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Specific Targeting of the Nrf2 Transcription Factor to Prevent Diabetic Kidney Disease Progression by Stimulating the Oxidative Stress Response: A Next-Generation Strategy

CAPSTONE

submitted in partial satisfaction of the requirements
for the degree of

MASTER OF SCIENCE

in Pharmacology

by

Allie M. Roach

Capstone Advisor:
Adjunct Professor Diana N. Krause, PhD

2021

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DEDICATION

To

R.T.M.

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CHAPTER I.

INTRODUCTION

Summary of Proposal

This research proposal outlines preclinical pharmacological approaches to elucidate the potential efficacy of Nrf2 activation to ameliorate diabetic kidney disease (DKD). A key vulnerability of the kidney in diabetes stems from its functional reliance on mitochondrial energetics and its susceptibility to oxidative stress. The focus on Nrf2 is linked to this transcription factor's induction of several antioxidant genes upon activation. The goals of this proposal are thus twofold. First, I seek to demonstrate target engagement of the novel small molecule Nrf2 activator, leading to improvement in functional and histological endpoints in an animal model of DKD. Next, I will evaluate whether selective Nrf2 agonism avoids adverse effects on the endothelin signaling pathway in a preclinical model of DOCA-salt nephropathy. This adverse effect, which can lead to heart failure, was seen in a subset of patients in clinical trials with the nonspecific Nrf2 activator Bardoxolone methyl. The ultimate purpose of this initiative is the development of effective strategies for prevention and management of human diabetic nephropathy, a renal disease demonstrating insufficient activation of the antioxidant stress response. The potential translational impact of this approach is high, as the Nrf2 activator Bardoxolone methyl has already demonstrated efficacy in mild to moderate DKD in clinical trials, though it has demonstrated toxic side effects in advanced DKD. To accomplish these goals, I propose three projects to demonstrate: (1) Nrf2 Dysregulation in a Preclinical Model of DKD; (2) Target Engagement and

Functional/Histological Improvement in a Preclinical Model of DKD with an Orally Dosed Small Molecule Nrf2 Activator; and (3) Lack of Toxic Liabilities of an Orally Dosed Specific Small Molecule Nrf2 Activator that are Otherwise Associated with Bardoxolone Methyl Treatment, a Nonspecific Nrf2 Activator.

Significance and Novelty of the Proposed Study

Type 2 diabetes mellitus (T2DM) continues to be the leading cause of chronic kidney disease (CKD) worldwide (KDOQI, 2007). Currently the number of treatment options for patients with CKD are limited to targeting the renin-angiotensin system and many patients still progress to end stage renal disease (Barnett et al., 2004; Navaneethan, Nigwekar, Sehgal & Strippoli, 2009; Onuigbo, 2011). Considering that the number of US adults with diagnosed diabetes is projected to nearly triple by 2060 (Lin et al., 2018), novel therapies are urgently needed to address this high unmet clinical need. The pathogenesis of DKD is still poorly understood. However, DKD is associated with high levels of oxidative stress, which is thought to be linked to mitochondrial dysfunction and insufficient cytoprotective response. Thus, one area of therapeutic promise involves targeting the antioxidant stress response pathway (Ratliff, Abdulmahdi, Pawar & Wolin, 2016).

Nrf2 is a key transcription factor in the kidney involved in the antioxidant stress response, activating a variety of cytoprotective genes related to redox and detoxification (Kensler, Wakabayashi & Biswal, 2007). Under basal conditions, Nrf2 is restricted to the cytoplasm by the bound protein Keap1. Nrf2 is only released and allowed to translocate to the nucleus to transcribe its target genes when electrophilic species induce covalent modification of the Keap1 cysteine residues that are responsible for sensing oxidative stress

(Wakabayashi et al., 2004). Studies over the past decade using the Nrf2 activator Bardoxolone methyl have demonstrated promising results in CKD, with improved renal function and preserved tissue structure in both mouse and man (Aminzadeh, Reisman, Vaziri, Khazaeli, Yuan & Meyer, 2014; Pergola et al., 2011). However, this compound's electrophilic mechanism of action results in non-specific interaction with over 500 molecular species (Yore, Kettenbach, Sporn, Gerber & Liby, 2011), which may partially explain the premature conclusion of the BEACON trial due to an increase in heart failure events in patients with T2DM and more advanced CKD (de Zeeuw et al., 2013). In this proposal, I will thus address the hypothesis that a potent novel, specific small molecule Keap1-Nrf2 interaction inhibitor with favorable drug-like properties, free of cardiovascular off-target effects, is useful in the prevention and treatment of DKD and associated mitochondrial dysfunction.

Research Hypotheses to be Tested

Specific Hypothesis 1: If Nrf2 is dysregulated in diabetic kidney disease, then kidney tissue from the ZSF1 rat model of DKD will demonstrate decreased target gene expression.

Specific Hypothesis 2: If increased Nrf2 activation is beneficial in the ZSF1 rat model of DKD, then in vivo treatment with the lead Nrf2 activator compound will increase renal target engagement, increase renal function, improve histological endpoints, and ameliorate mitochondrial dysfunction.

Specific Hypothesis 3: If the selected small molecule Nrf2 activator has pharmacological properties that enable its efficacy in vivo via oral dosing without the toxicity liabilities that limit the clinical use of Bardoxolone methyl, then oral dosing of the DOCA-Salt

Hypertensive rat model of CKD with this compound will result in renal target engagement, without significant decreases in endothelin receptor A (ET_A) and endothelin-1 expression, weight reduction, reduced food intake, increased blood pressure, or dyslipidemia.

CHAPTER II.

REVIEW OF THE LITERATURE

Chronic Kidney Disease Has High Unmet Clinical Need

Type 2 diabetes mellitus (T2DM) is the leading cause of chronic kidney disease (CKD) worldwide. In the United States, it is estimated that one in three diabetic adults has CKD (Centers for Disease Control and Prevention, 2019). As the number of US adults with diabetes is projected to nearly triple by 2060 (Lin et al., 2018), the levels of CKD diagnoses are expected to rise as well. Though over 30 million Americans currently have CKD, diagnosis of the disease and awareness amongst individuals with CKD are quite low. Amongst all CKD patients, it is estimated that only 10% are aware of their disorder. Even more striking, for those in the advanced stages of CKD (Stage 4), still only 57% have been diagnosed with CKD (Centers for Disease Control and Prevention, 2019). This lack of early awareness prevents early treatment and sufficient lifestyle changes to occur in a timely manner. As such, CKD is now the 8th leading cause of death in the United States, contributing to over 50,000 early deaths annually (Kochanek, Xu & Arias, 2020).

There are currently very limited treatment options for patients with CKD. Current standards of care focus mainly on altering the function of the renin-angiotensin system to exert blood pressure control (Remuzzi, Schieppati & Ruggenenti, 2002), while new drugs in clinical trials focus on lowering blood volume (Cowie & Fisher, 2020). Despite treatment, many patients with diabetes will still progress to end stage renal disease (ESRD). Of all patients with ESRD, an estimated 44% have diabetes (United States Renal Data System, 2017). ESRD patients must undergo regular dialysis while awaiting kidney transplant, a

process that is as costly as it is time consuming. In the US alone, the Medicare costs for CKD are over $84 billion annually. Furthermore, treating people with ESRD costs an additional $36 billion to Medicare every year (Centers for Disease Control and Prevention, 2019).

