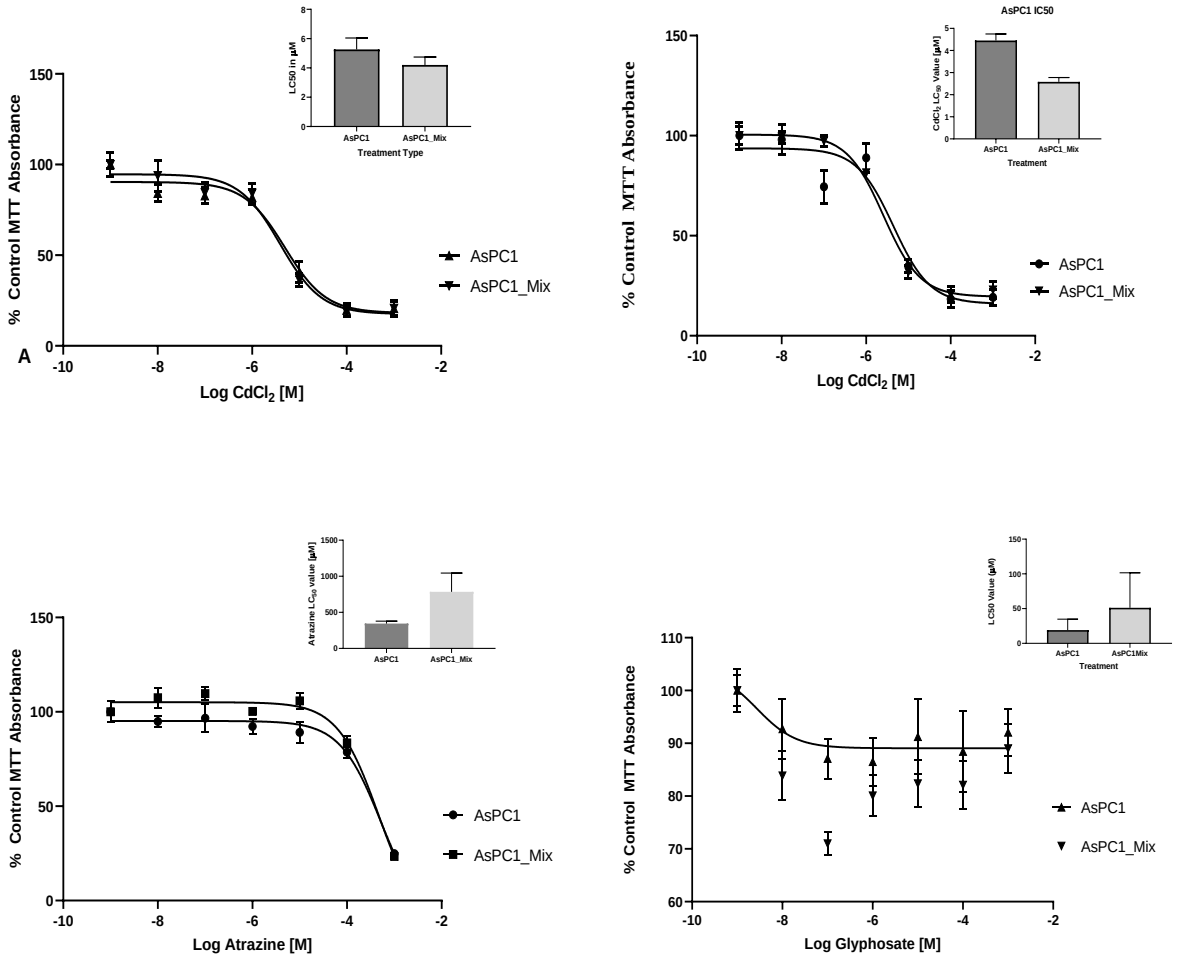

Figure 17: Non linear regression analysis of LD50 values for AsPC1 cells in response to (A) cadmium and its mixture with 500nM atrazine, (B) cadmium and its mixture with 500nM glyphosate, (C) atrazine and its mixture with 500nM Cd, and (D) glyphosate and its mixture with 500nM cadmium. While cytotoxicity threshold values appear to be around 1µM for both cadmium groups, neither group seems affected by the addition of pesticide. While glyphosate had no discernable curve at the tested concentrations, atrazine and its mixture exhibited a threshold around 100µM. N=6 and results are presented ±SEM.

Aim 2

Effects of toxicant exposure on apoptotic proteins

Experiment 2.1: p53

Examination of total p53 was done using Pierce p53 Colorimetric In-Cell Elisa kit #62216 from Thermo Fisher Scientific (Waltham, MA). The assay is analogous to a Western blot and works using target specific primary antibodies and a horseradish peroxidase conjugate as outlined in the methods section. All wells were also treated with Janus green to standardize results for differences in plating cell densities. Results were assessed using a ratio representing per cell fluorescence. Cells were treated after a 48 hour exposure to toxicant concentrations identified in the First Aim; 500 nM Cd, 1 µM pesticide, or mixtures of the two. Experimental groups (n=4) were tested in duplicate and seeded at an average of 1-5 x 10^4 cells per well. P53 fluoresence was divided by Janus green fluorescence to normalized data. Data was analyzed as percent control using two way ANOVA and Tukey's post hoc multiple comparisons tests on GraphPad Prism version 8.0. The results are shown in fgure 18.

There was a significant effect of treatment ($F_{(5,36)}$ =49.07; $p<0.0001$) and cell line ($F_{(1,36)}$=22.62, $p<0.0001$) on p53 expression. There was significant interaction between these variables as well ($F_{(5,36)}$=20.28; $p<0.0001$), highlighting the differences in treatment response between control HPNE cells and tumor AsPC1 cells. In post-hoc comparisons

with control cells, all treatment groups containing cadmium elicited an increase in p53 fluorescence of 54-71% in the HPNE cell line. Neither mixture produced significantly

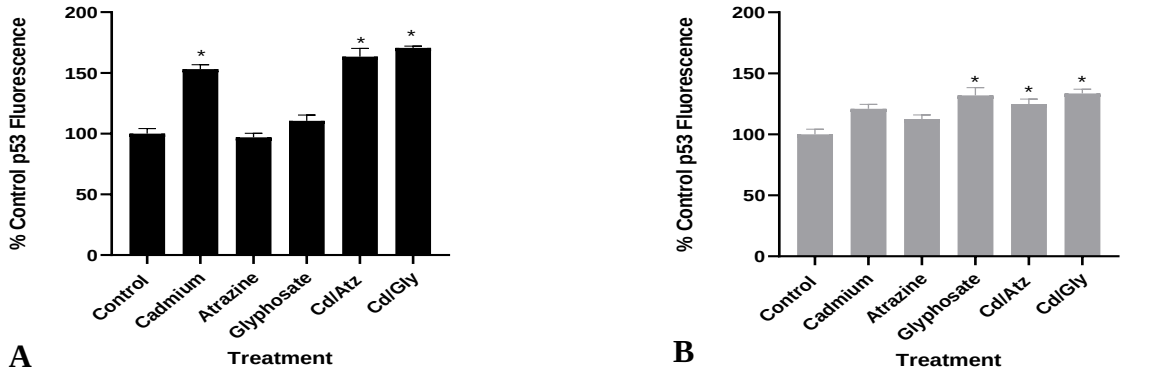

***Figure 18*:** p53 fluorescence response to 48 hour toxicant treatment in control HPNE cells (A) and tumor AsPC1 cells (B) expressed as percent control. Cadmium ($p<0.0001$), Cd/Atz ($p<0.0001$) and cadmium/glyphosate ($p<0.0001$) all showed significantly elevated p53 response in healthy cells. While cadmium values were not significantly different from the mixtures in this cell line, there was a slight but insignificant increase from the cadmium/glyphosate treatment ($p=0.1948$). AsPC1 tumor cells showed no increase in p53 response after incubation with cadmium, but glyphosate ($p=0.0004$), cadmium/atrazine ($p=0.0111$), and cadmium/glyphosate ($p=0.0002$) showed increased fluorescent signal. No treatment group showed statistical differences from any other treatment group in AsPC1 cells. Data was n=4 ± SEM

different p53 expression compared to cadmium treatment alone, but did differ from their respective pesticides ($p<0.0001$). This indicates cadmium could be the primary driver of the HPNE p53 response in this experiment. It is important to note that in the HPNE cell line, Janus Green testing showed all treatment groups had significantly more cells (10-21%) than controls, though no treatment groups differed from one another. In AsPC1 cells, all treatments containing cadmium showed significantly fewer cells compared to controls while pesticides had no effect. It is possible that cell number

may influence p53 response despite the normalization of the data. Whether cells are proliferating or not growing, both can potentially mediate response and should be explored.

Conversely, glyphosate (p=0.0004), and both mixtures increased p53 responses in AsPC1 tumor cells (Cd/Atz p=0.0111; Cd/Gly p=0.0002). There was a slight but insignificant increase in p53 response to cadmium (p=0.0580).AsPC1 cells produced significantly less p53 response compared to control HPNE cells across all treatment groups ($p<0.0001$).

Experiment 2.2: Caspase 3/7 Activity

Exposure to toxicants alone or as a mixture for 48 hours resulted in a significant effect on caspase 3/7 activity that was dependent on the treatment ($F_{(5,60)}=2.929$; p=0.0197) and the interaction between treatment and cell line ($F_{(5,60)}=4.827$; p=0.0009). Post hoc tests revealed no differences from controls in caspase 3/7 activity for any treatment group in either cell line. The two mixture groups in HPNE cells showed differences (p=0.0168) with Cd/Atz having decreased caspase activity compared to Cd/Gly. The only treatment group to show statistically significant differences between the cell lines was the Cd/Atz mixture (p=0.0107). Figure 19 shows the results comparing both cell lines side by side.

