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Modulation of cell death signaling and cell proliferation by the interaction of homoserine lactones and Paraoxonase 2.

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MODULATION OF CELL DEATH SIGNALING AND CELL PROLIFERATION BY THE INTERACTION OF HOMOSERINE LACTONES AND PARAOXONASE 2

By

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B.S., Paul Quinn College, Dallas, TX, 2007
M.S., University of North Texas, Denton, TX 2012

A Thesis

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DEDICATION

This Thesis is dedicated to my dear aunt, Sr. Jacinta Neely O.S.B and uncle Alpheus Neely Jr. for their unconditional love, support and encouragement. They taught me how to maximize limited resources to obtain a desired end goal. They encouraged me to transcend beyond socioeconomic limitation and exceed the expectations of others, whether negative or positive. Growing up, Uncle Junior instilled in me a sense of pride and self-worth. Aunt Jacinta showed me that I could be more than my environment allowed and imparted in me valuable lessons such as "Procrastination is the thief of time" and "Your writing is an accurate reflection of who you are". Aunt Jacinta has been a source of spiritual guidance and discipline and Uncle Junior was the only father figure I knew. Thank you both and may God bless guide and protect you.

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ABSTRACT

MODULATION OF CELL DEATH SIGNALING AND CELL PROLIFERATION BY THE INTERACTION OF HOMOSERINE LACTONES AND PARAOXONASE 2

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Pseudomonas aeruginosa produces N-(3-oxododecanoyl)-homoserine lactone (C12) as a quorum-sensing molecule that functions to facilitate bacteria-bacteria communication. C12 has also been reported to affect many aspects of human host cell physiology, including evoking cell death in various types of cells. However, the signaling pathway(s) leading to C12-triggerred cell death remains unclear. To clarify cell death signaling induced by C12, we examined mouse embryonic fibroblasts (MEFs) deficient in one or more caspases. Our data indicate that, unlike most apoptotic inducers, C12 evokes a novel form of apoptosis in cells, probably through the direct induction of mitochondrial membrane permeabilization. Previous studies indicate that C12 requires the lactonase/arylesterase paraoxonase 2 (PON2) to exert its cytotoxicity on MEFs. PON2 is known to function as a lactonase to cleave C12. We found that PON2 was overexpressed in tissues from non-small cell lung carcinoma (NSCLC) patients and oncogenically transformed human bronchia/tracheal epithelial

(NHBE) cells. Reducing PON2 expression in NSCLC cell lines as well as several non-transformed cell lines rendered them resistant to C12. However, PON2 expression is only important for the proliferation of NSCLC cell but not that of their untransformed counterparts, indicating that PON2 mediates apoptosis independently of its function to modulate cell proliferation. Overall, our results reveal a unique mitochondrial apoptotic signaling pathway triggered by C12/PON2 interaction and PON2 plays distinct roles in apoptosis signaling and cell proliferation.

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CHAPTER 1: INTRODUCTION

Tissue differentiation and homeostasis are tightly regulated by the BcL-2 regulated cellular suicide program, apoptosis (2,3). Many human tumor cells acquire resistance to conventional chemotherapeutic drugs that depend on BcL-2 proteins. These neoplastic cells have been found to have an increased ratio of anti-apoptotic to pro-apoptotic Bcl-2 proteins. Thus, the discovery of novel drugs that are capable of overcoming apparent tumor cell BcL-2 protein dependent resistance to cell death is a major challenge. A promising anti-tumor approach is the identification of small molecules (as well as their cellular targets) that preferentially trigger tumor cell apoptosis, independent of tumor cell Bcl-2 protein profile (4,5). While several candidate compounds have been identified, most if not all of these drugs could only induce apoptosis independent of either anti-apoptotic or pro-apoptotic Bcl-2 proteins; but not both (5-7). Conversely, the quorum-sensing molecule N-(3-oxododecanoyl)-homoserine lactone (C12) preferentially induces transformed cell apoptosis *in vitro* and inhibits transplanted tumor growth *in vivo* independent of both anti- and pro-apoptotic Bcl-2 proteins; thereby making it an ideal candidate drug. Our preliminary data indicates that C12 triggers Bcl-2 protein-independent apoptosis, likely mediated by Paraoxonase 2 (Pon2). PON2 is known to function as a lactonase to cleave C12. Previous studies indicate that C12 requires the lactonase / arylesterase PON2 to exert its cytotoxicity (8,9).

1.1. Programmed cell death-Apoptosis

The word Apoptosis derives from Greek origin meaning "dropping off or falling off," and initially referred to the process by which leaves fell from trees or petals from flowers. Apoptosis is a biological phenomenon that consists of a programmed sequence of biochemical events that culminate in the selective elimination of damaged, infected and potentially neoplastic cells from the bodies of multicellular organisms (10,11). Apoptosis is one of the most ubiquitously employed mechanisms by which the body disposes of cell debris or damaged cells without eliciting localized inflammation; due to leakage of cellular contents(12). This mode of cell death is a vital component of normal tissue development, disease progression and maintenance of tissue homeostasis. Additionally, it serves as a means of defense against the development and advancement of cancer (13). The biochemical events that result in the occurrence of Apoptosis also elicit a large number of morphological changes in cells including cell shrinkage, blebbing of the membrane, condensation and fragmentation of nuclear material and the formation of apoptotic bodies.

1.2. Regulation of apoptosis

Apoptosis is under stringent genetic control and can be activated by stimuli from multiple sources. It is initiated in response to specific developmental signals or in the presence of various stimuli including the reduction of essential growth factors, the activation of Tumor Necrosis Factor receptors (TNFR), DNA damage, loss of

cellular attachment, decreases in the local concentration of tissue morphogens and major alterations in homeostatic state of the cell(14,15).

Excess or limited apoptosis can disrupt tissue homeostasis of multicellular organisms. When apoptosis occurs more frequently than cell proliferation does, neurodegenerative disorders are exacerbated. Further to this, the dysregulation of apoptosis has been implicated in the ontogeny and progression of many disease states including many cancers and neurodegenerative disorders such as Alzheimer's, Huntington's and Parkinson's diseases (16-18). Conversely, insufficient apoptosis can precipitate cancer development and progression (19,20). Thus, tight regulation between apoptosis and cell proliferation is imperative for the viability of all multicellular organisms.

1.3. Biological significance of apoptosis

Apoptosis has long been recognized as a critical regulatory component of the development process. The role of apoptosis in development has been investigated and well established in three organisms: Nematodes (Caenorhabditis elegans), Fruit Flies (Drosophila melanogaster) and mouse (Mus musculus). Apoptosis has been found to drive hemaphroditic development during embryogenesis in *C. elegans* and promote the completion of development in *D. melanogaster*(21). Studies have indicated that the inhibition of apoptosis elicits developmental impediments, disorders and/or death (13,22).

In early mammalian development, apoptosis is instrumental in the formation of synapses between neurons in the brain and the spinal cord as it facilitates the removal of excess neurons cells(23). Additionally, Apoptosis assists in tissue remodeling, molding and shaping of the body and organs and the detailing/separation of extremities (fingers and toes) by the removal of excess tissues. Inhibition or insufficient apoptotic signaling elicits malformed limbs and digits joined by soft tissue (Figure 1.1) (1).

Figure 1.1 Apoptosis is required for proper development. (A) Cells between the digits undergo apoptosis to facilitate proper finger and toe formation. (B) Insufficient apoptosis elicits malformed digits that are joined by soft tissues yielding the persistence of interdigital web as seen in the duck (1).
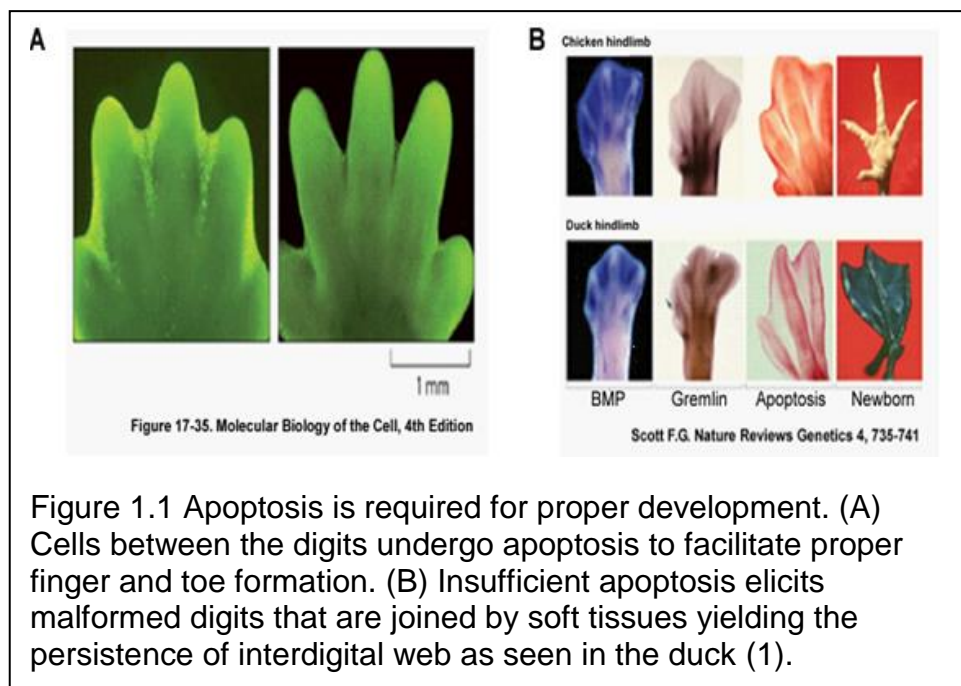
Apoptosis is also vital in the removal of those cells that may have deleterious effects on the organism. These include cells that are capable of participating in an autoimmune response as well as virally infected; and thus cytotoxic, T cells (24). Apoptosis facilitates the maintenance of tissue homeostasis. In the average adult, billions of cells die daily via apoptosis and are then replaced with new cells that originate from the body's stem cell populations (25). Apoptosis regulates the

constant internal environment and normal tissue homeostasis. Under healthy and normal conditions, the rate of cell proliferation is approximately the same as the rate of apoptosis. This paradigm is essential for ensuring the viability of multicellular organisms. If this balance is disrupted, such that the occurrence of cell death is more frequent than that of cell replacement, the probability of the onset of neurodegenerative disorders is amplified. Moreover, inadequate apoptosis may elicit cancer development (26).

1.4. The pathways of apoptosis

Apoptosis typically occurs through one of three signaling pathways, namely the mitochondrial (intrinsic), the death receptor (extrinsic) and the mixed pathways.