The Pathogenesis of Chronic Kidney Disease is Poorly Understood

The structure and function of the kidney are very complex. There are as many as twenty-one unique cell types that have been identified in the kidney (Park et al., 2018). These cells make up intricate structures such as the nephron, which is the functional unit of the kidney that includes the glomerulus (passive filter) and proximal tubules (active filtration mechanisms), as well as the loop of Henle and the distal tubule. Other cells in this tissue form the interstitium, including endothelial cells, pericytes/fibroblasts, and resident immune cells, as well as the larger blood vessels, collecting duct, and renal capsule.

Within the kidney, disease is characterized by several common phenotypes. First and foremost is the loss of the glomerular filtration barrier, which is a multi-layered structure that normally prevents large molecules within the bloodstream from passing into the renal filtrate. Thus, the loss of this barrier due to cell stress and damage results in leakage of protein, which can be measured in the urine. Another key feature is damage to the renal tubules, which play key roles in the body to actively remove waste from blood, reabsorb nutrients, regulate the balance of electrolytes and fluid, maintain acid-base homeostasis, and regulate blood pressure. There is also a substantial immune response in renal disease, resulting in the infiltration of inflammatory cells, which may promote active fibrogenesis induced by activated pericytes and fibroblasts (i.e., myofibroblasts) within the interstitium. Despite the varied structural and functional changes that occur with disease, the current

challenge for clinical trials is that approval of novel drugs rests on a primary endpoint with a high bar: Preservation and/or rescue of the glomerular filtration rate (Levey et al., 2014).

The primary therapeutic treatments for CKD are drugs that globally effect the RAS system, thus there is a lack of drugs that target disease-specific pathways. Many cellular pathways are altered in this disease, including lysophosphatidic acid receptor signaling (LPAR) (Lee, Kim, Oh & Jun, 2019), mTOR (Ma, Yung & Chan, 2018), and Hippo pathways (Szeto et al., 2016). As such, it has been historically difficult to understand which affected pathways are a primary contributor to disease and which are non-critical or simply a downstream effect of disease. Recent advancements in single cell RNAseq and bioinformatics, however, have the potential to greatly improve the translational power in drug discovery by elucidating key pathways in disease.

The Powerhouse: Function of Mitochondria in Health and Disease

Recent studies point to an increasingly important role for mitochondrial dysfunction in multiple disease states, including neurodegenerative diseases, cardiovascular disease, cancer, and renal disease (2014; Ballinger, 2005; Gamboa et al., 2016; Lin & Beal, 2006; Wallace, 2005; Wooller, Benstead-Hume, Chen, Ali & Pearl, 2017). In healthy tissue, the mitochondria act as the “powerhouses” of the cell, producing the majority of the cell’s ATP through oxidative phosphorylation (OXPHOS) via the electron transport chain (ETC). The ETC is composed of four protein complexes (I-IV). During OXPHOS, these complexes undergo sequential redox reactions that cause conformational changes in the protein machinery, thereby pumping protons into the intermembrane space, forming the membrane potential and the transmembrane proton gradient. This gradient is then harnessed by complex V,

which drives the phosphorylation of ADP to form ATP. Though the majority of mitochondrial oxygen consumption is efficiently coupled to ATP production, a small percentage of oxygen is reduced by complexes I and III to form potentially dangerous reactive oxygen species (ROS) (Baughman & Mootha, 2006; Muller, Liu & Van Remmen, 2004; Murphy, 2009). During homeostasis, these ROS act as second messengers (Baughman & Mootha, 2006). However, in disease states such as cancer, Leigh syndrome, Charcot-Marie-Tooth 2A disease and diabetic kidney disease, impaired mitochondrial function results in increased levels of ROS and decreased ATP production (Galvan, Green & Danesh, 2017; Petros et al., 2005; Trounce, Neill & Wallace, 1994; van Hameren et al., 2019), which are thought to contribute to the pathogenesis of these diseases. High levels of ROS are known to lead to activation of cell stress signalling pathways (Baughman & Mootha, 2006), as well as cause genomic damage (Parrinello, Samper, Krtolica, Goldstein, Melov & Campisi, 2003) and accelerate telomeric shortening (von Zglinicki, 2002). It is believed that these downstream targets of ROS potentially activate a positive feedback loop, thus amplifying and sustaining the elevated ROS levels (Meeus, Nijs, Hermans, Goubert & Calders, 2013).

In order to prevent or eliminate wayward mitochondria, cells have evolved a complex system that balances mitochondrial fusion and division with the mitophagy quality control pathway. Mitochondrial biogenesis, on the other hand, allows for increased cellular plasticity, enabling cells to respond to augmented energy demands, adapt to stress signalling pathways, or to replace damaged organelles. However, in chronic mitochondrial dysfunction states, this delicate balance can be undone. Though mitochondrial hyperfusion is able to transiently buffer the potentially detrimental effects of certain cell stressors, such as impaired function of complex IV (Rolland et al., 2013), this pathway cannot protect against

long-term defects in the ETC. Persistent OXPHOS dysfunction has been shown to block mitochondrial biogenesis (Viscomi et al., 2011; Wenz, Diaz, Spiegelman & Moraes, 2008), eventually culminating in an overall decrease in membrane potential in the remaining mitochondria. Normally, suppressed membrane potential triggers initiation of the mitophagy pathway via Pink1 and Parkin. However, cells with increased levels of mitochondrial fusion and/or decreased mitochondrial division demonstrate attenuated mitophagy, likely caused by the inability of the autophagy machinery to process the larger, hyper-fused organelles (Gomes, Di Benedetto & Scorrano, 2011; Nunnari & Suomalainen, 2012; Rambold, Kostelecky, Elia & Lippincott-Schwartz, 2011). These mitochondria are thus unable to be recycled, resulting in further defects in OXPHOS, increased ROS production, and mitochondrial DNA (mtDNA) loss during cell division (Hanekamp et al., 2002; Ishihara et al., 2009; Parone et al., 2008; Wakabayashi et al., 2009).

Mitochondrial Homeostasis in Kidney Health and Disease

Due to the numerous active functions conducted by the kidney, this organ has the second highest resting metabolic rate, mitochondrial content, and oxygen consumption in the human body (Bhargava & Schnellmann, 2017). As the glomerular cells (podocytes, endothelial cells, and mesangial cells) are able to filter the blood through a passive process that removes small molecules (i.e., glucose, urea, water, and salts) while retaining large molecules (i.e., haemoglobin), this step in the renal filtration process does not directly require ATP. Thus, the glomerular cells have the ability to perform aerobic and anaerobic respiration in order to produce enough ATP for basal cell processes. Proximal tubules (PTs), on the other hand, use multiple active transport mechanisms to reabsorb 80% of the filtrate

that passes through the glomerulus (Zhuo & Li, 2013). Thus, PTs contain more mitochondria than any other structure in the kidney (Bhargava & Schnellmann, 2017). Furthermore, as the PT has a low capacity for glycolysis, aerobic respiration is the primary mechanism of ATP production in the PTs (Weinberg & Molitoris, 2009). Therefore, disruption of mitochondrial homeostasis is an important factor that drives tubular injury and persistent renal dysfunction. Strikingly, patients with mitochondrial respiratory chain abnormalities have reported progressive renal disease, with some presenting renal abnormalities as their primary pathology (Finsterer & Scorza, 2017).