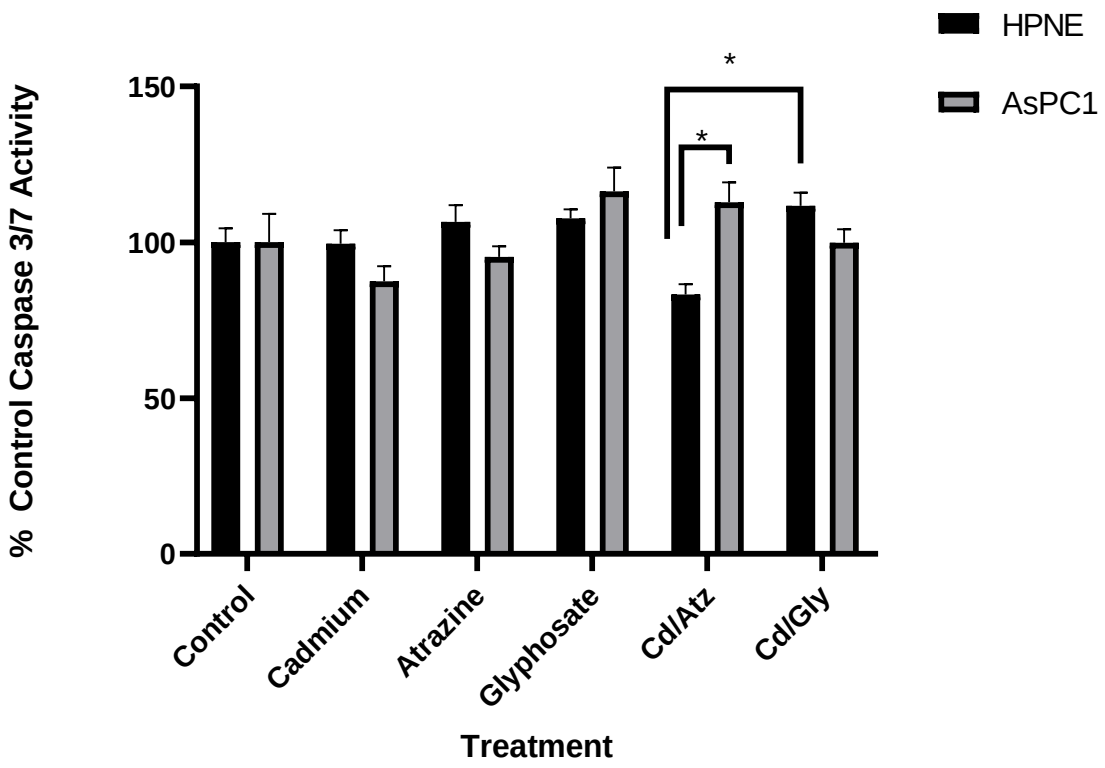

Figure 19: Measurement of caspase 3/7 activity in HPNE cells and AsPC1 cells shown as percent of control. Neither cell line showed any statistical variance from controls after a 48h exposure to treatments. Data was run using n=4 ± SEM.

Experiment 2.3: Annexin

Experiments (n=4) involved duplicate testing with an n=3 in for control wells, and one replicate set of no cell controls. This was done to establish baseline signals and results are expressed as percent control. After a 48 hour exposure, we observed a significant effect of treatment ($F_{(5,34)}$=95.11, $p<0.0001$), with an observed

significant interaction between cell line and treatment ($F_{(5,34)}$=81.80, p<0.0001). HPNE control cells had differing responses to each of the six treatments, though all responses were significant (p<0.0001). Cadmium exposure increased annexin expression up to 100% over control values while all other pesticide containing groups produced signals significantly lower with luminescence about 50% of control values. While cadmium induced significantly more PS expression than its mixtures in control cells (p<0.0001), neither pesticide differed from their mixtures. In contrast, AsPC1 tumor cells showed no variability between any treatment groups. All treatment groups expressed statistical differences across cell lines. Figure 20 highlights the resulting changes on the cell membrane in each cell line.

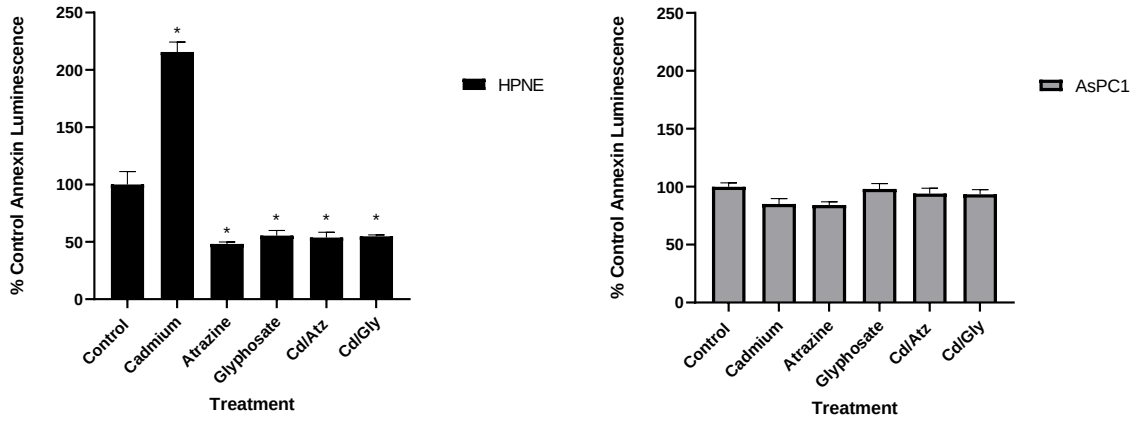

Figure 20: Annexin V assay for the presence of phosphatidylserine on the outer membrane of cells as a measure of apoptosis in HPNE control cells (A) and AsPC1 tumor cells (B). Cadmium elicited a 100% change in PS signal in control cells after a 48 hour incubation while all pesticide containing groups showed a significant reduction in PS membrane presence (A). AsPC1 tumor cells showed no deviations from control values for any treatment group (B). Data was n=4 ± SEM.

Aim 3

Bioenergetic response to toxicants

Experiment 3.1: DCFH

There were significant associations between ROS fluorescence and treatment ($F_{5,60}$=40.50; $p < 0.0001$), cell line ($F_{1,60}$=34.78; $p < 0.0001$), as well as their interaction ($F_{(5,60)}$=3.084; p=0.0152) after two way ANOVA. AsPC1 cells showed a 7-15% increase in ROS fluorescence compared to HPNE cells after a 48 hour incubation as described in general methods, and comparisons of each treatment between cell lines reflected that significant increase. All treatment groups in both cell lines produced slight but significant increases in ROS when compared to their untreated controls. Though there were no differences between cadmium and its mixture with atrazine in HPNE cells, the cadmium/glyphosate group differed from cadmium alone (p=0.0001). Both mixtures exhibited statistically significant differences from one another in HPNE cells ($p<0.0001$). AsPC1 cells revealed no differences between any treatment group, but treatments increased ROS production 5-10% compared to untreated controls. The only treatment groups to show differences between cell lines was the Cd/Gly mixture ($p<0.0001$). Figure 21 shows the production of ROS compared to controls for each cell line.

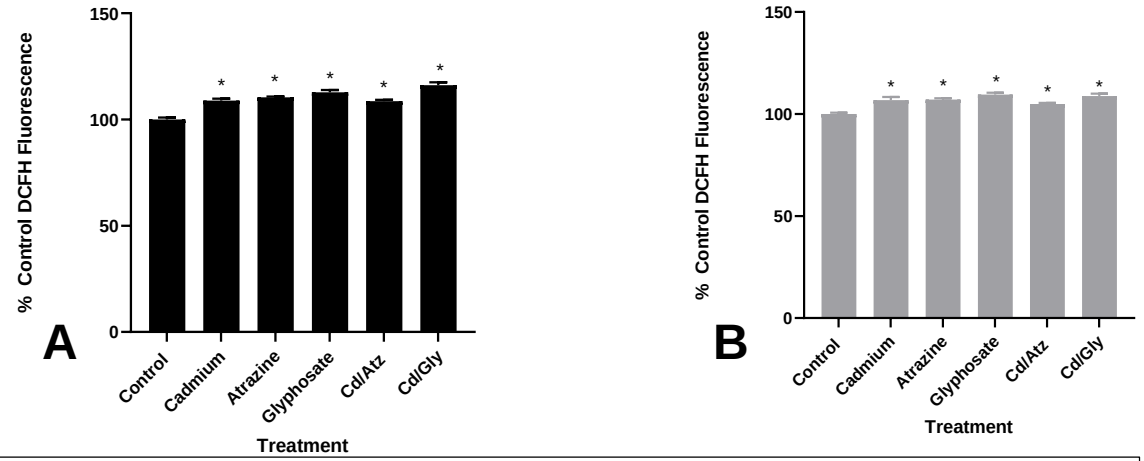

Figure 21: All treatment groups in both cell lines produced significantly more reactive oxygen species than untreated controls. HPNE cells yielded about 11% less ROS fluorescence than AsPC1 cells and comparisons between cell lines were significant for every treatment group. In the control group (A), the cadmium glyphosate mixture showed a statistical increase from both cadmium alone as well as from the other mixture. No mixture produced different results from their components or from one another in AsPC1 tumor cells (B). Data is expressed as n=4 ± SEM.

Experiment 3.2-3: Glutathione & Conjugated GluathioneTesting

Experiments measured both total glutathione protein as well as conjugated glutathione content to determine if the treatment groups had an effect on antioxidant response. No treatment group in either cell line appeared to up-regulate total glutathione expression despite the fact that slight increases in ROS were detected. Additionally, there was no statistical difference in basal levels between the cell lines. Glutathione levels were measured every 5 minutes for 30 minutes due to the quick degredation of glutathione ($t_{1/2}\approx$1 hour), but no differences were found.

The next objective was to assess conjugated glutathione in our samples. If total glutathione remains unchanged, but conjugated glutathione increases, the ability of free radicals to trigger apoptosis is impeded. Interestingly, although total glutathione was the same in both cell lines, AsPC1 tumor cells expressed roughly 50% more conjugated glutathione and the cell line dependent effect on the results was significant ($F_{(1,24)}$ = 72.90; $p<0.0001$). There were no differences between any treatment group in conjugated glutathione levels. Figure 22 shows the results for both total and conjugated glutathione.

