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Doxorubicin Cytotoxicity in a Human Proximal Tubular Epithelial Cell Line was Attenuated by the Natural Product Resveratrol

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DOXORUBICIN CYTOTOXICITY IN A HUMAN PROXIMAL TUBULAR EPITHELIAL CELL LINE WAS ATTENUATED BY THE NATURAL PRODUCT RESVERATROL

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Approved by
Dr. Monica Valentovic, Committee Chairperson
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APPROVAL OF THESIS

We, the faculty supervising the work of Morghan S. Getty, affirm that the thesis, *Doxorubicin Cytotoxicity in a Human Proximal Tubular Epithelial Cell Line was Attenuated by the Natural Product Resveratrol*, meets the high academic standards for original scholarship and creative work established by the Biomedical Sciences and Marshall University. This work also conforms to the editorial standards of our discipline and the Graduate College of Marshall University. With our signatures, we approve the manuscript for publication.

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DEDICATION

I dedicate this work to my parents, Ann and Greg, whose support has been unwavering, and love and faith in me endless. This journey would not have been possible without you and there are not words to express my gratitude for your steadfast support. I am beyond blessed to have you as my parents; thank you. To my editor, best friend and sister, Meghan, who amazes and inspires me every day. Thank you for all the late night "off the ledge" talks and encouraging me to do what is right rather than what is easy. To my grandmother, who inspires through action. As my grandfather said, "she doesn't see impossible things; she just sees things that take a little more ingenuity to get done." Grandmother, thank you for encouraging me to see the possible. To Steve and Kathy Turner, my neighbors, who became family. I will cherish your love and kindness forever and I cannot thank you enough for the endless encouragement, meals and swing talks. To Robby, mate of my soul, thank you for believing in me without question or hesitation. I cannot wait for our new adventure. Finally, I dedicate this work in loving memory of my "Pop," who told me not so long ago that you cannot run without first learning to walk confidently. My steps and life are more confident and purposeful because of his encouragement.

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"And, when you want something, all the universe conspires in helping you to achieve it."
– Paolo Coelho

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ABSTRACT

The cancer chemotherapeutic agent doxorubicin (DOX), Adriamycin, is part of the treatment regimen for breast, ovarian, small cell lung cancer and acute/chronic lymphoid leukemia. Adverse effects associated with DOX are cardiotoxicity and nephrotoxicity. Interventions are needed to reduce DOX nephrotoxicity. Resveratrol (RES) is a phytochemical contained in grapes, berries and nuts, which possesses antioxidant and anticancer properties. This study tested the hypothesis that RES will attenuate DOX renal cytotoxicity in human noncancerous renal proximal tubular epithelial (HK-2) cells and that RES will reduce DOX mediated changes in mitochondrial function. HK-2 cells were plated and grown for 48 hours (h). Cells were next pre-incubated for 1h with 0 (DMSO), 5 or 7.5 μM RES followed by a 24 h co-incubation with 0-5 μM DOX. RES did not alter cell growth or viability at the concentrations tested as indicated by comparable MTT values between DMSO and RES groups ($p>0.05$). Cell viability was further assessed by cell count using Trypan blue exclusion. DOX produced a concentration dependent decline in viability within a 24 h exposure. Pretreatment for 1 h with RES was sufficient to reduce DOX loss of cell viability. Studies were initiated to investigate the cellular mechanism of RES attenuation of DOX cytotoxicity. Western blot of cells following 24 h exposure examined increased protein carbonylation as an indicator of oxidative stress. Initial studies were begun to examine the DOX effects on mitochondrial oxygen consumption using a Seahorse platform. In summary, RES did not diminish cell viability at the concentrations tested in our HK-2 cells. DOX diminished cell viability within 24 h relative to vehicle control. A 1 h pretreatment with RES reduced DOX cytotoxicity in HK-2 cells. Prevention of mitochondrial impairment and oxidative stress by DOX are potential mechanisms for RES protection in HK-2 cells.

CHAPTER 1

INTRODUCTION: DOXORUBICIN

Doxorubicin: Cancer Chemotherapeutic Agent

Doxorubicin (DOX), trade name Adriamycin, is an anthracycline cancer chemotherapeutic that has been used in the treatment of a variety of cancers including: breast, ovarian and small cell lung cancers and both acute and chronic lymphoid leukemias (Figure 1). The first anthracycline, daunorubicin, was isolated in 1951 and its analogue, DOX, was developed in 1969 (Arcamone, 1967). DOX is an antitumor antibiotic that was originally isolated from the *Streptomyces peucetius* bacteria. The longevity and continued use of the drug, it was first approved for use in the United States in 1974, are a testament to the drug's efficacy. Typically DOX is administered in intravenous doses of 60 to 75 mg/m^2 every 21 to 28 days (National Library of Medicine, 2017). The dosage varies based upon: indication, body surface area and hepatic function (National Library of Medicine, 2017). The mechanism of action for DOX involves intercalating between base pairs of deoxyribonucleic acid (DNA) and inhibiting macromolecule biosynthesis (Gu et al., 2016). The effectiveness of DOX is related to its ability to inhibit DNA replication through the inhibition of topoisomerase II (Top2) by preventing the ligation of nucleotide strands after double strand breakage (Eissa, El-Naggar, El-Sattar, & Youssef, 2017). The DNA strand requires unwinding for both transcription and replication. However, the unwinding has to be compensated by overwinding elsewhere in the DNA molecule (Nitiss, 2009) This problem of supercoiling is addressed through topoisomerases that introduce DNA double strand breaks that eliminate the overwinding of the DNA molecule. Top2 introduces double strand breaks altering DNA structure, replication, transcription and chromosome segregation that are essential processes in preventing tumor growth (Nitiss, 2009).

The inhibition of Top2 triggered by DOX administration is directly correlated with the drug's effectiveness as a cancer chemotherapeutic agent.

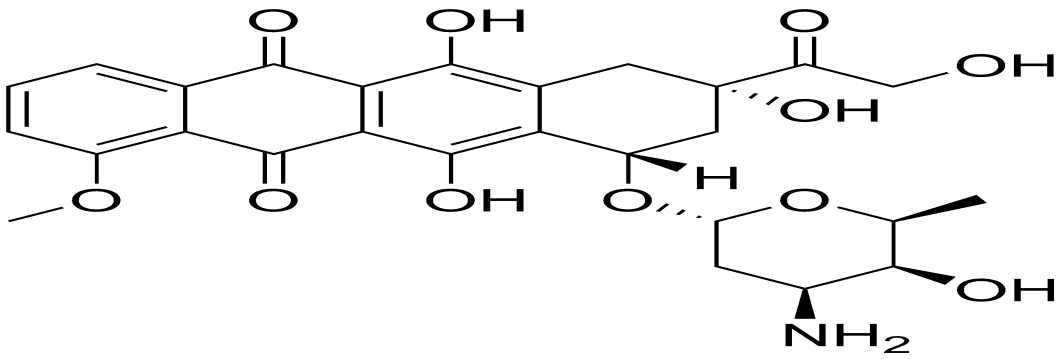

Figure 1: Chemical Structure of Doxorubicin

The same mechanisms that make DOX an effective antineoplastic agent are also in part responsible for the drug's adverse effects. Administration of DOX has been limited by dose-dependent, irreversible and progressive toxicity (Buzdar et al., 1992). DOX direct cytotoxic action on membranes leads to redox cycling, generation of reactive oxygen species (ROS) (Cappetta et al., 2017) and subsequent lipid peroxidation and DNA damage (Gewirtz, 1999). The metabolism of DOX (Figure 2) through reductive pathways leads to the generation of a semiquinone free radical, doxorubicinol (DOX-OL) and ROS (Riddick et al., 2005). Studies have shown that an intravenous bolus of DOX produces high plasma concentrations that quickly fall due to rapid and extensive distribution into tissues (Speth, Linssen, Holdrinet, & Haanen, 1987). Independent of the absolute drug concentration in plasma, 50 to 85% of plasma DOX is bound to protein, leaving 50% of DOX and its metabolite DOX-OL as free drug (Celio, Digregorio, Ruch, Pace, & Piraino, 1982). After 50% of DOX is removed from the body unchanged the remaining 50% is metabolized to DOX-OL via a two-electron reduction (Joerger, Huitema, Meenhorst, Schellens, & Beijnen, 2005). Other metabolites are formed but are detected at much lower concentrations (Joerger et al., 2005). Elimination primarily occurs in the bile (Takanashi &

Bachur, 1976) and over 50% is eliminated as part of the "first pass effect" through the liver (Harris & Gross, 1975)**.** There has been no evidence to indicate that enterohepatic recirculation occurs with DOX administration (Takanashi & Bachur, 1976). Patients have described a reddish discoloration of urine and further examination indicated only about 5% of a dose was discovered in urine (Di Fronzo, Lenaz, & Bonadonna, 1973). The mean half-life of DOX has been estimated to be between 1-3 hours. It should also be noted that DOX concentrations measured in healthy tissues such as liver, spleen, lymph nodes, muscle, hematopoetic cells, kidney and lung always exceed plasma concentrations (Chan et al., 1978; J. Cummings & McArdle, 1986; Y. T. Lee, Chan, Harris, & Cohen, 1980; Speth, van Hoesel, & Haanen, 1988). The unique features of drug accumulation and distribution associated with DOX are part of the reason it is an effective antineoplastic agent in the treatment of a variety of cancers. DOX has a propensity to accumulate in tissues over time and it is this accumulation that may predispose tissues to cytotoxicity.

Toxicities associated with DOX have limited its clinical usage. Common side effects include bone marrow suppression, nausea, vomiting, diarrhea, headache, skin rash, dizziness, confusion, neuropathy, alopecia and fever (National Library of Medicine, 2017). The more severe adverse reactions that are associated with high doses or prolonged therapy are cardiotoxicity, hepatotoxicity and nephrotoxicity and are major dose limiting effects. The mechanisms associated with various toxicities have not been fully elucidated but a disturbance in the antioxidant-oxidant system leading to free radical generation and iron-dependent oxidative damage has been implicated in previous studies (Liu, Li, Xia, Li, & Shao, 2007). Additionally, DOX has been shown to induce inflammatory changes in heart, liver and kidney tissue (Deepa & Varalakshmi, 2005). The tissue damage caused by DOX produces an imbalance between free radicals and antioxidants that is demonstrated by lipid peroxidation and protein modifications

leading to tissue damage (Ayla et al., 2011). The free radicals formed as a result of the conversion of DOX to DOX-OL cause damage by inducing oxidative stress and subsequent tissue damage. Increased levels of DOX-OL in human myocardium have been shown to cause impairment of hydrolase and/or reductase deglycosylation and to uncouple hydrolysis from the carbonyl reduction causing increased levels of the redox-cycling metabolite (Licata, Saponiero, Mordente, & Minotti, 2000). DOX cardiotoxicity has been linked to the DOX-OL metabolite which compromises both systolic and diastolic cardiac function more than the parent compound DOX (Olson et al., 1988). The NADPH: Cytochrome P450 reductase (P450R) enzyme catalyzes the one electron reduction of DOX to the semiquinone free radical, which can be directly cytotoxic leading to covalent modification of macromolecules (Riddick et al., 2005). This quinone-structured agent can be enzymatically reduced and may subsequently autoxidize, generating free radicals (Goodman & Hochstein, 1977). The free radicals produced are the superoxide anion, hydrogen peroxide and the hydroxyl radical (Goodman & Hochstein, 1977). However, it should also be mentioned that the P450R enzyme also plays a role in the DOX detoxification pathway involving reductive deglycosylation to the metabolite, 7-deoxyglycone (Niitsu, Kato, Shikoshi, & Umeda, 1997). The effectiveness of DOX as an antineoplastic agent is well documented but there is a need to find better methods to combat the drug's multiple toxicities.

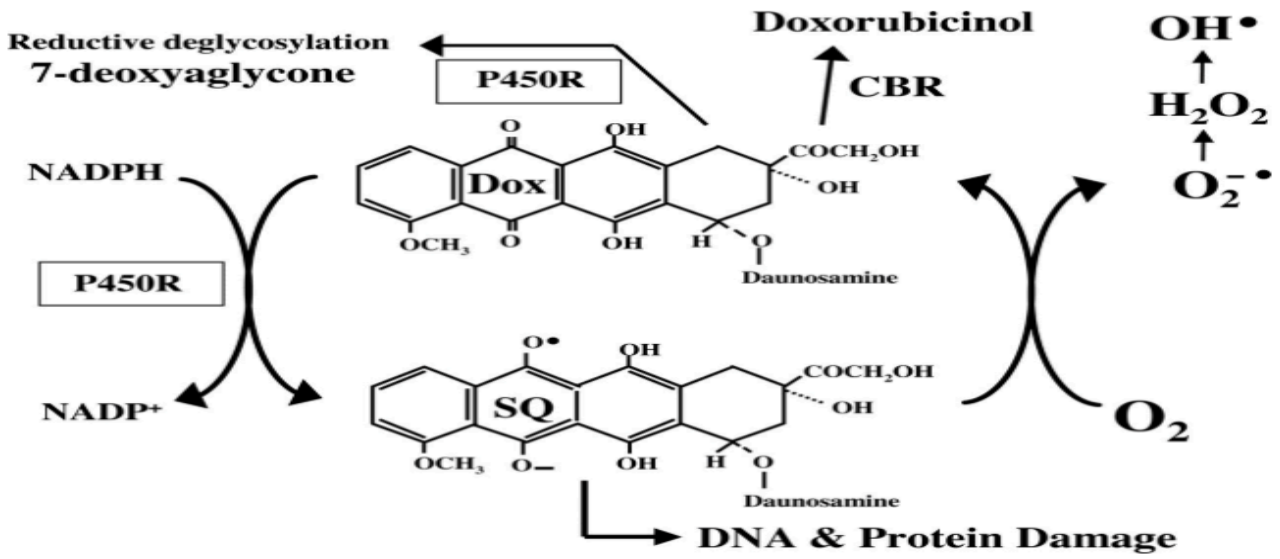

Figure 2: Major pathway of doxorubicin metabolism in humans

Doxorubicin Toxicity: Heart

The effects of DOX on the heart are both acute and chronic, meaning that even after the therapy is discontinued the accumulated drug can lead to left ventricular dysfunction, dilated cardiomyopathy and heart failure (Lipshultz et al., 2010). The effects on the heart are more pronounced in part due to its lower levels of protective antioxidant enzymes such as superoxide dismutase, which results in cardiomyocytes having a greater susceptibility to injury over other tissues (Barry, Alvarez, Scully, Miller, & Lipshultz, 2007). The severity of heart disease is linked directly to accumulated DOX dosage during the course of therapy ranging from 3-5% in patients who received a cumulative dose of 400mg/m^2 to 18-48% in patients receiving 700mg/m^2 (Lipshultz et al., 2010). DOX induced cardiotoxicities are clearly a dose-dependent response. Studies have shown that the various DOX manifested cardiotoxicities can be attributed to the excessive production of ROS causing damage to cardiomyocytes. Cardiomyocytes require mitochondria for extensive ATP production that ensures contractile function of the heart (Koleini & Kardami, 2017). Oxidative stress activates molecular pathways causing the loss of cardiomyocytes through necrosis and apoptosis (Licata et al., 2000). The oxidative stress

mechanism of DOX has also been shown to mediate mitochondrial toxicity, a key player in acute and long-term cardiac dysfunction (Zhang, Shi, Li, & Wei, 2009). Ichikawa and colleagues have shown there is also a preferential accumulation of Iron (Fe^{2+}) in the mitochondria following treatments and isolated cardiomyocytes showed an increase in Fe concentrations for both mitochondrial Fe^{2+} and cellular ROS levels (Ichikawa et al., 2014). The excessive amount of ROS and substantial increases in Fe^{2+} are two ways that DOX has been shown to cause acute and chronic cardiac toxicities.