CKD is associated with high levels of mitochondrial dysfunction and oxidative stress (Gamboa et al., 2016). Oxidative stress is caused by increased production and insufficient catabolism of ROS and superoxide (SO), and sustained oxidative stress is a major contributor to cell death and tissue damage. In a mouse model of CKD (*Col4a3*$^{-/-}$) conducted in the Duffield lab, it was found that mitochondrial function was the most dysregulated gene ontology pathway (Gomez et al., 2015). Importantly, in our recent unpublished work, we found that there is a significant loss of mitochondrial networks in human DKD (Duffield, *et al.*; *unpublished*). In this same study, we found that the cytoprotective response was insufficient, as noted by alterations in critical ROS and SO scavenging proteins in human PT epithelial cells (PTECs) from CKD patients. Specifically, there were increases in SDHA (a complex II ETC component) and COX10 (required for the expression of the functional complex IV ETC component), but a decrease in COX1 (a complex IV ETC component), SOD1, SOD2, and Thioredoxin (critical ROS/SO scavenging proteins). Interestingly, in our mouse model of CKD, it was also noted that changes in mitochondrial morphology preceded histological signs of disease, specifically a loss of mitochondrial network. Thus, one area of

therapeutic promise for treating CKD may involve improving mitochondrial health specifically through the antioxidant stress response pathway.

Nrf2 as a Potential Drug Target for Chronic Renal Disease

Nrf2 is a master transcription factor involved in the antioxidant stress response (Dinkova-Kostova et al., 2005). The primary target genes of Nrf2 are those that influence mitochondrial biogenesis, regulate oxidative stress responses, and regulate the expression of many anti-oxidative proteins (>200) (Zhu & Fahl, 2001). Nrf2 is normally limited to the cytoplasm by binding of the regulatory protein Keap1. Upon binding, this regulatory protein polyubiquitinates Nrf2, targeting it for proteasomal degradation, thus resulting in constant recycling of newly translated Nrf2 protein (Wakabayashi et al., 2004). In conditions of stress, however, electrophilic species will induce covalent modification of the Keap1 cysteine residues responsible for sensing oxidative stress (Zhang & Hannink, 2003). This modification thus leads to changes in the Keap1 protein tertiary structure that prevents both polyubiquitination of Nrf2 as well as the release of Nrf2 into the cytoplasm. As a consequence of Keap1 stalling, newly transcribed Nrf2 is able to remain unbound and thus translocate into the nucleus to activate its target genes by binding to antioxidant response elements (Kensler, Wakabayashi & Biswal, 2007). Nrf2 activation thus reinforces and strengthens the anti-oxidative cell defense.

In recent studies conducted in this lab, it has come to light that Nrf2 is dysregulated in kidney disease (Duffield, *et al.*; *Unpublished)*. Specifically, in a model of CKD (*Col4a3*$^{-/-}$), a promoter analysis identified Nrf2 as a potential therapeutic target. Investigating whole kidney tissue from this model, it was found that numerous key Nrf2 target genes were

downregulated in disease, specifically *Sod1*, *Nqo1*, *Pparg*, and *Cat*, to name a few. Through the use of a proprietary Nrf2 small molecule activator tool compound, we showed that treatment of cells in vitro could activate Nrf2 target genes, including *Nqo1*, *Prdx1*, and *Hmox1* in murine renal PTECs. Use of this tool compound in our mouse model of CKD also demonstrated numerous beneficial effects, including attenuation of disease progression (reduced urine albumin and blood urea nitrogen levels), reduced interstitial fibrosis (by picrosirius red staining), reduced glomerulosclerosis (by silver staining), reduced tubular injury (by H&E staining), and reduced myofibroblast and leukocyte expansion (by αSMA and F4/80 staining, respectively). Based on our preliminary studies, these data thus lend support for Nrf2 activating compounds as potential therapeutic agents for the treatment of CKD.

The Next Generation: Moving Past the Shortcomings of Bardoxolone Methyl

Bardoxolone methyl is a Nrf2 activating compound that has undergone clinical testing with mixed results. Much excitement first built around this compound when it was found in Phase 2 trial patients with T2DM and mild to moderate CKD that there was a significant improvement in the estimated glomerular filtration rate. Importantly, these effects were sustained for the complete 52 weeks of the trial period (Pergola et al., 2011). In a follow-up Phase 3 BEACON clinical trial, however, the targeted population was altered to include patients with T2DM and more advanced (Stage 4) CKD. In a major blow to the Nrf2 hypothesis in CKD, this bardoxolone trial was ended early due to a significant increase in heart failure events (Chin et al., 2014).

The mechanism of action of bardoxolone rests on its electrophilic activities, which can interact with the Keap1 cysteine residues responsible for sensing oxidative stress. However,

this mechanism of action is nonspecific, and bardoxolone is known to have the capacity to interact with over 500 molecular species (Yore, Kettenbach, Sporn, Gerber & Liby, 2011). Furthermore, bardoxolone is also known to inhibit NFκB signaling, a major transcription factor that regulates genes responsible for immune responses (Ahmad, Raina, Meyer, Kharbanda & Kufe, 2006). Thus, there is a mixed understanding of whether preliminary success seen in the Phase II clinical trials was linked mainly to Nrf2 activation, reduction of inflammation, or some other mechanism. To be noted, however, the potential off-target effects of bardoxolone due to its non-specific mechanism of action may explain failure of the Phase 3 BEACON trial. Specifically, the rapid increase in blood volume and sodium retention noted in patients may have induced a fluid overload that likely led to the observed heart failure events in patients (Chin et al., 2014). One proposed mechanism for why this may have occurred as a result of bardoxolone treatment may be related to the noted alterations in endothelin receptor A (ET_A) and endothelin-1 expression (Chin et al., 2014). This is further supported by the observation that bardoxolone's fluid overload phenotype is similar to advanced CKD patients treated with endothelin receptor antagonists (Mann et al., 2010), suggesting off-target modulation of endothelin pathway may be linked to heart failure events.

Thus, a specific small molecule inhibitor of Keap1:Nrf2 protein−protein interaction represents the next generation of promising therapeutic compounds for diseases involving oxidative stress (Davies et al., 2016). Utilizing such a compound will allow for tight and selective binding of Keap1 with potent activation of the Nrf2 antioxidant response. The long-term goal of this proposal is therefore to address the hypothesis that a potent novel, specific small molecule Keap1-Nrf2 interaction inhibitor with favorable drug-like properties, free of

cardiovascular off-target effects, is useful in the prevention and treatment of DKD and associated mitochondrial dysfunction.

CHAPTER III.

EXPERIMENTAL DESIGN

Specific Hypothesis 1: If Nrf2 is dysregulated in diabetic kidney disease, then kidney tissue from the ZSF1 rat model of DKD will demonstrate decreased target gene expression.

Here, we will use the male uninephrectomised (UNX) ZSF1 rat model of diabetic kidney disease, an animal model of DKD that recapitulates many of the comorbidities present in patients with T2DM, including hypertension, congestive heart failure, obesity, hypertriglyceridemia, and diabetic nephropathy. This is a highly translational model, with many clinical characteristics that follow what is seen in human disease, including a transient increase in renal filtration followed by a progressive decline in glomerular filtration rate, the presence of key urinary biomarkers, and progressive tubulointerstitial fibrosis. This animal model is especially relevant as it displays metabolic syndrome and elevated microalbumin-to-creatinine ratio by 12 weeks of age, and progressively worsening inflammatory cell infiltration and glomerulosclerosis that ultimately culminates in end stage renal disease (Dower et al., 2017; Su et al., 2016). Importantly, male ZSF1 rats appear to meet most if not all of the criteria put forth by the Animal Models of Diabetic Complications Consortium for rodent models of progressive diabetic nephropathy (Dower et al., 2017). We will not be using female ZSF1 rats in this study because, although they have been shown to demonstrate

symptoms of diabetic nephropathy, they are not the ideal model due to delayed disease manifestation and generally less severe disease (Su et al., 2016).