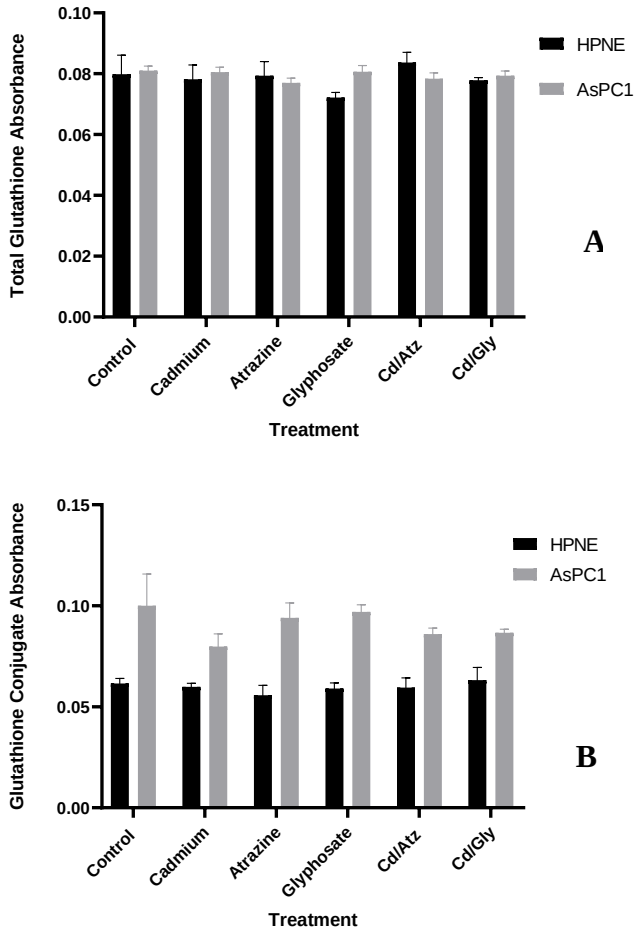

Figure 22: Analysis of total glutathione production. No treatment group showed statistical differences in controls from either cell line, and neither cell line exhibited statistically distinct levels of glutathione protein from the other (A). Though no treatment groups were different from controls, conjugated glutathione was increased in AsPC1 tumor cells an average of 40% (B). Data is expressed as n=4 ± SEM.

Experiment 3.4: JC10 Mitochondrial Membrane Potential

There were significant effects that were both cell line- ($F_{(1,36)} = 7688$; $p<0.0001$) and treatment-dependent ($F_{(5,36)} = 18.67$; $p<0.0001$), as well as their interaction ($F_{(5,36)} = 11.90$; $p<0.0001$), highlighting the differences between HPNE and AsPC1 cell line response. Although treatment groups had a significant change in mitochondrial membrane potential from untreated controls in the HPNE cell line, AsPC1 tumor cell

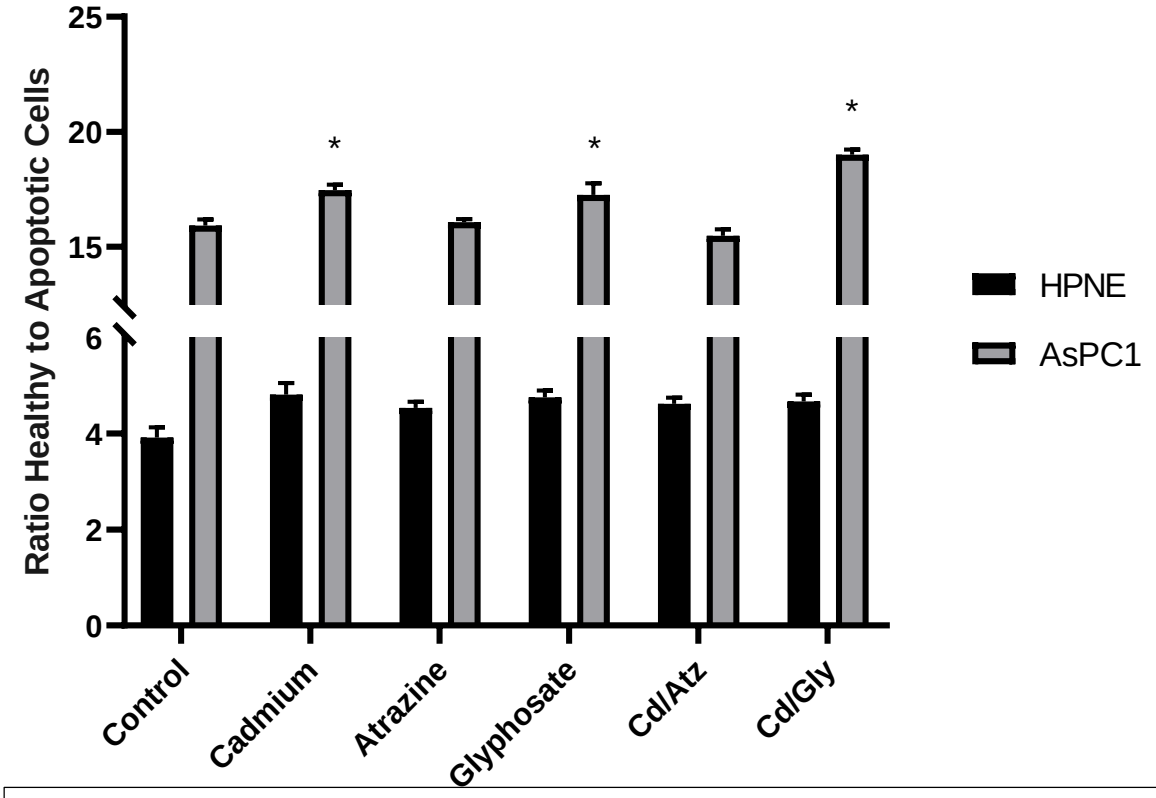

Figure 23: Cell line comparison of mitochondrial membrane potential in HPNE and AsPC1 cells after 48h exposure to toxicants. AsPC1 cells exhibited significantly higher ratios of healthy to apoptotic cells at basal levels and across all treatment groups. No HPNE treatment groups showed differing MMP from control values. In tumor AsPC1 cells, cadmium, glyphosate, and the Cd/Gly mixture all showed increased MMP from no treatment controls. The differences between AsPC1 treatment groups are outlined in table 2. Data is expressed as n=4 ± SEM.

ratios between healthy and impaired cells were significantly increased for treatments with cadmium ($p=0.004$), glyphosate ($p=0.0209$), and the cadmium/glyphosate mixture ($p<0.0001$) compared to control cells. Both cadmium and glyphosate treatments alone differed from their mixtures ($p=0.0035$ and $p=0.0006$) respectively, though the difference was less than additive. Additionally, cadmium alone was associated with an increased mitochondrial membrane potential compared to its mixture with atrazine ($p<0.0001$). Comparisons with atrazine and the mixture were not significant. This could indicate enhanced viability and heterogeneity of the cell (Zorova et al. 2018). JC10 results for both cell lines can be found in figure 23.

Experiment 3.5: Oxygen Consumption

Oxygen consumption experiments were performed using the Oxygen Consumption Rate Assay Kit from Cayman Chemical (Ann Arbor, MI, product # 600800), and fluoresence was read and transformed to percent control. The assay was read kinetically every 5 minutes for 2 hours. Non-linear regression analysis was used to determine slope that correlates to the rate of oxygen consumption. There were no significant findings between treatments or cell lines. V_{max} was analyzed to determine if a

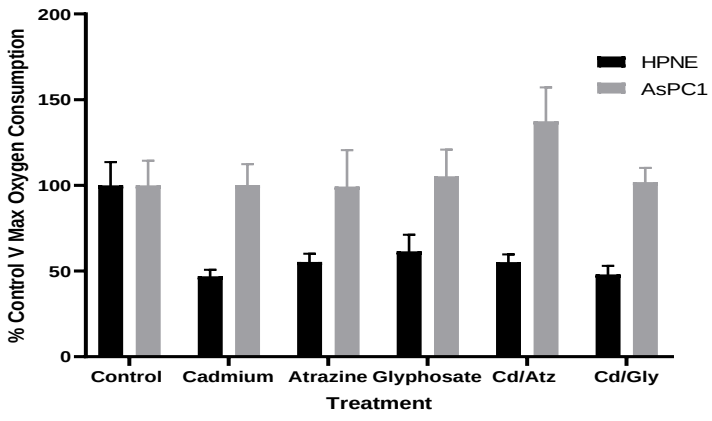

***Figure 24*:** Percent control cell line comparison of Vmax oxygen consumption. Treatment differed from controls in either cell line. The only treatment to show significance between cell lines was the Cd/Atz mixture ($p=0.0017$). Data is expressed as n=4 ± SEM.

new baseline rate had been set post exposure, and the cell line comparison is found in figure 24. After the 48 hour incubation, there was no treatment dependent effect in V_{max} values ($F_{(5,34)}$=1.1571, p=0.1946), but effects dependent on cell line were observed ($F_{(1,34)}$=41.15, p<0.0001). AsPC1 cells exhibited 69-127% increases in V_{max} over that of HPNE cells for all treatment groups, even though control cells did not exhibit a change. The only treatment to show statistical differences between cell lines was the Cd/Atz mixture (p=0.0017).