1.4.1 The intrinsic pathway of apoptosis

Intracellular death signals are translocated to the mitochondria where they induce the mitochondria dependent intrinsic pathway of apoptosis. These signals activate the pro-apoptotic Bcl-2 proteins, which in turn lead to the formation of permeation channels on the outer mitochondrial membrane (OMM). These permeation channels facilitate the release of apoptogenic proteins (Diablo/Smac and cytochrome c) from the mitochondrial inter-membrane space (IMS) into the cytosol. Upon release into the cytosol, Diablo/Smac and cytochrome c induce a cascade of caspase reactions that culminate in the occurrence of apoptosis(27). Cytochrome c activates apoptotic protease factor 1 (apaf1), which promotes the transition of procaspases to their active caspase form. Diablo/Smac promotes

apoptosis through direct interaction with inhibitors of apoptosis proteins (IAPs),

thereby impeding their ability to inactivate caspases (Figure 1.2) (28,29).
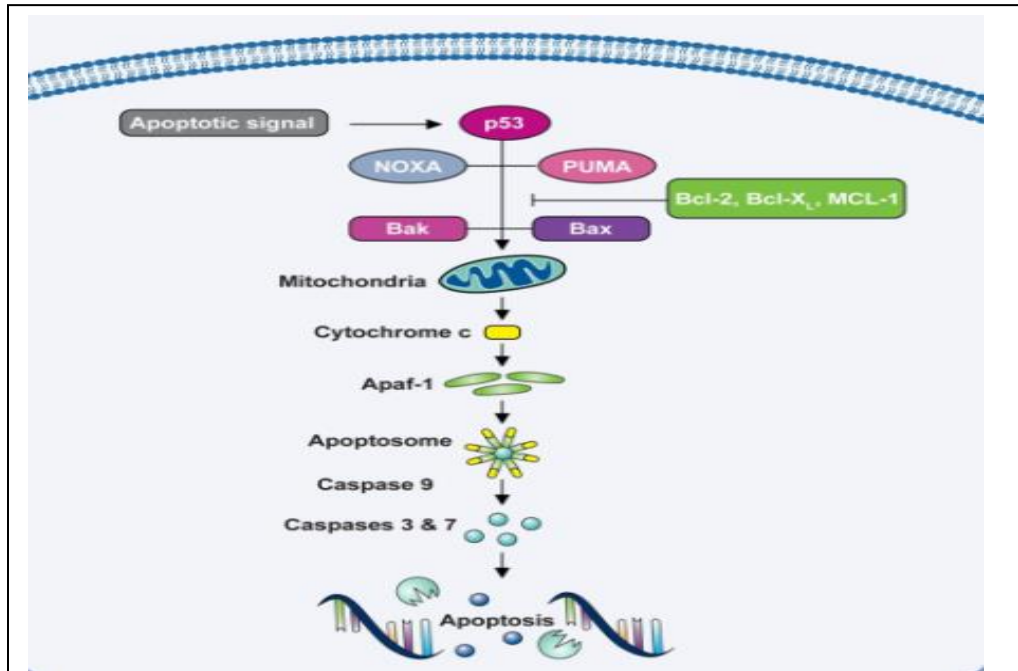
Figure1.2. The intrinsic (mitochondrial) pathway of apoptosis.
Apoptotic stimuli; such as DNA damage, elicits p53 activation.
Activated p53 activates pro-apoptotic Bcl-2 proteins which in turn
forms permeation channels on the OMM. These permeation
channels facilitate the transport of cytochrome C and Diablo/Smac
into the cytosol. While in the cytosol, cytochrome C initiates a
caspase cascade that culminates in the occurrence of apoptosis
while Diablo/Smac interacts with IAPs impeding their ability to inhibit
caspases thereby promoting apoptosis.   The figure is from Dr. Mohd
Saquib Khan at Pondicherry University.

1.4.2 The extrinsic pathway of apoptosis

In contrast to the intrinsic apoptotic pathway, death signals for extrinsic apoptotic pathways are initiated from the outside of the cell. Induction of the extrinsic apoptotic pathways does not involve the mitochondria, involvement of the Bcl-2 protein family or the release of cytochrome c forms the mitochondrial IMS to the cytosol. In this pathway, specific death ligands such as tumor necrosis factor (TNF), Fas Ligand (FasL) or TNF-related apoptosis-inducing ligand (TRAIL) binds to their respective specific transmembrane death receptors including FAS, tumor necrosis factor receptor 1 (TNFR1), p75, DR4(30).

The binding of death ligands to their respective specific death receptor promotes the collection and recruitment of the adaptor protein Fas-associated death domain (FADD) and the inactive forms of the initiator caspases 8 and 10 (pro-caspases 8 and 10), thus allowing for the formation of the death-inducing signaling complex (DISC). The DISC transports the procaspase molecules in close proximity, thereby ensuring access for their autocatalysis and eventual release into the cytosol (31-35). Caspase 8 or 10 will then activate the effector caspases 3/7 in a cascade precipitated by caspase-mediated reactions that will culminate in the occurrence of apoptosis.
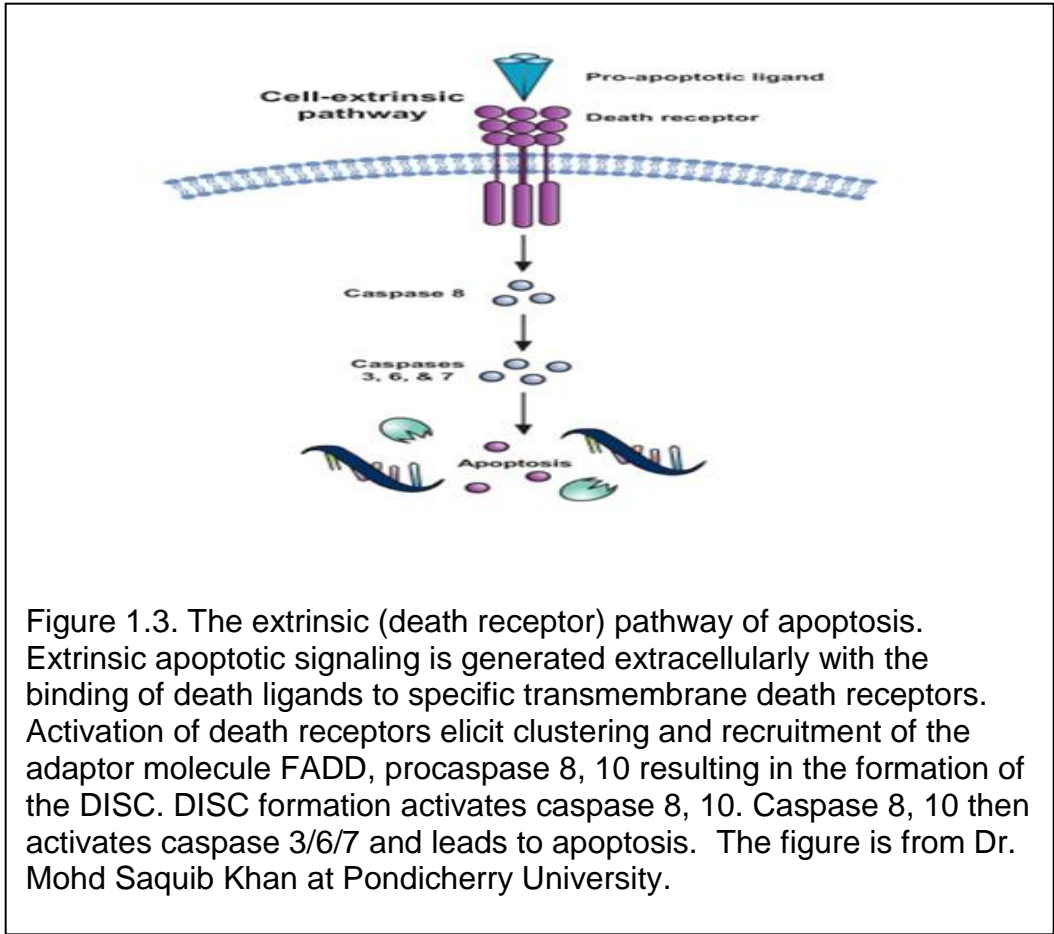
Figure 1.3. The extrinsic (death receptor) pathway of apoptosis. Extrinsic apoptotic signaling is generated extracellularly with the binding of death ligands to specific transmembrane death receptors. Activation of death receptors elicit clustering and recruitment of the adaptor molecule FADD, procaspase 8, 10 resulting in the formation of the DISC. DISC formation activates caspase 8, 10. Caspase 8, 10 then activates caspase 3/6/7 and leads to apoptosis. The figure is from Dr. Mohd Saquib Khan at Pondicherry University.

1.4.3 The mixed pathway of apoptosis

There exist certain instances where the induction of apoptotic signaling cascades from external sources can also elicit the activation of both intrinsic and extrinsic apoptotic pathways. This phenomenon is known as the mixed apoptotic pathway and entails death stimuli that are generated extracellularly causing the activation of pro caspase 8, pro caspase 10, and formation of the DISC; in the same manner as observed in the extrinsic apoptotic pathway. The DISC formation will ultimately lead to autocatalysis and the eventual activation of caspase 8 and 10 (31-35). When caspase 8 is activated, it can enter one of two routes: (i) it can activate the effector caspases 3, 6 and 7, thereby eliciting apoptosis via the extrinsic pathway or (ii) it can enter the intrinsic apoptotic pathway via interaction with the Bcl-2 protein family. In this instance, caspase 8 will cleave the inactive pro-apoptotic BH3-only protein "Bid" into its truncated and active form tBid. The tBid then activates the Bcl-2 proteins Bax and Bak at the OMM. Bak and Bax then undergo conformational changes as well as oligomerization thereby yielding the formation of permeation pores on the OMM. Permeation pore formation elicits the release of cytochrome c and Diablo/Smac from the mitochondrial IMS into the cytosol. Once in the cytosol, cytochrome c and Diablo/Smac exert their pro-apoptotic effects in the same fashion as they do in the intrinsic apoptotic pathway (27,36).
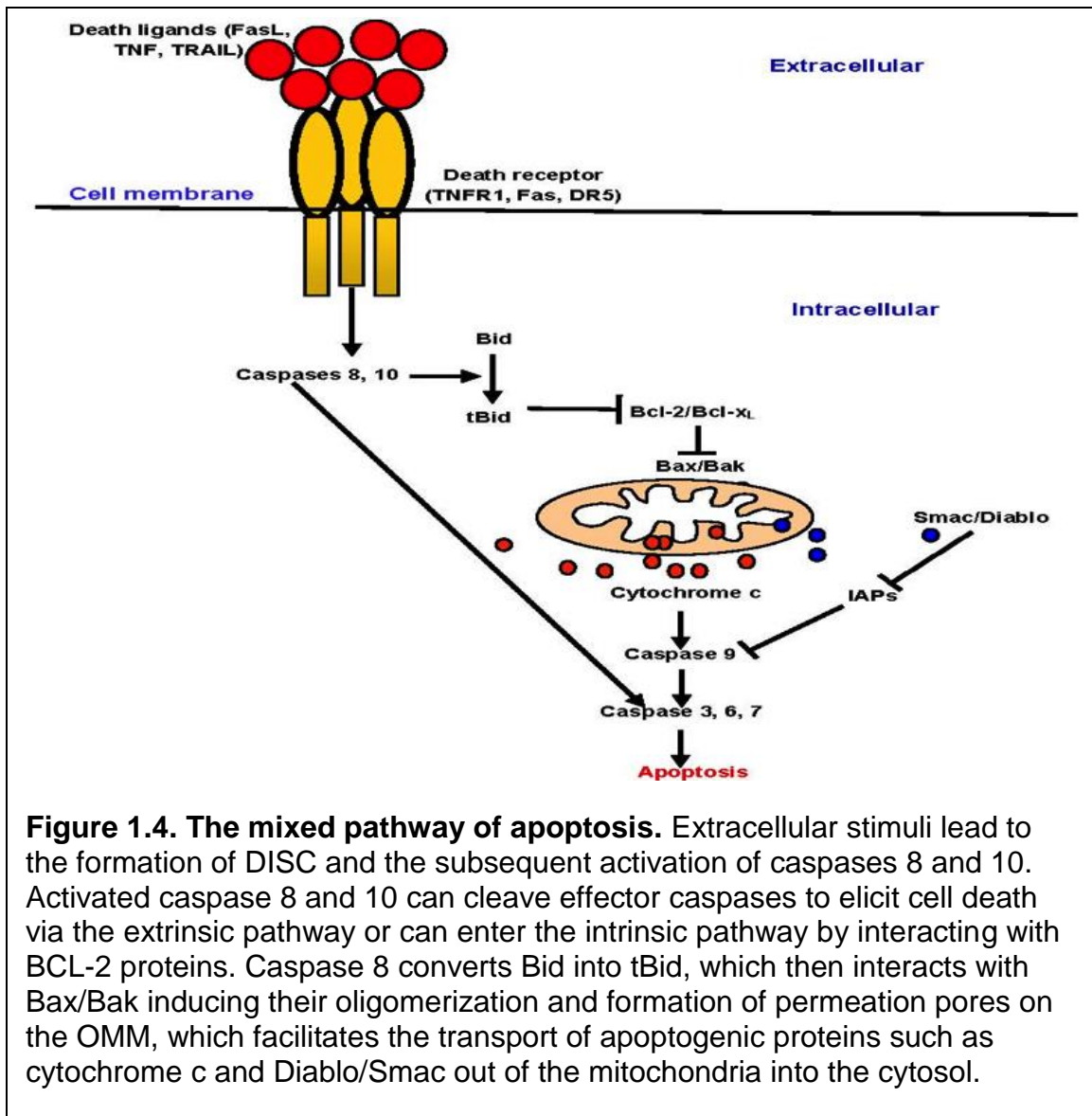
Figure 1.4. The mixed pathway of apoptosis. Extracellular stimuli lead to the formation of DISC and the subsequent activation of caspases 8 and 10. Activated caspase 8 and 10 can cleave effector caspases to elicit cell death via the extrinsic pathway or can enter the intrinsic pathway by interacting with BCL-2 proteins. Caspase 8 converts Bid into tBid, which then interacts with Bax/Bak inducing their oligomerization and formation of permeation pores on the OMM, which facilitates the transport of apoptogenic proteins such as cytochrome c and Diablo/Smac out of the mitochondria into the cytosol.