ZSF1 rats will be obtained from Charles River Laboratories (Lean: *fa*/+ or *fa*/?, Strain code #379; Obese: fa/fa^{CP}, Strain code #378) at 4 weeks of age. Animals will be housed in our lab's associated vivarium in pairs and acclimated under 12-hour light-dark cycles with free access to water and Lab Diets 5008 chow. All animal studies will be performed under protocols approved by the Institutional Review Board.

The primary aim of this natural history study is to demonstrate that in this model there is decreased Nrf2 activation in the kidney during T2DM. The experimental groups include male lean and obese ZSF1 rats (n = 10 Lean rats per group; n = 10 Obese rats per group; Total n = 20 rats). It is important to note that lean animals do not progress to any manifestations of DKD, whether intact or uninephrectomised, thus they will act as the negative control here.

To prepare for this study, all rats will undergo a partial nephrectomy, whereby one kidney will be surgically removed at 8 weeks of age by experienced technicians according to standard procedure (to accelerate the progression of diabetic nephropathy in obese animals). Following the UNX surgeries, the muscle and fascia will be closed with absorbable sutures and the skin incision will be closed with wound glue and clips that will be removed by veterinary staff 7-10 days after surgery. Additionally, rats will receive daily IP injections for 3 days of Animalgesic, which is a suspension of extended-release buprenorphine, to reduce post-surgical discomfort. Cage-side assessments will occur at least twice weekly (starting at 8 weeks through 32 weeks of age) to monitor for standard signs of pain and

discomfort, including measurement of body weight, body condition scoring (BCS) (Table 1), and Animal Health Assessment. The latter is a descriptive assessment, which is indicative of declining health, and includes inspecting for signs of lethargy, moribund behavior, dehydration, abnormal locomotion, and ruffled fur. If animals lose weight (15% from peak body weight), then they will be monitored daily. However, if an animal reaches over 20% body weight loss or shows a poor body condition score of less than or equal to two, or if the animal is moribund (as defined by a general unresponsiveness and lack of awareness of external stimuli), the rat will be euthanized immediately.

Table 1: Body Conditioning Score Chart

BCS	Scoring Criteria
1	Rat is emaciated: Skeletal structure extremely prominent; little or no flesh cover. Vertebrae distinctly segmented.
2	Rat is underconditioned: Segmentation of vertebral column evident. Dorsal pelvic bones are readily palpable.
3	Rat is well-conditioned: Vertebrae and dorsal pelvis not prominent; palpable with slight pressure.
4	Rat is overconditioned: Spine is a continuous column. Vertebrae palpable only with firm pressure.
5	Rat is obese: Rat is smooth and bulky. Bone structure disappears under flesh and subcutaneous fat.

At 32 weeks of age, all animals will be anesthetized by isoflurane inhalation, then euthanized by bilateral pneumothorax followed by exsanguination via incision of the inferior vena cava. Rats will then be perfused with ice cold phosphate buffered saline solution to flush the blood from the tissue, and the remaining kidney will be harvested for analysis.

The dependent variables will be the gene expression of *Nqo1* and the protein expression of Nqo1 in the kidney, a key Nrf2 target gene and protein product. The independent variables will be the rat genotypes (Obese: fa/fa^{CP}; Lean: $fa/+$ or $fa/?$).

The potential confounding variables of this study are also the important ethical considerations: the potential for pain and distress in the animals. This includes increased stress following shipment of animals to the final vivarium and the UNX surgeries. To reduce the possibility of such confounding variables affecting the experiment and ensure ethical treatment of the study animals, the rats will be allowed to acclimate to the new vivarium for two weeks before experimental procedures are performed on them. Additionally, following surgery at 8 weeks of age, as mentioned above, the animals will be monitored for signs of pain, distress, and morbidity.

The primary endpoints of this study include measuring gene expression (*Nqo1*) and protein levels (Nqo1) of a key Nrf2 target gene in the kidney. These will be measured using standard RT-qPCR and Western blot techniques from whole tissue, as detailed in the 2019 paper from Ji and colleagues (Ji, Xiong, Zhao, Liu & Yu, 2019).

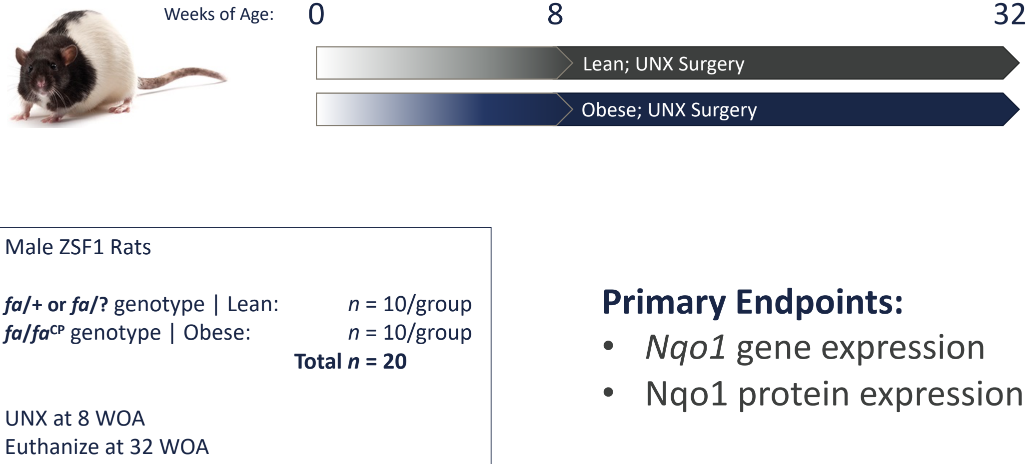


Figure 1. A 24-week Natural History Study Measuring Nrf2 Activation in the Male Uninephrectomised ZSF1 Rat

In order to control bias during the experiment and analysis, the animal and sample ID's will be anonymized so that only the project manager is aware of their true group assignments, and the laboratory scientists performing and analyzing the experiments will be blinded.

During the data analysis for the RT-qPCR experiments, the data will be normalized to a housekeeping gene, such as *GAPDH*, to control for pipetting error. Furthermore, we will also normalize the ΔC_T values to the Lean UNX group to determine the fold change of gene expression for each measured gene product. During the data analysis for the Western blot experiments, the data will be normalized to a loading control, such as beta actin, to control for pipetting error. This method will determine the relative protein expression level changes for each protein lysate tested. These data sets can then both be statistically analyzed using a Student's t-test, since we will be comparing two substrains (Lean vs. Obese). The Null

Hypothesis here will be that all the qPCR fold changes for the Obese UNX group will be equal to 1 and all the densitometry plot measurements will also show no difference as compared to Lean UNX group. The level of significance will be 5%. I will report the error of my data using SEM. Here, it is expected that Obese UNX animals will have significantly decreased levels of Nqo1 protein and *Nqo1* transcript in tissue collected at 32 weeks of age, as compared to Lean UNX rats, demonstrating a lack of activation of the antioxidant stress response pathway in a preclinical model with demonstrated oxidative stress (Prabhakar, Starnes, Shi, Lonis & Tran, 2007). One limitation is that these assays require fresh kidney tissue, thus it cannot be conducted using in-life collected biosamples such as blood or urine, thereby limiting the time points in which this assay can be performed to time of necropsy.

Specific Hypothesis 2: If increased Nrf2 activation is beneficial in the ZSF1 rat model of DKD, then in vivo treatment with the lead Nrf2 activator compound will increase renal target engagement, increase renal function, improve histological endpoints, and ameliorate mitochondrial dysfunction.