Doxorubicin Toxicity: Liver

The liver achieves the highest concentration of DOX compared to all other organs studied (Y. T. Lee et al., 1980). This distribution of DOX was observed in patients about 1-1.5 hours after an intravenous bolus dose with the highest levels of uptake, 2.3-19.8μg/g, occurring in the liver compared to plasma levels of 0.2-0.53 μg/g (Y. T. Lee et al., 1980). The aforementioned distribution would indicate that the liver plays a central role in DOX metabolism and distribution. In the past, hepatic impairment has led to a dose reduction or removal of the medication entirely from a chemotherapeutic regimen. Hepatic dysfunction is also mediated through the generation of free radicals causing oxidative damage to tissue. The high levels of P450 enzymes in the liver make it a direct target for drug accumulation and potential damage. The P450R facilitated conversion of DOX to its toxic metabolite DOX-OL occurs in the liver. The toxic effects are seen with increases in aspartate aminotransferase (AST) and alanine aminotransferase (ALT) in stressed erythrocytes (Hamlaoui et al., 2012). High malondialdehyde (MDA) has also been used as a diagnostic marker of hepatic impairment. The increased levels of MDA, AST and ALT within erythrocytes have been accompanied by a concomitant decrease of antioxidant enzymes in both regions of the liver (Hamlaoui et al., 2012). The increase in free iron

(Fe^{2+}), hydrogen peroxide (H_2O_2), coupled with depressed Ca^{2+} levels, and followed by the induction of the hydroxyl radical (•OH) that can in turn affect Ca^{2+} homeostasis in both erythrocytes and plasma (Bengaied, Ribiero, Amri, Scherman, & Arnaud, 2017). Kassner and colleagues reported that the extrinsic and intrinsic apoptotic responses mediated by Fas and Bax were both associated with DOX-induced acute hepatic damage (Kassner et al., 2008). The reduction of DOX to DOX-OL detected in the cytosol of the liver has the highest V_{max} and clearance values making it a primary target for the drug's metabolism as well as the adverse effects associated with DOX (Kassner et al., 2008).

Doxorubicin Toxicity: Kidney

The effects of DOX associated toxicities have been studied much less extensively in the kidney as compared to other organs. However, DOX-induced mechanisms of nephrotoxicity are postulated to be as a result of free radical generation similar to what has been reported to contribute to hepatotoxicity and cardiotoxicity, along with preferential accumulation of DOX in the kidney leading to direct renal damage (Refaie, Amin, El-Tahawy, & Abdelrahman, 2016). The preferential accumulation of DOX in the kidney leads to increased capillary permeability and glomerular atrophy (Injac et al., 2008). Although the exact mechanism is unknown, there is ample data to suggest that free radical formation, iron-dependent oxidative damage of biological macromolecules and membrane lipid peroxidation are again the key players in DOX-induced toxicity (Liu et al., 2007). The possible role of DOX in nitric oxide synthase (NOS) metabolism is through direct or indirect stimulation of nitric oxide (NO) production and could be attributed to the increase in free radical generation mediated by the drug (Ayla et al., 2011). Nitric oxide (NO) can act as a cytoprotective or cytotoxic agent and is generated by endothelial nitric oxide synthase (eNOS) or inducible nitric oxide synthase (iNOS) (Nathan & Xie, 1994). Free radical

production and/or NO release induced by DOX may lead to toxicity in the kidney. The significant change in kidney tissue triggered by DOX administration is exemplified by the cytotoxic effects on human noncancerous renal proximal tubular epithelial cells (HK-2) causing alterations in caspase pathways (Y. Wu et al., 2009). Alterations in caspase pathways alter apoptosis.

Damage to the liver as well as the heart may also indirectly effect DOX induced nephropathy and nephrotoxicity. The kidneys receive nearly 25% of cardiac output and as one of the major organs of excretion, are exposed to a greater proportion of circulating drugs (Y. Wu et al., 2009). The known cardiac toxicities associated with the drug may more directly affect the kidney than previously believed and should be examined more thoroughly. DOX has also been shown to accumulate at high levels in the mitochondria increasing Fe^{2+} levels within the mitochondria as well as increasing cellular reactive oxygen species (Ichikawa et al., 2014). DOX has the ability to interact with Fe directly and forms a DOX-Fe^{2+} complex, resulting in Fe^{2+} cycling between Fe(II) (Niitsu et al., 1997) and Fe(III) forms producing a substantial amount of ROS (Ichikawa et al., 2014). The same study showed that DOX mitochondrial content was significantly greater than DOX content in the cytosol, 0.8μM and 0.01 respectively upon administration of 20 μM/mg dose. Additionally, DOX-Fe^{2+}, measured as Mito ^{55}Fe content, in the mitochondria was almost twice that of the control. DOX toxicity to the mitochondria in the heart, as well as the kidney, shows that mitochondrial damage plays a central role in DOX-induced complications. Attenuating DOX mitochondrial toxicity may play a central role in mitigating nephrotoxicity and will be examined in this study.

INTRODUCTION: RESVERATROL

Resveratrol (3,4',5-trihydroxystilbene) (RES) is a naturally occurring polyphenolic compound found in various nuts, berries and grapes and exhibits antioxidant properties and anti-inflammatory activity (Valentovic et al., 2014) (Figure 3). RES is a phytoalexin that is produced by plants as a defense mechanism triggered by conditions of fungal growth, specifically powdery mildew. The main enzyme responsible for RES biosynthesis is stilbene synthase that condenses one *p*-coumaroyl-CoA and three molecules of malonyl-CoA (Park & Pezzuto, 2015). Despite its early isolation in 1939, RES has only gained attention relatively recently for its numerous antioxidant and anti-inflammatory benefits. In 1997, Jang and colleagues discovered the chemopreventive effects of RES and its ability to inhibit events associated with tumor initiation e.g. free radical formation (Jang et al., 1997). Further studies have concluded that RES has both anti-angiogenic and anti-tumor effects in skin, breast, colorectal, prostate and lung cancers (Park & Pezzuto, 2015). RES's anti-oxidant and anti-inflammatory properties have been shown to exert positive effects on the heart, liver and kidney in addition to its known chemopreventive effects.

The pharmacological properties of RES can make research somewhat limiting. RES is known to have high absorption but extremely poor bioavailability (<1%) when administered orally. RES undergoes rapid metabolism and excretion and most of the oral dose is recovered in urine unchanged. However, oral intake remains the major route administration and has been

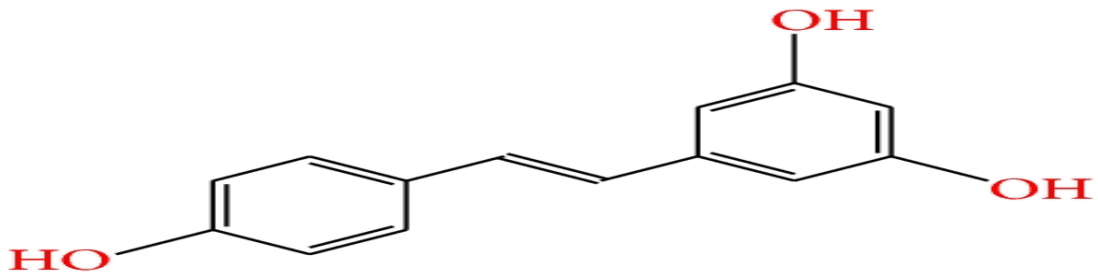

Figure 3: Chemical structure of Resveratrol

studied extensively. RES exists as both *cis* and *trans* isomers; however, most studies use the *trans* isomer due to greater stability. Improvements have been made in identifying RES and its metabolites using high performance liquid chromatography (HPLC) analytical methods. HPLC has enabled direct identification of metabolites and the position of the hydroxyl substitution. This new method led to the development of a metabolite standard and has enabled the measurement of RES derivatives (Cottart, Nivet-Antoine, Laguillier-Morizot, & Beaudeux, 2010). The advancement of analytical methods has also contributed to identifying free and bound forms of RES in plasma. RES is lipophilic, and therefore, it is necessary to take into account LDL-and protein bound fractions (Cottart et al., 2010). In 2008 it was described *in vitro*, that more than 90% of free *trans* RES is bound to human plasma lipoproteins in a non-covalent manner in healthy human subjects (Burkon & Somoza, 2008). A full understanding of the pharmacokinetics and bioavailability is essential in determining proper dose and response of RES, and only then can it be utilized to its full potential in the treatment of disease. RES's ability in slowing or halting disease progress has been widely studied in the past few years and will be discussed in the following subsections. There is little doubt that the potential clinical usage of antioxidant RES could have far reaching applications in the mitigation of a number of disease pathologies.

Resveratrol and Cancer

Jang and colleagues discovered the chemopreventive properties of RES by demonstrating the anti-initiation, anti-promotion and anti-tumor progression actions of RES in different models (Jang et al., 1997). Potential mechanisms proposed for RES anticancer activity include action as an antioxidant and/or anti-inflammatory agent, interference with signal transduction pathways, modulation of cell cycle regulating proteins and selective induction of apoptosis, which has been characterized in various cell lines (Ulrich, Wolter, & Stein, 2005). RES anti-tumor effects and

pathways have been studied broadly *in vitro* on various cell lines but there are still very limited clinical applications available. The need for further studies on human cell lines is imperative in determining the possible uses of RES as a chemotherapeutic agent.

Resveratrol and the Heart

Resveratrol has long been part of the "French paradox" and in 1992, Renaud and colleagues showed that the French population had a lower risk of cardiovascular disease (CVD) even though their diet was high in saturated fats. The intake of a moderate amount of RES containing red wine was thought to play a potential protective role against CVDs. Further studies showed RES as being a good candidate for prevention of CVD due to its apparent protection of vascular walls on oxidation, inflammation, platelet oxidation and thrombus formation (Delmas, Jannin, & Latruffe, 2005). Cardioprotective mechanisms of RES are in part due to its ability to upregulate eNOS which in turn favors NO mediated vasodilation (Leikert et al., 2002; Wallerath et al., 2002). The known physiological roles of NO show that it improves vasodilation and decreases platelet aggregation as well as stimulating leukocyte recruitment and proliferation of smooth muscle cells (H. Li & Forstermann, 2000). Previous studies have also shown that RES can protect against oxidative stress by scavenging the hydroxyl and superoxide radical (Bonnefont-Rousselot, 2016). These same RES scavenging properties that prevent against oxidative stress are also involved in the prevention of lipid peroxidation. The identification of multiple targets, as well as the known protective benefits against CVDs, make naturally occurring RES a noteworthy compound for future study in disease prevention.

Resveratrol and the Liver

Hepatotoxicity is not only a major problem in the development of drugs, but is also one of the major reasons certain drugs are withdrawn from the market. Clinically, drug-induced liver

injury is the most frequent cause of acute liver failure (Pagliarini et al., 2008) in the west (E. S. Lee, Shin, Yoon, & Moon, 2010). RES has been shown to protect against numerous *in vitro* and *in vivo* rodent models of liver injury, including hepatotoxicity caused by drugs and other xenobiotics (Bishayee, Darvesh, Politis, & McGory, 2010). The proposed mechanism of RES-mediated protection against liver injury is that it acts as an antioxidant and decreases oxidative stress by directly scavenging free radicals or by upregulating cellular antioxidant enzymes such as superoxide dismutase (SOD), catalase and glutathione peroxidases (McGill, Du, Weemhoff, & Jaeschke, 2015). Inflammation often accompanies liver disease and the anti-inflammatory properties of RES have been demonstrated by the downregulation of inflammation-inducing biomarkers including proinflammatory mediators, oxidative stress markers and endogenous vasoconstrictors, while accompanied by the upregulation of inflammation-reducing biomarkers and anti-inflammatory proteins (Park & Pezzuto, 2015). The poor bioavailability of RES and its rapid and extensive metabolism in the liver continues to be an obstacle in clinical application and further metabolic studies are needed to validate its potential as a prospective therapeutic option.

Resveratrol and the Kidney

The positive effects that RES could potentially exert on the kidney have not been examined as closely as with other organs. Pharmacokinetic studies indicated that RES levels are higher in the kidney and liver compared to other organs suggesting that RES has a greater potential to induce its effects on these organs respectively (Aggarwal et al., 2004). RES has been shown to alleviate oxidative stress in a diabetic kidney and attenuate increases in proinflammatory mediators (Palsamy & Subramanian, 2011), but little is known about its potential to reduce or protect against xenobiotic-induced nephrotoxicity. Previous studies completed in our lab have shown that pretreatment with RES, when co-administered with

cisplatin (a well-known cancer chemotherapeutic agent) demonstrated positive effects in the mitigation of nephrotoxicity. The results have shown that pretreatment with RES was protective for cisplatin renal cytotoxicity in an *in vitro* mouse model. Additionally, RES protection was associated with prevention of lipid peroxidation and oxidative stress that is normally associated with cisplatin administration (Valentovic et al., 2014).

