Developing A Preclinical Model Of Human Sunitinib Cardiotoxicity To Assess The Role Of Mechanical Loading Using Engineered Cardiac Microtissues

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Abstract

ABSTRACT

DEVELOPING A PRECLINICAL MODEL OF HUMAN SUNITINIB CARDIOTOXICITY TO ASSESS THE ROLE OF MECHANICAL LOADING USING ENGINEERED CARDIAC MICROTISSUES

Rachel Elizabeth Truitt
Kenneth B. Margulies, MD

Sunitinib, a multi-targeted oral tyrosine kinase inhibitor used to treat many solid tumors, has led to important survival gains. However, this agent carries a significant risk of cardiotoxicity, with left ventricular dysfunction reported in up to 9.7% of treated individuals, and hypertension in 11-43%. There are a number of proposed mechanisms for sunitinib cardiotoxicity, however the relative contribution of each remains poorly understood. In particular, the relationship between increased left ventricular afterload toward inducing cardiac dysfunction remains unknown. Shortcomings of conventional cell culture and rodent models have hampered the identification of pivotal mechanisms of cardiotoxicity such as increased afterload. We instead chose to utilize a recently developed 3D in vitro microtissue model, where rat myocytes self-assemble to form microtissues.

Our model of human sunitinib cardiotoxicity recapitulated characteristics observed by other research groups, specifically, cardiomyocyte death, decreases in force generation and spontaneous beating, and demonstrated the dependence of these characteristics on sunitinib dose and treatment duration. Additionally, we observed decreases in mitochondrial membrane potential consistent with findings of mitochondrial abnormalities in patient biopsies. We demonstrated that increased in vitro afterload augments sunitinib cardiotoxicity. Finally, we created microtissues from cardiomyocytes derived from human pluripotent stem cells and found that afterload is required for sunitinib induced apoptosis at clinically relevant exposure concentrations.

Our finding that afterload is a key mediator suggests that anti-hypertensive therapy may be important for avoiding eventual LV dysfunction in patients treated with sunitinib.

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DEVELOPING A PRECLINICAL MODEL OF HUMAN SUNITINIB CARDIOTOXICITY TO ASSESS THE ROLE OF MECHANICAL LOADING USING ENGINEERED CARDIAC MICROTISSUES

Rachel Elizabeth Truitt

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Chapter 1: Introduction

The rise of small molecule inhibitors targeting receptor tyrosine kinases (RTKs) that regulate angiogenesis and proliferation has resulted in important gains in cancer survival. However, many of these “targeted” therapies have unintended consequences on the cardiovascular system. Sunitinib is a multi-targeted TKI that is used widely in the treatment of renal cell carcinoma, gastrointestinal stromal tumors, and neuroendocrine tumors and is currently under investigation in over 100 open clinical trials. Specifically, as it relates to sunitinib, hypertension occurs in 11-43% of patients and left ventricular (LV) dysfunction in 9.7%. These toxicities, although often manageable, can result in dose delays, treatment interruptions, or dose reductions.

Cardiovascular toxicity with sunitinib has been hypothesized to be a result of off-target inhibition of RTKs required for normal physiologic function. Sunitinib, used in the treatment of many solid tumors, demonstrates inhibitory activity across a number of RTKs including: vascular endothelial growth factor receptors (VEGFR1-3), platelet derived growth factor receptor (PDGFR-B), FMS-like tyrosine kinase (FLT-3/CD135), the stem cell receptor c-kit (KIT/CD117), and 5' AMP-activated protein kinase (AMPK), all of which have been shown to be either cardioprotective during times of stress or important for maintaining cardiovascular homeostasis. However, the relative contributions of each of these factors remains poorly understood. In particular, the direct effects of increased afterload on cardiac function in the setting of sunitinib remain speculative. Given the established link between increased afterload and the eventual development of LV dysfunction, we hypothesized increased afterload will augment the cardiotoxic effects of sunitinib.
Testing the hypothesis that increased afterload exacerbates sunitinib toxicity would require a large in vivo clinical trial and substantial expense, and may face various ethical roadblocks if we were to have cohorts of patients with uncontrolled hypertension. Current in vitro cell culture and animal models suffer from limitations that minimize their usefulness for modeling human sunitinib cardiotoxicity. The overall goal of this thesis was to create a preclinical model of human sunitinib cardiotoxicity using an in vitro microtissue model in which 3D cardiac microtissues (CMT) from neonatal rat cardiomyocytes self-assemble on silicone (PDMS) cantilevers. We used this system to characterize sunitinib cardiotoxicity using metrics for cell viability, mitochondrial dysfunction and contractile function, and examined how these characteristics are impacted by sunitinib dose, treatment duration, and degree of loading. We also performed preliminary experiments using CMTs composed of human pluripotent (iPS) derived cardiomyocytes and compared our results to neonatal rat CMT results.