Following the validation of the ZSF1 rat model of DKD as an appropriate preclinical model to mimic the decline in Nrf2 activation following onset of kidney disease (Specific Hypothesis 1), we will now expand the study to include a group of obese UNX rats that have been treated with the lead Nrf2 activator compound (i.e., Lead Compound), while the other two groups will be treated with Vehicle (n = 10 Lean + Vehicle rats; n = 10 Obese + Vehicle rats; n = 10 UNX Obese + Lead Compound rats; Total n = 30 male ZSF1 rats). Beginning at 6 weeks of age and occurring every 4 weeks thereafter until the conclusion of the study, urine will be collected from all animals over an 8-hour period via a metabolic chamber and blood will be collected via tail vein. Beginning at 7 weeks of age and occurring every 4 weeks thereafter until the conclusion of the study, the glomerular filtration rate (GFR) of rats will be measured by experienced technicians using a MediBeacon transdermal device and fluorescent tracer agent (Scarfe et al., 2018). At 8 weeks of age, the rats will undergo a partial nephrectomy, as described above. Additionally, beginning at 8.5 weeks of age and continuing through the completion of the study, the rats will be orally dosed twice daily with either Vehicle or Lead Compound at a pre-determined concentration to achieve EC_{90} at 12 hours post-dose. At 32 weeks of age, all animals will be anesthetized by isoflurane inhalation, then euthanized by bilateral pneumothorax followed by exsanguination via incision of the inferior vena cava. Rats will then be perfused with ice cold phosphate buffered saline solution to flush the blood from the tissue, and the remaining kidney will be harvested for analysis.

In addition to the dependent variables for the groups described in Specific Hypothesis 1, here we will also include the GFR, blood urea nitrogen (BUN) levels, urine albumin-creatinine ratio (UACR), histological endpoints, urinary 8-OH-dG levels, and mitochondrial SO generation in PTECs. The independent variables will again be the rat genotypes (Obese: fa/fa^{CP}; Lean: *fa*/+ or *fa*/?), as well as the treatments (i.e., Vehicle or Lead Compound).

In addition to the potential confounding variables listed above, here we must also consider the effect of the Lead Compound treatment. In order to control for the effect of the compound's vehicle, we will have treated all other study groups with an equal volume of vehicle on the same dosing schedule as the Lead Compound treated group. This will thus help to ensure that any differences seen in the readouts are due to the Lead Compound itself, and not because of handling or vehicle effects.

Here, the primary endpoints will again be the Nrf2 target gene and protein expression levels in the kidney (*Nqo1*/Nqo1), as well as functional readouts (GFR measurements, BUN, UACR) and histology (silver stain, picrosirius red, and Periodic acid-Schiff). The secondary endpoints will be renal ROS/SO generation (urinary 8-OH-dg, PTEC mitochondrial SO generation in vitro) and mitochondrial network fragmentation (Transmission Electron Microscopy; TEM).

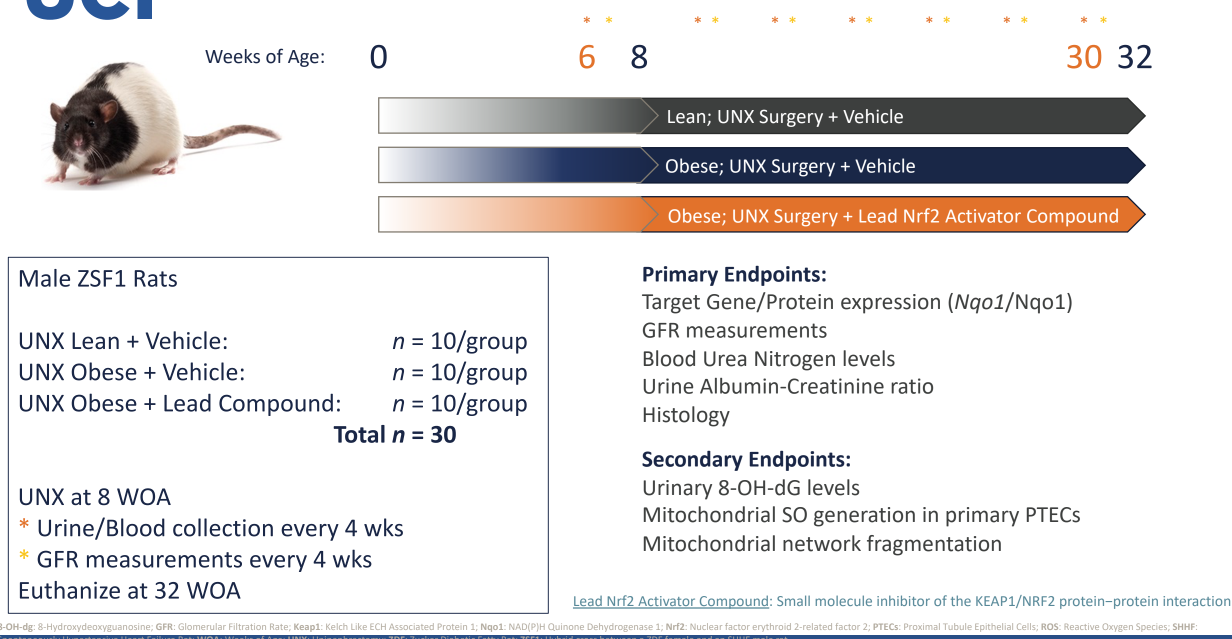


Figure 2. A 24-week Oral Dosing Diabetic Kidney Disease Model Pharmacology Study in the Male Uninephrectomised ZSF1 Rat

In order to control for bias during the experiment and analysis, the test articles, animals, and sample ID's will remain anonymized so that only the project manager is aware of their true group assignments, and the laboratory scientists performing and analyzing the experiments will be blinded.

The measurement of *Nqo1* gene expression and Nqo1 protein levels in the kidney will be performed as described above, with the addition of the UNX Obese + Lead Compound samples. Here we will normalize the ΔC_T values to the UNX Obese + Vehicle group to determine the fold change of gene expression for each measured gene product. These gene expression and Western blot data sets can then both be statistically analyzed using a One-Way ANOVA with Tukey's test, since we will be comparing two substrains (Lean vs. Obese) and two treatments (Vehicle vs. Lead Compound). The Null Hypothesis here will be that the

UNX Obese + Lead Compound qPCR fold changes will equal to 1 and there will be no difference in the densitometry plot measurements when comparing the UNX Obese + Lead Compound and UNX Lean + Vehicle groups to the UNX Obese + Vehicle group. The level of significance will be 5%. I will report the error of my data using SEM. Here, it is expected that UNX Obese + Lead Compound animals will have significantly increased levels of Nqo1 protein and *Nqo1* transcript in tissue collected at 32 weeks of age, as compared to UNX Obese + Vehicle rats. One limitation is that these assays require fresh kidney tissue, thus it cannot be conducted using in-life collected biosamples such as blood or urine, thereby limiting the time points in which this assay can be performed to time of necropsy.

During the data analysis for the GFR measurements, the serial measurements of FITC-sinistrin half-life over 24 weeks will be monitored and recorded. These levels will be fitted to linear mixed-effects models, according to Scarfe and colleagues, in order to characterize changes of FITC-sinistrin half-life over time (Scarfe et al., 2015). These data sets will be statistically analyzed using a One-Way ANOVA with Tukey's test, since we will be comparing two substrains (Lean vs. Obese) and two treatments (Vehicle vs. Lead Compound). The Null Hypothesis here will be that all the FITC-sinistrin half-lives of the UNX Obese + Vehicle rats will be equal to UNX Lean + Vehicle and UNX Obese + Lead Compound rats. The level of significance will be 5%. I will report the error of my data using SEM. Here, it is expected that Obese UNX Vehicle animals will initially show significantly increased FITC-sinistrin half-life, as compared to Lean UNX vehicle rats, indicating renal hyperfiltration. It is next expected to progressively decline until late in the study period when the rats will have significantly reduced GFR as compared to Lean UNX Vehicle animals, thus showing a time-dependent decline in overall renal function (Su et al., 2016). With Lead Compound treatment in the

Obese UNX rats, however, significant preservation of GFR is expected over time as compared with the Obese UNX + Vehicle rats. As this is a minimally invasive, transdermal procedure conducted in conscious animals, there are no foreseen limitations.