Resveratrol and the Mitochondria

Mitochondria are cytoplasmic double-membraned organelles that play a crucial role in cell physiological processes, e.g. energy generation via formation of adenosine triphosphate (ATP) through oxidative phosphorylation (Trumbeckaite et al., 2006). Oxidative phosphorylation leads to two electron transfer through the electron transport chain that is organized into five different trans-membrane protein complexes I-V (Dias & Bailly, 2005). Mitochondria are a common target of toxicity for drugs and other chemicals, and damage results in decreased aerobic metabolism and cell death (Beeson, Beeson, & Schnellmann, 2010). Mitochondrial dysfunction plays a role in the pathophysiology of many human diseases, as well as in the initiation and progression of apoptosis and the production of ROS in cellular systems. The roles of these tiny powerhouses range from apoptosis to energy homeostasis and cell signaling to lipid metabolism, making it evident that proper mitochondrial function is imperative to a variety of biological processes. RES may modulate mitochondrial function by regulating transcription factors that activate or repress mitochondria-related genes, causing alterations in mitochondrial physiology (de Oliveira et al., 2016). Proper mitochondrial function is essential but mitochondrial biogenesis, which restores cell vitality, is imperative. It has been proposed that recovery of organ and cellular injury following insult, may be limited by the remaining mitochondrial function and ATP levels, and that stimulation of mitochondrial biogenesis may

promote organ recovery and restore cellular function in the short and long term (Beeson et al., 2010). RES has previously been shown to induce mitochondrial biogenesis as assessed through respirometric measurement when compared to untreated control cells (Beeson et al., 2010). The mechanism by which RES can induce mitochondrial biogenesis is not known but it is possible that it may work through inducing peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC1α), the "master regulator" of mitochondrial biogenesis (Beeson et al., 2010). The anti-inflammatory and antioxidant properties of RES may serve to protect mitochondria against dysfunction induced by xenobiotics as well as to promote mitochondrial biogenesis. This two-fold protective response could potentially mitigate mitochondrial toxicity seen with not only DOX administration but also a variety of other drugs. Although RES has been shown to have effects on mitochondrial function, the exact mechanism and dynamics by which RES modulates function are not fully understood (de Oliveira et al., 2016). As such, there is a need for further study to examine the effects of RES on modulating and possibly protecting mitochondrial function in the presence of oxidative stress induced by anthracyclines, e.g. DOX.

Statement of Hypothesis

The effectiveness of DOX as a cancer chemotherapeutic agent is well documented in a variety of different types of cancers. However, it is clear that the various toxicities associated with the drug limit its clinical use; thus, new methods designed to reduce toxicity are vital to its continued clinical usage. The previous discovery by our lab, showing that RES decreased the extent of oxidative stress and associated modification of proteins, has led us to consider the possible effects of using RES to mitigate DOX induced nephrotoxicity. Additionally, we wanted to determine if RES' antioxidant and anti-inflammatory properties would also protect against mitochondrial dysfunction. Previous studies have shown that DOX accumulates at high levels in

the mitochondria; therefore, we hypothesized that RES would reduce DOX renal cytotoxicity in HK-2 cells and that RES would attenuate DOX-mediated changes in oxidative stress and mitochondrial function.

Chapter 2

Materials and Methods

Chemicals and Reagents

DOX and RES were purchased from Sigma Aldrich (Item No. D1515 and Item No. R5010) and were used throughout the experiment. All other chemicals were purchased from Fisher Scientific or Sigma Aldrich Company and were of the highest analytical quality. Antibody sources and dilutions are described below in the appropriate subsection(s).

Cell Line and Treatment

Human immortalized epithelial (HK-2) cells were purchased from the American Type Culture Collection (ATCC) and cultured according to the manufacturer's guidelines. Cells were grown in keratinocyte-free media with 50μg/mL bovine pituitary extract and 5 ng/mL recombinant epithelial growth factor from Invitrogen (Carlsbad, CA, USA, Item No. 17005-042). Cells were grown in a humidified incubator under constant conditions of 37° and 5% CO_2. HK-2 cells were plated into six-well tissue culture plates ($1.5x10^6$ cells/well) (Corning, Sigma Aldrich Item No. CLS3516) and allowed to grow for 48 h. Media was replaced and cells were treated with 0.5 or 7.5 μM of RES or 0, 1 or 2 μM of DOX for 24 h. Vehicle controls were equal amounts of Dimethyl sulfoxide (DMSO) (Item No. TS-20688) for RES or purified water for DOX, respectively. Following the 24 h treatment period, cells were collected with Trypsin-EDTA (0.25%) (Invitrogen, Item No. 25200072) for sample analysis.

Cell Viability

Cells were plated in 48-well tissue culture plates (39,000 cells/mL) (Cyto One, USA Scientific, Ocala, FL, USA, Item No. CC7682-7548) and allowed to grow for 48 h in the manner listed in the above sub-section. In later experiments, cells were plated in 96-well tissue culture

plates (3700 cells/mL) (Thermo Fisher Scientific, Item No.12565501) and allowed to grow for 48 h in the same manner. Following the treatment period, cell viability was assessed using the MTT assay (van Meerloo, Kaspers, & Cloos, 2011). The MTT assay relies on the conversion of tetrazolium dye 3-(4,5 dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (Sigma Aldrich, Item No. M5655-5X1G) to formazan by NAD(P)H-dependent oxidoreductases. The cells were incubated at room temperature for 4 h with MTT, and quantity was specified in 25mL of HBSS (Hank's buffered salt solution) (Gibco, Thermofisher Scientific, Pittsburgh, PA, USA, Item No. 14025076). Following the 4 h incubation with MTT, cell viability was assessed using a plate reader (Synergy 2, Biotek Instruments, Winooski, Ca, USA) at 540 nm by measuring formazan concentration as reflected in optical density (OD). Cells not exposed to DOX or RES were compared to values of the wells that were exposed to the drugs.

Western Blot

Western blot analysis was conducted to assess the expression of cytochrome-C oxidoreductase IV. Protein concentration in each sample was determined using the Bradford protein assay (Bradford, 1976). A 40 μg aliquot of each sample was denatured by boiling for 5 min. Proteins were then separated on a 12.5% polyacrylamide gel and transferred to a nitrocellulose membrane (Bio-Rad, Hercules, CA, USA, Item No. 162011). Successful transfer and unified protein loading were verified using MemCode Reversible Protein Stain Kit (Pierce Biotechnology, Rockford, Il, USA, Fisher Scientific, Item No. PI-24580). Membranes were blocked in 5% milk/TBST solution (10mM Tris-HCl, 150mM NaCl, 0.1% Tween-20; pH 8.0) for 1 h. Membranes were then incubated with continuous shaking overnight at 4°C with cytochrome C (Cell Signaling, Item No.4272, 1:1000 dilution). The membranes were washed four times with TBST, and goat anti-rabbit HRP-linked secondary antibody (Santa Cruz, Item

No. sc-2004) was diluted 1:5000 in 5% milk/TBST and added for 1 h with continual shaking. The membranes were washed again with TBST in the same manner listed above. Membranes were developed using Amersham ECL Western Blotting Detection Agent (GE Healthcare Life Sciences, Malborough, MA, USA, Item No. RPN2232). A Bio-Rad Chemidoc system was to capture the gel image for densitometry analysis. All western analyses were conducted as three independent experiments. Western blot was also conducted as described above using rabbit polyclonal antibody to peroxisome proliferator-activated receptor gamma coactivator 1-alpha (anti-PGC-1α) (Abcam, Item No. ab191838) and sirtuin 1 (anti-SIRT1) (Abcam, Item No. ab7343) both diluted 1:1000 in 5% w/v milk/TBST. Human heart tissue whole cell lysate, (Abcam**,** Item No.7919 dilution 1:1000) a positive control known to express PGC-1α, and SIRT1 was used in both PGC-1α and SIRT1 analyses at a dilution of 1:1000 and boiled for five minutes prior to loading. Following washing with TBST, membranes were treated with goat anti-rabbit HRP-linked secondary antibody for 1 h (Santa Cruz, Item No. sc-2004) and secondary was diluted 1:3000 in 5% w/v milk/TBST. Membranes were washed and developed as previously described. PGC-1α positive control was replaced with mouse heart total protein lysate (Abcam, Item No. Ab30291). SIRT1 positive control was replaced by human embryonic kidney whole cell lysate (HEK293). Dilutions for both positive controls were 1:1000 and were boiled for 5 minutes prior to loading. Secondary antibodies and dilutions remained unchanged.

Oxidative Stress

Protein carbonylation is an indicator of oxidative stress that produces an aldehyde or ketone side chain on amino acids. Protein carbonylation was assessed using the Oxyblot Protein Oxidation Detection Kit (EMD Millipore, Billerica MA, USA, Item No. S7150). Following a 24 h treatment with vehicle(s), DOX, RES or DOX and RES, cells were trypsinized, pelleted and

stored in lysis buffer at -80°C until the blot was performed. The Bradford assay measured protein content and a 25 μg aliquot was derivitized as described in the previous sub-section. Proteins were separated on a 12.5% polyacrylamide gel, transferred to a nitrocellulose membrane and uniform protein loading was verified using MemCode Reversible staining as described in the previous sub-section. Protein carbonyl moieties on amino acids generated by oxidative stress were derivitized in the presence of 2,4-dinitrophenylhydrazine to stable 2,4-dinitrophenylhydrazone groups. The membrane was blocked for 1 h in 1% BSA in PBST (Phosphate buffered saline, 0.05% Tween-20 pH 7.2-7.5). The primary antibody used recognizes 2,4-dinitrophenylhydrazine groups on proteins and was used at a dilution of 1:150 and incubated with constant shaking overnight at 4°C. The membrane was washed in PBST four times for 10 minutes and incubated with secondary antibody at a dilution of 1:300 in 1%BSA/PBST for 1 h at room temperature. The membrane was washed again in the same manner listed above. Results were analyzed with BioRad Chemidoc densitometry software (version 4.0.1, Catalog No. 170-9690, BioRad, Hercules, CA, USA).

Seahorse XFp Assays

Seahorse XFp assays allow measurement of basal Oxygen Consumption Rate (OCR) and Extracellular Acidification Rate (ECAR) following injection of compounds to identify sources of mitochondrial impairment. Mitochondrial function was measured using Agilent mitochondrial stress and glycolytic stress assays. Cells were cultured in XFp miniplates (75,000 cells/mL) (Agilent Technologies, Item No. 103025-100). Prior to the assay, cells were washed with assay media (Agilent Technologies, Item No. 103334-100) supplemented with 1mM pyruvate, 2 mM glutamine and 0 mM glucose and equilibrated in 180 μL pre-warmed assay media at 37°C with no CO_2 for 1 h. Cells were treated and incubated for 24 h with the varying drug concentrations or

vehicle controls. In each assay, three basal OCR/ECAR measurements were taken at 7-minute intervals using the Seahorse XFp instrument system. Following basal measurements, various probes were injected and additional OCR and ECAR measurements were taken. Maximal respiration was stimulated by the addition of carbonyl cyanide-4-(trifluoromethoxy) phenylhydrazone (FCCP), a mitochondrial uncoupler. The mitochondrial stress test assay (Agilent Technologies, Item No. 103015-100), injections of oligomycin (0.05μM), and a mixture of rotenone/antimycin A (0.05μM) were added and OCR and ECAR were measured. In the Glycolysis Stress Test (Agilent Technologies, Item No. 103020-100), glucose (10mM), oligomycin (1μM), and 2-DG were injected, and OCR and ECAR were measured. Upon completion of each assay, cells were washed with 200μL PBS and lysed. Protein concentration was determined using the Bradford assay as previously described. Results were normalized to total protein concentration and analyzed using Wave Software (Agilent Technologies, Wave for Desktop, Version 2.3.0.19).

Statistical Analysis

Values represent mean ± SEM with 2-4 independent experiments conducted with 2-4 biological replicates. Differences between groups were determined with one-way or two-way ANOVA followed by a Bartlett's post-hoc test with or Tukey's multiple comparison test respectively with $p<0.05$ (Prism 7 for Mac OS X, Graph Pad Software, Inc. La Jolla, CA 92037).

CHAPTER 3

RESULTS

Doxorubicin and Resveratrol Effects on Cell Viability

The MTT (MTT, (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium Bromide)) concentration response assays showed DOX reduced cell viability at concentrations of 3μM ($p<0.0385$) and 5 μM ($p<0.0071$) within 24 h compared to control (Figure 4A). DOX at concentrations of 0.5, 1 and 2 μM did not have significant effects on cell viability. A dose response assay performed at 48 h showed toxicity at all concentrations of DOX ($p<0.0001$) relative to control (Figure 4B). MTT assays performed at 8 h with 1 h RES pretreatment indicated that 5 μM RES pretreatment was ineffective in mitigating DOX induced cytotoxicity while the higher RES concentration of 7.5 μM was slightly more effective (Figure 5A)**.** DOX concentration of 1 μM coupled with 5 μM RES pretreatment had significantly reduced cell viability ($p<0.0011$) when compared to control but 1 μM DOX coupled with 7.5 μM RES pretreatment did show a significant decline. DOX at 2 μM had significantly reduced cell viability in both cells treated with 5μM RES ($p<0.0003$) and 7.5 μM RES ($p<0.0004$). Cells treated with 4 μM DOX and 5 μM RES showed reduced cell viability ($p<0.0246$) while cells pretreated with 7.5 μM RES did not show a significant decline in viability. MTT performed at 24 h showed a significant decline between control vs. 4 μM DOX ($p<0.0481$) (Figure 5B). Additional declines were observed between 5 μM RES vs. 5 μM RES+2 μM DOX ($p<0.0453$) and 5 μM RES+ 4 μM DOX ($p<0.0347$). Additional declines were also observed between 7.5 μM RES vs. 7.5 μM RES+4 μM DOX ($p<0.0066$) and 7.5 μM RES+1 μM DOX vs. 7.5 μM RES+4 μM DOX ($p<0.0396$).