1.5 Capases

The caspases and the Bcl-2 protein family are primary regulators of Apoptosis. The caspases are a group of cysteine proteases, which cleave proteins at sites that are proximal to aspartic acid residues(37). Caspase activation is typically considered the molecular hallmark of apoptosis(38). Caspases exist in their inactive forms (pro caspase), and become activated during apoptosis by pro-apoptotic proteins(39).

While 12 caspases have been identified to date, not all play a role in the regulation of apoptosis. For instance, caspase 1 is instrumental in the regulation of biological processes that are unrelated to cell death including red blood cell and skeletal muscle myoblast maturation. In contrast, caspase 14 is critical in skin cell development(40).

There are two classes of caspases involved in apoptosis namely the initiator (apical) and the effector (executioner) caspases. The initiator caspases include caspases 2, 8, 9 and 10 and are activated in response to upstream apoptotic stimuli. The initiator caspases cleave and process the effector caspases 3, 6 and 7. Upon activation, the effector caspases trigger cell death by degrading critical intracellular structural proteins.

1.6 The Bcl-2 proteins

The Bcl-2 family of proteins is a major regulator of the intrinsic pathway of apoptosis. Their names derived from B-cell lymphoma/leukemia 2, and are the second member of a collection of proteins initially discovered during chromosomal translocations involving chromosomes 14 and 18 in human follicular lymphomas (41). There are approximately 20 different members of the Bcl-2 protein family. Bcl-2 protein family members all share one to four homologous Bcl-2 homology (BH) domains that are important for homo and hetero-dimeric interactions among different family members. Bcl-2 proteins are either pro-apoptotic or anti-apoptotic (2,3,14) depending on the amount of BH domains and their ability to regulate apoptosis(42).

Pro-apoptotic Bcl-2 proteins are important for the initiation and stimulation of the intrinsic apoptotic pathway. These proteins are divided into two structurally and functionally distinct categories: multi-domain and BH3-only Bcl-2 proteins(43). Multi-domain pro-apoptotic Bcl-2 proteins Bak, Bax and Bok share three BH domains (BH1-BH3). They are responsible for the formation of permeation channels on the OMM that disturbs mitochondrial membrane integrity during apoptosis. This facilitates the release of apoptogenic proteins from the mitochondria into the cytosol (44). Bak and Bax are ubiquitously expressed in all tissues while Bok is present only in reproductive cells (44). Bax and Bak are localized in different subcellular compartments of healthy cells. In the presence of death stimuli Bax undergoes conformational changes at both the amino- and

carboxyl- termini eliciting its translocation from the cytosol to the OMM and the eventual formation of large oligomeric complexes. Bak is localized solely in the mitochondria and upon apoptotic stimuli, will undergo conformational changes elicit the formation of oligomeric complexes (45,46). The oligomeric form of Bax and Bak form permeation pores on the OMM (47). These permeation channels mediate the release of pro-apoptotic proteins cytochrome c and Smac/Diablo from the IMS of the mitochondria to the cytosol where they exert their effects.

1.7 Other cell death modalities - Autophagy and Necrosis

Autophagy is a tightly regulated, ordered cell death process. It is self-degradative and essential for the maintenance of the balance of sources of energy at critical stages of development as well as in response to nutrient deprivation(48). Autophagy is also responsible for the removal of damaged organelles, misfolded and aggregated proteins(49) and the elimination of intracellular pathogens(50). The deregulation of autophagy has been implicated in non-apoptotic cell death. Autophagy can be selective or non-selective in the removal of specific organelles, ribosomes and protein aggregates(51). Furthermore, autophagy promotes cellular senescence and cell surface antigen presentation. It protects against genomic instability and is critical in the prevention of necrosis(50). Thus, autophagy has a key role in the prevention of diseases including cancer, neurodegeneration, liver diseases, autoimmune diseases, cardiomyopathy and infections. Increased endocytosis, vacuolation, membrane blebbing and nuclear condensation are all characteristic morphological hallmarks of autophagy. This

type of cell death is categorized as a defensive reaction that can elicit cell death or cell survival(50).

Another modality of cell death is necrosis. Necrosis is the irreversible loss of plasma membrane integrity(52). It is a form of cell injury that results in the premature death of cells in living tissue by autolysis. This type of cell death lacks the features of apoptosis and autophagy, and typically is considered uncontrolled (52,53). Necrosis is typically connected to immoderate cell loss in human pathologies and can lead to local inflammation (54-56), thought to occur through the liberation of factors from dead cells that alert the innate immune system (53,54,57). Necrosis is signaled by irreversible cytoplasmic alterations (condensation, fragmentation and loss of structure) and nuclear changes (pyknosis, karyolysis and karyorhexis) (52,53). Infection, toxins and trauma can result in the unregulated digestion of cell components leading to necrosis.

1.8 N-(3-oxododecanoyl)-homoserine lactone (C12**)**

The gram-negative opportunistic bacterium *Pseudomonas aeruginosa* produces N-(3-oxododecanoyl)-homoserine lactone (C12) as a quorum-sensing molecule used to facilitate bacteria-bacteria communication (7). Quorum sensing is a bacterial communication system that releases and detects small diffusible autoinducers (8,58). This system is responsible for the regulation of bacterial gene expression in response to changes in cell population density(58). Gram positive and gram-negative bacteria employ quorum-sensing communication to

regulate variety of physiological functions including: symbiosis, antibiotic

production, conjugation, motility and biofilm formation(58). Mounting data has

shown that C12 is involved in the regulation of bacterial virulence genes and also

interacts with eukaryotic cells (59,60).   As a small, lipid-soluble and diffusible

molecule, C12 readily enters cells of multiple tissues in the lungs of cystic fibrosis

patients including fibroblasts, epithelial cells, leukocytes, and endothelial cells

(9). Additionally C12 alters many aspects of eukaryotic cell physiology including

the inhibition of the secretion of proinflammatory cytokines (61-64), activation of

p53, and inhibition of events commonly associated with cell death (65-68). C12

has been shown to induce apoptosis in multiple types of cancer cells (66,69-71).

C12 induces apoptosis by inhibiting phosphatidylinositide3-kinases, arresting

Akt/PKB pathway and attenuating STAT3 activity in breast carcinoma cells

(8,66). In pancreatic carcinoma cells, C12 induces apoptotic signaling and

inhibits cell migration (8,70). In colorectal cancer cells, C12 reduces the

expression of thymidylate synthase while enhancing the activity of otherwise

conventional chemotherapeutic agents including 5-fluorouracil (5-FU) (69).

 Comparative SAR analysis has indicated that long acyl side chains with a 3-oxo

substitution are essential for C12's anti-cancer effect (69). However, the exact

signaling pathway(s) leading to C12-triggered cell death remains unclear.   Our

preliminary studies indicate that C12-triggered tumor cell apoptosis occurs by

selectively activating the mitochondria-specific intrinsic pathway through a novel

mechanism that is independent of activities of both anti- and pro-apoptotic Bcl-2

proteins in human tumor cells. We also find that C12 induces apoptosis

preferentially in oncogenically transformed but not in non-transformed human bronchial epithelial cells. Importantly, we discovered that C12 cytotoxicity is mediated through the lactonase activity of paraoxonase 2 (PON2).

1.9 Paraoxonase 2 (PON2)

PON2 is a ubiquitously expressed mammalian protein with anti-oxidant properties and lactonase/arylesterase activities (72,73), and it rapidly hydrolyzes C12 to C12-acid, which becomes trapped and accumulates within human bronchial epithelial cells, particularly in mitochondria (74-77). PON2 is upregulated in many types of cancer, including lung cancer, enabling cancer cells to resist conventional therapeutic drugs (78,79). PON2 expression also prevents oxidation and inflammation, but the detailed mechanisms remain unclear. This membrane-bound protein's expression is markedly elevated in several human non-small cell lung carcinoma (NSCLC) cell lines. Mutations in the Pon2 gene may be associated with vascular disease and a number of phenotypes related to diabetes.

An important anti-tumor approach is the identification of small molecules that preferentially trigger tumor cell apoptosis regardless of the Bcl-2 protein profile in tumor (4,5). The quorum-sensing molecule C12 preferentially induces transformed cell apoptosis *in vitro* and inhibits transplanted tumor growth *in vivo* independent of both anti- and pro-apoptotic Bcl-2 proteins. The apoptosis cascade induced by C12 in tumor cells is unique, evident by its rapid pro-

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apoptotic effects, such as depolarization of mitochondrial membrane potential within minutes, release of cytochrome c into the cytosol within one hour and detection of maximal activation of caspases within four hours. This distinctive pro-apoptotic feature of C12 has not been observed in any other apoptosis paradigms, which might be attributed to the ability of C12 or its metabolite(s) to directly permeabilize mitochondria (within minutes) without involving Bcl-2 proteins. Furthermore, lung tumor cells are resistant to conventional therapeutic drugs partially due to overexpression of paraoxonase 2 (PON2), a protein with anti-oxidant properties and lactonase/ arylesterase activities (80,81).

CHAPTER 2: MATERIALS AND METHODS

2.1. Reagents

N-(3-oxododecanoyl)-homoserine lactone (C12) was purchased from Sigma (St. Louis, MO). Propidium iodide (PI) was obtained from Invitrogen (Carlsbad, CA). Unless otherwise stated, all reagents were dissolved in dimethyl sulfoxide (DMSO). Dulbecco's Modified Eagle's Medium (DMEM), penicillin/streptomycin, trypsin, and L-glutamine were obtained from Mediatech (Manassas, VA), and fetal bovine serum (FBS) was purchased from Gemini (Broderick, CA). Caspase-Glo assay 3/7 kit was purchased from Promega (Madison, WI). Antibodies (Abs) used for western blot analysis were anti-β-actin mAb (Sigma), anti-caspase-3 pAb (Cell signaling; Danvers, MA), anti-caspase-7 pAb (Cell signaling), anti-caspase-8 pAb (Cell signaling), anti-caspase-9 pAb (Cell signaling), anti-Bax pAb (Santa Cruz; Dallas, TX), anti-Bak pAb (Millipore; Billerica, MA), anti-Bcl-2 mAb (Santa Cruz), anti-human PON2 (Abcam; Cambridge, MA), anti-murinePON2 (Antibodies-on-line; Atlanta, GA), peroxidase-conjugated goat anti-rabbit IgG (Thermo; Waltham, MA) and peroxidase-conjugated goat anti-mouse IgG (Thermo).

2.2. Cell lines and cell culture

Immortalized mouse embryonic fibroblasts (MEFs) deficient in the expression of caspase-8 and their wild-type counterparts were provided by Professor David

Vaux (Walter and Eliza Hall Institute of Medical Research, Parkville, VIC Australia). MEFs lacking caspase-9 and their wild-type counterparts were obtained from Professor Jerry Adams (Walter and Eliza Hall Institute of Medical Research). MEFs lacking caspase-3, caspase-7, caspase-3 and caspase-7, or their wild-type counterparts were obtained from Professor Richard Flavell (Yale University). HCT116 cells expressing different levels of Bak and Bax were obtained from Dr. Richard Youle (National Institutes of Health). NSCLC cell line A549 and NCI-H1299 cells were obtained from ATCC. A549 cells overexpressing Bcl-2 were produced by retroviral infection as described previously (8). To generate NCI-H1299 or A549 cells with reduced PON2 expression or vector control, we infected cells using respective lentiviral supernatants with 10 μg/ml polybrene. Stable cell lines were obtained by culturing cells in the medium containing1.5 μg/ml puromycin. NHBE cells were purchased from Lonza (Walkersville, MD). hT/LT/Ras HBE cells were obtained from Professor Barrett Rollins (Harvard Medical School). Lewis Lung Carcinoma cells, NCI-H1299 and A549 cells were cultured as described previously (8). NHBE and hT/LT/Ras cells were grown in BEGM supplemented with SingleQuots (LONZA). HCT116 cells were grown as described previously (8). Cells were all cultured in a 5% CO2 humidified incubator at 37°C.