Experiment 3.6: Mitochondrial Toxicity

In our cytotoxicity experiments there were significant effects of treatment ($F_{(5,36)}$ = 4.075; p=0.0049) and media ($F_{(1,36)}$=117.3; p<0.0001) as well as treatment X media ($F_{(5,36)}$=9.145; p<0.0001) in HPNE cells. These results are summarized in figure 25 for HPNE and 26 for AsPC1. In the presence of glucose in HPNE cells, both atrazine (p=0.006) and glyphosate (p=0.0124) showed decreased membrane integrity compared to untreated controls. In the presence of galactose, no treatment decreased membrane integrity. However, all treatment groups containing glyphosate appeared to show increased membrane integrity in the HPNE cell line (p=0.0395 for glyphosate and p=0.0053 for its mixture) with galactose supplementation. ATP production for the control cell line also showed significant effects from treatment ($F_{(5,36)}$=8.552, p<0.0001), media ($F_{(1,36)}$=267.8;p<0.0001), and their interaction ($F_{(5,36)}$=11.17; p,0.0001). In the presence of glucose, cadmium appeared to have no impact on ATP production in HPNE cells. All other treatment groups showed significant declines in ATP production. In the presence of galactose, no treatment group was different from control in ATP production. Holistic examination of the HPNE data sets suggests that both pesticides alone are

showing both decreased membrane integrity concurrent with decreased ATP production, which is consistent with primary necrosis.

Both mixtures analyzed showed no membrane integrity effects, but diminished ATP production, indicating that they are acting as mitochondrial toxins in the presence of glucose. While cadmium had no effect on either parameter in these experiments, it is important to note that while membrane integrity was similar to untreated values ($p=0.9994$), it showed a slight but insignificant decrease in ATP production ($p=0.0921$). It is possible cadmium could be a weak mitochondrial toxin.

MitoTox testing in AsPC1 tumor cells again showed significant effects from both treatment ($F_{(5,36)} = 225.7$; $p<0.0001$) and media ($F_{(1,36)} = 300.8$; $p<0.0001$) as well as their interaction ($F_{(5,36)} = 18.59$; $p<0.0001$) in membrane integrity analysis. In contrast to HPNE cells, cadmium ($p<0.0001$), as well as its mixtures with atrazine ($p<0.0001$) and glyphosate ($p<0.0001$) exerted significant effects on membrane integrity concurrent with significant decreases in ATP production ($p<0.0001$ for all groups). Cadmium appeared to be driving the effect, and it was not significantly different from its mixtures by either parameter. The experimental profile implicates primary necrosis is occurring in these treatment groups in the presence of glucose. The individual pesticides, however, reacted oppositely, showing reinforced membrane integrity for atrazine ($p=0.0003$) and glyphosate ($p<0.0001$), but with no effect on ATP production. ATP production in glucose experiments mirrored membrane integrity effects of treatment ($F_{(5,36)} = 169.2$; $p<0.0001$), media ($F_{(1,36)} = 205$; $p< 0.0001$), and their interaction ($F_{(5,36)} = 18.18$; $p< 0.0001$).

AsPC1 response with galactose supplementation showed similar responses to the glucose group with notable exceptions. Treatment, media, and their interaction was significant for both membrane integrity and ATP production. Treatment values were ($F_{(5,36)}$=225.7; p<0.0001) for membrane integrity and ($F_{(5,36)}$=169.2; p<0.0001) for ATP production. Media values were ($F_{(1,36)}$=300.8; p<0.0001) and ($F_{(1,36)}$=205; p<0.0005) respectively, and treatment effects were ($F_{(5,36)}$=18.59; p<0.0001) and ($F_{(5,36)}$=18.18; p<0.0001). Rather than having no effect from pesticide exposure as in glucose supplemented media, the addition of galactose appeared to reinforce membrane integrity for both atrazine and glyphosate (p<0.0001) while increasing ATP production (p=0.0003 and p<0.0001 respectively). It is important to note that in the presence of glucose, controls for both cell lines did not demonstrate a difference in membrane viability, but AsPC1 cell groups containing cadmium showed a 3 to 5 fold increase in membrane degradation compared to HPNE cells. Pesticide groups showed less membrane degradation in AsPC1 cells than HPNE cells, but glyphosate narrowly missed significance (p=0.0619). At the same time, all AsPC1 groups produced significantly more ATP than HPNE groups in the presence of glucose, with the pesticides producing up to 300% more ATP than their HPNE counterparts. Conversely, with galactose supplementation, AsPC1 control cells expressed significantly more necrotic protein than HPNE controls, and cadmium groups mirrored their glucose results. ATP production in galactose was similar to glucose, with AsPC1 groups expressing more ATP across the board. It is important to note that in the presence of glucose, untreated AsPC1 cells expressed nearly three times the signal of untreated HPNE controls. In galactose, that differential decreased to two-fold.

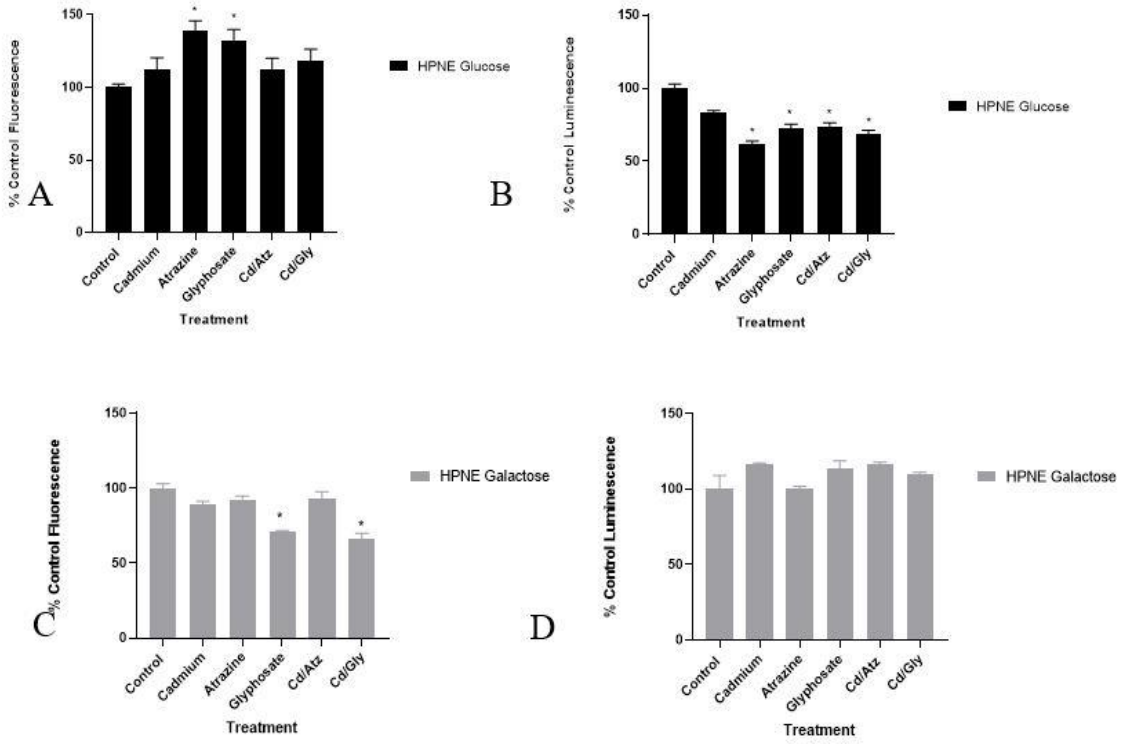

Figure 25: HPNE response to toxicant 48h incubation with supplemented glucose for (A) membrane integrity and (B) ATP production and galactose for membrane integrity (C) and ATP production (D). In glucose, atrazine and glyphosate exhibited decreased membrane activity and decreased ATP production, indicating primary necrosis. The mixtures however, had no membrane effects while producing statistically less energy, indicating they are toxic to the mitochondria under those conditions. No treatment group expressed compromised membrane integrity or differential ATP production with glucose supplementation. Groups containing glyphosate did show increased membrane integrity in the galactose group.

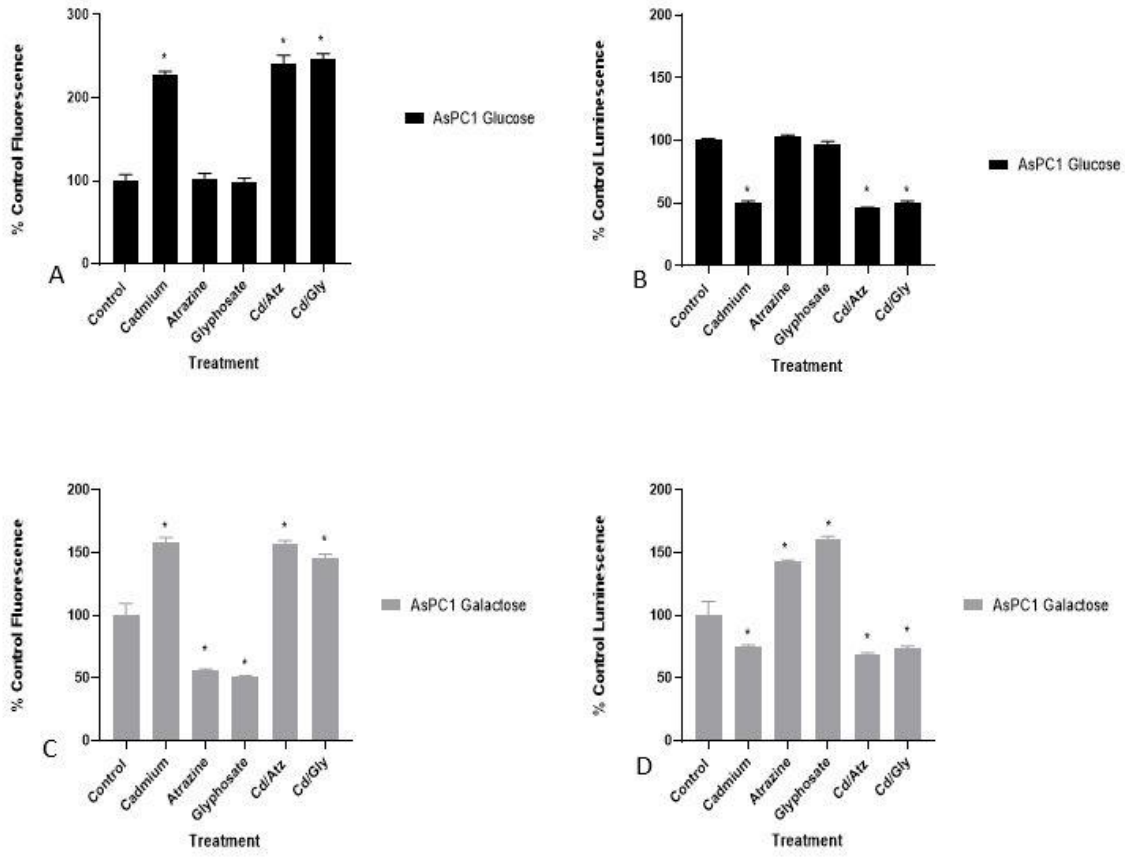

***Figure 26*:** AsPC1 response to toxicant 48h incubation with supplemented glucose for (A) membrane integrity and (B) ATP production and galactose for membrane integrity (C) and ATP production (D). In glucose, all cadmium groups expressed significantly elevates signal of necrotic associated proteins concurrently with decresed ATP production, indicating the presence of primary necrosis and highlighting AsPC1's sensitivity to cadmium. In the presence of galactose, the groups containing cadmium behaved the same, but the pesticide groups both expressed a decreased signal for necrotic protein concurrently with increased ATP production. Data is expressed as n=4 ± SEM.