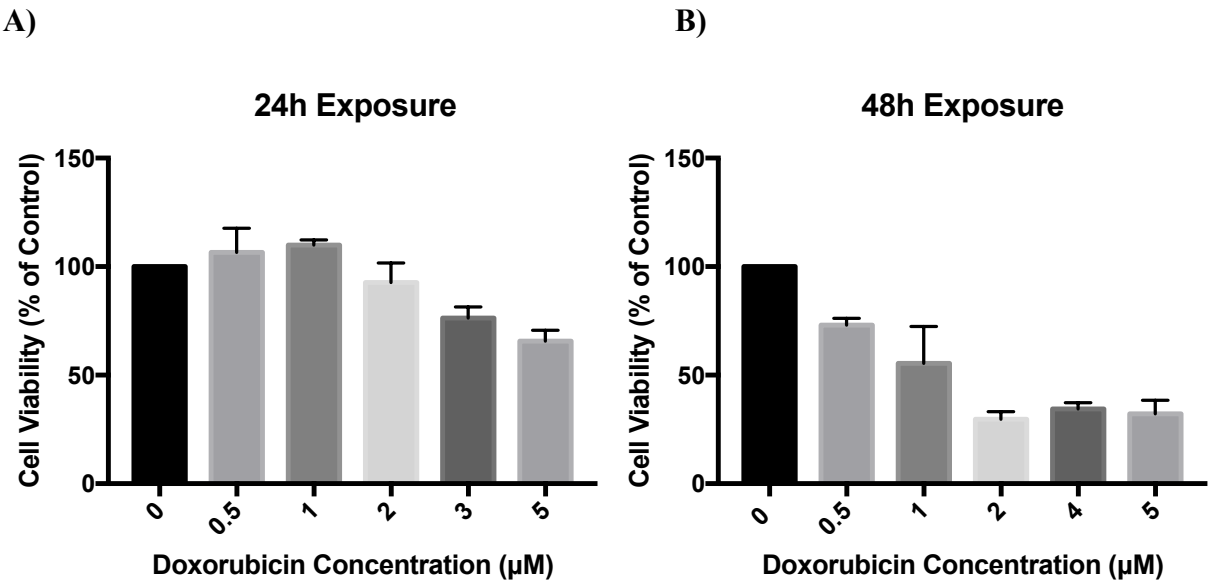

Figure 4: DOX cytotoxic effects HK-2 cell viability using MTT. DOX dose response at 24 h (A) and DOX dose response at 48 h (B). Bar graph values represent Mean ± SEM for two independent experiments.

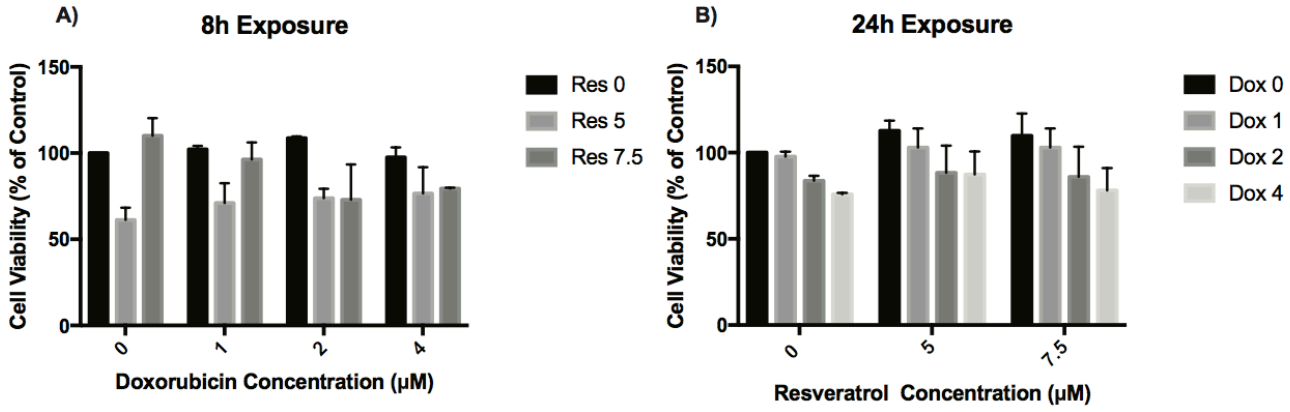

Figure 5: Protective effects of RES against DOX cytotoxic effects HK-2 cell viability using MTT. RES was unable to protect against DOX induced cytotoxicity at (A) 8 h. RES was able to protect against significant loss of cell viability at 24 h (B) with no significant decreases between groups. Bar graph values represent Mean ± SEM for four independent experiments.

Oxidative Stress: Protein Carbonylation

Preliminary data indicated that RES decreased protein carbonylation at both concentrations relative to control (Figure 6). DOX alone appears to increase protein carbonylation relative to control and RES co-administered with DOX showed a decline in protein carbonylation relative to control (Figure 6). These results are based on one replicate and further experiments are required to determine statistical significance.

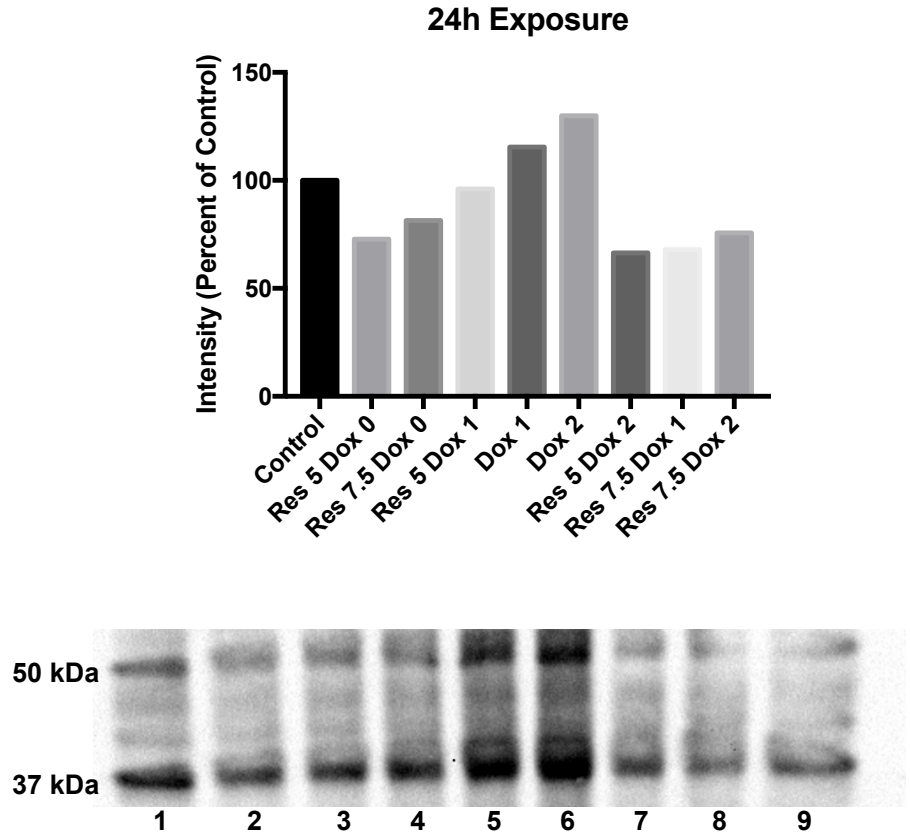

Figure 6: DOX treatment increased protein carbonylation in HK-2 cells. Western blot lanes correspond to the above graph (1-9). Bar graph values represent Mean ± SEM for three independent experiments run with one biological replicate.

Western Blot: Cytochrome C Leakage

DOX at 1 and 2μM alone did not cause significant leakage of cytochrome C (cyt C) from the mitochondria into the cytosol relative to control as confirmed by western blot (Figure 7). Additionally, RES alone or RES co-administered with DOX did not significantly decrease cyt C leakage from the mitochondria into the cytosol relative to control within the 24 h treatment period. Cyt C is located in the inner membrane of the mitochondria and initiate apoptosis. The lack of an observed difference in cyt C leakage may be attributed to the short experimental time course and the localization of the protein.

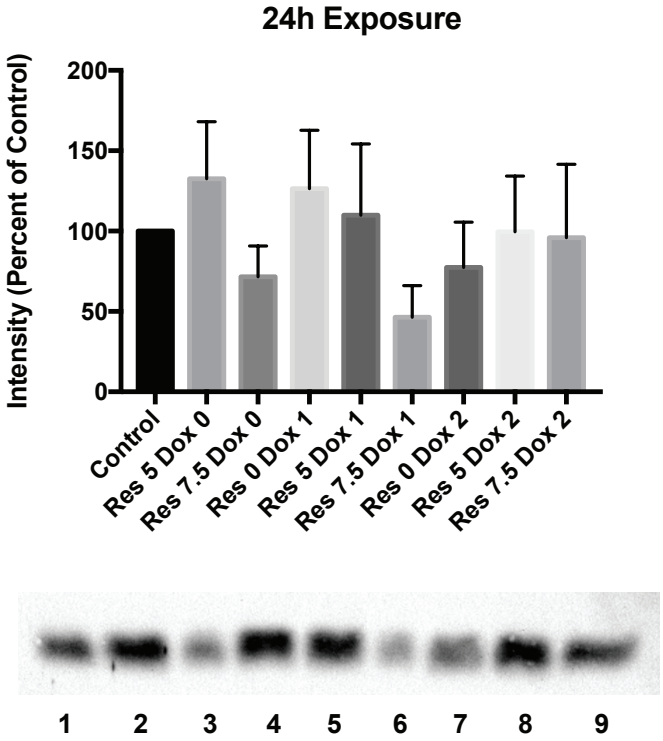

Figure 7. Cytochrome C leakage from HK-2 cells treated with various concentrations of DOX and RES. Cytochrome C leakage was not significantly increased or decreased relative to control or among treatment groups. Bar graph values represent Mean ± SEM for three independent experiments.

Western Blot: SIRT1 and PGC1α

SIRT1 expression was decreased significantly in 2 μM DOX ($p<0.0142$) treated cells relative to control (Figure 8A). Multiple comparisons revealed significant decreases between 5 μM RES alone and 1 μM and 2 μM DOX alone ($p<0.0033$ and $p<0.0045$) respectively (Figure 8A). Significant reductions in SIRT1 were also observed between 7.5 μM RES alone and 1 μM and 2 μM DOX ($p<0.0045$ and $p<0.0020$) respectively (Figure 8). Increases in SIRT 1 expression were observed between 1 μM DOX vs. 7.5 μM RES+1 μM DOX ($p<0.0020$) and 7.5 μM RES+2 μM DOX ($p<0.0403$) (Figure 8A). Increases were also observed between 5 μM RES+1 μM DOX vs. 2 μM DOX ($p<0.0304$) and 7.5 μM RES+1 μM DOX vs. 2 μM DOX ($p<0.0004$) (Figure 8A). These results indicate that SIRT1 is expressed to a higher degree in cells treated with 5 μM and 7.5 μM RES regardless of the addition of DOX (Figure 8A).

PGC1α expression was higher in RES treated groups relative to control and DOX alone treated cells although the increases were not significant (Figure 8B). The large variance within groups indicates that these tests should be repeated but do seem to support increases in SIRT1 mediate increases in PGC1α.

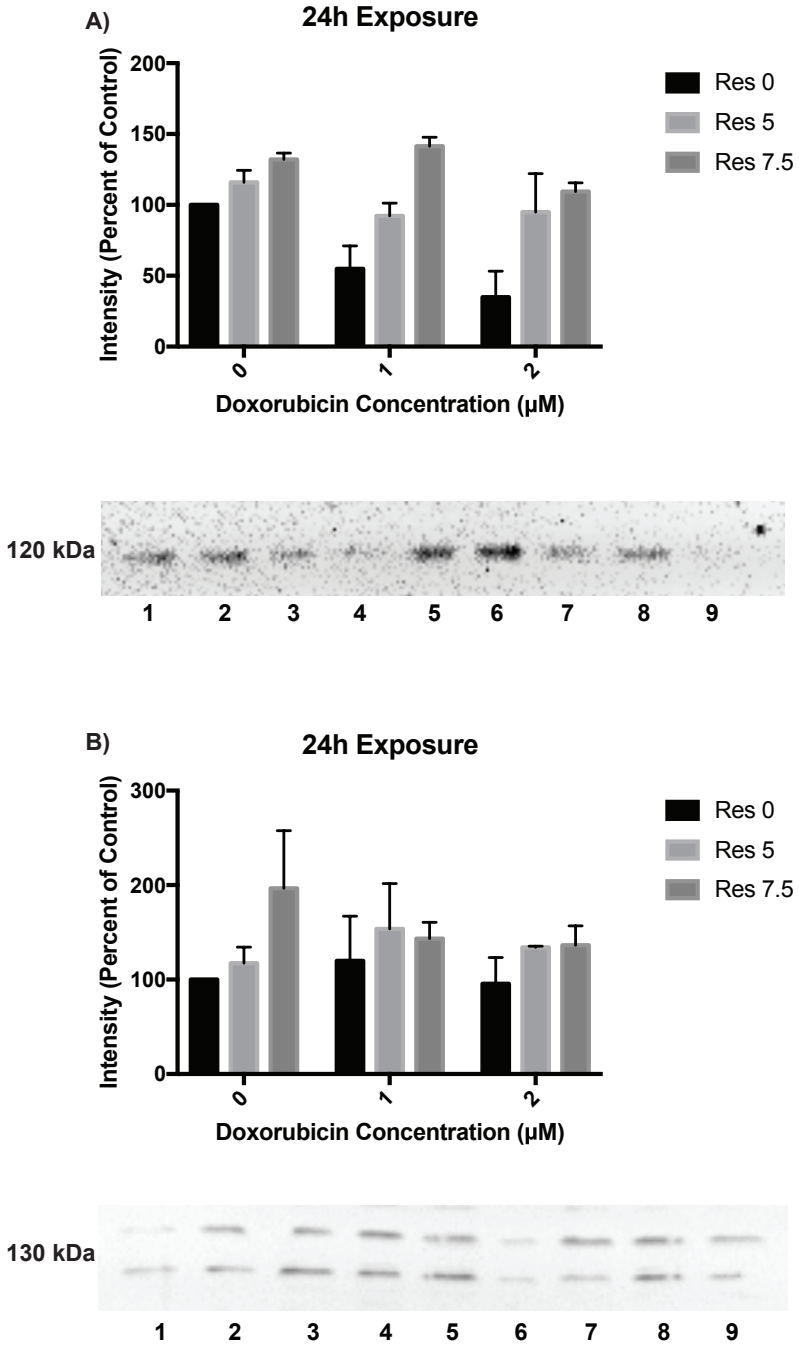

Figure 8. SIRT1 and PGC1α western blot of HK-2 cells treated with varying concentrations of DOX and RES. Western blot of SIRT1 (A) and PGC1α (B) and corresponding treatments: Control (1), RES 5 (2), RES 7.5 (3), DOX 1 (4), RES 5+DOX 1 (5), RES 7.5+ DOX 1 (6), DOX 2 (7), RES 5+DOX 2 (8), RES 7.5+DOX 2 (9) ($p<0.05$). Bar graph values represent Mean ± SEM for two independent experiments.