2.3. Cell viability/Death assays

The indicated MEF cell lines were plated in a 48-well tissue culture plate with 20,000 cells in each well and cultured for 24 hours. Following the treatment with different concentrations of C12, cells were harvested in the presence of 1.0 µg/ml propidium iodide (PI). Cell viability was measured by PI exclusion using flow cytometry (FACSCalibur, Becton Dickinson) (FACScalibur, Beckon Dickinson; San Jose, CA). The percentage of cell death is determined as 100 minus the cell viability measurement.

2.4. Caspase-3/7 activity

Caspase-3/7 activities were measured using a Caspase-Glo assay kit (Promega, Madison, WI, USA) (82). In this assay, the proluminescent substrate containing the amino acid sequence Asp-Glu-Val-Asp (DEVD) is cleaved by activated caspase-3/7, resulting in the release of a luciferase substrate (aminoluciferin) and the production of luminescent signal. Briefly, 24 hours before the treatment, cells were plated in white-walled 96-well plates. At the indicated time points following treatment with various molecules, cells were mixed with CellTiter-Glo reagent and the luminescence was quantified by a Gemini EM microplate spectrofluorometer (Molecular Devices; Sunnyvale, CA) according to the manufacturer's protocols. Data were presented as relative fluorescence units (RFUs).

2.5. Western blot analysis

Equal amounts of proteins (30 µg) were separated on a 4-12% Bis-Tris gel (Bio-Rad; Hercules, CA) and transferred onto PVDF membrane (Millipore; Billerica, MA). The membrane was incubated with appropriate primary or secondary antibodies either overnight at 4^oC or at room temperature for 3 hours in 1X phosphate-buffered saline (PBS) containing 5% (w/v) nonfat dry milk (Bio-Rad) and 0.2% (v/v) Tween 20. Protein levels were detected using the enhanced chemiluminescent detection system (Pierce; Rockford, IL) as described previously[83].

2.6. Measuring $\Delta\psi_{mito}$ using imaging microscopy of JC1

For imaging experiments to measure mitochondrial membrane potential ($\Delta\psi_{mito}$), cells were incubated with growth media containing the $\Delta\psi_{mito}$ probe JC1 (10 µM) for 10 minutes at room temperature, and then washed three times with Ringer's solution to remove the extra dye. JC1-loaded cells were placed onto a chamber on the stage of a Nikon Diaphot inverted microscope. Cells were maintained at room temperature during the experiment. Treatments were made by diluting stock solutions into Ringer's solution at the concentrations stated in the text. Fluorescence imaging measurements of $\Delta\psi_{mito}$ were performed using equipment and methods that have been reported previously (7,14). Briefly, a Nikon Diaphot inverted microscope with a Fluor 20 X objective (0.75 numerical apertures) was used. A charge coupled device camera collected JC-1 emission images (green: 510–540 nm; red: 580–620 nm) during excitation at 490 +/- 5 nm using filter

wheels (Lambda-10, Sutter Instruments, Novato, CA). Axon Imaging Workbench

5.1 (Axon Instruments, Foster City, CA) controlled filters and collection of data.

Images were corrected for background (region without cells). Quantitative data

are reported as JC1 fluorescence ratios normalized to minimal JC1 ratios

obtained at the start of the experiment and maximal JC1 ratios obtained after

treatment with 5 µM FCCP.

2.7. Detection of the release of cytochrome c from mitochondria

Mitochondria were purified from MEF cells as described previously (84). Isolated

mitochondria were resuspended in buffer containing 12 mM HEPES (pH 7.5), 1.7

mM Tris-HCl (pH 7.5), 100 mM KCl, 140 mM mannitol, 23 mM sucrose, 2 mM

KH_2PO_4, 1 mM $MgCl_2$, 0.67 mM EGTA, and 0.6 mM EDTA supplemented with

protease inhibitors (Complete; Roche Diagnostics, Indianapolis, IN). After one

hour incubation with C12 at 30°C, mitochondrial vesicles were centrifuged at

10,000×g for 10 min, and vesicles were dissolved in 1×SDS-PAGE loading buffer.

Proteins in the vesicle fractions were detected by Western blotting.

2.8. Electrophysiological experiments

Solvent free planar phospholipid membranes were formed across a 0.1 mm hole

in a Saran partition by the monolayer method (60,61). The monolayers were

formed by layering the lipid solution (0.5% (w/v) diphytanoylphosphatidylcholine,

0.5% (w/v) asolectin (polar extract of soybean phospholipids), and 0.05% (w/v)

cholesterol in hexane) on the surface of the aqueous solutions (1.0 M KCl, 1 mM

MgCl2, and 5 mM PIPES, pH 6.9) on either side of the partition. The phospholipids were purchased from Avanti Polar Lipids. Calomel electrodes were used to interface with the aqueous phase. The membrane voltage was clamped using a high-quality operational amplifier in the inverted mode and the current recorded using Clampex 10.3 software. Data was low-pass filtered at 500 Hz when recorded. Typically 20-50µL of 0.7 mg/ml C12 (dissolved in 95% isopropanol, 5% DMSO) was dispersed with rapid stirring into a 5mL aqueous solution on one side of the membrane, labeled "cis" side. All voltages referred to the cis side, the trans being held at virtual ground by the amplifier. Vehicle controls produced no conductance.

2.9. Immunofluorescence microscopy

MEF cells plated onto cover glasses 24 hours earlier were rinsed with Ringer's solution and incubated for 4 hours with either vehicle (DMSO) or 50 µM C12 in Ringer's solution. The immunofluorescence staining of cytochrome c and tom20 were carried out as described previously (Zhao et al., JBC, 2015). Images were captured using a Nikon Eclipse Ti confocal microscope (Nikon; Melville, NY) equipped with a PlanApo 60x, 1.42 NA oil immersion objective. To minimize variability for quantitative assessment, the same microscope settings were used across vehicle control and C12-treated samples for three individual experiments. Four to nine fields of view were captured to acquire a sample size of at least 100 cells for each individual experiment. Quantification of cytochrome c and tom20 percentage of overlap was performed using ImageJ (NIH). For each image,

Maximum Intensity Projections (MIPs) were first created for both the cytochrome c and tom20 channels. Using tom20 as a guide, regions of interest (ROIs) were drawn around the cytoplasm of the cells, and these ROIs were copied onto the cytochrome c and tom20 MIPs. The percent area of pixels above a set threshold was calculated for every ROI (cell) for both cytochrome c and tom20. The thresholds, although set differently for cytochrome c and tom20, were kept the same for every image across control and C12-treated samples to reduce bias. Percent overlap between cytochrome c and tom20 was calculated as (percent area cytochrome c/percent area tom20)*100.

For immunofluorescence staining of tumor sections, tumor sections (5 µm) were treated with antigen retrieval procedure by boiling in 10% Triton x-100, then slowly cooled down at room temperature. After incubating with the blocking buffer (1× PBS, 0.2% Triton X-100, 5% goat serum), the slides were incubated with antibodies against activated caspase-3 (Cell signaling) overnight at 4°C. Following three 10-minute washes, slides were incubated with goat anti-rabbit IgG (Alexafluor-568, Invitrogen) for 1 hour. The fluorescence was visualized by confocal microscopy using a 40x CFI Plan Fluor objective (NA 0.6)

2.10. Cell cycle analysis and cell proliferation assay

For cell cycle analysis, 5×10^{5} cells were sedimented (300 x g for 5 minutes) and washed twice with 500 µl 1 x PBS. Cells were then fixed with 1 ml 70% ethanol in 1 x PBS at 4°C overnight. After centrifugation, cells were washed twice with 1

x PBS and resuspended in 500 µl 1 x PBS. 50 U RNase A (Qiagen, Valencia, CA) were added to samples and incubated at 37^oC for 1 hour. Five µg propidium iodide was added to samples which were incubated for 30 minutes at 37^oC before flow cytometric analysis. To evaluate cell proliferation, 1.5×10^4 cells were plated in wells of a 12-well plate and the total cell number was determined by using a hemocytometer.

2.11. *In vivo* animal studies

For transplanted tumors in C57BL/6 mice, eight-week old C57BL/6 female mice (Jackson Laboratories; Bar Harbor, ME) were inoculated subcutaneously (s.c.) with 1×10^6 Lewis Lung Carcinoma cells on the right flank. Tumors were measured daily with dull edged Vernier calipers ($V = L \times W^2/2$). After tumor size reached around 100 mm^3, animals with size-matched tumors were divided into control group and C12 group. DMSO or C12 was administered intraperitoneally each day. At the end of the experiments, tumors were excised for apoptosis evaluation. TUNEL labeling was carried out by the Pathology Research Services Laboratory at University of Washington. The slides were scanned by a ScanScope CS digital slide scanner (Aperio; Vista, CA).

2.12. Statistical analysis

All experiments were performed in triplicate at least three times. Results are presented as mean ± standard deviation. Statistical analysis was performed using Student's two tail t-test. A p value < 0.05 was considered significant.

CHAPTER 3: RESULTS

3.1 Caspase-3 and caspase-7 were required for C12-induced cell death.

In multicellular organisms, cell death is a highly heterogeneous process in which several distinct, in some cases partially overlapping, cell signaling cascades can be activated (1). Although C12's ability to trigger the events commonly linked to apoptosis has been reported (References 56, 60, 63, 65, 66), it is unclear whether other cell death signaling is involved. To thoroughly explore C12-induced cell death signaling, we first investigate whether caspase-3 and caspase-7 are essential to mediate cytotoxic effects of C12 Cytotoxicity of C12 was examined in MEF cells lacking only caspase-3 (caspase-3-KO), only caspase-7 (caspase-7-KO), or both of them as well as their wild-type (WT) counterparts (Figure 3.1A). C12 induced significant cell death in WT, caspase-3-KO and caspase-7-KO MEF cells, whereas caspase-3/7-DKO MEF cells were completely resistant to C12 exposure (Figure 3.1B). Moreover, less cell death was observed in caspase-3-KO or caspase-7-KO MEFs than their wild-type counterparts, indicating that both caspase-3 and caspase-7 are involved in apoptosis signaling initiated by C12, although caspase-3 appears to play a more prominent role. The essential role of caspase-3 and caspase-7 indicates that cell death induced by C12 is largely attributed to apoptosis.

Figure 3.1. Caspase-3 and caspase-7 were required for C12-induced cell death (A) Caspase-3 and caspase-7 expression in MEF cells was examined via western blot analysis. **(B)** The indicated MEFs were treated with various concentrations of C12 for 48 hours and cell viability was measured by propidium iodide exclusion using flow cytometry. Cell death data are shown as mean ± standard deviations of 3 independent experiments. Asterisks indicate P < 0.05 (*); Student's unpaired t test.

3.2. Caspase-3 and caspase-7 were not required for mitochondrial depolarization.

Mitochondrial outer membrane permeabilization (MOMP) has been recognized to be a "no-return" step in both intrinsic and extrinsic apoptotic pathways (1). To further explore C12-initiated apoptotic signaling, we first studied the involvement of caspase-3 and caspase-7 in the key event of MOMP: depolarization of mitochondrial membrane potential ($\Delta\psi_{mito}$). Depolarization of $\Delta\psi_{mito}$ was evaluated by determining the changes in fluorescence with the voltage-dependent dye JC1 being released from mitochondria into the cytosol and nucleus. Within minutes of C12 exposure, mitochondria in MEFs were largely depolarized to a degree close to the complete depolarization of $\Delta\psi_{mito}$ induced by the ionophore FCCP (Figure 3.2A). Importantly, depolarization of $\Delta\psi_{mito}$ occurred at similar levels in WT and caspase-3/7-DKO MEF cells upon C12 exposure, indicating that MOMP induced by C12 occurs upstream of "effector" caspase activation (Figure 3.2B).

Figure 3.2. Caspase-3 and caspase-7 were not required for mitochondrial depolarization. (A) C12's effect on mitochondrial potential is independent of caspase-3 and caspase-7. MEFs were loaded with the mitochondrial potential dye JC-1, and its fluorescence was measured using imaging microscopy during the treatment with 50 µM C12 and 10 µM FCCP. Typical results are shown. **(B)** C12 caused equivalent depolarization of mitochondrial potential in both WT and caspase 3/7-DKO MEF cells. Means +/- standard deviations for 3 experiments are shown. Responses of WT and caspase-3/7- DKO MEFs are not significantly different (p>0.05). Student's unpaired t test.