CHAPTER V

DISCUSSION AND CONCLUSIONS

Of the known elements, transition metals are some of the most toxic. Cadmium specifically has been identified as potentially tumorigenic and has been correlated to several cancers including pancreatic adenocarcinoma (Wätjen et al. 2002; Waalkes 2003; Kundu et al. 2011). Although it does not switch valence states as easily as other known carcinogenic metals like mercury, cobalt, and chromium, it can produce ROS and operate through similar cellular mechanisms. Cadmium's presence as a pollutant ensures its presence in the food supply and it preferentially sequesters in the leaves of plants (Shacklette 1972). Additionally, cadmium is considered to be the only metal toxic to humans and animals at plant tissue concentrations that are not phytotoxic to the plant itself (Ismael et al. 2019). Since cadmium is ubiquitous throughout the environment, interactions with glyphosate or atrazine would be likely considering the prevalence of pesticide use. These pesticides are identified in over 90% of the food supply in trace amounts, and little is known about their long term effects (Mesnage et al. 2013; Séralini et al. 2014). While most toxicological reports are limited to individual compounds, the interaction of these toxicants can change the toxicokinetic and toxicodynamic properties specific to the mixture (Morya and Vachhrajani 2014; Wallace and Buha Djordjevic 2020). This study aimed to establish sub toxic concentrations of both individual toxicants

and their mixtures to use in subsequent apoptotic and bioenergetics analyses in order to explore molecular changes being affected. It was imperative when identifying sub-toxic concentrations that they be biologically relevant and within or below recommended minimum exposure limits set by regulatory agencies. Previously published studies from this laboratory indicated the serum concentration in test media impacted neither cell growth nor viability, allowing us to eliminate that confounder (Wallace et al. 2019).

The importance of concentration in mixture toxicity studies is paramount when assessing risk. Contrary to real life exposure scenarios, the majority of current risk assessment data is compiled from the analysis of independent chemicals. A particular insult may not be carcinogenic individually, but combined with concurring insults with other chemicals may produce a synergistic or potentiative effect. The EPA has focused additional attention on the importance of mixture profiling, and new models are being devised to facilitate this goal. The primary benchmark for mixture analysis is dose addition (Ilboudo et al. 2014; Nelms et al. 2018). A mixture is defined as synergistic when their toxic effects are in excess of their predicted additive values, or antagonistic when these values are less than their predicted additive toxicity. Further, mixtures may exert effects differentially between both organism and tissue type within the same organism, necessitating viability analysis subjective to particular models. Non-linear regression curves can pinpoint sub toxic concentration values and identify LC50 values. Using viability as the measure of toxicity for an *in vitro* system, seemingly harmless concentrations can be tested for their effects on specific proteins and pathways to determine if alternative consequences exist. There are several alternatives for measuring cell viability, and each has their own pros and cons. A wonderful comparative study of

these options by (Bopp and Lettieri 2008) examined the potential differences in sensitivity between MTT, LDH, Alamar Blue, and CFDA-AM in zebrafish liver, and determined all options had comparable and reproducible results. Two tests determine if alternative consequences exist. Two tests were used in our determinations, lactate dehydrogenase (LDH) and tetrazolium dye 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT). LDH is a cytosolic enzyme present in most all cell types. LDH catalyzes the formation of pyruvate from lactate and rapidly leaks out of the damaged cell membranes of non-viable cells. The assay uses an enzymatic reaction that converts resazurin into a fluorescent resofurin product that can be read by a plate reader. The addition of matched groups of lysed cells has the additional benefit of allowing us to examine total cell counts to account for cell stasis, which could be equally damaging for a population over time. In contrast, MTT is taken up by living cells and reduced to formazan in the mitochondria. Therefore, while LDH assays measure viability based on membrane integrity, MTT viability assessment is considered to be a measure of metabolic activity. In these experiments, LDH was used in preliminary testing to determine the effect between Roundup™ formulation and its active ingredient, glyphosate, and to identify an optimal subtoxic threshold concentration to use in future studies. The combination of both the LDH and MTT tests will permit a more complete picture of treatment effects.

LDH testing using identical concentrations of toxicants confirmed that 50µM cadmium had significant effects on the viability of both HPNE and AsPC1 cells. This concentration of cadmium is accepted to be above the concentration that will kill 50% of the sample (LC50) in human cells and predictably, we saw significant reductions in both

cell lines after cadmium exposure at that concentration. Exposure to the toxicant mixture did not alter viability compared to controls. The mixture concentration tested produced no differences from controls in all viability tests for HPNE cells, potentially indicating that 1µM cadmium mixtures are below the toxic threshold for cadmium. Additionally, exposure to neither pesticide elicited viability effects in control cells. AsPC1 cells exhibited a much more pronounced viability decline in response to the cadmium test concentration, and this value was significantly different from all mixture groups. No pesticide was significantly different from its mixture. Interestingly, Roundup™ appeared to have little impact on viability in either cell line, and produced no cell number declines compared with glyphosate alone. Since exposure to Roundup™ did not result in any discernable viability changes, it was eliminated from further testing, to avoid interactions with unknown components of the proprietary formula. Serial dilutions of each toxicant proceeded to both serve as a comparison for further MTT testing and to assess cell number perturbations at multiple concentration points for each toxicant. This helped us to determine if disruption of the cell cycle might occur upon exposure to the compounds. In both cell lines, cadmium showed decreased cell counts at 100µM and 1mM, the highest two concentration points. These values are in line with previous cytotoxicity studies for cadmium (Wallace et al. 2019; Hinkle and Osborne 1994; Tchounwou et al. 2001; Sarabia et al. 2002; Goulet and Hontela 2003). Atrazine mirrored these results with significant decreases at the same concentrations while glyphosate showed no impact on decreased cell numbers at any tested concentration. Keeping LDH data in mind, MTT viability testing was performed on both individual toxicants as well as mixtures. Similarly to LDH, all compounds were tested individually using serial dilutions from

1mM to 1nM concentration, then in mixtures with 500 nM pesticide or 500 nM cadmium. This concentration was chosen for uniformity between toxicants, as a large body of scientific literature and testing in this lab indicates an expected NOAEL for cadmium at 1 µM. LC50 values in cell culture can show variability between cell lines and exposure times. A 48 hour exposure time was set to represent a more chronic exposure. As anticipated, cadmium LC50 values for AsPC1 cells increased over HPNE cells approximately tenfold, highlighting their greater sensitivity to cadmium. This is not surprising as HPNE cells are more equipped to mitigate potential toxicant damage as their defense mechanisms are intact. Conversely, AsPC1 cells were much less sensitive to atrazine exposure than HPNE cells, with LC50 values differing by a factor of four. Our LC50 values for atrazine were in alignment with testing performed with embryonic kidney cells after a 24 hour incubation (Benachour et al. 2007). Pesticide exposure in general was much less toxic than metal exposure, with atrazine roughly three times less toxic to HPNE cells and more than 100 times less toxic in AsPC1 cells. Glyphosate, showing no cytotoxic effects in either cell line at tested concentrations up to 1mM, produced no LC50 values in these experiments. These findings are in alignment with current glyphosate research on hepatic, embryonic, and placental cell lines as well as systemic research in both rats and zebrafish (Kim et al. 2013; Mesnage et al. 2013; Pereira et al. 2018). Mixtures were preliminarily set at 500nM for all compounds, strictly to see potentiative effects, and no mixture differed significantly from its parent toxicant at that concentration. Although concentrations in this project were set, it is important to note that both ratio and sequence of exposure could have profound implications in mixture studies. MTT analysis allowed us to identify LC50 values for all pollutants and to

generate dose response curves permitting clear visualization of concentrations below the adverse response thresholds. Our experiments, coupled with current literature, identified a cadmium concentration of 1µM to be beyond the adverse effect limit for both cell lines. In recognition of the bioaccumulative nature of cadmium metal and in the interest of surety, a half concentration point below that value was chosen as our test concentration for that metal. This value was subjected to analysis using the Fractional Occupancy Equation and roughly 1.2% of cell death could be contributed to the 500nM exposure. For comparison, the daily intake of cadmium metal is estimated to be 32.58 µg per day and the Provisional Tolerable Daily Intake Level (PTDI) established by the World Health Organization is set at 62.3 µg (Rahmdel et al. 2015). Concentrations of 1µM were chosen for each pesticide. Atrazine potentially accounted for up to 0.9% of cell death at this concentration, and although glyphosate exhibited no noticeable toxic effects at concentrations much greater, consistent concentrations were used for both pesticides for examination of molecular apoptotic and bioenergetic endpoints. Mixtures were prepared using the determined individual toxicant concentrations. These concentrations were used in subsequent experiments.