Seahorse Analysis: Measurement of Mitochondrial Function

A mitochondrial stress test was performed following a 1 h pretreatment with RES (7.5 μM) and 24 h incubation with DOX (2 and 4 μM). Following treatment, cells were serially injected with probes to identify parameters of mitochondrial function. The first injection of oligomycin inhibits the ATP synthase enzyme and results in a decrease in OCR. This decrease represents mitochondrial respiration linked to ATP production. FCCP uncouples mitochondrial respiration and increases the cell's oxygen consumption to its maximum level and enabled us to calculate the spare or reserve respiratory capacity. The final injection of rotenone/antimycin A inhibits complex I and III and was used to calculate non-mitochondrial respiration.

Cells exposed to DOX at a concentration of 4 μM showed a significant decrease in basal respiration ($p<0.0083$) and 7.5 μM RES+4 μM DOX ($p<0.0471$) at 24 h but not cells exposed to 2 μM DOX when relative to control (Figure 9A). Basal respiration was significantly decreased between DOX 2 μM vs. Dox 4 μM ($p<0.0047$), 7.5 μM RES +2 μM DOX vs. 4 μM DOX ($p<0.0246$) and 7.5 μM RES vs. 7.5 μM RES+4 μM DOX ($p<0.005$) (Figure 9A).

ATP-linked OCR was calculated and results analyzed through a two-way ANOVA and multiple comparisons (Figure 9C). DOX 4 μM was significantly decreased ($p<0.0205$) relative to control (Figure 9C). There were significant declines observed in 2 μM DOX vs. 4 μM DOX ($p<0.0041$) (Figure 9C). Multiple comparisons revealed maximal OCR was not significantly affected among any treatment groups relative control and the only significant decline observed was between RES 7.5 μM vs. DOX 4 μM ($p<0.0022$) (Figure 9B). Proton leak was not significantly affected in any treatment group relative to control (Figure 9E). Surprisingly, proton leak was decreased between 7.5 μM RES vs. 4 μM DOX ($p<0.0402$) and vs. 7.5 μM RES +4 μM

DOX ($p<0.0464$) (Figure 9E). Proton Leak was also diminished significantly between control and DOX 4 μM ($p<0.0337$).

Spare Capacity was not effected in any treatment groups compared to control, but was decreased in 7.5 μM RES vs. 4 μM DOX ($p<0.0022$) (Figure 9D).

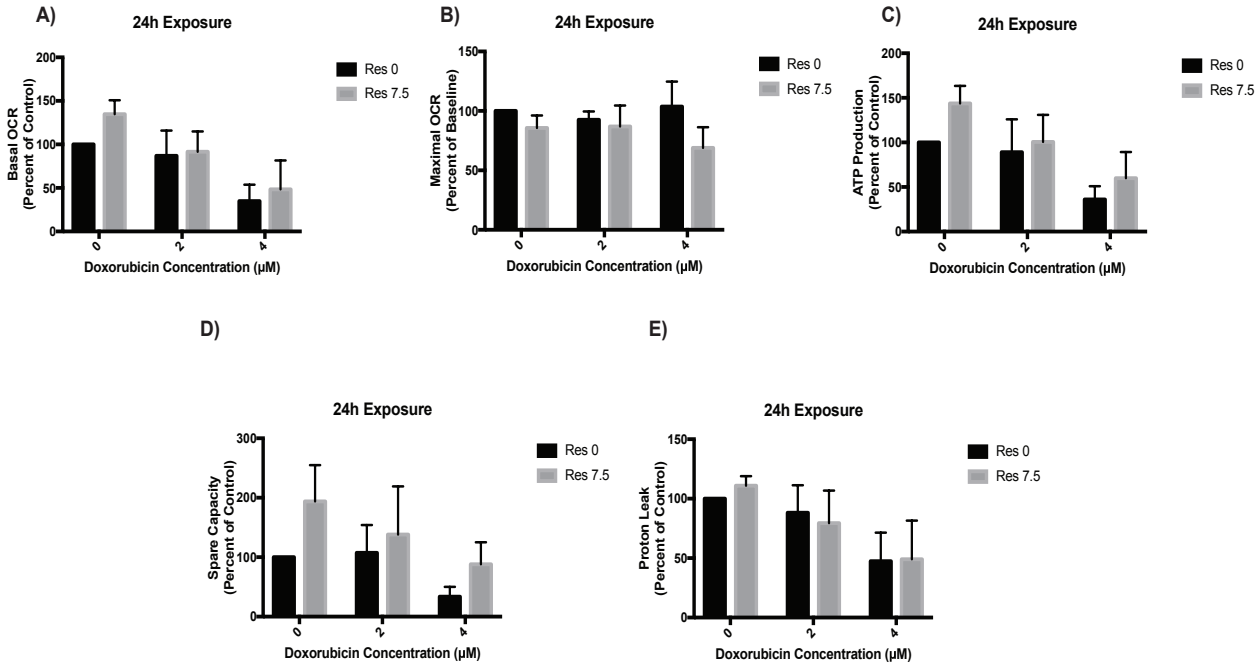

Figure 9: Mitochondrial Stress Test, effects of DOX and RES on ATP production and mitochondrial respiratory capacity. Basal (A) and Maximal (B) OCR following 24 h exposure to RES, DOX or RES+DOX. ATP Production (C) spare respiratory capacity (D) and proton leak (E) following 24 h exposure to RES, DOX or RES+DOX. Bar graph values represent Mean ± SEM for three independent experiments run with two biological replicates.

Glycolytic stress tests were performed after 24 h treatments with the various concentrations of RES and DOX. The cells are saturated with glucose and basal ECAR is assessed. Following basal ECAR assessment, oligomycin is injected to inhibit ATP synthase thereby driving glycolysis to its maximal capacity. The third and final injection of 2-DG, a glucose analog, inhibits the first enzyme in the glycolytic pathway known as hexokinase. The

following observed decrease in ECAR indicates that the ECAR produced in the experiment is due to glycolysis.

Basal ECAR was assessed but the only significant decline relative to control was in 7.5 μM RES+4 μM DOX group ($p<0.0131$) (Figure 10A). A significant basal ECAR drop was observed in 7.5 μM RES vs 2 μM DOX ($p<0.0020$) and 4 μM DOX ($p<0.0032$) treated groups (Figure 10A). There was also significant decrease among 7.5 μM RES treated cells vs. 7.5 μM RES+4 μM DOX ($p<0.0002$) and a decrease between 7.5 μM RES+2 μM DOX and 7.5 μM RES+4 μM DOX ($p<0.0157$) (Figure 10A).

Glycolytic capacity was reduced in cells treated with 4 μM DOX alone ($p<0.0106$) and 7.5 μM RES+4 μM DOX ($p<0.0098$) relative to control cells (Figure 10B). Additionally, cells treated with 7.5 μM RES alone had significantly higher glycolytic capacity when compared to 2 μM DOX ($p<0.0051$) or 4 μM DOX ($p>0.001$) alone (Figure 9B). A significant decline was observed in cells treated with 7.5 μM RES alone vs. either 7.5 μM RES+ 2 or 4 μM DOX ($p<0.0149$, $p<0.0001$) respectively (Figure 10B).

Glycolytic reserve indicated a significant increase in cells treated with 7.5 μM RES alone ($p<0.0203$) relative to control (Figure 10C). Significant decreases in glycolytic reserve were demonstrated between 7.5 μM RES alone vs. 2 μM DOX ($p<0.0019$), 4 μM DOX ($p<0.0001$) and 7.5 μM RES+4 μM DOX ($p<0.0001$) respectively (Figure 10C).

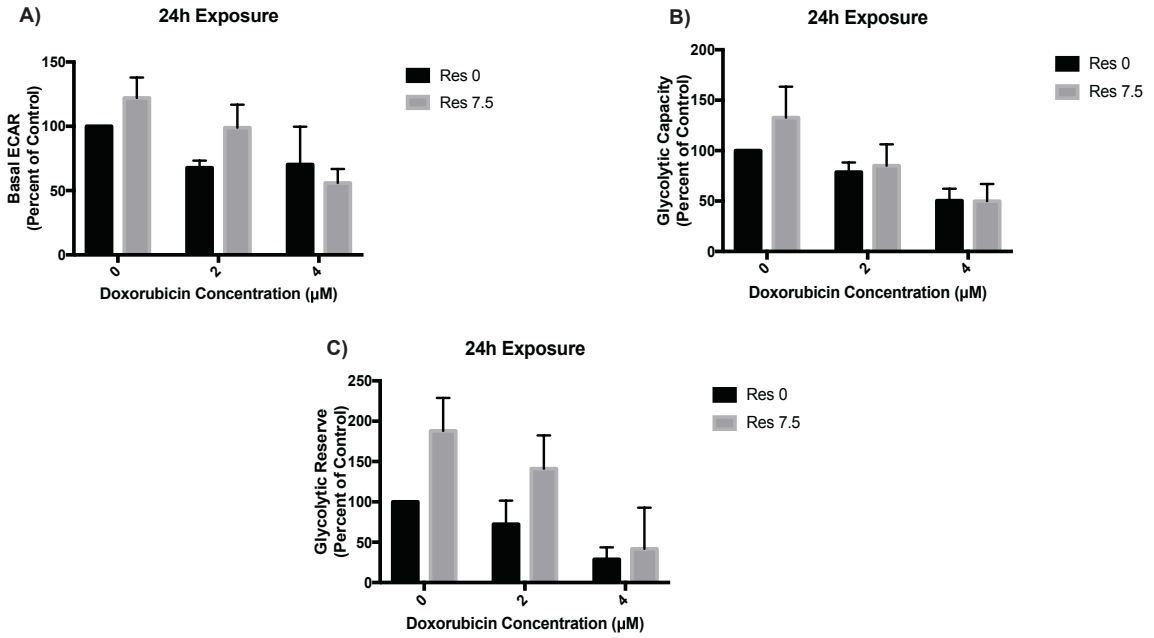

Figure 10. Glycolytic stress test, effects of RES, DOX or RES+DOX on glycolytic capacity and reserve. Basal (A) and Maximal (B) ECAR following and Reserve Capacity (C) following 24 h exposure to RES, DOX or RES+DOX. Bar Graph values are presented as a Mean ± SEM for two independent experiments run with two biological replicates.

CHAPTER 4

DISCUSSION

Doxorubicin: Cancer Chemotherapeutic

Doxorubicin has been in use as a cancer chemotherapeutic agent since the 1970s despite the known toxicities associated with administration. The high anti-tumor capability of DOX and its power to prevent DNA replication is largely why it is still part of the treatment regimen for breast, ovarian and small cell lung cancer and both acute and chronic lymphoid leukemias. There is a clear need for better interventions to treat the numerous toxicities associated with DOX, which could ultimately be responsible for its removal from a patient's therapy regimen. As stated previously DOX-associated nephrotoxicity has been studied to a much less extent than other adverse side effects linked to the anthracycline. DOX progressive and dose-dependent toxicity is a result of the drug's accumulation in tissue over time. The preferential accumulation of DOX in the kidney causes increased capillary permeability and glomerular atrophy (Injac et al., 2008). The high cytochrome P450 levels in the renal proximal tubule would make the kidney a direct target for the conversion of DOX to toxic DOX-OL and subsequent formation of ROS (B. S. Cummings, Zangar, Novak, & Lash, 1999). ROS formation leads to oxidative stress, lipid peroxidation and subsequent renal damage. Additionally, the high numbers of mitochondria in the proximal tubule lead to increased damage and mitochondrial dysfunction. The need to combat renal toxicity is imperative for the continued usage, and use of the natural product RES in mitigating kidney toxicity would have strong clinical applications.

RES's anti-oxidant and anti-inflammatory properties have been well documented in the literature. The additional anti-cancer and anti-tumorigenic properties associated with RES show its potential in mitigating cancer metastasis in addition to its function as an antioxidant. The

unique ability of RES to scavenge free radicals could be a potential mechanism against oxidative stress and lipid peroxidation. At present, it is difficult to determine if RES reduces oxidative damage by scavenging radicals or if it is capable of inhibiting the generation of free radicals. A more comprehensive mechanistic study should be performed in order to determine the exact mechanism by which RES exerts its positive effects. The ability of RES to potentially reduce oxidative stress and successive damage will be discussed in the following subsections.

Resveratrol Protection Against Doxorubicin Induced Oxidative Stress and Cytotoxicity

Oxidative stress and successive damage are responsible for the irreversible and progressive toxicity associated with DOX administration. Under normal conditions ROS are produced at low enough concentrations where the cell is able to detoxify the radicals through innate antioxidant mechanisms. Excessive production of free radicals, as seen with administration of DOX, triggers eventual damage that the cell is unable to overcome. However, it should be noted that even subtle changes due to oxidation of amino acid residues in a protein polypeptide chain may cause significant alteration in protein higher order structures and cellular functions (Aryal, Jeong, & Rao, 2014). These alterations indicate that oxidative stress can begin to cause damage virtually upon onset.

Toxicity mediation through free radical generation, Fe^{2+}-dependent oxidative damage of biological macromolecules and membrane lipid peroxidation are major players in progressive toxicity (Tian, Li, Wang, Xie, & Li, 2012). The inability to mitigate protein oxidation and stress will eventually lead to cell death. Protein carbonylation is a non-specific technique that allowed us to examine oxidative stress among various treatment groups (Figure 6). Stressed cells produce an aldehyde or ketone side chain on amino acids, which are then derivitized (see pg. 12) and analyzed. Proteins are often the first targets of cellular oxidative stress and are commonly used as

a marker of injury. Cellular dysfunction and disease progression occur as a result of protein carbonylation (Aryal et al., 2014).