3.3. C12-induced mitochondrial outer membrane permeabilization occurs upstream of caspase-3/7 activation.

To validate these observations, we studied the involvement of caspase-3 and caspase-7 in another key event of MOMP, cytochrome c release from mitochondria into the cytosol. We performed immunofluorescent studies to evaluate C12-evoked redistribution of cytochrome c to the cytosol/nuclei. Upon C12 treatment, intracellular distribution of cytochrome c was diffuse in the cytosol and nuclei in both WT and caspase-3/7-DKO MEFs, whereas Tom20 maintained its characteristic mitochondrial distribution, demonstrating that mitochondria in WT and caspase-3/7-DKO MEFs were permeabilized with cytochrome c released into the cytosol and diffused into the nuclei (Figure 3.3A). The redistribution of cytochrome c was evaluated by calculating the percentage of overlapping between cytochrome c and Tom20, and the results indicate that C12 caused equivalent cytochrome c release in WT and caspase-3/7-DKO MEFs (Figure 3.3B). Overall, our results provide more evidence that C12 triggers MOMP independent of caspase-3 and caspase-7 activation.

Figure 3.3 C12-induced mitochondrial outer membrane permeabilization occurs upstream of caspase-3/7 activation. (A) Representative confocal images of MEF cells treated with either DMSO (control) or 50 µM C12 for 4 hours. The mitochondrial marker Tom20 is shown in red, cytochrome c is in green and DAPI in blue. Following the treatment with C12, staining of cytochrome c became diffuse and lost its co-localization with Tom20. **(B)** Cytochrome c is released from both WT and caspase-3/7-DKO MEFs to the similar degree upon C12 exposure. Threshold intensity of cytochrome c is compared to that of Tom20 for over 100 cells across control and C12 treated samples. Data represent means +/- standard deviations for three independent experiments. Student's unpaired t test.

3.4. Caspase-8 and caspase-9 plays distinct roles in C12-induced apoptosis.

While it has been shown that C12 induces an apoptotic modality of cell death, the exact signaling pathway remains unclear. It is well accepted that caspase-8 activity is characteristic of the extrinsic apoptotic pathway while caspase-9 activity is hallmark of the intrinsic apoptotic pathway. To elucidate which apoptotic pathway is triggered in C12 induced cell death, we studied two pairs of MEF cells deficient in either caspase-8 or caspase-9 and their wild-type counterparts. Upon treatment with C12, similar levels of cell death were detected in both WT and caspase-8-KO MEF cells (Figure 3.4B). In agreement with cell death data, caspase-3/7 was activated regardless of caspase-8 expression, indicating that caspase-8-mediated extrinsic pathway is not involved in C12-induced apoptosis in MEFs. In contrast, MEFs deficient in caspase-9 expression were completely resistant to C12 treatment (Figure 3.4E). Furthermore, C12 failed to evoke any significant activation of cspase3/7 (Figure 3.4F). Overall, these data suggest that C12 induces apoptotic signaling largely through activating the mitochondria-dependent intrinsic apoptotic pathway in MEFs.

Figure 3.4. Caspase-8 and caspase-9 plays distinct roles in C12-induced apoptosis. (A) Caspase-8 expression in MEF cells was examined by western blot. **(B)** Caspase-8 activation is not involved in C12-induced cell death. The cytotoxicity of C12 on MEFs was assessed 24 hours following the exposure by a propidium iodide DNA dye exclusion approach. **(C)** Caspase-3/7 activities were measured 24 hours after C12 treatment. **(D)** Caspase-9 expression in MEF cells was determined by western blot. **(E)** Wild-type and caspase-9-KO MEFs were treated with C12, and cell viability was measured 48 hours later. **(F)** Upon C12 treatment for 24 hours, caspase-3/7 activities were determined. All data are shown as means ± standard deviations of 3 independent experiments. Asterisks indicate P < 0.05 (*); Student's unpaired t test. ns, no significance.

3.5. C12-induced mitochondrial potential decrease is independent of caspase-8 and caspase-9.

To further explore the roles of the "initiator" caspases in MOMP mediated by C12, depolarization of $\Delta\psi_{mito}$ was examined in caspase-8-KO or caspase-9-KO MEF cells or their WT counterparts respectively. Consistent with its effects on cell viability and caspase-3/7 activation (Figures 3.4B-C), deficiency in caspase-8 expression did not affect quick depolarization of $\Delta\psi_{mito}$ (Figures 3.5A-B). Similarly, C12 caused the same levels of $\Delta\psi_{mito}$ depolarization in WT and caspase-9-KO MEF cells (Figures 3.5C-D), indicating that the "initiator" caspases are not involved in C12- induced MOMP.

Figure 3.5 C12-induced mitochondrial potential decrease is independent of caspase-8 and caspase-9. (A) The mitochondrial potential of the WT and caspase-8-KO MEF cells loaded with JC-1 was determined by fluorescent microscopy upon the treatment with 50 μM C12 and 10 μM FCCP. Representative results were shown. (B) C12 caused equivalent mitochondrial depolarization in WT and caspase-8-KO MEF cells. (C) Mitochondria in WT and caspase-9-KO MEF cells were depolarized to the similar degrees upon the treatment of 50 μM C12 and 10 μM FCCP. (D) Summary of the data shown in (I). All data are shown as means ± standard deviations of 3 independent experiments. Student's unpaired t test. ns, no significance.

3.6. C12-induced caspase-9 activation occurs downstream of mitochondrial membrane permeabilization.

We have demonstrated that caspase-9 activation is involved in C12-induced apoptosis. To further elucidate the involvement of caspase-9 activation, we examined cytochrome c redistribution from mitochondria to the cytosol/nuclei upon C12 exposure using immunofluorescence staining. While C12 evoked the release of cytochrome c from mitochondria to the cytosol/nuclei regardless of caspase-9 expression, Tom20 displayed typical punctate and perinuclear mitochondrial distribution following C12 treatment (Figure 3.6A). Moreover, C12 caused similar level of cytochrome c release from mitochondria in WT and caspase-9-KO MEF cells (Figure 3.6B). Taken together, these data indicate that C12 causes acute MOMP independent of any "initiator" caspase, suggesting that the effects of C12 on MOMP might be attributed to its direct action of on mitochondria.

Figure 3.6. C12-induced caspase-9 activation occurs downstream of mitochondrial membrane permeabilization. (A) Representative confocal images of MEF cells treated with either DMSO (control) or 50 µM C12 for 4 hours. The mitochondrial marker Tom20 is shown in red, cytochrome c in green, and DAPI in blue. Following the treatment with C12, staining of cytochrome c became diffuse and lost its co-localization with Tom20. **(B)** Cytochrome c is released from both WT and caspase-3/7-DKO MEFs to the similar degree upon C12 exposure. Threshold intensity of cytochrome c is compared to that of Tom20 for over 100 cells across control and C12-treated samples. Data represent means +/- standard deviations of three independent experiments. Student's unpaired t test.

3.7. C12 directly induces mitochondrial outer membrane permeabilization *in vitro*.

Since C12 depolarized $\Delta\psi_{mito}$ within minutes independent of both "initiator"

caspases and "effector" caspases (Figures 3.2 and 3.5), we reasoned that C12

could possess activities directly permeabilize mitochondria. To this purpose, we

examined the effects of C12 on mitochondrial outer membrane integrity *in vitro*.

Mitochondria isolated from WT MEF cells were incubated various concentrations

of C12. We assessed the amount of cytochrome c released from the

mitochondria using western blot analysis (Figure 7A). In a manner dependent of

C12 doses, less cytochrome c was detected in mitochondrial fractions with

concurrent increase of cytochrome c in released fractions, indicating that C12 is

able to permeabilize mitochondria directly *in vitro*.

Figure 3.7. C12 directly induces mitochondrial outer membrane permeabilization *in vitro.* **(A)** Mitochondria was isolated from wild-type MEFs and incubated with C12. The cytochrome c release from mitochondria induced by C12 was determined by western blot. **(B)** The intensities of cytochrome c in mitochondrial fractions and released fractions shown in (A) were quantified using the software ImageJ (NIH). Cytochrome c release is represented as a percentage of the sum of the protein intensity mitochondrial fractions and released fractions. Mean ± standard deviation for three independent experiments are shown. Asterisks indicate P values of < 0.05 (*) or < 0.01 (**) by Student's unpaired t test.

3.8. C12 forms channels with large conductance in phospholipid membranes *in vitro.*

The amphipathic lipid ceramide forms large stable permeation channels in the mitochondrial outer membrane capable of releasing proteins (85,86). As C12 directly induce MOMP *in vitro* (Figure 3.7), we postulated that amphipathic C12 might possess similar activities. Thus, we studied whether C12 is capable of forming large conductance pathways in phospholipid membranes lacking any proteins. Following the addition of C12 to planar phospholipid membranes, the conductance increased slowly reaching steady levels but with frequent increases and decreases in conductance (Figure 8A). The formation of the conductance consisted of discrete conductance increments that are characteristic of channels with various magnitudes. Upon the addition of $LaCl_3$, conductance formed by C12 was rapidly lost, suggesting that it was not caused by defects in the membrane (Figure 3.8A). This was reversed by chelation of the lanthanide with EDTA. Furthermore, the discrete conductance changes showed a log-normal distribution, which is typical of substances of varying size (Figure 3.8B). However, the distribution of conductance does not seem to be a continuum but rather shows indications of preferential conductance that is multiples of 16 nS. The results are consistent with C12 forming large channels of variable size that grow and shrink by incorporating or losing assemblies of C12 lipids, suggesting that C12 might directly function on mitochondria to induce MOMP (Figure 3.8D).

Figure 3.8. C12 forms channels with large conductance in phospholipid membranes *in vitro.*
(A) Formation and reversible disassembly of C12 channel(s) in a planar phospholipid membrane. At the indicated time points, C12, $LaCl_3$, or EDTA was added to the aqueous phase on one side (cis side) of the membrane. **(B)** Distribution of C12-induced conductance changes in phospholipid membranes. Sudden changes in conductance were measured and grouped into bins in log scale as appropriate for the apparent log-normal relationship of the data. The data are pooled from 7 separate experiments. **(C)** Distribution of C12-induced conductance measured on occasions of sudden conductance changes. Four nanoSiemen bins were used for the histogram. These are pooled data from 7 separate experiments. **(D)** The proposed model of C12 or C12 metabolite(s) functioning as a mitolytic molecule to directly cause MOMP.

3.9. C12 inhibits LLC tumor growth and induces tumor cell apoptosis *in vivo* in a dose-dependent fashion.

The cytotoxic effects of C12 on tumor cells have been reported previously (66,69,74,80), but whether they are selective for transformed cells was unknown. To investigate whether oncogenic transformation influences the cytotoxicity of C12, we studied normal human bronchia/tracheal epithelial (NHBE) and corresponding HBE immortalized and transformed successively by telomerase, SV40 large T antigen and activated Ras (H-ras V12). This is a well-established epithelial cell malignant transformation system related to human lung cancer (87). Upon C12 treatment, we observed higher levels of cell death and caspases-3/7 activation in transformed HBE cells than in their untransformed counterparts. This indicates that C12 induces apoptosis preferentially in transformed cells (Figure 3.9A-B). To investigate the relevance of C12 cytotoxicity on transformed cells to tumor growth in animals, we examined the effects of C12 on the growth of established Lewis Lung Carcinoma (LLC) tumors. We found that transplanted tumors grew much more slowly in C12-treated mice than in vehicle-treated mice, revealing a dose dependent anti-tumor activity of C12 as a single agent (Figure 3.9C). By evaluating caspase3/7 activation and TUNEL labeling, we found that apoptosis is involved in the inhibitory activity of C12 *in vivo* (Figure 3.9D-F).

Figure 3.9. C12 inhibits LLC tumor growth and induces tumor cell apoptosis *in vivo* in a dose-dependent fashion. (A-B) Cytotoxicity of C12 is affected by oncogenic transformation. C12's effects on HBE cell viability (A) and caspase-3/7 activation (B) were examined. All data shown are mean ± standard deviation of 3 independent experiments. Asterisk indicates P < 0.05 (*) or P< 0.01 (**) by student's unpaired t test. **(C)** The inhibitory effects of C12 on the growth of LLC tumors were studied. Tumors were measured daily and tumor tissues were removed at the end of treatments. Data are shown as mean ± standard deviation of tumor volumes of 7 animals in either vehicle control or C12-treated group. Asterisk indicates P < 0.05 (*) by student's unpaired t test. **(D)** Apoptotic cells in tumor sections were detected by immunofluorescence staining of activated caspase-3. Representative images of tumor sections are shown. Scale bar, 50 μm. **(E)** TUNEL staining of apoptotic cells in control or C12-treated tumor sections. Representative images are shown. Scale bar, 60 μm. **(F)** The percentage of apoptotic cells shown in (E) was quantified using ImageJ software. Data are mean ± standard deviation of three independent tumor sections. Asterisk indicates P < 0.05 (*) or P< 0.01 (**) by student's unpaired t test.