To further determine the function of the kidney, we will perform two additional tests. First, we will measure BUN, which quantifies the levels of the urea waste product in the blood that is normally cleared by the kidneys. Second, we will measure UACR, which quantifies the levels of albumin in the urine (a small protein that passes the glomerular filtration barrier and is normally reabsorbed by the tubules, therefore avoiding excretion in the urine) as compared to the levels of creatinine in the urine (a normal waste product found in urine). For the BUN and UACR analyses, these tests will be performed using commercially available plate assays, as described by Gomez *et al*. (Gomez et al., 2015). These data sets will then be statistically analyzed using a One-Way ANOVA with Tukey's test, since we will be comparing two substrains (Lean vs. Obese) and two treatments (Vehicle vs. Lead Compound). The Null Hypothesis here will be that all the BUN and UACR levels of the UNX Obese + Vehicle rats will be equal to UNX Lean + Vehicle and UNX Obese + Lead Compound rats. The level of significance will be 5%. I will report the error of my data using SEM. It is expected that the UNX Obese + Lead Compound rats and UNX Lean + Vehicle rats will have significantly decreased BUN and UACR, as compared to UNX Obese + Vehicle rats. As these are standard assays that can be ran using small amounts of blood serum and urine, there are no foreseen limitations.

For the histological endpoints, Periodic acid-Schiff (PAS), methenamine silver, and picrosirius red stains will be performed on formalin-fixed paraffin-embedded tissue sections, as previously described (Gomez et al., 2015). The stained sections will then be

evaluated by a renal pathologist to quantify differences in glomerulosclerosis (silver stain), interstitial fibrosis (picrosirius red stain), and tubule injury (PAS stain). Briefly, to quantify glomerulosclerosis, the extent of sclerosis in 30-40 sequential glomeruli per animal in silver-stained kidney sections will be determined (scarring plus capillary loop destruction) and scored from Grade 0 to 4 where 0 = none, 1 = 0-25%, 2 = 25-50%, 3 = 50-75% and 4 = >75% (Ryu, Mulay, Miosge, Gross & Anders, 2012). Interstitial fibrosis will be quantified in picrosirius red-stained paraffin sections as described (Humphreys et al., 2010). Finally, epithelial injury will be quantified in PAS-stained kidney sections as described (Ren et al., 2013). As we will be comparing two substrains (Lean vs. Obese) and two treatments (Vehicle vs. Lead Compound), these data sets will then be statistically analyzed using a One-Way ANOVA with Tukey's test. The Null Hypothesis here will be that the glomerulosclerosis, interstitial fibrosis, and tubule injury levels of the UNX Obese + Vehicle rats will be equal to UNX Lean + Vehicle and UNX Obese + Lead Compound rats. The level of significance will be 5%. I will report the error of my data using SEM. It is anticipated that the UNX Obese + Lead Compound rats and UNX Lean + Vehicle rats will have significantly decreased glomerulosclerosis, interstitial fibrosis, and epithelial injury, as compared to UNX Obese + Vehicle rats. One limitation is that these assays require fresh kidney tissue, thus it cannot be conducted using in-life collected biosamples such as blood or urine, thereby limiting the time points in which this assay can be performed to time of necropsy.

Finally, the secondary endpoints of this study will focus on measuring changes in mitochondrial form and function, specifically focusing on mitochondrial fragmentation (TEM), as well as ROS/SO production in vivo (urine 8-OH-dG levels) and in vitro (mitoSOX staining of primary PTECs). The level of mitochondrial fragmentation will be determined

using TEM and appropriate quantification, as previously described (Cassina, Chiaravalli & Boletta, 2020), where round shaped organelles were defined as more fragmented than those that appeared elongated. Urinary 8-OH-dG levels, a biomarker of oxidative stress in the kidneys, will be assessed via ELISA (Zhou et al., 2006). Finally, upon necropsy, PTECs will be collected from a portion of the rat kidneys as previously described (Gomez et al., 2015). They will then be cultured in vitro, stained using mitoSOX (a marker of mitochondrially-produced superoxide), and analyzed according to standard protocol (Gomez et al., 2015). As we will be comparing two substrains (Lean vs. Obese) and two treatments (Vehicle vs. Lead Compound), these data sets will then be statistically analyzed using a One-Way ANOVA with Tukey's test. The Null Hypothesis here will be that the mitochondrial fragmentation and urinary 8-OH-dG levels, as well as the number of mitoSOX-positive PTECs, of the UNX Obese + Vehicle rats will be equal to UNX Lean + Vehicle and UNX Obese + Lead Compound rats. The level of significance will be 5%. I will report the error of my data using SEM. It is anticipated that the UNX Obese + Vehicle rats will have significantly increased mitochondrial fragmentation, urinary 8-OH-dG concentrations, and mitoSOX-positive cells as compared to Lean Vehicle-treated groups. With treatment, however, the UNX Obese + Lead Compound group is expected to have significantly reduced mitochondrial fragmentation and urinary 8-OHdG concentrations, as well as fewer mitoSOX-positive cells, as compared to the UNX Obese + Vehicle group. One limitation is that the TEM and in vitro assays require fresh kidney tissue, thus it cannot be conducted using in-life collected biosamples such as blood or urine, thereby limiting the time points in which those assays can be performed to time of necropsy.

The main ethical considerations for this study are in regard to the urine collection protocol, which occurs in a metabolic caging system with wire flooring. To limit the stress

and discomfort of the rats, commercially available and industry-accepted metabolic cages will be used. Additionally, the urine collection timeframe will be limited to eight hours and a red-tinted igloo will be placed in the metabolic cage to promote natural behaviors. Additionally, the rat will be allowed free access to food and water during this time, using special systems included with the metabolic cage that prevent contamination of the urine samples. As the safety and tolerability of the Lead Compound has already been validated in previous studies, there are no additional ethical considerations regarding compound treatment for this study.

Specific Hypothesis 3: If the selected small molecule Nrf2 activator has pharmacological properties that enable its efficacy in vivo via oral dosing without the toxicity liabilities that limit the clinical use of Bardoxolone methyl, then oral dosing of the DOCA-Salt Hypertensive rat model of CKD with this compound will result in renal target engagement, without significant decreases in endothelin receptor A (ET_A) and endothelin-1 expression, weight reductions, reduced food intake, increased blood pressure, or dyslipidemia.

Here, we will use the DOCA-Salt Hypertensive rat model of CKD to investigate the potential for the lead Nrf2 activator compound to induce or avoid the adverse cardiovascular findings from the BEACON clinical trial. This rat model of cardiovascular oxidative and inflammatory stress demonstrates excessive Na^+ retention. Thus, it possesses the potential to recapitulate the rapid increase in blood volume and sodium retention noted in human patients treated with Bardoxolone methyl, which led to fluid overload and subsequent heart failure events (Rossing, 2013). In this model, young male rats will undergo a UNX followed by the subcutaneous implantation of a synthetic mineralocorticoid derivative (DOCA) in combination with salt loading in the drinking water. This design thus induces hypertension with cardiovascular remodeling that is characteristic of human volume-overload induced hypertension (Iyer, Chan & Brown, 2010), and is expected to potentiate the toxic liabilities that occur with Bardoxolone methyl treatment.