A study by Toldo and colleagues in 2013 demonstrated, *in vivo* and *in vitro*, DOX treatment causes significant damage in cardiomyocytes, over production of ROS and total protein carbonylation (Toldo et al., 2013). Additionally, Aryal and colleagues demonstrated that major serum proteins, serum albumin and serotransferrin, were found to be highly carbonylated under DOX-induced acute cardiotoxicity. DOX effects on cardiomyocytes, as well as cardiac tissue, has been widely studied, but little is known about DOX-induced protein carbonylation in HK-2 cells (Aryal et al., 2014). Cardiac tissue is known to have lower levels of antioxidant enzymes and high levels of mitochondria making it the primary target of toxicity and the reason it has been heavily studied. Nonetheless, examination of DOX-induced damage in other organs, i.e. kidney. gave new insight into DOX-induced multi-organ damage. By examining oxidative damage to the kidney, we were able to better assess and examine a potential way to curb kidney damage. These findings may lead to new ways to prevent DOX-induced kidney damage and potentially organ damage as a whole.

DOX has been shown to cause renal damage, but the exact mechanism has remained elusive. In the past DOX-induced kidney damage has been examined by observing levels of enzymes capable of detoxifying free radicals. DOX has been shown to induce a glutathione (GSH) and oxidized-glutathione (GSSG) imbalance as well as lipid peroxidation in kidney tissue of rats (Injac et al., 2008). The GSH/GSSG ratio is a critical regulator of cellular redox states and declines in this ratio are closely associated with oxidative stress and disease (Zhou et al., 2014). Additionally, administration of DOX causes a decrease in GSH, SOD and glutathione-S-transferase (GST) (Liu et al., 2007). GSH and SOD are major players in free radical

detoxification and decreased levels may indicate the cell's inability to detoxify ROS at the rate in which they are being formed. GSTs are enzymes which are strictly specific for epithelial cells of the proximal and distal tubules in the kidney and are detected in urine when tubular damage has occurred causing decreased tissue levels (Polak et al., 1999). The imbalance between GSH/GSSG coupled with decreases in GSH, SOD and GST is indicative of damage and will lead to toxicity. A potential way to alleviate damage is through the use of RES which has previously been shown to reduce oxidative stress by increasing expression of SOD and glutathione peroxidase (GPx1) in human endothelial cells (Spanier et al., 2009). Assessing DOX injury and potential protection by RES via protein carbonylation analysis was our first step in examining and conceivably combatting DOX-induced oxidative injury. Oxidative damage was not assessed at 8 h based on RES's apparent inability to combat cell damage in the short amount of time (Figure 5A). Preliminary data showed that DOX alone increased oxidative stress relative to control and that RES reduced stress when compared to control cells. Cells pretreated with RES and various concentrations of DOX had decreased protein carbonylation within a 24 h time period (Figure 5B). It is of interest that like the heart, the proximal tubule of the kidney is high in mitochondria. RES may have the ability to exert protective effects on cardiac tissue as well as renal tissue and should be studied further. It is generally accepted that ROS and subsequent oxidative stress are key players in the initiation of DOX-induced toxicities. This is the first study to assess protein carbonylation in DOX and RES treated HK-2 cells and demonstrate RES' capability in reducing oxidative stress caused by DOX which lead us to question if RES could potentially protect against DOX-induced cytotoxicity by ameliorating decreases in cell viability.

Prolonged oxidative stress and depletion of cellular antioxidant enzymes leads to irreparable damage and subsequent activation of pro-apoptotic pathways. Apoptosis is one of the

major mechanisms of cell death in response to cancer therapies (Danial & Korsmeyer, 2004). The major apoptotic pathway of anticancer drugs is through the loss of mitochondrial membrane integrity (Kaufmann & Earnshaw, 2000). The loss of mitochondrial membrane integrity causes the release of cytochrome c from the mitochondria, which in turn activates effector caspases. Loss of mitochondrial membrane integrity and the subsequent activation of apoptotic pathways are essential to the drug's efficacy. However, the need for interventions to prevent DOX from damaging non-cancerous cells is imperative to its continued use. There have been notably fewer reports on the effects of DOX-toxicity on non-cancerous cell lines and we endeavored to correct that with this study.

Previous findings have suggested that DOX induces apoptosis in normal cells vs. tumor cells by distinctively different mechanisms (Wang et al., 2015). This analysis demonstrated DOX-induced apoptosis in endothelial cells and cardiomyocytes was a result of a H_2O_2-mediated mechanism that was largely independent of p53 activation. However, this was not the case for tumor cell lines, as p53 tumor suppressor played a crucial role in inducing apoptosis in DOX-treated cells (Wang et al., 2015). The determination that tumor cell lines and normal cell lines activate apoptosis differently gives us a potential conduit to treat toxicity in non-cancerous cells without altering the effectiveness of DOX on cancer cells.

DOX has previously been shown to produce dose-dependent and progressive toxicity. Assessing HK-2 cell's response to varying concentrations of DOX was the first phase in quantifying dose-dependent progression of toxicity. HK-2 cell's mitochondrial activity is constant and therefore an increase or decrease in the number of viable cells is linearly related to mitochondrial activity (van Meerloo et al., 2011). The concentration of formazan was measured to determine changes in cell viability in the MTT assay. HK-2 cells exposed to DOX for 24 h

showed a significant decrease only at the two highest concentrations (Figure 4A). At 48 h, cell viability was decreased even at the lowest concentration of 0.5 μM (Figure 4B). These results confirmed that over time DOX, even at low concentrations, will lead to dose-dependent progressive toxicity confirming previous studies. We attempted to mitigate the decrease in cell viability through the use of RES. As discussed previously RES has the ability to upregulate endogenous antioxidants. The upregulation of SOD, GST, catalase and GPx1 provide a defense against oxidative stress (Y. Li, Cao, & Zhu, 2006). It is not known whether RES is directly cytoprotective but the upregulation of cellular antioxidants indirectly protects against cell damage and death.

We were able to determine RES's ability to reduce or protect against oxidative stress relative to cell death. Cells pretreated with 5 μM RES and 1, 2 and 4 μM DOX were not able to prevent a significant decline in cell viability within the 8 h time course (Figure 5A), indicating that a concentration of 5 μM RES is not significant enough to protect against DOX induced cytotoxicity. However, 1 h pretreatment with RES at the higher concentration of 7.5 μM was able to prevent significant reductions in cell death in DOX treatments of 1 and 4 μM but interestingly not at 2 μM. The results show that the 8 h exposure is not enough time for lower concentrations of RES to mitigate cytotoxicity and is observed by the inability of the cell to reduce oxidative stress and subsequent damage. Even at higher concentrations, a longer exposure time would be more ideal to determine protective effects of RES on DOX treated cells. Cells exposed to RES pretreatments (5 and 7.5 μM) for 24 hours and accompanying concentrations of DOX (1, 2 and 4 μM) showed no significant decline in cell viability (Figure 5B). No significant decline in cell viability shows RES is capable of protecting HK-2 cells against DOX-induced cytotoxicity. Cells treated with RES alone had higher cell viability than control cells which

confirms previous studies that RES itself is cytoprotective. We can conclude that pretreatment with the chosen RES concentrations shows potential to decrease cytotoxicity that accompanies DOX administration within 24 h. RES should be assessed after longer exposure (48 h and 72h) to determine if it is better able to protect against DOX-induced oxidative stress and cytotoxicity and to confirm that it does not lose its ability to protect against cell death. There is evidence that RES's ability to induce pro-oxidant effects and resist cytotoxicity is time-dependent (Martins et al., 2014) and should be further examined in longer experimental time courses. These initial results verify RES's ability to decrease oxidative damage and subsequent cell death within a 24 h time period and further experiments should be performed.

Resveratrol Protection Against Doxorubicin Induced Cytochrome C Leakage

The key role that mitochondria play in the regulation of apoptosis have been established in various studies. The release of different pro-apoptotic proteins that are normally present in the intermembrane space of these organelles has been observed during the early stages of apoptotic cell death (Cai, Yang, & Jones, 1998). Among the proteins released from the mitochondrial inner membrane is cyt C. Cyt C is an essential component of the electron transport chain (ETC) and a key regulator of apoptosis in the mitochondria. The cyt C protein functions as a single electron carrier in the final step of the ETC (Huttemann et al., 2011). During programmed cell death cyt C is released from the mitochondria into the cytosol where it binds to Apaf1 to activate a series of caspase cascades (Cai et al., 1998). As previously discussed, mitochondrial outer membrane permeabilization is a key initial step in apoptosis.

Mitochondrial membrane permeabilization occurs through several mechanisms and is also referred to as the mitochondrial permeability transition (MPT). The first pathway involves the induction of MPT through the opening of non-specific pores in the inner membrane leading

to osmotic swelling of the mitochondrial matrix, mitochondrial uncoupling, rupture of the outer membrane and release of proteins like cyt C (Robertson, Orrenius, & Zhivotovsky, 2000; Yang & Cortopassi, 1998). A second mechanism capable of inducing mitochondrial membrane permeabilization is through the Bcl-2 family of proteins. Bcl-2 proteins, specifically Bax, are capable of inducing the release of cyt C, independent of MPT, through the induction of voltage-dependent ion channels (VDAC). VDAC is associated with most of the metabolite flux across the mitochondrial outer membrane (Colombini, 1983). The third pathway is also MPT-independent and initiates cyt C release in a volume-dependent manner through mitochondrial swelling which leads to permeabilization of the mitochondrial outermembrane (Gogvadze, Orrenius, & Zhivotovsky, 2006). Last, there is recent evidence to suggest that caspase-2 is also capable of mediating the release of cyt C and subsequent activation of downstream caspases (Gogvadze et al., 2006). It is clear that mitochondrial membrane permeabilization and subsequent cyt C release is a key step in the initiation of apoptosis. Understanding and modulation of these pathways experimentally may result in new techniques to mitigate apoptosis and should be studied more extensively.

Previous *in vivo* findings have shown DOX treatment causes cyt C release and apoptosis at much higher levels than untreated animals (Childs, Phaneuf, Dirks, Phillips, & Leeuwenburgh, 2002). Green and colleagues demonstrated that DOX increased cyt C efflux indicating mitochondrial dysfunction which lead to a change in mitochondrial membrane permeability that was not observed in the mitochondria of control animals (P. S. Green & Leeuwenburgh, 2002). Additionally, both studies showed a marked increase in caspase-3 activity indicating apoptosis and the likelihood that caspase-3 is the primary effector caspase involved. *In vitro* studies of cardiac myocytes treated for 24 h with DOX showed increased leakage of cyt C into the

cytosolic compartment relative to untreated cells (Kotamraju, Konorev, Joseph, & Kalyanaraman, 2000). Once again various studies have been performed using animal cardiac tissue and cardiac cell lines but little information is available on DOX-induced cyt C leakage regarding other organs and cell lines.

The therapeutic effects of RES as a potential anti-cancer agent as well as a cardioprotective agent have been studied extensively. In a study completed this year, RES was given prophylactically and therapeutically with DOX and results compared in rats (Shoukry et al., 2017). Prophylactic administration was defined as pre-treatment with RES before DOX and therapeutic defined as RES treatment post DOX administration. The study demonstrated that DOX induced cardiac fibrosis but when RES was administered therapeutically cardiac fibrosis was decreased. Additionally, prophylactic supplementation of RES was more effective in reducing fibrosis than when RES was administered therapeutically (Shoukry et al., 2017). Apoptosis was examined by looking at Bax, Bcl-xl and caspase 3 expression. DOX led to an increase in myocardial apoptosis and a significant increase in caspase 3. Therapeutic treatment with RES reduced myocardial apoptosis but not as significantly as when RES was administered prophylactically (Shoukry et al., 2017). RES, like DOX, has been studied widely in respect to its cardioprotective abilities but less so in other cells and tissues.

For the first time HK-2 cells treated with DOX, RES and DOX+RES were examined to determine if DOX increases cyt C leakage from the mitochondrial inner membrane. The results indicated that HK-2 cells treated with DOX alone had no increase or decrease in cytosolic levels of cyt C and cells treated with RES alone or co-administered with DOX showed no significant increase or decrease relative to control (Figure 7). Cyt C is normally bound to the inner mitochondrial membrane by an association with anionic phospholipid cardiolipin, where it can

reversibly interact with complexes III and IV of the respiratory chain and dissociation from of cyt C from cardiolipin may be the critical first step in caspase activation (Petrosillo, Ruggiero, Pistolese, & Paradies, 2001). Previous studies done by our lab showed no discernible changes in cardiolipin levels within 24 h supporting our results that cyt C has not yet dissociated and translocated within this time period.

Within the 24 h time course it is possible that this terminal step of the ETC is not affected or that DOX at concentrations of 1 and 2 μM are slower to effect apoptosis initiation and translocation of the protein from the inner mitochondrial membrane to the cytosol. Additionally, the kidneys have higher levels of detoxifying enzymes than the heart. It is possible that within 24 h cells are able to detoxify radicals and protect against the initiation of apoptosis. Eventually these enzyme levels will be depleted and apoptosis initiated and a longer period of study is indicated to ascertain RES potential protection against apoptosis. These findings are confirmed by our cell viability assay that DOX administered at concentrations of 1 and 2μM are not yet toxic to the cell within 24 h time period (Figure 5).

Resveratrol: A Mechanistic Approach

Sirtuin 1 (Sirt1) functions as a protein deacetylase to remove acetyl groups on proteins in a NAD-dependent manner. Sirt1 is known to modulate a number of transcription regulators, including PGC1α, a nuclear hormone receptor coactivator that promotes mitochondrial biogenesis (Koo & Montminy, 2006). PGC1α is required for mitochondrial biogenesis but previous assays of PGC1α activation of upstream effectors have not consistently predicted the subsequent biogenic process (Beeson et al., 2010). Previous studies have demonstrated that SIRT1 modulates the activity of a number of transcriptional regulators in mammals, most notably, PGC1α (Koo & Montminy, 2006). Studies have also shown that resveratrol improves

energy balance and increases mitochondrial function in mice by stimulating SIRT1-mediated deacetylation of PGC1α (Baur et al., 2006; Lagouge et al., 2006).

We endeavored to examine the capability of RES to potentially induce PGC1α through SIRT1 increases and in turn promote mitochondrial biogenesis. Our results demonstrated that RES treated groups had a higher expression of SIRT1 than cells treated with either concentration of DOX (Figure 8A). RES-mediated SIRT1 increases are supported by previous studies (Baur et al., 2006; Koo & Montminy, 2006; Lagouge et al., 2006) and indicate that RES is capable of improving mitochondrial function through SIRT1 activation. However, based on our results we are unable to definitively determine that it is through SIRT1-mediated modulation of PGC1α. The increases in PGC1α (Figure 8B) observed indicate that this is a distinct possibility but further experiments are required to positively determine the extent to which PGC1α is involved. It is clear that SIRT1 induction is a probable mechanism for RES to protect against DOX-induced toxicity.