3.10. C12 induces tumor cell apoptosis independent of anti-apoptotic Bcl-2 proteins.

Anti-apoptotic Bcl-2 proteins are frequently overexpressed in human cancers and associated with chemotherapeutic resistance and relapse(88). To investigate the involvement of anti-apoptotic Bcl-2 proteins in C12-induced human tumor cell apoptosis, Bcl-2 was stably overexpressed in A549 cells by retroviral infection (Figure 3.10A). The anti-tumor drug actinomycin D caused less cell death and less caspases-3/7 activation in Bcl-2-overexpressing cells than in cells expressing the empty vector. In contrast, C12 induced similar levels of cell death and caspase-3/7 activation in cells overexpressing Bcl-2 and the vector control cells (Figure 3.10B-C). We also investigated whether C12's effect on mitochondrial membrane potential is dependent of Bcl-2. A549-vector and A549-Bcl-2-overexpressing cells were loaded with JC1, and its fluorescence was measured using imaging microscopy (Figure 3.10D-E). Within minutes of C12 exposure, mitochondria in A549-vector cells and A549-Bcl-2 overexpressing cells were depolarized to the same degree, providing more evidence that C12 evokes apoptosis independent of anti-apoptotic Bcl-2 proteins.

Figure 3.10. C12 induces tumor apoptotic cell death independent of anti-apoptotic Bcl-2 proteins. (A) Retrovirally overexpressed Bcl-2 in A549 cells was examined by western blot. **(B)** Cell viability was measured 48 hours after C12 or actinomycin D (ActD) exposure. **(C)** Caspase-3/7 activities were determined following 2 hour exposure to C12 and 24 hour exposure to actinomycin D. **(D)** C12's effect on mitochondrial membrane potential is independent of Bcl-2. A549-vector and A549-Bcl-2-overexpressing cells were loaded with JC1, and its fluorescence was measured using imaging microscopy during the treatment with 100 µM C12 and 5 µM FCCP. Typical results from three independent experiments are shown. **(E)** C12 caused equivalent depolarization of mitochondrial potential in vector and Bcl-2-overexpressing A549 cells. All data are shown as mean ± standard deviation of three independent experiments. Asterisks indicate P < 0.05 (*); ns, no significant by student's unpaired t test.

3.11. C12-induced tumor cell apoptosis is independent of Bak and Bax.

Previous studies show that C12 induces apoptosis in MEFs independent of

Bak and Bax (9), two pro-apoptotic Bcl-2 members required for MOMP in

almost all apoptotic paradigms (3). To elucidate whether Bak and Bax are also

involved in C12-induced tumor cell apoptosis, human colon carcinoma HCT116

cell lines deficient in Bak alone (Bak-KO), Bax alone (Bax-KO), or both Bak

and Bax (Bak/Bax-DKO) were investigated (Figure 3.11A). We found that C12

caused equivalent cell death and caspase-3/7 activation in all the HCT116 cell

lines examined (Figure 3.11B-C). This indicates that deficiency of Bak or Bax in

HCT116 cells did not influence their responses to C12. Furthermore, deficiency

in Bak/Bax expression did not affect C12's effect to depolarize $\Delta\psi_{mito}$ (Figure

3.11D-E).

Figure 3.11. C12-induced tumor cell apoptosis is independent of Bak and Bax. (A) Bak and Bax expression in the indicated HCT116 cells was examined by western blot. **(B-C)** C12 induced similar levels of cell death (B) and caspase-3/7 activation (C) among WT, Bak-KO, Bax-KO and Bak/Bax-DKO HCT116 cells after 24 hours treatment. (D) The mitochondrial potential of the WT and Bak/Bax-DKO HCT116 cells loaded with JC1 was determined by fluorescent microscopy upon the treatment with 50 µM C12 and 5 µM FCCP. Representative results are shown. (E) Summary of the data shown in (D). All data are presented as mean ± standard deviation of three different experiments. ns, no significant.

3.12. PON2 expression is enhanced in human lung tumor tissues

and oncogenically transformed HBE cells.

It has been shown that PON2 upregulation in some cancer cells, including lung

cancer cell lines, enables cancer cells to become resistant to conventional

therapeutic drugs (80). To determine whether PON2 expression is enhanced in

human lung cancer, we examined PON2 protein levels in tumor tissues of non-

small cell lung carcinoma (NSCLC) patients by western blot. Among eleven

samples from patients, we found that PON2 was overexpressed in eight of lung

cancer tissues compared with corresponding adjacent normal tissues, whereas

its expression was slightly decreased in three of them (Figure 3.12A). As Ras-

transformed HBE displayed higher levels of apoptosis compared with their

untransformed counterparts upon C12 treatment (Figure 3.9A-B), PON2

expression was also increased in transformed HBE cells. These observations

provide more evidence that oncogenic transformation enhances PON2

expression (Figure 3.12C).
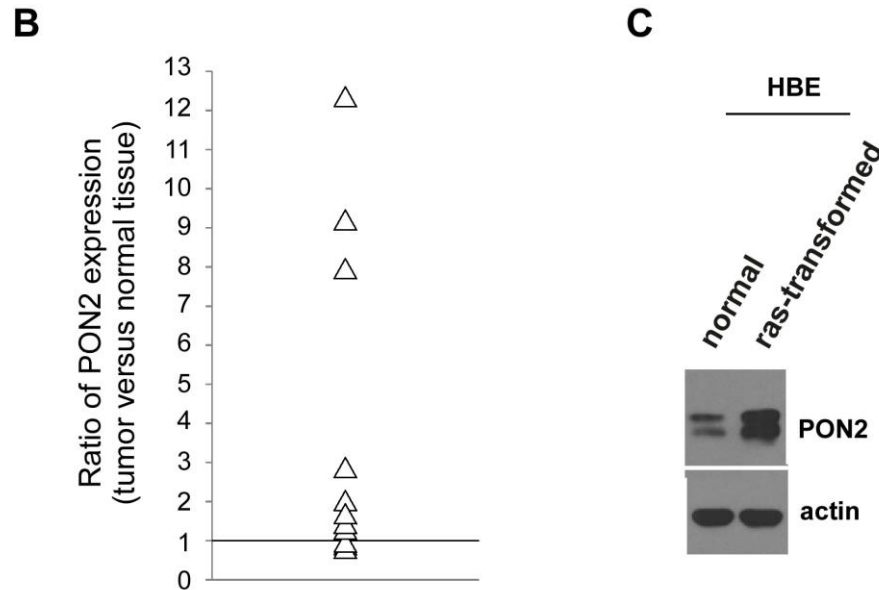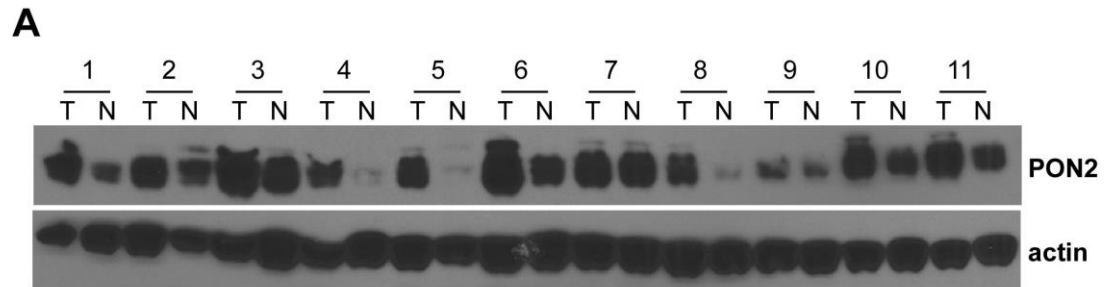
Figure 3.12. PON2 expression is enhanced in human lung tumor tissues and oncogenically transformed HBE cells. (A) Expression of PON2 in NSCLC tissue specimens and corresponding adjacent normal tissues from 11 patients were evaluated by western blot. Samples 1-4, 6, 8, 9, 11 were from adenocarcinoma patients, whereas samples 5, 7, 10 were from squamous cell carcinoma patients. T, tumor; N, normal. **(B)** The intensities of bands in (A) were quantified using ImageJ software (NIH). To normalize loading variation, the relative levels of PON2 were calculated by dividing the PON2 value into the corresponding value for actin. The data were shown as a ratio of PON2 levels in a tumor tissue sample versus its corresponding normal tissue, and the value bigger than 1 indicates that PON2 expression is increased in tumor tissues. Differential expression of PON2 in tumor versus normal tissues is significant with the value of "P" smaller than 0.01 as calculated by student's paired t test. **(C)** The expression of PON2 and PON3 in primary HBE cells and their transformed counterparts was determined by western blot.

3.13. PON2 is required in C12 cytotoxicity in human lung tumor cells.

Overexpression of PON2 promotes cytotoxicity of C12 in non-transformed MEF and HEK293T cells (9), but the role of endogenous PON2 in C12-induced apoptotic signaling is unclear. To further investigate the mechanism of C12-triggered apoptosis, we investigated the prospective involvement of endogenous PON2 in C12 cytotoxicity in tumor cells. We employed shRNA to stably reduce PON2 expression in human NSCLC cell lines A549 and NCI-H1299. Treatment with C12 elicited less cell death and caspase-3/7 activation in A549 and NCI-H1299 cells lacking PON2 expression (Figure 3.13). Conversely, increased cell death and caspase-3/7 activation observed in PON2-deficient cells in response to the conventional apoptotic stimuli actinomycin D and tunicamycin, suggesting that PON2/C12 interaction induces a novel form of apoptosis distinct from that evoked by classical apoptotic stimuli (8) .

Figure 3.13 PON2 is required in C12 cytotoxicity in human lung tumor cells.
(A) PON2 expression in A549 cells was stably reduced by shRNA. The expression levels of PON2 were determined by western blot. **(B)** C12 induced less cell death in A549 cells with reduced PON2 expression than in control vector cells. Cell death was assessed after 32 hour incubation. **(C)** Upon treatment with different doses of C12 for 32 hours, less caspase-3/7 activation was detected in cells with reduced PON2 expression than control vector cells. **(D)** Stable reduction of PON2 expression in NCI-H1299 cells was evaluated by western blot. **(E)** C12 induced less cell death in NCI-H1299 cells with reduced PON2 following 24-hour treatment. **(F)** Less apoptosis was detected in NCI-H1299 cells with reduced PON2 expression than control vector cells induced by C12. All data shown are mean ± standard deviation of three independent experiments. Asterisks indicate P values of < 0.05 (*) or < 0.01 (**) by Student's unpaired t test.

3.14. Murine PON2 sensitizes human lung tumor cells with reduced endogenous PON2 expression to C12.

To validate the role of PON2 in mediating C12-induced apoptosis, we stably overexpressed murine PON2 cDNA in A549 cells deficient in PON2 expression by retroviral infection (Figure 3.14A). Upon treatment with C12, we observed more cell death and caspase-3/7 activation in PON2-deficient A549 cells overexpressing murine PON2 compared to vector control and parental cells (Figure 3.14B). Similarly, stable overexpression of murine PON3 in PON2-knockdown NCI-H1299 cells was confirmed by western blot (Figure 3.14C). We found that C12 induced more cell death and elicited higher caspase-3/7 activation in PON2-knockdown NCI-H1299 cells expressing mouse (Figure 3.14D).

Figure 3.14 Murine PON2 sensitizes human lung tumor cells with reduced endogenous PON2 expression to C12. (A) Murine PON2 cDNA was stably overexpressed in A549 cells with reduced PON2 expression by retroviral infection. Expression levels of PON2 were determined by western blot. **(B-C)** After treating with different doses of C12 for 24 hours, more cell death (B) and caspase-3/7 activation (C) were detected in PON2-knockdown A549 cells with increased mouse PON2 expression comparing to vector control and parental cells. **(D)** Stable overexpression of murine PON3 in PON2-knockdown NCI-H1299 cells was examined by western blot. **(E-F)** C12 induced more cell death (E) and caspase-3/7 activation (F) in PON2-knockdown NCI-H1299 cells expressing mouse PON2 after 24 hours treatment. All data shown are mean ± standard deviation of three independent experiments. Asterisks indicate P values of < 0.05 (*) or < 0.01 (**) by Student's unpaired t test.