Cell death occurs when cells are irreversibly dying and eliminated, and can have multiple causes. The two most common mechanisms leading to cell death are apoptosis and necrosis. Necrosis is considered a response to external stimuli, while apoptosis is a self-generated process designed to benefit the organism (Fink and Cookson 2005). Figure 27 showcases the differences between the two primary cell death mechanisms. Apoptosis

is a primary and fundamental cellular mechanism designed to serve multiple functions and is an innate response to the suppression of tumorigenesis. Many cancer drugs target the apoptotic machinery, which is disrupted in many cancers (Kaczanowski 2016),

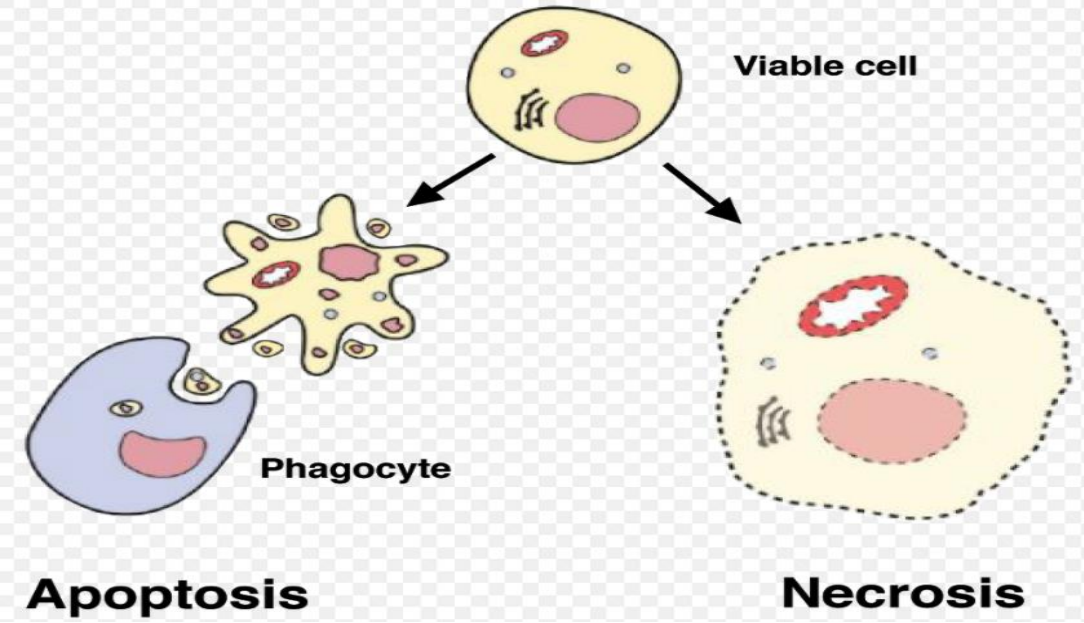

Figure 27: Pictoral representation of the differences between apoptotic and necrotic cell death. Necrosis is associated with a loss of membrane integrity and cellular swelling, while apoptosis is associated with membrane blebbing and cellular shrinkage.

primarily because apoptosis causes little to no inflammatory response (Jan and Chaudhry 2019). This dysregulation is thought to play significant roles in both tumor development as well as chemotherapeutic resistance (Pistritto et al. 2016). Apoptosis can occur naturally as during aging, or as part of immunity or response to toxic insults. Although apoptosis can be initiated both extrinsically and intrinsically, experiments in this aim focused on the intrinsic response. The intrinsic pathway is normally triggered by cellular stress either in the form of ROS, DNA damage, oncogene activation, or toxic chemicals (Jan and Chaudhry 2019). These stressors initiate the apoptotic machinery primarily

through p53. Tumor suppressor protein p53 is known as the guardian of the genome and has been highly conserved in eukaryotes for over a billion years (Lane et al. 2010). Half of all cancers have one or more p53 mutations, and that number increases to between 60 and 80% in pancreatic tumors (Dong et al. 2003; Wanebo and Vezeridis 1996). Once activated, p53 can itself mobilize multiple pathways, including DNA repair, cellular senescence, and apoptosis. These are summarized in figure 28.

Multiple proteins play a role in the modulation of apoptosis at varying levels, but for the

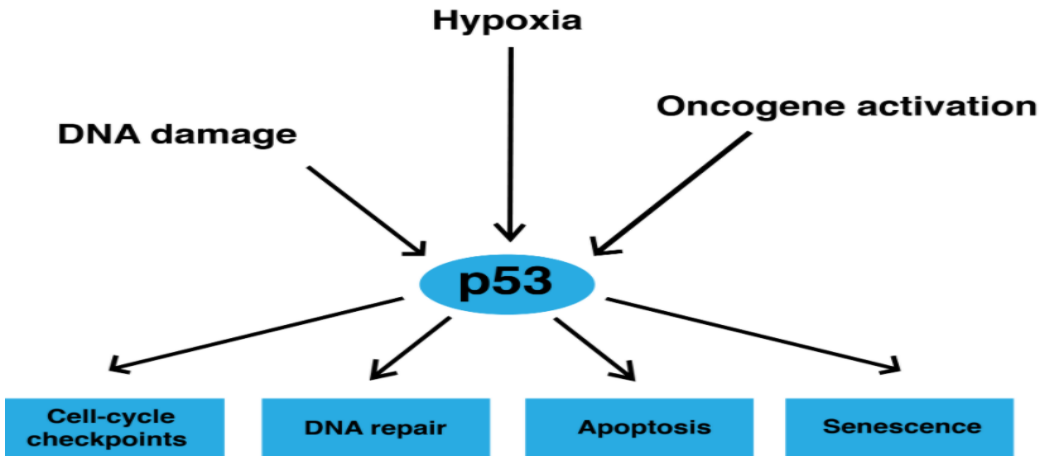

Figure 28 : p53 upregulation and its biological endpoints. p53 is involved in multiple pathways and can determine whether a cell becomes apoptotic, or senescent.

focus of this project, p53 recruits Bax which in turn initiates the release of cytochrome c from the mitochondria. Once released, cytochrome c activates initiator caspase 9 which in turn activates executioner caspases. Experiments in this aim looked at the initiation phase of apoptosis, exemplified by the upregulation of tumor suppressor protein p53, as well as the end of the pathway represented by activation of effector caspases 3 and 7. Additionally, potential early manifestation of apoptosis was examined by evaluating

differential presence of phosphatidylserine flipped to the external plasma membrane from its normal internal position by annexin V labeling.

Expression of p53 was increased in HPNE, and the significant differences we saw in expression between the cell lines could be attributed to the p53 mutation in AsPC1 cells. Cadmium appeared to be the driver in HPNE response, and has also been reported to stimulate accumulation of p53 in other studies (Chang et al. 2013; Lee et al. 2016). Neither pesticide interfered with p53 expression, though their mixtures with cadmium elevated p53 expression in HPNE cells. AsPC1 cells did not show an elevated response to cadmium alone despite the increased sensitivity in these cells as shown in viability testing. Rather, glyphosate and both mixtures initiated an elevated p53 response. Little literature exists on the relationship of the pesticides tested here and p53, and the literature that exists is conflicting. Glyphosate has been suggested to suppress p53 expression at much higher concentrations in blood cells (Kwiatkowska et al. 2017). A study done in rats showed atrazine increased p53 expression after exposure for six months in blood cells, but that the expression significantly decreased over longer term exposures (Cantemir et al. 1997). Our results indicated statistically significant increases in p53 response to glyphosate in the tumor cell line, even at sub-toxic concentrations. Atrazine alone had no effect on p53 expression in either HPNE or AsPC1 cells after 48 hours.

We then measured executioner caspases 3and 7 as the apoptotic endpoint. Caspases are a group of proteolytic enzymes active in apoptosis and are responsible for both cell signaling and the dismantling of cellular components (Lie et al. 2011). While many caspases are part of distinct extrinsic or intrinsic apoptotic pathways, the executioner caspases are ultimately responsible for the degradation of the cell regardless

of the stimulus (Walsh et al. 2008). The Apo-ONE Homogenous Caspase 3/7 Assay (Promega, Madison, WI) uses an assay buffer to facilitate entrance of the the non-fluorescent substrate rhodamine 10, bis-(N-CBZ-L-aspartyl-L-glutamyl-L-valyl-L-aspartic acid amide; Z-DEVD-R110) into the cell. Once inside, it is recognized and cleaved by caspases 3 and 7, and the DEVD peptides are removed from the molecule. Rhodamine 110 then acquires intense fluorescence at wavelength 499nm excitation and 521nm emission and the signal read by the plate reader is directly proportional to the amount of caspases 3/7 present in the sample. No treatment groups in either cell line appeared to interfere with caspase 3/7 activity, despite p53 upregulation.

The last assessment of this aim focused on analysis of membrane protein phosphotidylserine (PS) as a marker of early apoptosis. PS is known to re-orient from the inner membrane of a healthy cell to the outer membrane once the cell becomes apoptotic for recognition and removal (Gardai et al. 2006). The RealTime-Glo Annexin V Apoptosis Assay uses Annxin V fusion proteins LgBiT luciferase and SmBiT in the presence of calcium to bind to PS and produce a luminescent signal. Interestingly, HPNE cells did express an increased PS presence on the outer membrane in response to cadmium, despite the absence of caspase activity. Additionally, all pesticides and mixtures indicated a significantly decreased PS presence on the outer membrane, suggesting a role in increased membrane integrity in the HPNE line. No AsPC1 treatment group had any annexin response different from controls. Table 2 below helps summarize the apoptotic effects the six treatment groups had on both cell lines.

	HPNE			AsPC1		
	p53	Annexin	Caspase	p53	Annexin	Caspase
Cadmium	↑	↑	—	—	—	—
Atrazine	—	↓	—	—	—	—
Glyphosate	—	↓	—	↑	—	—
Cd/Atz	↑	↓	—	↑	—	—
Cd/Gly	↑	↓	—	↑	—	—

Table 2: Summary of Aim 2 apoptotic data. Arrows represent significant increases (gray) or decreases (red). Empty boxes indicate no change from control values.