Resveratrol Protection Against Doxorubicin Induced Mitochondrial Damage

Mitochondria play a major role in Ca^{2+} homeostasis, ROS generation, redox balance, cellular proliferation and apoptosis (Whitaker et al., 2015). The kidneys are second only to the heart in mitochondrial abundance and oxygen consumption at rest (Pagliarini et al., 2008). The proximal tubules of the kidney use the majority of oxygen consumption for ATP generation and contain most of the mitochondria that power the active reabsorption of glucose, ions and other metabolites as well as synthesize protein (Forbes, 2016). Damage to the mitochondria of proximal tubule cells, i.e. HK-2 cells, have the potential to cause significant damage to the kidney. In the past mitochondrial damage has been difficult to quantify but in this novel study we

were able to compute mitochondrial changes during both oxidative phosphorylation and glycolysis.

Mitochondrial dysfunction is an early indicator of DOX-induced apoptosis and subsequent oxidant production (P. S. Green & Leeuwenburgh, 2002). The ability to restore mitochondrial function and/or to prevent initial damage would have strong clinical implications. RES has been shown to produce mitochondrial biogenesis. However, the ability to do so may be limited by the remaining mitochondrial function and ATP levels but through stimulation of mitochondrial biogenesis RES may promote organ recovery and return of cellular functions (Beeson et al., 2010). Ideally a pre-treatment with RES would protect against initial injury while simultaneously stimulating mitochondrial biogenesis.

The Seahorse XFp Analyzer is a unique tool allowing the simultaneous monitoring of the two energy pathways of the cell. The majority of cells possess the capability to shift between the two pathways allowing cells to adapt to environmental changes. Previously there were only high-throughput assays assessing changes in mitochondrial gene expression but these assays lacked the capability to evaluate mitochondrial biogenesis. The mitochondrial and glycolytic stress tests are the first high-throughput assays to examine the changes in mitochondrial respiration and an improved approach for examining mitochondrial toxicity (Beeson et al., 2010). This study is the first to look at the direct effects of DOX on mitochondria as well as the ability of RES to potentially mitigate mitochondrial toxicity and promote mitochondrial biogenesis.

Primary cultured HK-2 cells are a valuable model for evaluating mitochondrial toxicity because they acquire their energy from oxidative phosphorylation and maintain differentiated functions and membrane polarization (Nowak & Schnellmann, 1996). The kidneys primarily undergo aerobic metabolism and mitochondrial damage leads to decreased aerobic metabolism

and ATP, disruption of cellular functions, cell injury and death (Beeson et al., 2010). HK-2 cells exhibit *in vivo* levels of aerobic metabolism, are not glycolytic and retain higher levels of differentiated functions making them an appropriate experimental model for study (Beeson et al., 2010). Using seahorse technology and primary culture HK-2 cells we were able to examine the cellular targets of toxicity specific to a primary renal cell line and the potential protective effects of RES against DOX induced kidney damage.

The cell mitochondrial stress test was performed and for the first time we were able to quantify basal OCR, ATP-linked OCR with proton leak, maximal respiration, spare capacity and non-mitochondrial OCR in control and treated HK-2 cells. The basal measurements established a baseline prior to serial injection with various inhibitors and electron transport chain uncouplers. The baseline readings establish the threshold below which the cells would be unable to sustain oxidative phosphorylation to meet energy demand. Living cells do not store ATP, they produce it continually and on demand. Therefore, cells constantly consume oxygen and fuel substrates and the demand for ATP in cells controls the rate of OCAR (Pike Winer & Wu, 2014).

Cells treated with 7.5 μM RES tended to increase basal respiration relative to control (Figure 9A). Although it was not a statistically significant increase it does show RES may potentially increase the threshold and therefore the cell's ability to meet an increased energy demand. On the other hand, baseline readings showed that cells treated with 4 μM DOX alone had a significantly lower threshold than control cells and there was a significant decline in basal respiration between cells treated with 2 μM vs. 4 μM DOX (Figure 9A). Additionally, 7.5 μM RES+4 μM DOX treated cells had reduced basal respiration relative to 7.5 μM RES alone as well as control cells. It would appear that RES is capable of protecting against a significant decline at 2 μM DOX but the threshold is too low for it to exert protective effects at 4 μM and a higher

concentration of RES may be indicated to adequately protect against a decline in basal respiration.

Metabolism strips electrons from fatty acids, sugars and amino acids, and they accumulate on the soluble electron carrier NADH and on protein-bound $FADH_2$ (K. Green, Brand, & Murphy, 2004). The electrons are then passed down the mitochondrial respiratory chain to drive ATP synthesis through oxidative phosphorylation (K. Green et al., 2004). The first injection of oligomycin halts the electron passage down the chain by acting as a sort of roadblock against transport. The corresponding change in respiration quantifies the amount of basal respiration actually being used for ATP production and is expressed as ATP-linked OCR. Previous studies reveal that "healthy" individuals have a high ATP linked OCR or an increased ATP demand. A decrease would indicate low ATP demand, a lack of substrate and/or severe damage to oxidative phosphorylation, impeding the flow of electrons resulting in a lower ATP-coupled OCR (Chacko et al., 2014). Chacko's study supported our results that RES treatment increases ATP-linked OCR and ATP demand. RES when coupled with DOX treatment reduced declines in ATP-coupled OCR that were observed in cells only exposed to DOX (Figure 9C). The protective effects of RES against this drop indicate protection against damage to oxidative phosphorylation.

The remaining basal respiration represents the "proton leak" which has two possible implications for the mitochondria and both will be discussed. Functional mitochondria move electrons down the gradient from NADH/$FADH_2$ to O_2 while conserving redox energy by simultaneously pumping protons across the intermembrane to build up the proton electrochemical potential that is used by ATP synthase to make ATP (K. Green et al., 2004). The above is an example of the mitochondria using protons as a regulatory mechanism for ATP

production. However, in some instances of mitochondrial damage, protons leak back into the matrix without ever forming ATP thereby reducing energy production. Higher levels of proton leak indicate fewer protons available to drive ATP synthase to make ATP and may correspond to a decrease in energy production. ATP production tended to increase in RES treated cells alone relative to control, although not significantly (Figure 9C). Cells treated at the higher concentration of DOX did show a significant decline in ATP production relative to control but when pre-treated with RES this decline was alleviated. Further declines were only observed in comparison to RES treated cells alone and confirmed that pre-treatment with RES protects against a decline in ATP production.

The most interesting results were observed when quantifying proton leak. There was no change in proton leak in any treatment groups relative to control but RES treated cells alone had increased proton leak when compared to the higher concentration (4 μM) of DOX treated cells alone and those pre-treated with RES (Figure 9E). Previous studies have shown that increased calcium transport can manifest changes in proton leak (Chacko et al., 2014). It is possible that administration of RES may increase calcium transport and account for the increase in proton leak shown rather than as an indicator of damage. The lack of decline in ATP production in RES treated groups would support that there is another mechanism at work and should be examined in future studies. Additionally, diminished proton leak may be a potential compensatory mechanism in mitigating the decline in ATP production in response to DOX-induced stress. Maximal activity was examined further by uncoupling ATP production from the electron transport chain to examine OCR capacity.

The electron roadblock was offset by injection of FCCP allowing us to examine the effects of uninhibited electron flow through loss of the proton gradient. The uncoupling of the

electron transport chain from ATP production enabled us to quantify its maximal activity. As previously mentioned, metabolism strips electrons from fatty acids, sugars and amino acids. The stimulation by FCCP and resultant drive to maximal capacity causes the rapid oxidation of those metabolic substrates to meet the increased energy demands. Interestingly, there was no effect on maximal OCR capacity in any treatment group when compared to control indicating that DOX and RES, whether co-administered or alone, do not appear to significantly affect the maximal activity of the electron transport chain relative to control (Figure 9B). No change in maximal activity would imply that maximal OCR was not yet effected by administration of DOX and/or RES, and that cells were able to meet the metabolic challenge within the exposure period.

Spare capacity was calculated to determine how closely the cell is respiring to its theoretical maximum. Spare capacity was calculated by subtracting maximal respiration from basal respiration and the difference quantified the cell's ability to respond to an increase in energy demand. When damage to the mitochondria occurs, spare capacity is called upon to meet increased energy demand and prevent damage. Studies have shown that depletion of this reserve leads to excessive damage and cell death (Hill, Dranka, Zou, Chatham, & Darley-Usmar, 2009). Oxidative stress and formation of ROS can increase OCR and deplete reserve capacity and inhibit respiration (Hill et al., 2009).

Intriguingly, spare capacity was not significantly increased or decreased in any of the treatment groups relative to control (Figure 9D). RES alone did not increase significantly when compared to control but it should be noted that there was a tendency to increase. The only significant decrease observed was between RES alone and the higher concentration of DOX and we would expect to see diminished spare capacity in these cells. These results indicate RES's potential to mitigate a decline in spare capacity by improving the cells ability to respond to an

increased energy demand. RES appears to be protecting against a reduction in spare capacity allowing the cells to function closely to their theoretical maximum. Non-mitochondrial respiration was assessed by an injection of rotenone/antimycin A that shut down electron transport complexes I and III respectively. Rotenone coupled with antimycin A effectively turned off mitochondrial respiration and allowed us to delineate between mitochondrial respiration and non-mitochondrial respiration.

The second energy pathway of the cell was assessed using the Glycolytic stress test assay and was performed to measure metabolic activity through ECAR. Glycolysis is the conversion of glucose to pyruvate and then pyruvate to lactate in the cytoplasm or CO_2 and H_2O in the mitochondria. Cells utilize glucose to produce pyruvate which in turn produces ATP, H_2O, NADH and protons. The relationship between ECAR and glycolytic rate can be confounded by other acidification mechanisms, i.e. CO_2 (Mookerjee, Goncalves, Gerencser, Nicholls, & Brand, 2015). It should be noted that in this study the baseline OCR/ECAR ratio was <4 signifying that CO_2 made a negligible contribution to ECAR and the observed results were from glycolysis alone. A major component or ECAR is the glycolytic production of lactate (M. Wu et al., 2007). Lactate increases acidification and lowers pH; therefore the media is devoid of glucose for the first measurements to determine accurate acidification unrelated to glycolysis (Mookerjee et al., 2015). The saturating injection of glucose leads to the conversion of glucose to pyruvate and pyruvate to lactate triggering proton extrusion into the medium and acidification of the medium surrounding the cell. The extrusion of protons from glucose catabolism permitted ECAR to be measured a second time and basal level glycolysis calculated.

Basal glycolysis was increased in RES alone treated cells, and cells treated with DOX alone had a severe decline in basal ECAR relative to RES treated cells at both concentrations

(Figure 10A). However, when the lower concentration of DOX was pre-treated with RES this decline was ameliorated but it was not able to prevent a significant decline when coupled to the higher concentration of DOX. The inability to prevent a decline in basal glycolysis was observed in OCR as well. A higher concentration of RES may be indicated when administered with the higher concentration of DOX. RES appears to be protective against a decrease in ECAR when co-administered with 2 μM DOX cells showing that RES is capable of protecting against diminished basal glycolysis at this concentration (Figure 10A).

Oligomycin was injected to "max out" glycolysis by shutting down oxidative phosphorylation and forcing cells to use the glycolytic pathway to capacity. When cells experience loss of mitochondrial ATP production due to inhibition of oxidative phosphorylation or by oligomycin, they augment their glycolytic flux to make more ATP from glycolytic pathways in order to maintain cellular ATP homeostasis (M. Wu et al., 2007). The increased glycolytic flux in response to deficiency in mitochondrial ATP production is known as the glycolytic capacity (Pike Winer & Wu, 2014). The glycolytic capacity of cells subjected to 4 μM DOX was significantly decreased compared to control as were cells treated with RES plus the higher concentration of DOX (Figure 10B). Treatment with RES alone increased capacity and, although RES+ 2 μM DOX was decreased compared to RES alone, it was not decreased when compared to control demonstrating conceivable protection against a loss of ATP production. Again, we observed the inability of RES to protect against loss of ATP production.

The glycolytic reserve was calculated to indicate how well the cell was able to respond to its theoretical maximum. Cells treated with RES alone functioned well above their theoretical max compared to control (Figure 10C). Although RES+2 μM DOX and RES+4 μM DOX treatment groups were decreased in comparison to RES alone, neither was decreased relative to

control. Furthermore, the RES+2 μM DOX treatment group was increased relative to control. RES appears to be better able to mitigate damage at the lower DOX concentration but still exhibits a partial protection between 4 μM DOX and RES+4 μM DOX.

In order to confirm the experimental ECAR was produced by glycolysis, 2-deoxy-glucose (2-DG) was injected. 2-DG is a glucose analog that inhibits glycolysis via inhibition of glucose by competitively binding to glucose hexokinase, the first enzyme in the glycolytic pathway inhibitor. We observed a dramatic drop in ECAR that confirmed the experimental ECAR was produced by glycolysis.

When comparing OCR and ECAR, it appears that RES exerts a more protective effect on the glycolytic pathway than on oxidative phosphorylation. During oxidative phosphorylation, it appears that basal respiration and ATP production are so severely decreased between DOX 2 and 4 μM that RES is not able to overcome and protect against toxicity. These decreases may be a direct result of the cell using proton leak pathways to regulate ATP production allowing those treatment groups to still function relatively close to their theoretical max. Basal ECAR was decreased in control vs. 2 and 4 μM DOX as well as between RES+2 μM DOX and RES+4 μM DOX. Just as in OCR, it appears that the damage occurs between 2 and 4 μM DOX and RES is not able to protect against damage. RES was better able to protect against significant decreases in glycolytic capacity and reserve overcoming the initial decrease of basal ECAR. The results suggest that RES has less of a protective effect on mitochondrial rate (OCR) than glycolytic rate (ECAR).

The utilization of Seahorse technology has potential widespread clinical implications. The ability of RES to stimulate mitochondrial biogenesis even in the presence of cytotoxic DOX is a potential therapeutic option to mitigate DOX induced nephrotoxicity through recovery and

promotion of cellular function. The Seahorse XFp Analyzer provides us with the first tool to quantify bioenergetics in different cellular models. This high-throughput assay quantified mitochondrial function and permitted a way to predict a response to a given treatment. We obtained a comprehensive bioenergetics profile for HK-2 cells and their response to treatments with RES, DOX and RES + DOX respectively. By showing RES's positive effect on mitochondrial energetics we have introduced a clinically relevant way to mitigate potential DOX-induced mitochondrial toxicity in the epithelium of mitochondria-rich renal proximal tubular cells.