3.15. PON2 is essential for C12-triggered cell death in HEK-293T and HBE cells.

To further explore the involvement of PON2 in apoptotic signaling in non-transformed cells, endogenous PON2 expression was stably reduced in Human Embryonic Kidney-293T (HEK-293T) cells, whose viability was assessed upon treatment with C12 (Figure 3.15A-B). Reducing PON2 expression in HEK-293 cells enabled cells resistant to C12. Furthermore, we stably decreased PON2 expression in immortalized human bronchial epithelial (HBE) cells (89) and measured cell viability upon C12 exposure (Figure 3.15C-D). Reducing PON2 expression in HBE cells de-sensitized cells to C12. Overall, these data provide evidence that PON2 plays a similar role in apoptotic signaling in both transformed and non-transformed cells.

Figure 3.15 PON2 is essential for C12-triggered cell death in HEK-293T and HBE cells. (A) PON2 expression was stably reduced in HEK-293Tcells. **(B)** The viability of HEK-293Tcells was measured 24 hours after C12 treatment. **(C)** Stable decrease of PON2 expression in human bronchial epithelial (HBE) cells was determined by western blot. **(D)**The viability of HBE was evaluated 24 hours following C12 exposure. Mean ± standard deviation for three independent experiments are shown. For all the data, *, P < 0.05, Student's unpaired t test.

3.16. PON2 is essential for human lung tumor cell proliferation but not non-transformed cell proliferation.

During the process of generating A549 and NCI-H1299 cells lacking PON2 expression, we observed that those cells proliferated much slower than their vector control counterparts. To determine whether or not PON2 is essential for human lung tumor cell proliferation, we measured the proliferation of A549 cells and NCI-H1299 expressing PON2 shRNA or the empty vector control. We found that proliferation of NCI-H1299 and A549 cells with reduced PON2 expression was slower compared to that of their empty vector expressing counterparts (Figure 3.16A-B), implicating a role of PON2 in lung tumor cell proliferation. To determine whether or not PON2 is involved in the proliferation of non-transformed cells, we measured the proliferation of HEK-293T and HBE expressing PON2 shRNA or the empty vector control. It was found that HEK-293T and HBE cells lacking PON2 expression grew at the same rate as their counterparts expressing the empty vector (Figure 3.16C-D), indicating that PON2 is not involved in non-transformed cell proliferation. Overall, these data indicate that PON2 mediates apoptosis independently of its function to modulate cell proliferation.
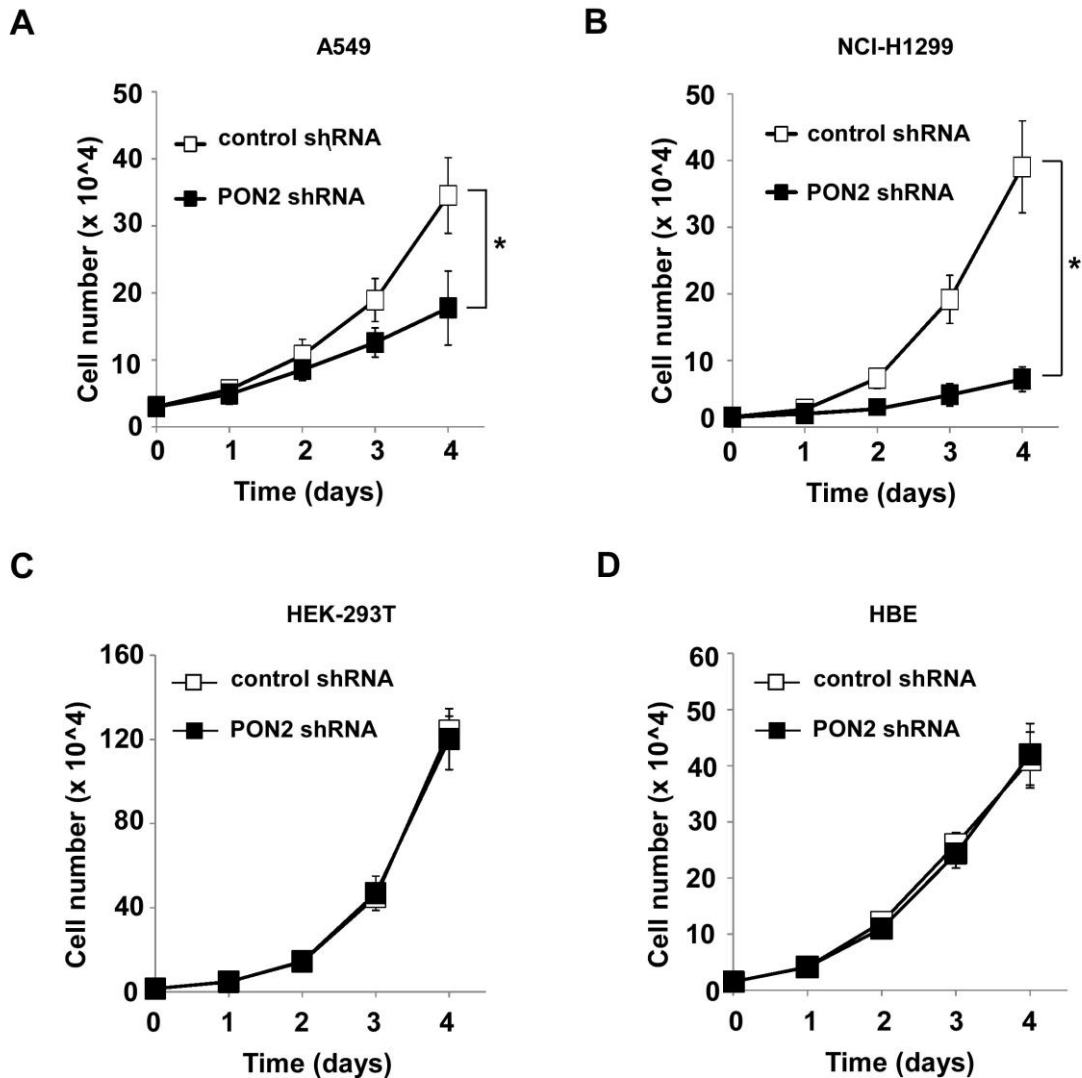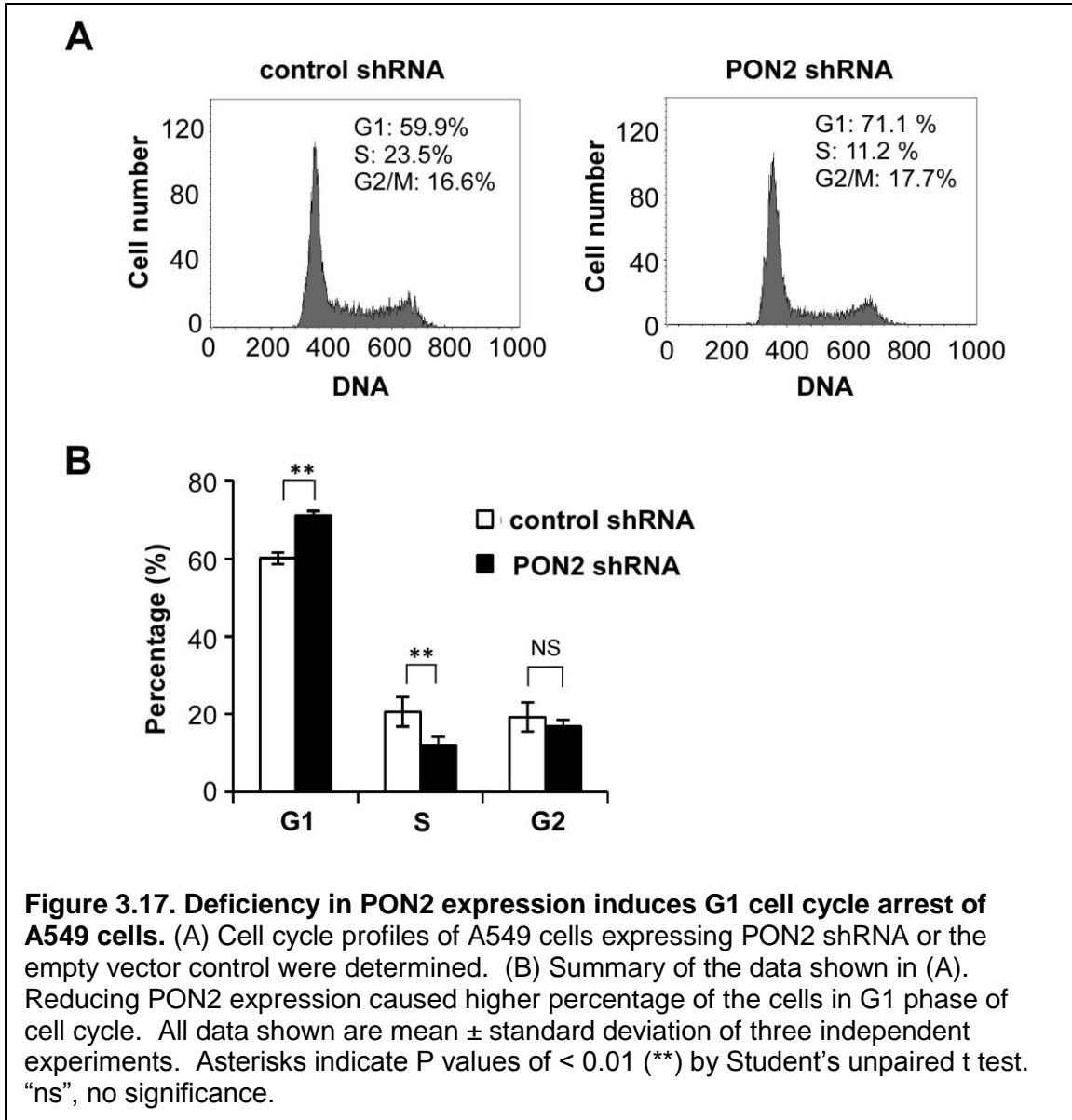
Figure 3.16 PON2 is essential for human lung tumor cell proliferation but not non-transformed cell proliferation. The proliferation of A549 cells **(A)**, NCI-H1299 cells **(B)**, HEK-293T cells **(C)** and HBE cells **(D)** expressing PON2 shRNA or the empty vector control was measured. Proliferation of NCI-H1299 and A549 cells with reduced PON2 expression was slower compared with their counterparts expressing the empty vector. HEK-293T cells (C) and HBE cells with reduced PON2 expression proliferated at the same rate as their vector control counterparts. All data shown are mean ± standard deviation of three independent experiments. Asterisks indicate P values of < 0.05 (*) by Student's unpaired t test.

3.17. Deficiency in PON2 expression induces G1 cell cycle arrest of A549 cells.

To investigate the effects of deficient PON2 expression, we performed cell cycle analysis to determine the cell cycle profile of A549 cells expressing PON2 shRNA or the empty vector. We found that deficiency in PON2 expression induces cell cycle arrest at G1 phase in A549 cells (Figure 3.17).

Figure 3.17. Deficiency in PON2 expression induces G1 cell cycle arrest of A549 cells. (A) Cell cycle profiles of A549 cells expressing PON2 shRNA or the empty vector control were determined. (B) Summary of the data shown in (A). Reducing PON2 expression caused higher percentage of the cells in G1 phase of cell cycle. All data shown are mean ± standard deviation of three independent experiments. Asterisks indicate P values of < 0.01 (**) by Student's unpaired t test. "ns", no significance.

CHAPTER 4: DISCUSSION

The quorum-sensing molecule C12 evokes apoptosis in a variety of mammalian cells (62,65,68,90). Several signaling pathways leading to apoptosis have been associated with C12 cytotoxicity. In addition to the activation of the intrinsic apoptosis pathway, C12 is also shown to induce caspase-8 activation, suggesting a role of the extrinsic apoptosis cascade in C12-induced apoptosis (9). The interplay between these pathways and the functions of key molecules involved were still unclear. In this study using cells deficient in one or more caspases, we present evidence that caspase-3/7 and caspase-9 but not caspase-8 are essential for C12-induced apoptotic cell death (Figures 3.1-3.2), indicating that C12 selectively triggers the mitochondria-dependent intrinsic apoptotic pathway. Previously reported C12-caused caspase-8 activation; in addition to the events associated with activating a plasma membrane receptor (e.g. TNF receptor), are likely secondary responses to MOMP. The dispensable roles of both "initiator" caspase (e.g. caspase-9) and "effector" caspase (e.g. caspase-3/7) in C12-indcued MOMP demonstrate that C12 may directly damage mitochondria without the involvement of other pathways triggering caspase activation.