Sprague Dawley rats will be obtained from Charles River Laboratories (Strain Code 001) at approximately 4 weeks of age. Animals will be housed in our lab's associated vivarium in pairs and acclimated under 12-hour light-dark cycles with free access to water

and Lab Diets 5008 chow. All animal studies will be performed under protocols approved by the Institutional Review Board.

The primary aim of this study is to demonstrate whether treatment with the lead Nrf2 activating compound induces or worsens the adverse cardiovascular events associated with fluid overloading, as compared to a Bardoxolone methyl analog (RTA 405) or Vehicle treatment. It should be noted that the use of RTA 405 is required in this preclinical study as rodents may metabolize Bardoxolone methyl to toxic metabolites, thereby confounding results. The four experimental groups here include male Sprague Dawley rats that have been randomized into treatment groups and have undergone a UNX and either subcutaneous implantation with DOCA (200 mg/rat) with 1% saline drinking water or subcutaneous implantation with a rubber implant without DOCA and tap water to drink (n = 10 UNX + rubber implant + tap water; n = 10 UNX + DOCA implant + 1% saline drinking water + Vehicle; n = 10 UNX + DOCA implant + 1% saline drinking water + RTA 405; n = 10 UNX + DOCA implant + 1% saline drinking water + Lead Compound; Total n = 40 rats). Beginning at 6 weeks of age and occurring every week thereafter until the conclusion of the study, animal blood pressure will be monitored using the tail-cuff method on conscious semi-restrained rats after warming of the rat (Deng, Day & Schiffrin, 1996). At 8 weeks of age, the rats will undergo a partial nephrectomy, as described above. Additionally, beginning at 8.5 weeks of age and continuing through the completion of the study, the rats will be orally dosed with either Vehicle (twice daily), RTA 405 (once in the morning at 20 mg/kg body weight; once in the afternoon with Vehicle), or Lead Compound (twice daily at a pre-determined concentration to achieve EC_{90} at 12 hours post-dose). Rats will be euthanized as described above three weeks after the average of when the UNX + DOCA implant + 1% saline drinking

water + RTA 405 group becomes hypertensive (i.e., blood pressure > 150 mm Hg). Immediately following euthanasia, rats will be perfused with ice cold phosphate buffered saline solution to flush the blood from the tissue, and the remaining kidney will be harvested for analysis.

As described previously, rats will receive daily IP injections of Animalgesic, which is a suspension of extended-release buprenorphine, for three days following uninephrectomy to reduce post-surgical discomfort. Cage-side assessments will occur at least twice weekly (starting at 8 weeks until the end of the study period) to monitor for standard signs of pain and discomfort, including measurement of body weight, BCS (Table 1), and Animal Health Assessment. If animals lose weight (15% from peak body weight), then they will be monitored daily. However, if an animal reaches over 20% body weight loss or showed a poor body condition score of less than or equal to two, or if the animal was moribund, the rat will be euthanized immediately.

The dependent variables will be the gene expression of *Nqo1* and the protein expression of Nqo1 in the kidney (a key Nrf2 target gene and protein product), ET_A and endothelin-1 expression in the kidney (protein changes that occurred with Bardoxolone methyl treatment as a result of off-target effects of the drug), and weight reductions, reduced food intake, increased blood pressure, and dyslipidemia (signs of worsening heart failure). The independent variables will be the implantation status of the rats [i.e., DOCA or Rubber implants], drinking water [i.e., 1% saline water or tap water], and test article treatments [i.e., Vehicle, RTA 405, or Lead Compound].

In addition to the potential confounding variables listed above, here we must also consider the effect of the RTA 405 and Lead Compound treatments. In order to control for

the effect of the Lead Compound's vehicle, we will have treated the negative control group with an equal volume of vehicle on the same dosing schedule as the Lead Compound treated group. Additionally, as the RTA 405 group receives only once daily dosing in the morning, this group will receive an oral dose of vehicle in the afternoon at an equal volume and on the same dosing schedule as the other two groups. These measures will thus help to ensure that any differences seen in the readouts are due to the Lead Compound itself, and not because of handling or vehicle effects.

The primary endpoints of this study include measuring gene expression (*Nqo1*) and protein levels (Nqo1) of a key Nrf2 target gene in the kidney. These will be measured using standard RT-qPCR and Western blot techniques from whole tissue, as detailed above. Other primary endpoints include ET_A and endothelin-1 expression in kidney tissue, as measured by RNAscope (Deng, Day & Schiffrin, 1996), Western blot, and ELISA (Chin et al., 2014). Finally, changes in weight, food intake, blood pressure, and dyslipidemia will be measured according to standard procedure (Zoja et al., 2013).

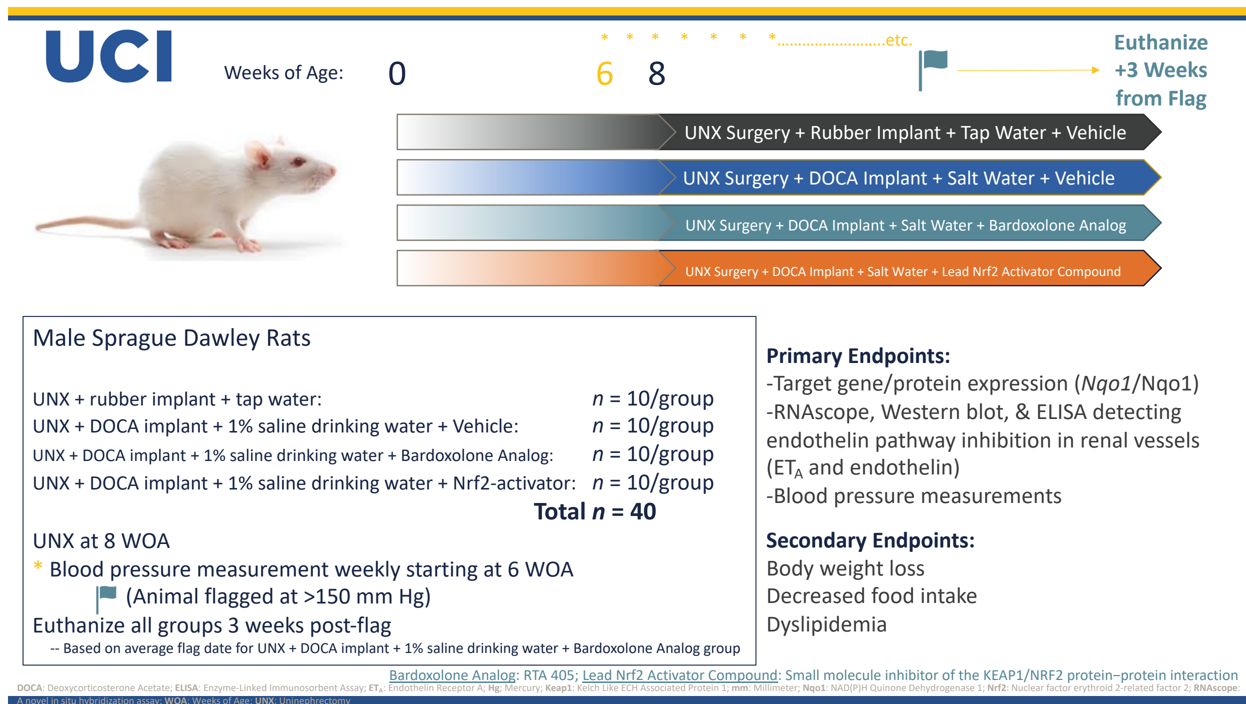


Figure 3. Effect of Lead Nrf2 Activator Compound Treatment on Target Gene Expression and Adverse Effects in the DOCA-Salt Hypertensive Rat Model of CKD

In order to control bias during the experiment and analysis, the test articles, drinking water solutions, animals, and sample ID's will be anonymized so that only the project manager is aware of their true group assignments, and the laboratory scientists performing and analyzing the experiments will be blinded.