The organization of this thesis is as follows: Chapter 2 provides background information on tyrosine kinase inhibitors and how we evaluate their cardiotoxic effects, such as hypertension and LV dysfunction, in a clinical setting. Next, we specifically focus on sunitinib, and discuss its observed cardiotoxic effects in patients and our current insights into the primary mechanisms governing sunitinib cardiotoxicity. We also discuss some of the limitations with current models being utilized to assess sunitinib cardiotoxicity. The final part of chapter 2 focuses on how we can take advantage of recent advances in human pluripotent stem cell derived cardiac cell types and tissue engineering to create novel preclinical models for evaluating drug induced cardiotoxicity.

Chapter 3 describes the experimental methods utilized for this thesis. We included information about isolation and cell culture techniques for both neonatal rat cardiac cell types as well as information on human pluripotent culture and cardiac differentiation.
methods. We also discuss microfabrication and seeding protocols for creating cardiac microtissues (CMTs). In the final part of this chapter, we discuss analytical methods used for detecting cell viability, cardiac function, and mitochondria function (membrane potential, ATP levels).

Chapter 4 discusses the in vitro model established to study sunitinib cardiotoxicity. Using the rat microtissue model, we characterized sunitinib cardiotoxicity in terms of its effects on cell viability and CMT function. We looked at caspase 3/7 activation and late apoptosis/necrosis to characterize cell viability. When examining effects on cardiac function, we analyzed changes in tissue force generation (diastolic and systolic) as well as electrophysiology (spontaneous beating rate, excitation threshold, maximum capture rate). We assessed the dependence on these factors on sunitinib dose and/or treatment duration.

In Chapter 5 we focused on elucidating some of the mechanisms of sunitinib cardiotoxicity. As mentioned above, we hypothesized that increased afterload would augment sunitinib cardiotoxicity. We created microtissues under different degrees of afterload using CMT model by changing the material properties of the pillars, which serves as the major source of afterload, and assessed caspase 3/7 activation in response to sunitinib treatment. We also examined changes in mitochondria function in response to sunitinib. Specifically we looked at differences in mitochondrial membrane potential in rat cardiomyocytes, and evaluated the dependence of this response on treatment duration. To see how changes in mitochondria function affect downstream energy production, we also measured cellular ATP levels. Finally, we evaluated the ability of an AMPK activator to reverse sunitinib induced caspase activation.
In Chapter 6 we show preliminary results with human CMTs created from pluripotent stem cells derived cardiomyocytes (iPS-CMs). We evaluate caspase 3/7 activation with sunitinib treatment and compared our findings to rat CMTs. We also measured caspase 3/7 levels in human CMTs subjected to different degrees of afterload to determine whether increased afterload augments sunitinib cardiotoxicity in human cells.

Chapter 7 summarizes the results of the present work and discusses their clinical relevance. We also outline future research plans for this project.
Chapter 2: Background Information

2.1 Tyrosine Kinase Inhibitors and their Cardiotoxic Effects

2.1.1 Development of Tyrosine Kinase Inhibitors (TKIs) as Anti-Neoplastic Agents:

Tyrosine Kinase Inhibitors (TKI’s) entered the drug landscape at a time when researchers were looking for more targeted therapies for treating cancer, as such drugs were predicted to have less overall toxicity compared to conventional anti-neoplastic treatments (anthracyclines, anti-metabolites) that were currently available on the market. Advances in genomics as well as our understanding of cell signaling networks allowed researchers to elucidate the differences between healthy and cancerous cells. From this body of research, receptor tyrosine kinases (RTK) emerged as a therapeutic target, as many cancers were found to be associated with erroneous activation of these receptors [Gschwind et al. 2004]. This finding was confirmed on the DNA level [Robinson et al. 2000]. RTKs play a major role in signaling networks associated with normal cell function, regulating processes associated with growth and differentiation, and are present in most tissues [Lodish et al. 2000]. Because cancer is associated with erroneous activation of these receptors, anti-neoplastic drugs were targeted towards partially silencing these RTKs, and hence the class of TKI drugs was born. The amount of cancer research dedicated to identifying effective TKI’s has increased dramatically since 1970 (Figure 2.1 panel A) [NCBI, PubMed].