CHAPTER 5

CONCLUSIONS AND FUTURE DIRECTIONS

This thesis has shown the ability of RES to protect DOX-induced nephrotoxicity and may potentially have many clinical implications. RES has shown promise in its ability to improve mitochondrial function and mitigate DOX toxicity by decreasing oxidative stress, improving cell viability and protecting against mitochondrial damage in both oxidative phosphorylation and glycolytic pathways. This thesis has also proposed a potential mechanism that RES's ability to induce SIRT1 is responsible for its protective abilities against DOX-induced damage.

RES shows potential in reducing oxidative stress and cell death but the exact mechanisms are still unknown. Given that proteins are often the immediate targets of cellular oxidative stress, it is of utmost importance to determine how adductions of reactive nucleophiles irreversibly alter protein structure and function. I would propose to examine 4-hydroxynonenal (4-HNE) to examine the extent of lipid peroxidation in DOX treated cells and the capability of RES to protect against it. Additionally, I would like to determine if DOX induces the cleavage of caspases 3 and 9 to determine the exact initiation of the pro-apoptotic pathway.

Western blotting indicated that there was no change in cyt-C leakage among any groups relative to control. I would propose examination of cyt C leakage after 48 and 72 h to determine if the 24 h time course was not enough time for the protein to translocate from the inner mitochondrial membrane to the cytosol. The examination of ATP synthase in mitochondrial and cytosolic fractions would further determine the extent to which mitochondrial membrane integrity is compromised and should be assessed within the same time courses as cyt C.

Our initial study demonstrated that SIRT1 levels are higher in RES treated cells but we were unable to determine definitively if SIRT1 modulates increases in PGC1α. Additionally, the

variability within groups is a concern and should be addressed through further experimentation. However, the increases in PGC1α indicate that this is still a viable potential mechanism and longer experimental time courses may reveal statistically significant increases that would confirm our proposed mechanism.

Examination of both energetic pathways gave us unique insight into DOX effects on oxidative phosphorylation as well as the glycolytic pathway. The observed protection of RES at the lower concentration of DOX supported our hypothesis, but indicated higher concentrations of RES may be necessary when DOX is administered at higher concentrations. Additionally, performing the mitochondrial stress test and the glycolytic stress test at 48 h and 72 h time courses would give us more definitive data on RES ability to protect against DOX-induced toxicity.

Limitations

RES and its metabolites pose both physiological and pharmacokinetic problems. The low bioavailability of RES as well as the various routes of administration has made it difficult to uniformly quantify proper dose and subsequent response. Furthermore, RES is not regulated by the FDA because it is a natural compound. Controlled regulation and formulation of the compound is imperative for clinical application.

Time, as in most cases, has been my greatest limiting factor. The data have supported our hypothesis that RES is capable of preventing DOX-induced damage, but further experiments are required to further elucidate the mechanisms of protection.

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APPENDIX A: OFFICE OF RESEARCH INTEGRITY APPROVAL LETTER

MARSHALL UNIVERSITY
www.marshall.edu

Office of Research Integrity

October 30, 2017

Morghan Schuyler Getty
300 Wilson Court
Huntington, WV 25701

Dear Ms. Getty:

This letter is in response to the submitted thesis abstract entitled *"Doxorubicin cytotoxicity in a human proximal tubular epithelial cell line was attenuated by the natural product resveratrol"* After assessing the abstract, it has been deemed not to be human subject research and therefore exempt from oversight of the Marshall University Institutional Review Board (IRB). The Code of Federal Regulations (45CFR46) has set forth the criteria utilized in making this determination. Since the information in this study does not involve human subjects as defined in the above referenced instruction, it is not considered human subject research. If there are any changes to the abstract you provided then you would need to resubmit that information to the Office of Research Integrity for review and a determination.

I appreciate your willingness to submit the abstract for determination. Please feel free to contact the Office of Research Integrity if you have any questions regarding future protocols that may require IRB review.

Sincerely,

Bruce F. Day, ThD, CIP
Director

APPENDIX B: LIST OF ABBREVIATIONS

DOX...doxorubicin

DNA…deoxyribonucleic acid

Top2…topoisomerase II

ROS…reactive oxygen species

DOX-OL…doxorubicinol

NADPH…nicotinamide adenine dinucleotide phosphate

P450R…cytochrome P450 reductase

Fe…iron

H_2O_2…hydrogen peroxide

OH…hydroxyl radical

AST…aspartate aminotransferase

ALT…alanine aminotransferase

MDA…malondialdehyde

V_{max}…maximum velocity

NOS…nitric oxide synthase

NO…nitric oxide

iNOS…inducible nitric oxide synthase

eNOS…endothelial nitric oxide synthase

RES…resveratrol

HPLC…high performance liquid chromatography

CVD…cardiovascular diseases

ALF…acute liver failure

ATP…adenosine triphosphate

HK-2…human noncancerous renal proximal tubular epithelial cells

ATCC…American type culture collection

DMSO…dimethylsulfoxide

MTT…3-4,5-dimethylthiazol-2-yl-2,5-diphenyltetrazolium Bromide

HBSS…hank's buffered saline solution

OD…optical density

SIRT1…sirtuin 1

TBST…tris-buffered saline-tween

HEK293…human embryonic kidney whole cell lysate

PGC1α…peroxisome

BSA…bovine serum albumin

PBST…phosphate-buffered saline-tween

OCR…oxygen consumption rate

ECAR…extracellular acidification rate

FCCP…carbonyl cyanide-4-trifluoromethoxy phenylhydrazone

SEM…standard error of the mean

ANOVA…analysis of variance

Cyt C…cytochrome C

ETC…electron transport chain

MPT…mitochondrial permeability transition

VDAC…voltage-dependent ion channels

2-DG…2-deoxy-glucose

GSH…glutathione

GSSG…oxidized glutathione

SOD…superoxide dismutase

GST…glutathione-s-transferase

P53…tumor suppressor protein

GPx1…glutathione peroxidase 1

4-HNE…4-hydroxynonenal

APPENDIX C: VITA

Morghan S. Getty
300 Wilson Court
Huntington, WV 25701
301-876-0591
MorghanSchuyler@yahoo.com

Education

2015-2017 Masters of Biological Science, Marshall University School of Medicine, Huntington, WV
Graduated with Distinction and Highest Honors

2003-2006 Bachelor of Science, University of North Carolina, Wilmington, NC

Research

2016-current "Doxorubicin Cytotoxicity in a Human Proximal Tubular Cell Line was Attenuated by the Natural Product Resveratrol"

- Collaborators: Monica Valentovic, PhD; Kathleen Brown, MS
- Research conducted at Marshall University School of Medicine as part of the requirement for the degree of Master of Biological Science
- Funding: Supported by NIH Grant P20GM103434 to the West Virginia IDEA Network for Biomedical Research Excellence and NIH Grant 5P20RR016477

2013-2015 "pNaktide Inhibits Na/K-ATPase Reactive Oxygen Species Amplification and Attenuates Adipogenesis"

- Collaborators: Joseph Shapiro, MD; Komal Sodhi, MD; Kyle Maxwell, MS III; Yangling Yan, PhD; Jiang Liu, PhD; Muhammad Choudry, MS; Zijian Xie, PhD; Nader Abraham, PhD
- Research conducted at Marshall University School of Medicine
- Funding: NIH Grants: HL109015, HL071556, HL105649, HL55601and HL34300

"Uric Acid-Induced Adipocyte Dysfunction is Attenuated by HO-1 Upregulation: Potential Role of Antioxidant Therapy to Target Obesity"

- Collaborators: Komal Sodhi, MD; Jordan Hilgefort, MD; George Banks, MD; Chelsea Gilliam, MS IV; Sarah Stevens, BS; Hayden Ansinelli MS IV; Nader Abraham, PhD, Joseph Shapiro, MD; Nader Abraham, PhD; Zeid Khitan, MD
- Research conducted at Marshall University Joan C. Edwards School of Medicine
- Funding: NIH Grants: HL109015, HL105649 and HL071556

"The Association of Metabolic/Inflammatory Biomarkers and Insulin Resistance in Obese Children from West Virginia"

- Collaborators: Yoram Elitsur, MD; Deborah Preston, BS; Komal Sodhi, MD

- Research conducted at Marshall University School of Medicine

"Su1885 Insulin Resistance is a Key Factor for Metabolic and Inflammatory Biomarkers in Children with Obesity"

- Collaborators: Yoram Elitsur, MD; Deborah Preston, BS; Komal Sodhi, MD
- Research conducted at Marshall University School of Medicine

"20-HETE and Circulating Endothelial Cells, a Unique Vascular Profile Related to CVD in Morbidly Obese Appalachian Women"

- Collaborators: Kathleen O'Hanlon, MD; Komal Sodhi, MD; Eamonn Maher, MD; Zeid Khitan, MD; Nitin Puri, MD, PhD; Edith Hochhauser, PhD, Joseph Shapiro, MD; Nader Abraham, PhD
- Research conducted at Marshall University School of Medicine

"The Existence of a Strong Correlation between Leptin, TNFα and BMI in Morbidly Obese Appalachian Females"

- Collaborators: Ellen Thompson, MD; Kathleen O'Hanlon, MD; Joseph Shapiro, MD

Publications

Sodhi, K.; Maxwell, K; Yan, Y.; Liu, J.; Chaudhry, M.; Getty, M.; Xie, Z.; Abraham, N.; Shapiro, J. pNaKtide Inhibits Na/K-ATPase Reactive Oxygen Species Amplification and Attenuates Adipogenesis. *Sci. Avd.* 2015, Oct 15; 1:e1500781.

Sodhi, K.; Pesce, P.; Stevens, S.; Getty, M.; Puri, N.; Favero, G.; Rezzani, R.; Hutcheson, R.; Sacerdoti, D.; Shapiro, J. Upregulation of Heme Oxygenase-1 Attenuates Ischemic Heart Myocyte Cell Death and Improves Heart Function in Immunosupressed Mice. *J. of Cardiology And Therapy* 2015, Vol 2, No 2.

Sodhi, K; Hilgefort, J; Banks, G; Gilliam, C; Stevens, S; Ansinelli, H, Getty, M; Abraham, N., Shapiro, J; Khitan, Z. Uric-Acid Induced Adipocyte Dysfunction is Attenuated by HO-1 Upregulation: Potential Role of Antioxidant Therapt to Target Obesity. *Stem Cells International*. 2015, June 11; ID 453839.

Recent Presentations
Oral

December 2017 "Doxorubicin Cytotoxicity in a Human Proximal Tubular Cell Line was Attenuated by the Natural Product Resveratrol" Presented to faculty at Marshall University, Huntington, WV

March 2017 "Doxorubicin and Nephrotoxicity: Clinical Implications" Lecture Presented to Marshall University graduate students and faculty as part of the Spring 2017 Seminar Series, Huntington, WV

September 2014 "Targeting Endothelial Cells with HO-1 Attenuated Vascular and

Adipocyte Dysfunction in Mice Fed High Fat Diet" Presented at The American Heart Association Hypertension Conference, New Orleans, LA

April 2014 "20-HETE and Circulating Endothelial Cells: Clinical Implications in Cardiovascular Disease" Presented as Grand Rounds Lecture for Cardiology Attending's and fellows at Joan C. Edwards School of Medicine, Huntington, WV

Poster

December 2017 "Doxorubicin Cytotoxicity in a Human Proximal Tubular Cell Line was Attenuated by the Natural Product Resveratrol" Presented at a OVSOT Conference, West Lafayette, IN

March 2017 "Doxorubicin Cytotoxicity in a Human Proximal Tubular Cell Line was Attenuated by the Natural Product Resveratrol" Presented at Marshall University Research Day, Huntington, WV

March 2015 "20-HETE and Circulating Endothelial Cells, a Unique Vascular Profile related to CVD in morbidly obese Appalachian Women. Marshall University Research Day, Huntington, WV

September 2015 "The Existence of a Strong Correlation between Leptin, TNFα and BMI in Morbidly Obese Appalachian Females. American Heart Association Hypertension Conference, San Francisco, CA

Professional/Academic Activities

2016-Present Society of Toxicology, Member

2015-Present Marshall University Graduate Student Organization, Member

- Treasurer 2016-2017

2013-2015 American Heart Association, Member

Work Experience

2012-2015 Research Associate
Marshall University Research Corporation

- Research Assistant
- Assisted in management of day-to-day lab activities
- Directed incoming Medical students regarding lab experience and opportunities
- Point person for Clinical studies involving University Physicians in Various Departments
    - Cardiology
    - Obstetrics and Gynecology
    - Family Medicine
    - Pediatric Gastroenterology
    - Pulmonology

2009-2012 Marketing Coordinator
DARCO International

- Management responsibilities and International coordination of National Marketing Campaigns

- Creation of marketing plans in line with company objectives
- Creation of a wide range of marketing materials
- Market goods at national and international conferences
- Planned and coordinated strategies with European and Asian counterparts
- Prepared online and print marketing campaigns
- Maintain effective internal communications to ensure all are kept informed of company marketing objectives

2007-2008 Financial Advisor
Edward Jones Investments

- Advised clients and provided customized financial plans catered to individual financial goals and needs
- Sought new clients using various methods such as cold-calling, door to door sales, and professional networking events
- Utilized various data entry methods to report accurate profitability and sales to corporate office
- Completed professional objectives by preparing daily, weekly, and monthly schedules
- Maintained a well-organized and time-sensitive account management style
- Completed extensive training to prepare for state licensing exams as well as financial planning

Licenses/Certifications

- Licensed Series 7 as a Securities Trader per SEC –July 2007
- Licensed Series 66 as a Financial Advisor for the State of North Carolina- October 2007
- Licensed Emergency Medical Technician – January 2005
- Certified in CPR- January 2005

Skills/Proficiencies

- Office Proficiency (Word, Excel, and Power Point)
- Type 65 words per minute
- Proficient in various lab techniques including: Western Blot, Cell Culture, PCR, Seahorse XFp analyzer and various assays including: Bradford, MTT, caspase, ELISA and Boyden assays
- Proficient in IRBnet as well as writing, editing and submitting IRB's