During the data analysis for the RT-qPCR experiments assessing *Nqo1*, the data will be normalized to a housekeeping gene, such as *GAPDH*, to control for pipetting error. Furthermore, we will also normalize the ΔC_T values to the UNX + DOCA implant + 1% saline drinking water + Vehicle group to determine the fold change of gene expression for each measured gene product. During the data analysis for the Western blot experiments, the data will be normalized to a loading control, such as beta actin, to control for pipetting error. This method will determine the relative protein expression level changes for each protein lysate

tested. These data sets can then both be statistically analyzed using a One-Way ANOVA with Tukey's test, since we will be comparing two challenges (rubber implant + tap water; DOCA implant + 1% saline drinking water) and three treatments (Vehicle; RTA 405; Lead Compound). The Null Hypothesis here will be that the UNX + DOCA implant + 1% saline drinking water + Lead Compound group will have qPCR fold changes equal to 1 and densitometry plot measurements equal to UNX + DOCA implant + 1% saline drinking water + Vehicle. The level of significance will be 5%. I will report the error of my data using SEM. Here, it is expected that both the UNX + DOCA implant + 1% saline drinking water + Lead Compound and UNX + DOCA implant + 1% saline drinking water + RTA 405 groups will have significantly increased levels of Nqo1 protein and *Nqo1* transcript in tissue collected at necropsy, as compared to UNX + DOCA implant + 1% saline drinking water + Vehicle animals. One limitation is that these assays require fresh kidney tissue, thus it cannot be conducted using in-life collected biosamples such as blood or urine, thereby limiting the time points in which this assay can be performed.

Next we will assess the tissues for signs of endothelin pathway inhibition by measuring protein and transcript levels of ET_A and endothelin-1 in the renal tissue. During the analysis for the RNAscope experiments assessing ET_A and endothelin-1 levels in the kidney, whole slides will be scanned, and images will undergo semi-quantitative and quantitative analyses using HALO software by trained Advanced Cell Diagnostics, Inc. scientists. During the data analysis for the Western blot experiments, the data will be normalized to a loading control, such as beta actin, to control for pipetting error. Analysis of ET_A and endothelin-1 from whole kidney tissue by ELISA will be conducted using commercially available kits. These two methods will determine the relative protein

expression level changes for each protein lysate tested. These data sets can then be statistically analyzed using One-Way ANOVA with Tukey's test, since we will be comparing two challenges (rubber implant + tap water; DOCA implant + 1% saline drinking water) and three treatments (Vehicle; RTA 405; Lead Compound). The Null Hypothesis here will be that the UNX + DOCA + 1% saline drinking water + RTA 405 RNAscope quantifications, ELISA values, and densitometry plot measurements will be equal to UNX + DOCA implant + 1% saline drinking water + Vehicle and UNX + DOCA implant + 1% saline drinking water + Lead Compound. The level of significance will be 5%. I will report the error of my data using SEM. Here, it is expected that the UNX + DOCA implant + 1% saline drinking water + Vehicle group will show a significant increase of ET_A and endothelin-1 transcript and protein in tissue collected at necropsy, when compared to UNX + rubber implant + tap water + Vehicle animals, as the endothelin pathway is activated in the kidney by albumin and other noxious stimuli (Chin et al., 2014). It is also expected that the UNX + DOCA implant + 1% saline drinking water + RTA 405 group will show significant decreases of both ET_A and endothelin-1 transcript and protein in tissue collected at necropsy, as compared to UNX + DOCA implant + 1% saline drinking water + Vehicle rats. Finally, no significant difference between the ET_A and endothelin-1 transcript and protein levels is expected between the UNX + DOCA implant + 1% saline drinking water + Vehicle group and the UNX + DOCA implant + 1% saline drinking water + Lead Compound group. One limitation is that these assays require fresh kidney tissue, thus it cannot be conducted using in-life collected biosamples such as blood or urine, thereby limiting the time points in which this assay can be performed.

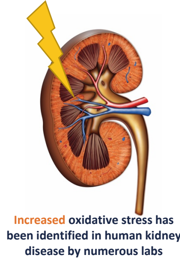
Finally, we will assess the animals for signs of worsening heart failure. During the analysis for body weight, food intake, blood pressure, and plasma lipid levels, raw data

values will be statistically analyzed using One-Way ANOVA with Tukey's test, since we will be comparing two challenges (rubber implant + tap water; DOCA implant + 1% saline drinking water) and three treatments (Vehicle; RTA 405; Lead Compound). The Null Hypothesis here will be that all the body weight, food intake, blood pressure, and plasma lipid levels measurements from the UNX + DOCA implant + 1% saline drinking water + RTA 405 group will be equal to UNX + rubber implant + tap water + Vehicle, UNX + DOCA implant + 1% saline drinking water + Vehicle, and UNX + DOCA implant + 1% saline drinking water + Lead Compound groups. The level of significance will be 5%. I will report the error of my data using SEM. Here, it is expected that UNX + DOCA implant + 1% saline drinking water + RTA 405 group will show significant increases of blood pressure and plasma lipid levels and significant decreases in body weight and food intake, as compared to UNX + DOCA implant + 1% saline drinking water + Vehicle rats. It is also expected that the UNX + DOCA implant + 1% saline drinking water + Lead Compound group will show no significant decrease in body weight or food intake nor increase in blood pressure or plasma lipid levels, as compared to UNX + DOCA implant + 1% saline drinking water + Vehicle animals. One possible limitation is that blood pressure measurements are sensitive to stress of the animal, so they must be performed quickly and by an experienced animal technician to reduce stress level increases.

GRAPHICAL ABSTRACT OF CAPSTONE

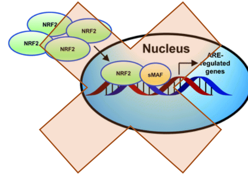
UCI

Summary of Capstone



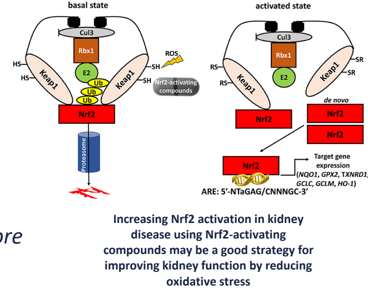
Increased oxidative stress has been identified in human kidney disease by numerous labs

But



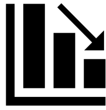
There is reduced activation of Nrf2 in human kidney disease,
despite Nrf2's published role in responding to increased oxidative stress which thus normally improves cell function

Therefore



Increasing Nrf2 activation in kidney disease using Nrf2-activating compounds may be a good strategy for improving kidney function by reducing oxidative stress

In This Capstone I Seek to Validate:



Decreased Nrf2 activation in a rat model of diabetic kidney disease,
thereby identifying an ideal animal model in which to test a Nrf2-activator compound

Increased Nrf2 activation, Increased renal function, Improved histology, and Reduced reactive oxygen species in a rat model of diabetic kidney disease following treatment with a Nrf2-activator compound,
thereby demonstrating amelioration of disease following successful activation of Nrf2 using pharmacological means

Absence of a worsened heart failure phenotype following treatment with a Nrf-2 activator compound,
thereby establishing a lack of toxic liabilities that were found in the clinically tested, non-specific Nrf2 activator: Bardoxolone methyl

Figure 4. Graphical Abstract of Capstone

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