The first TKI to be approved by the FDA was trastuzumab (Herceptin®, Genentech); a humanized mouse monoclonal antibody targeted against the human RTK epithelial growth factor 2 receptor (EGF2/ERbB2) in 1998 [Paul MK et al. 2004]. Its primary use was to treat metastatic breast cancer. Since the approval of trastuzumab, there
have been 27 TKIs approved by the FDA for a variety of different solid tumors (breast, colon, renal, etc.) since 2001 (Fig 2.1 panel B) [Wu et al. 2015].

**Figure 2.1: The rise of Tyrosine Kinase Inhibitors (TKIs).** A) Article count of TKI research in field of cancer over time (NCBI, PubMed). B) Timeline of FDA approval of protein and lipid kinase inhibitors. Reprinted from Trends in Pharmacological Sciences: P. Wu et al, “FDA-approved small-molecule kinase inhibitors”, Volume 36 Issue 7, 422-29, Copyright 2015 with permission from Elsevier. License no. 3980390063464

TKI drugs can either be single-targeted or multi-targeted. Looking at equilibrium dissociation constants (K_d) across a number of different substrates is useful for determining TKI specificity, with lower K_d constants being associated with increased binding affinity. In the figure below the authors determined dissociation constants for
numerous chemotherapy agents across numerous kinases (Figure 2.2) [Fabian et al. 2008]. Drugs like Lapatinib (labeled as GW-2016) only show binding affinity for a few substrates indicating a high degree of specificity. On the other hand, drugs like staurosporine and sunitinib (labeled as SU11248) have the ability to bind to numerous targets, giving them lower degrees of specificity. Multi-targeted TKIs with a low degree of specificity such as sunitinib have the potential to become problematic, as the drug may interfere with kinase activity required for normal physiological function, thus leading to unwanted side effects such as cardiotoxicity [Broekman et al. 2011]. Currently, there are over 300 open clinical trials [clinicatrials.gov] focused on TKI’s and tens of thousands of patients receiving these drugs, hence their impact will continue to grow, so it is important to identify any potential negative side effects associated with these drugs so we can maximize their use and safety.
Figure 2.2: Specificity profiles of various chemotherapy compounds. A plot of dissociation constants ($K_d$) of chemotherapy agents against a variety of target molecules. [Adapted by permission from Macmillan Publishers Ltd on behalf of Cancer Research UK: Nature Biotechnology, Miles A Fabian, William H Biggs, Daniel K Treiber, Corey E Atteridge, Mihai D Azimioara et al., “A small molecule kinase interaction map for clinical kinase inhibitors” Vol. 23 Issue 3: Copyright (2008); License no. 3979410866899]
2.1.2 Types of Cardiotoxicity

One major consequence of chemotherapy can be cardiotoxicity. Cardiotoxicity is defined as dysfunction relating to the heart (ischemia, arrhythmias, heart failure, myocarditis, pericarditis), as well as alterations in hemodynamics (hypertension, acute coronary syndrome) [Rodriguez 2015]. We will primarily focus our discussion on arrhythmias, hypertension, and heart failure as they are the most prevalent forms of chemotherapy induced cardiotoxicity observed in the clinic.