Experiments to establish toxicant effects on bioenergetics and mitochondrial health were also performed. Recent literature suggests that flux between oxidative phosphorylation and aerobic glycolysis may be indicative of a cell's transition to a cancer state (Devic 2016; Zheng 2012; Smith et al. 2016). Impacts on mitochondrial health and the efficiency of ATP production have extensive consequences for cell survival. Otto Warburg's realization that cancer cells' dependence on aerobic glycolysis for energy production despite the low ATP yield, led him to surmise that a dysregulation of oxidative phosphorylation must be occurring (Zong et al. 2016; Jose et al. 2011; Fan et al. 2013). Recent research indicates that though this is not the case, a definite metabolic reprogramming occurs in tumor cells despite the presence of functional mitochondria and oxidative phosphorylation (Smith et al. 2016; Jose et al. 2011; Fan et al. 2013), and there may be a coordinated regulation of the alternative energy pathways (Ma et al. 2007; Mookerjee et al. 2017), though a decrease in oxidative phosphorylation is not compulsory with the upregulation of glycolysis in tumor cells (Fadaka et al. 2017). Assessment of

multiple measures of mitochondrial health and energy production are necessary to evaluate a toxicant's effects on energetics. This study examined production of reactive oxygen species (ROS) as well as glutathione antioxidant response, mitochondrial membrane potential, oxygen consumption, and mitochondrial toxicity assessments employing both cell membrane integrity and ATP production endpoints.

One important consideration in evaluating apoptosis and bioenergetics, is the presence of reactive oxygen species and oxidative stress. We looked at both ROS production as well as glutathione, the most prolific antioxidant protein (Bansal and Simon 2018). While the production of ROS by each of these toxicants has been studied, little or no work has been done to date on their combinations, and literature on these concentrations is scant. Cadmium's ability to induce the production of ROS is not as well defined as for other metals in its class, and cadmium may only weakly promote ROS production (Wallace et al. 2019; Djordjevic et al. 2019). Little work has been done on pancreatic cell lines with respect to glyphosate and atrazine exposure, but Martinez et al. determined 5mM glyphosate caused an ROS increase in neuroblastoma cells after a 48h exposure (Martínez et al. 2020). Our study identified increases in ROS across both cell lines and all treatment groups, but though significant, the 7-12% increases are unlikely to be of primary biological significance other than contributing to the overall body burden of free radicals. Because these experiments were done in a 48 hour timepoint, initial free radical production could have been mediated prior to testing.

The body's primary response to ROS production are free radical scavengers. Glutathione is considered to be the first line of defense against toxic insult, and the redox balance between ROS and antioxidants may play an important role in

homeostasis (Singhal et al. 1987; Forman et al. 2009; Sobrino-Plata et al. 2014). Glutathione is a thiol that works to neutralize free radicals in the body by making toxicants more water soluble for elimination (Forman et al. 2009). The ratio of oxidized glutathione (GSSH) to its reduced form (GSH) is an indicator of oxidative stress. Although we would expect most toxicants to elicit a change in the ratio of conjugated GSSH to unconjugated GSH, that is not necessarily the case. Downregulation of glutathione activity has been documented in response to cadmium, and decreased antioxidant potential is a proposed method of toxicity that can lead to a cancer state (Li et al. 2016; Ivanina et al. 2008). Despite the differences in free radical production, or perhaps due to the fact that those increases were slight, we found total glutathione production to be unaffected in our experiments. In concordance with multiple studies performed on pesticide and pesticide mixtures, we did not see any detectable increases in glutathione production in any of our treatment groups (Ivanina et al. 2008; Lushchak et al. 2009; Astiz et al. 2012; Li et al. 2016). While there is some discussion that glyphosate specifically may decrease intracellular glutathione levels, the concentrations and exposure times used in this analysis cannot support those findings (Li et al. 2016). What we can say, is that we did not see a noticeable impact on glutathione production based on the parameters of our analysis, necessitating the examination of conjugated glutathione to make a full assessment of the response.

The ratio of oxidized glutathione (GSSH) to its reduced form (GSH) is another indicator of oxidative stress. Though there were no changes in glutathione responses compared to untreated controls in either cell line, conjugated glutathione was 40% greater in AsPC1 tumor cells than HPNE healthy cells. This supports the theory that the ratio of

reduced to oxidized glutathione may be of great importance in the cancer state (Traverso et al. 2013). It could also support the suggestion that conjugated toxicants may enter cells more easily and may have enhanced toxicity. Some studies have suggested cadmium may be more likely to be deposited once complexed, and may show increased toxicity upon its release (Nordberg et al. 1975). While these observations were made in association with metallothionein, similar studies with other agents have yet to be documented. Glutathione response may not be the primary compensatory mechanism for these toxicants, and it could be that these concentrations and exposure times in these cell lines recruit an alternative response protein. Other researchers have suggested alternative antioxidant responses could be significant in mediating response to these toxicants as well, and more work should be done in that area (Lushchak et al. 2009; Bhatti et al. 2011; Guilherme et al. 2012).

The link between mitochondrial health and apoptosis has been well established, and both cadmium and glyphosate are postulated to be mitochondrial toxicants (Chang et al. 2013; Wu and Bratton 2013; Wang D. et al. 2018; Pereira et al. 2018; Wallace et al. 2019). The mechanisms through which these toxicants operate remain unclear, and very little is known about how these mixtures affect mitochondrial membrane potential and mitochondrial health. Mitochondrial membrane potential (Ψ_M) is a measure of mitochondrial health and increased MMP is associated with healthy cells. The JC10 Mitochondrial Membrane Potential Assay (Abcam, Cambridge, UK) uses the JC10 cationic, lipophilic dye to detect changes in the mitochondrial membrane and is summarized in figure 29. In healthy cells, it concentrates in the mitochondrial matrix and forms red fluorescent aggregates (Abcam 2020). In damaged cells, the dye diffuses out

of the mitochondria and the red aggregates convert to monomers that emit green fluorescence. Mitochondrial membrane potential is a measure of efficient oxidative

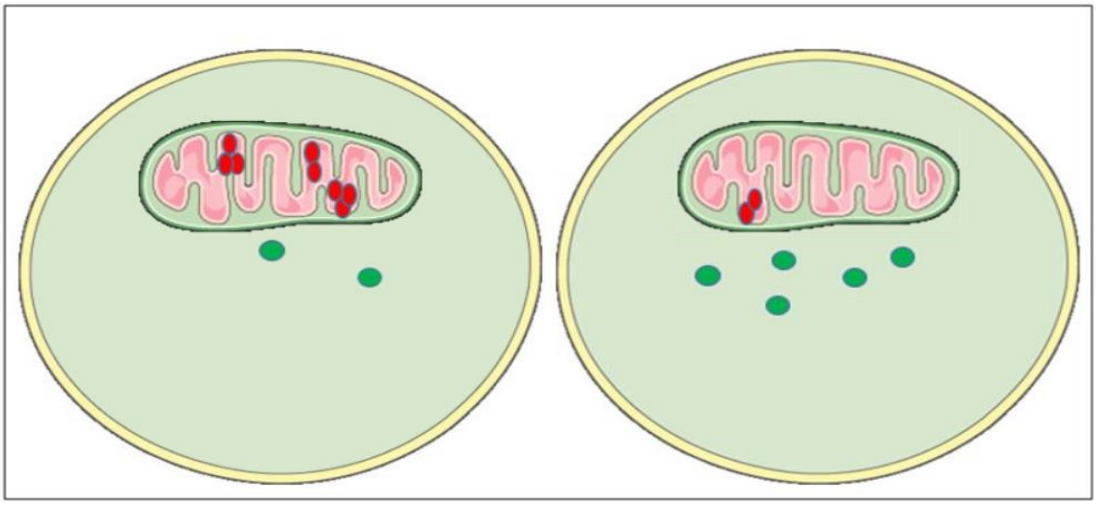

Figure 29: JC10 is a cationic, lipophilic dye that selectively permeates the mitochondria. In healthy cells, JC10 aggregates inside the mitochondrial matrix and fluoresce red when exposed to specific wavelengths (540ex/590em). In unhealthy apoptotic cells, JC10 diffuses out of the mitochondria where it transitions to its monomeric form which fluoresces green at specific wavelengths (490ex/525em). The ratio between the fluorescence of the healthy vs unhealthy cells is reflective of changes in mitochondrial membrane potential and permeability.

phosphorylation in the cell and can also indicate mitochondrial health. No toxicant showed decreased MMP at this concentration and incubation time. Significantly, AsPC1 cells showed an increased MMP in response to cadmium, glyphosate, and their mixture. These results are in agreement with the results published by Pereira et al., indicating low concentrations of glyphosate exposure is associated with hyperpolarization of the mitochondrial membrane (Pereira et al. 2018). Additionally, AsPC1 cells showed a much higher ratio of healthy to unhealthy cells across all treatment groups, hinting at a potential protective mechanism activated in tumor cells. Even at these concentrations, it is apparent there are some bioenergetic implications of these toxicants and mixtures. Further testing on ATP production and oxidative phosphorylation will examine the potential pathways being employed. It is possible there is a narrow, undetermined

concentration range of exposure for these toxicants and mixtures that differentially impact the mitochondria, increasing activity at some points and decreasing activity with higher concentrations, with both likely impacting mitochondrial homeostasis.

ATP production maintains cellular homeostasis, and the most efficient means of ATP production occurs through oxidative phosphorylation in the mitochondria. Oxidative phosphorylation is oxygen dependent and assessing the rate of oxygen consumption is an indicator of cellular function. Dysfunctional mitochondria will consume oxygen at a slower rate than healthy mitochondria. Oxygen consumption analysis coupled with MMP examination helps identify irregularities one test or the other may be insensitive to. MMP is sensitive to uncoupling of electron transport, but unaffected by changes in ATPase, which would have effects on oxygen consumption testing (Hynes et al. 2006). In this study, no HPNE treatment group interfered with mitochondrial membrane potential. AsPC1 cells had much greater membrane potentials, highlighting their increased metabolic needs to sustain growth. Neither cell line showed differences compared to controls for oxygen consumption, suggesting negligible impact on ATP production. Correlations of MMP with oxygen consumption can be imprecise, and their relationship to ATP production is not always predictable (Suzuki et al. 2018). Mitochondrial toxicity studies were completed next, to identify changes in ATP production and bioenergetics.