Consistent with its ability to induce depolarization of $\Delta\psi_{mito}$ within 20 minutes (Figures 3.2 and 3.5), amphipathic C12 molecules assemble permeation

channels in phospholipid membranes without any proteins (Figure 3.8). The characteristics of channels formed by C12 are similar to those of ceramide (85,85). Ceramide has been demonstrated to suppress tumors through inhibiting tumor cell proliferation, triggering apoptosis, autophagy and/or senescence (85,86). Ceramide-induced apoptosis signaling is modulated by both anti- and pro-apoptotic Bcl-2 proteins (91,92). In agreement with their functions in apoptotic signaling, Bcl-2 proteins also regulate ceramide channels *in vitro* *(93,94)*. It appears that C12 and ceramide evoke distinct apoptotic signaling with C12-triggered apoptotic responses independent of both anti- and pro-apoptotic Bcl-2 proteins in non-transformed as well as tumor cells.

Several signaling pathways linked to apoptosis initiation, including JAK/STAT pathway (66), MAPK and eIF2α pathways (60), and ER stress pathway (63,65), have been implicated in C12-evoked cell death. These apoptosis-associated signaling pathways are normally involved in multiple steps of signal transduction, and the biological events reflective of these signaling cascades are observed hours following C12 incubation. In contrast, depolarization of $\Delta\psi_{mito}$, the earliest step of MOMP (6), is always detected within minutes upon C12 exposure and reaches its maximal levels in 20 minutes as reported here (Figures 3.2 and 3.5) and in our earlier studies (8,9). Thus, it appears that activation of these signaling pathways is secondary to MOMP initiation in C12-triggered apoptosis cascade. It is conceivable that C12 or its metabolite(s) acts directly on mitochondria to permeabilize them. The *in vitro* studies in this paper support the notion that C12

might directly assemble permeation channels on mitochondria, leading to cytochrome c release, apoptotic cascade activation, and ultimately apoptosis.

Many neoplastic cells show an increased ratio of anti-apoptotic to pro-apoptotic Bcl-2 proteins, which enables them to survive even under the conditions that would normally initiate apoptotic signaling (95). An emerging strategy for cancer therapy is to overcome the resistance to apoptosis caused by aberrant Bcl-2 signaling in tumor cells (96,97). Recently, several small molecules triggering apoptosis independent of either pro- or anti-apoptotic Bcl-2 proteins have been identified as potential anti-tumor drugs. Among them, the pentacyclic triterpenoid betulinic acid induces Bax/Bak-independent MOMP and subsequent apoptosis (98,99). Unlike C12, cytotoxic effects of betulinic acid are influenced by Bcl-2 overexpression and it is ineffective against epithelial tumors. Similarly, Bax/Bak is also nonessential in apoptotic signaling induced by chelerythrine (100) or titanium dioxide (TiO_2) (101). The polyphenolic compound gossypol evokes Bax/Bak-independent apoptosis and inhibits Bcl-2-overexpressing human B lymphoblast tumor growth in nude mice (102). Furthermore, Bcl-2 expression fails to influence human tumor cell apoptosis induced by the antibiotic agent Tetrocarcin-A (103,104). Compared with those molecules, C12 is the first small molecule compound, to the best of our knowledge, inducing human tumor cell apoptosis *in vitro* as well as blocking tumor growth *in vivo* independent of both pro- and anti-apoptotic Bcl-2 proteins.

The apoptosis cascade induced by C12 in tumor cells is unique, evident by its rapid pro-apoptotic effects, such as depolarizing mitochondrial membrane potential within minutes (Figures 3.10 and 3.11), releasing cytochrome c into the cytosol and maximally activating caspases. These distinctive pro-apoptotic features of C12 have not been observed in any other apoptosis paradigms of cancer cells, which might be attributed to the ability of C12 or its derivatives generated in tumor cells to directly permeabilize mitochondria without the involvement of pro- and anti-apoptotic Bcl-2 proteins. This study also shows that endogenous PON2 is essential for C12's cytotoxicity in human lung tumor cells (Figure 3.13), which is consistent with our previous observation of PON2 overexpression in non-transformed fibroblasts and HEK293T cells (9).

It has been reported that lung tumor cells are resistant to conventional therapeutic drugs partially due to enhanced expression of PON2, which is thought to be associated with anti-oxidant activities of PON2 (78,79). Data presented in Figure 3.13 show decreased killing of A549 and NCI-H1299 cells with shRNA for PON2; which is consistent with previous results (9). Additionally, data in Figure 3.13 indicate that killing activities of C12 on lung tumor cells is mediated through PON2. Thus, it is conceivable that C12 or compounds derived from C12 could trigger rapid and Bcl-2 protein-independent apoptosis in lung tumors that are resistant to traditional chemotherapeutic drugs, whereas normal tissues are spared due to their lower PON2 expression.

PON2 is known to associate with the mitochondrial electron transport chain

Complex III component coenzyme Q10 (CoQ10), and it is optimally positioned

with appropriate enzymatic activity to cleave oxidized mitochondrial lipids

(73,105). One possibility is that enhanced PON2 expression in tumor cells

functions by interacting with CoQ10, leading to increased mitochondrial electron

transport, and subsequent mitochondrial bioenergetics, which generate enough

ATP and metabolites to sustain the rapid proliferation of lung tumor cells.

CHAPTER 5: CONCLUSION

Overall, our study reveals that C12 induces a unique mitochondrial apoptotic signaling pathway, in which C12 or C12 metabolite(s) acts on mitochondria as a mitolytic molecule to permeabilize mitochondria, leading to activation of apoptosis signaling independent of both pro- and anti-apoptotic Bcl-2 proteins. These properties of C12 enable it inhibit tumor growth as a single agent regardless of Bcl-2 protein expression in tumors, making it an ideal candidate of a lead compound for novel therapeutic agents for cancer. Further to this, our studies indicate that PON2 expression in non-small cell lung carcinoma cells is essential for their rapid proliferation. Therefore, C12 is an ideal candidate of a lead compound for novel therapeutic agents for cancer.

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APPENDIX: LIST OF ABBREVIATIONS

5-FU	5-Fluorouracil
Apaf-1	Apoptotic Protease Factor 1
BH	Bcl-2 homolog
C12	N-(3-oxododecanoyl)-homoserine lactone
COQ10	Coenzyme Q10
DISC	Death Inducing Signaling Complex
FADD	Fas Associated Death Domain
FasL	Fas Ligand
FBS	Fetal Bovine Serum
IAPs	Inhibitor of Apoptosis Proteins
LLC	Lewis Lung Carcinoma
MEFs	Mouse Embryonic Fibroblasts
MIPs	Maximum Intensity Projections
MOMP	Mitochondrial Outer Membrane Permeabilization
NHBE	Human Bronchi/Tracheal Epithelial Cells
NSCLC	Non-Small Cell Lung Carcinoma
OMM	Outer Mitochondrial Membrane
PBS	Phosphate Buffered Saline
PI	Propidium Iodide
PON2	Paraoxonase 2

ROIS	Regions of Interest
TLR4	Toll-Like Receptor 4
TNF	Tumor Necrosis Factor
TNFR	Tumor Necrosis Factor Receptor
TNFR1	Tumor Necrosis Factor Receptor 1
TRAIL	TNF-Related Apoptosis Inducing Ligand
WT	Wild Type

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 Department, Dallas, Texas (Supervisor Jordan Schnieder)
06/2011-08/2012: Teacher's Assistant/Lab Instructor-Introduction to Biology I,
 University of North Texas, Denton, Texas
01/2011-05/2011: Teacher's Assistant/Lab Instructor-Animal Physiology,
 University of North Texas, Denton, Texas
01/2010-12/2010: Teacher's Assistant/Lab Instructor- Human Anatomy &

Physiology I, University of North Texas, Denton, Texas
01/2009-12/2009: Teacher's Assistant/Lab Instructor- Human Anatomy and Physiology II, University of North Texas, Denton, Texas
01/2009- 05/2012: Graduate Research assistant, Department of Biology, University of Texas, Denton, Texas (Advisor Edward Dzialowski)
09/2001-12/2008: Customer Service Representative/ Personal Banker, The Royal Bank of Canada, Nassau, Bahamas (Supervisor Natasha, Huyler)
07/2000-08/2001: Office Clerk, The Private Trust Corporation (PTC), Nassau, Bahamas (Supervisor Kenya Pickersgill)

PROFESSIONAL MEMBERSHIPS:
2015- present: Society of Toxicology
2004-present: Phi Eta Sigma Honor Society

MAJOR AWARDS AND SCHOLARSHIPS:
2013-2015: University of Louisville Integrated Program in Biomedical Sciences (IPIBS) Doctoral Fellowship
2009-2012: University of North Texas Physiology Graduate Assistantship, Denton, Texas
University of North Texas Biology Teacher's Assistantship, Denton, Texas
The Good Neighbor Scholarship, Denton, Texas
2003-2007: International Student Scholarship, Paul Quinn College, Dallas, Texas, Track and Field Scholarship, Paul Quinn College, Dallas Texas

PUBLICATIONS:
Christian Schwarzer, Zhu Fu, Takeshi Morita, Aaron G. Whitt, Aaron M. Neely, Chi Li and Terry E. Machen (2015) Paraoxonase 2 mediates *N*-(3-oxododecanoyl)-homoserine lactone-triggered apoptosis in fibroblasts. *Journal Biological Chemistry.* 290:8949-8963. PMID:25697356.

Guoping Zhao, Aaron M. Neely, Christian Schwarzer, Huayi Lu, Aaron G. Whitt, Joseph A. Burlison, Terry E. Machen and Chi Li. (2016) N-(3-oxo-acyl)homoserine lactone inhibits tumor growth independent of Bcl-2 proteins. *Oncotarget.* 10.18632/oncotarget.6827. PMID: 26758417.

ABSTRACTS SUBMITTED TO REGIONAL AND NATIONAL MEETINGS:
Neely, A.M., Zhao, G., Whitt A., and Li, C. Distinct roles of paraoxonase 2 in apoptosis signaling cell proliferation. *Research!Louisville. September 2015.*

Neely, A.M., Zhao, G., Stivers, N.S., and Li, C. The *Psuedomonas aeruginosa* quarum-sensing molecule homoserine lactone triggers a unique mitochondrial

apoptotic signaling pathway. *Ohio Valley Society of Toxicology Student/PostDoc Summer Meeting.* June 2015.

Neely, A.M., Zhao, G., Stivers, N.S., and Li, C. The Psuedomonas aeruginosa quarum-sensing molecule homoserine lactone triggers a unique mitochondrial apoptotic signaling pathway. *University of Louisville Graduate Student Research Symposium. November 2014.*

Neely, A.M., Zhao, G., Stivers, N.S., and Li, C. The Psuedomonas aeruginosa quarum-sensing molecule homoserine lactone triggers a unique mitochondrial apoptotic signaling pathway. *James Graham Brown Cancer Retreat. November 2014.*

Neely, A.M., Zhao, G., Stivers, N.S., and Li, C. The Psuedomonas aeruginosa quarum-sensing molecule homoserine lactone triggers a unique mitochondrial apoptotic signaling pathway. *Research!Louisville. October 2014.*

TECHNICAL SKILLS:
CELL AND MOLECULAR BIOLOGY TECHNIQUES:
  Polymerase chain reaction (PCR), reverse transcriptase PCR (rt-PCR), quantitative real time PCR (qPCR), molecular cloning, Mitochondrial Purification, western blot analysis, flow cytometry analysis, immunoprecipitation (IP), immunofluescence analysis, retroviral infection, microscopy, cell culture.

ANIMAL HANDLING TECHNIQUES:
  Measurement of Metabolic Rates (Oxygen Consumption): Chicken (*Gallus gallus*), Alligator and Muscovy ducks

COMPUTER SKILLS:
  Microsoft office (MS word, MS Excel, MS power point), Adobe Photoshop, Canvas, EndNote 9, Reference Manager