2.1.2.1 Arrhythmias:

Arrhythmias are defined as any abnormality in the heart’s electrical system that results in an abnormal heart rhythm. These electrical impulses may happen too quickly, too slowly, or erratically – causing the heart to speed up, slow down, or beat erratically [The American Heart Association Sept 2016]. The main consequence of this abnormal beating is ineffective pumping of blood to the rest of the body. While most arrhythmias are harmless, some can be life threatening, such as long QT syndrome, a condition that causes prolongation of the QT interval resulting from a mutation(s) in one or more ion channels (sodium, potassium, or calcium) [The American Heart Association Sept 2016]. In the context of drug development, identifying any changes in the QT interval is vital as a measure of the pro-arrhythmic potential of a candidate drug [FDA Center for Drug Evaluation and Research (CDER) 2005]. Pre-clinical studies utilizing mammalian cells (CHO, HEK293) transfected with the human Ether-à-go-go-Related Gene (hERG; potassium ion channel) and large animal models are widely utilized to assess the pro-arrhythmic potential of drugs [FDA CDER 2005], but have their limitations as we will explain later. Later in the drug approval process, arrhythmias are closely monitored
during clinical trials with healthy and diseased patients. Despite these efforts, chemotherapy induced arrhythmias are still an issue today. Table 2-1 gives a detailed list of the types of arrhythmias and which chemotherapy agents are associated with causing these arrhythmias [Tarmargo et al. 2015]. To summarize, atrial fibrillation occurs in patients taking alkylating agents, cisplatin, and anti-metabolites (7.9-10%, 12-32%, 0.55-12% respectively). Taxanes have a high incidence of sinus bradycardia (slowing of heart beat), about 30% according to the National Cancer Institute [Tarmargo et al. 2015]. Treatment with anthracyclines carries a risk, about 24%, of premature ventricular contractions [Tarmargo et al. 2015]. As for tyrosine kinase inhibitors, many have been demonstrated to block hERG channel, however their effects on QT prolongation are mild (<15ms) and the overall occurrence of arrhythmias is low (<2%) [Tarmargo et al. 2015; Shah RR et al. 2014&2015]. Large effects on the QT interval (>500ms) have been observed in <2.3% of patients for the TKI sunitinib [Shah RR et al 2014&2015]. Additionally there have been only isolated cases of atrial fibrillation with TKI therapy [Tarmargo et al. 2015; Shah RR et al. 2014&2015]. In summary, arrhythmia disorders are often times a consequence of chemotherapy treatment, but depending on the agent, the type and risk of arrhythmias can vary significantly.
Table 2-1: Types of cardiac arrhythmias induced by chemotherapy drugs. [Original Source: Drug Safety, Cancer Chemotherapy and Cardiac Arrhythmias: A Review, Volume 38 Issue 2, 2015, Juan Tamargo. Reproduced with permission from Springer: License no. 3979440363625]

<table>
<thead>
<tr>
<th>Type of proarhythmia</th>
<th>Causative dng</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sinus bradycardia</td>
<td>Amsacrine, arsenic trioxide, bortezomib, capecitabine, cisplatin, combretastatin, crizotinib, cyclophosphamide, cytarabine, daunorubicin, fludarabine, 5-FU, mitoxantrone, paclitaxel, ponatinib, rituximab, taxanes, thalidomide, vinca alkaloids, vorinostat</td>
</tr>
<tr>
<td>AV block</td>
<td>Amsacrine, arsenic trioxide, bortezomib, capecitabine, cisplatin, cyclophosphamide, daunorubicin, doxorubicin, epirubicin, 5-FU, ifosfamide, IL-2, interferon-α, mitoxantrone, ponatinib, rituximab, taxanes, thalidomide</td>
</tr>
<tr>
<td>Intraventricular conduction block</td>
<td>Cisplatin, 5-FU, imatinib, paclitaxel, trastuzumab</td>
</tr>
<tr>
<td>Sinus tachycardia</td>
<td>Amsacrine, arsenic trioxide, bortezomib, bosutinib, capecitabine, carbustine, cyclophosphamide, epirubicin, 5-FU, paclitaxel, romidepsin, vorinostat</td>
</tr>
<tr>
<td>Atrial fibrillation</td>
<td>Aminotumuban, amsacrine, bortezomib, cetuximab, cisplatin, cyclophosphamide, doxorubicin, gemcitabine, ifosfamide, IL-2, interferon-α, melphalan, ponatinib, rituximab, sorafenib, sunifilin, taxanes, vinca alkaloids</td>
</tr>
<tr>
<td>Supraventricular tachycardias</td>
<td>Amsacrine, capecitabine, cisplatin, cyclophosphamide, daunorubicin, doxorubicin, ifosfamide, interferon-α, IL-2, melphalan, ponatinib, taxanes</td>
</tr>
<tr>
<td>QT prolongation</td>
<td>Amsacrine, arsenic trioxide, bosutinib, cabozantinib, capecitabine, combretastatin, daunorubicin, doxorubicin, enzastaurin, erulinexilate, HDAC inhibitors (dacinostat, panobinostat, romidepsin, vorinostat), rituximab, small-molecule PTK (dasatinib, lapatinib, nilotinib, ponatinib, sorafenib, sunifilin, vandetanib)</td>
</tr>
<tr>
<td>Premature ventricular contractions</td>
<td>Capecitabine, bortezomib, 5-FU, ifosamide, interferon alpha/gamma, methotrexate, rituximab, taxanes, vincristine</td>
</tr>
<tr>
<td>Ventricular tachycardia/fibrillation</td>
<td>Alkylating agents (Cisplatin, cyclophosphamide, ifosfamide, melphalan), amsacrine, antimetabolites (capecitabine, 5-FU, gemcitabine, methotrexate), anthracyclines (daunorubicin, doxorubicin), arsenic trioxide, dasatinib, HDAC inhibitors (panobinostat, romidepsin), interferon-α/γ, IL-2, monoclonal antibodies (alemtuzumab, rituximab, trastuzumab), taxanes</td>
</tr>
<tr>
<td>Torsades de pointes</td>
<td>Amsacrine, arsenic trioxide, cabozantinib, capecitabine, cisplatin, doxorubicin, 5-FU, interferon-α, nilotinib, romidepsin, rituximab</td>
</tr>
<tr>
<td>SCD</td>
<td>Amsacrine, arsenic trioxide, cabozantinib, capecitabine, cisplatin, doxorubicin, 5-FU, interferon-α, nilotinib, romidepsin, rituximab</td>
</tr>
</tbody>
</table>