Metabolic reprogramming has come to be one of the fundamental hallmarks of cancer, and ground zero of that phenomena lies within the mitochondria. The incidence of tumor formation is tightly associated to mitochondrial functionality, and shifts in ATP production from oxidative phosphorylation may signal transition to the cancer state, a

phenomena known as the Warburg Effect. Normal healthy cells prefer to use oxidative phosphorylation to produce 38 ATP per cycle. Cancer cells have a tendency to upregulate glycolysis to produce energy, producing 2 ATP even in the presence of oxygen and functional mitochondria. The inclination of tumor cells to use aerobic glycolysis for a substantial portion of their ATP production has been observed by many researchers, though the mechanism through which this proceeds is undetermined. Mitochondrial toxicity testing can help elucidate that transition by multiplexing membrane integrity fluorescence with ATP production luminescence. The Mitochondrial ToxGlo Assay (Promega, Madison, WI) is a multiplexed assay that examines cell membrane integrity and ATP production to establish an energetic profile that either identifies mitochondrial dysfunction or cytotoxic mechanisms that do not involve the mitochondria.

Tests were performed with two different nutritive sources supplementing the cytotox media. The first was 25mM glucose to allow cells full choice between oxidative phosphorylation and aerobic glycolysis. Cytox media were also supplemented with 10mM galactose in order to enhance oxidative phosphorylation and highlight compounds that could be mitotoxicants. There are several alternatives in data interpretation: *i.)* A lack of change in membrane integrity or ATP levels means the compound is not a mitochondrial toxin. *ii.)* If there is a reduction in ATP with commensurate changes in membrane activity, the compound is not a mitochondrial toxin, but is causing primary necrosis. *iii.)* Reduction in ATP with no change in membrane activity means the compound is a mitochondrial toxin. *iv.)* Reduction in ATP with discordant changes in membrane activity means the compound is a mitochondrial toxin. (Promega)

In the presence of glucose, both pesticides alone resulted in decreased membrane integrity and decreased ATP production in HPNE cells, sugesting the cells were undergoing primary necrosis. The mixtures, however, showed intact membrane integrity with decreased ATP production, identifying them as mitochondrial toxins and indicating a potential shift to aerobic glycolysis for energy production. When in the presence of galactose, HPNE cell treatments had no effects on ATP production, and both glyphosate groups showed increased membrane integrity. Cadmium alone showed no significance for either measured endpoint, but other studies with slightly higher concentrations of cadmium have identified its potential for mitochondrial toxicity, and more work should be done to elucidate those effects (Wallace et al. 2019). In AsPC1 cells, all treatment groups containing cadmium indicated they were experiencing primary necrosis, despite which media supplement they were assayed in. Atrazine and glyphosate actually appeared to have a protective effect on tumor cells, increasing their membrane integrity and increasing ATP production in the presence of galactose.

In the presence of glucose, where cells may produce energy via either pathway, energy production is where our experiments determined mixtures were behaving differently from their constituent parts. Though cadmium treatment showed no effects on mitochondrial health, and both pesticides indicated they initiated primary necrosis, the mixtures were identified as mitochondrial toxins. Both mixtures exhibited unaffected membrane integrity and marked decreases in ATP production, indicating a shift to aerobic glycolysis. When forced into oxidative phosphorylation with galactose supplementation, no effects were seen with the cadmium atrazine mixture, and increased membrane integrity was seen with cadmium glyphosate with no impact on ATP

production. In tumor cells, both mixtures were determined to be necrotic despite changes in nutrient supplementation.

In conclusion, due to the widespread existence of cadmium, glyphosate, and atrazine in the environment, it is likely that we, as humans, will be exposed to one or a combination of the toxicants. Early toxicological studies examined the toxicity of individual compounds with no regard for the toxicity of chemical mixtures, further emphasizing the importance and need for toxicological studies on chemical mixtures like the one reported here (Nelms et al. 2018; Bopp et al. 2019; Wallace and Buha Djordjevic 2020). Our data establishes baseline and threshold toxicity levels for cadmium, glyphosate and atrazine exposure to pancreatic cell lines and is the first to assess the toxicity of metal-pesticide mixtures in pancreatic cells after chronic (48 hour) single concentration exposure. Interestingly, our data suggest that neither glyphosate nor atrazine is overtly toxic in the HPNE and AsPC-1 cell lines with LC50 values in excess of 500 μM, whereas cadmium is moderately toxic with an LC50 values of 30-40 μM. Further analysis of the data revealed a minimum concentration that would elicit toxicity and from this value, we were able to examine the toxicity of cadmium-pesticide mixtures after 48 hour exposure.The sub-toxic test concentrations of toxicants and mixtures are not mobilizing the p53 apoptotic pathway in either pancreatic cell line. Though cadmium metal did generate p53 response and annexin labeling of increased PS in the outer membrane of HPNE cells, it did not activate caspases. The importance of calcium in annexin experimentation and its similarity to cadmium may have exaggerated annexin signaling in control cells. No other HPNE treatment group exhibited increased annexin signaling with or without increased p53 expression. Coupled with the absence of caspase

activation, results indicate that p53 mediated apoptosis is either impaired or non-existent in response to these treatments. Mitochondrial toxicity combined with decreases in apoptotis associated proteins suggest a potential pathway toward tumorigenity, as the presence of p53 is insufficient to identify apoptotic activation (Mashima et al. 1998). p53 could recruit DNA repair proteins or initiate cellular senescence instead. More testing would need to be done to determine its role. In AsPC1 cells, our results demonstrate that apoptosis is not a factor in treatment response. Neither annexin labeling nor caspase activation experiments yielded responses in the tumor cell line. It is possible that response is operating via an alternative pathway, or that chronic exposures may induce some manner of apoptotic tolerance to allow damaged cells to proliferate, and the absence of p53 mediated apoptosis may play a role in tumor resistance to chemotherapy and enhanced metastatic cancer of the pancreas (Mashima et al. 1998).

Bioenergetics and mitochondrial health were impacted by acute mixture exposure at the sub toxic concentrations used in this study. In most experiments, there appeared to be one component driving the response, and the toxicity of the mixtures mirrored at least one of its parent compounds. Mixtures did, however, effect the bioenergetics of the cell differently than either of their parent compounds in HPNE cells. The combined mitochondrial toxicity with apparent anti apoptotic effects provide a potential pathway toward tumorigenity and highlights the complexity of mixture analysis. HPNE's upregulation of p53 in the mixtures coupled with their mitochondrial toxicity indicates a potential role for p53 in bioenergetics pathways in the pancreas. Recent studies suggest p53 may play a role in regulation of the glycolytic pathway, though the literature is conflicting. p53 has been suggested to inhibit glycolysis through regulation of glucose

transporters, while others studies suggest p53 metabolic regulation occurs via a promotion of oxidative phosphorylation (Matoba et al. 2006; Ma et al. 2007; Moulder et al. 2018). In our experiments, exposures leading to increased p53 protein expression in response to toxicant mixtures may divert energy production in part to aerobic glycolysis in HPNE cells, accounting for their low ATP production. AsPC1 cells are mirroring this data with increased p53 resulting in decreased ATP production despite increases in the polarization of the mitochondrial membrane. Importantly, upregulated p53 was not associated with energy changes in samples exposed to individual toxicants. Further experimentation is warranted to determine what role p53 may play in the initiation of Warburg energetics in the pancreas. Taken together, the exposure to sub-toxic concentrations of these test toxicants appears to exert small effects that subtly accumulate to cause damage in pancreatic cells. Collectively, this data enhances our understanding of mixture toxicity and leads us to further questions. Toxicant exposures are complex and dynamic, requiring various combinations, exposure periods, and sequence testing to fully elucidate their impacts on cellular health.

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APPENDICES

PC	***Pancreatic Cancer***
EPA	***Environmental Protection Agency***
NOAEL	***No Observable Adverse Effect Limit***
LDH	***Lactate Dehydrogenase***
MTT	***3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide***
ROS	***Reactive Oxygen Species***
AMPA	***α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid***
DEA	***Diethanolamine***
IARC	***International Agency for Research on Cancer***
GSH	***Glutathione***
EDTA	***Ethylenediaminetetraacetic acid***
DFOA	***Deferoxamine***
DMSA	***Meso-2, 3-dimercaptosucccinic acid***
DMPS	***2,3-dimercaptopropanesulfonic acid***
VX	***Venomous Agent X***
DDT	***Dichlorodiphenyltrichloroethane***
TCDD	***2,3,7,8-tetrachlorodibenzo-p-dioxin***
POEA	***Polyoxyethylene tallow amine***
KRAS	***Kirsten rat sarcoma viral oncogene***

MYC	***Myelocytomatosis proto oncogene***
Bcl	***B-cell lymphoma***
Bax	***Bcl like protein***
MDM2	***Mouse double minute 2***
AKT	***Protein kinase B***
GLUT1	***Glucose transporter 1***
MT	***Metalothionein***
DNMT	***DNA methyltransferase***
OP	***Organophosphate***
ESPS	***5-enolpyruvalshikimate-3-phosphate synthase***
US ADI	***US Acceptable Daily Intake***
ATSDR	***Agency of Toxic Substances and Disease Registry***
TSH	***Thyroid stimulating hormone***
ATCC	***American Type Culture Collection***
RFU	***Relative Fluoresence Units***
TBS	***Tris buffered saline***
HRP	***Horseradish peroxidase***
MMP	***Mitochondrial membrane potential***
DCFH	***Dichlorofluorescin***
CFDA AM	***5-Carboxyfluorescein diacetate***
RDI	***Recommended Daily Intake***
PS	***Phosphatidylserine***
GSSH	***Oxidized Glutathione***
PTDI	***Provisional Tolerable Daily Intake Level***

VITA

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Dissertation: APOPTOTIC AND BIOENERGETIC CHANGES IN PANCREATIC CELLS AFTER CHRONIC EXPOSURE TO INDIVIDUAL TOXICANTS AND THEIR MIXTURES

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