5-FU 5-fluorouracil, AV atrioventricular, HDAC histone deacetylase, IL-2 interleukin-2, PTK protein kinase inhibitor, SCD sudden cardiac death

2.1.2.2 Hypertension and its Link to Cardiac Function

Hypertension is a relatively common cardiovascular toxicity caused by chemotherapy. Hypertension is defined as a systemic blood pressure greater than 140/90 mmHg. Increased blood pressure can cause damage to arteries, leading to long-term consequences including abnormal vasomotion, atherosclerosis, and ultimately result in inadequate blood flow to organs (heart, kidneys, brain) and increase the risk for myocardial infarction, kidney failure, and stroke [The American Heart Association Oct 2016]. TKI’s are especially known for inducing hypertension, as many of these drugs were designed to inhibit angiogenesis, via vascular endothelial growth factor receptor (VEGFR) and/or platelet derived growth factor receptor (PDGFR) inhibition. Thus, TKIs
carry a high risk for causing vascular dysfunction and increased oxidative stress [Shah RR et al 2015; Chintalgattu et al. 2010; Di Siena et al. 2016; Zentilin et al. 2010]. Studies report an 11-43% incidence of hypertension with anti-angiogenic TKI therapy [Guverich et al. 2009]. These patients have a 7-8 fold increased relative risk for hypertension [Guverich et al. 2009]; thus blood pressure should be monitored during a patient’s treatment with TKI therapy.

Increased blood pressure also has direct effects on the heart. The left ventricle of the heart, the “pump” responsible for ejecting blood into the aorta, must generate enough pressure to match circulating blood pressure in order for aortic valve to open and eject blood. Thus, during a hypertensive state, ventricles are required to generate more systolic (active) force. We use the term afterload to describe the external load the myocardium (heart) must overcome before shortening (ejection) can begin [Norton JM 2001]. Therefore hypertension causes increased afterload on the heart. This is significant because increased demands for force generation will also have effects on the amount of volume pumped out by the ventricle (stroke volume, SV) [Klabunde 2015]. Figure 2.3 (panel A) depicts what is known as the force-velocity relationship for cardiac muscle. When force is at a maximum, such as when the heart is under high degrees of afterload, contraction velocity is approaching zero. As the amount of afterload/force decreases, the velocity of contraction increases. Therefore if the ventricle is forced to pump harder, it can’t pump as quickly and less blood ultimately gets ejected, hence stroke volume decreases (Figure 2.3 panel B) [Klabunde 2015]. If not compensated for, this would be a major problem as organs wouldn’t be receiving enough blood. Luckily hearts have another mechanism to compensate for decreased SV due to increased afterload. The increased volume of blood remaining in the ventricle due to the afterload increase will cause the ventricle to stretch more during filling process; this stretch on the ventricle is called preload. According to
the Frank-Starling mechanism, this increase in preload will lead to increase in stroke volume [Brady JM 1991]. Hence, increases in afterload can be partially compensated for by increases in preload. However, if a patient has defects in the Frank-Starling mechanism due to an underlying cardiovascular condition, a hypertensive (increased afterload) state could be lead to decreased cardiac output and left ventricular dysfunction, which I will be discussing in the next section [Fernandes-Silva et al. 2016; Ozkan et al. 2011].

Figure 2.3: Effect of Afterload on Cardiac Force Generation. A) Force-Velocity Relationship. B) Increased afterload decreases stroke volume (SV) which results in increased filling pressures (LVEDP) during next cardiac cycle. [Original Source: Richard E. Klabunde, CV Physiology 2015. “Cardiac Afterload” and “Cardiac Muscle Force and Velocity Relationship” www.cvphysiology.com ]
2.1.2.3 Left Ventricular (LV) Dysfunction

The previous section already alluded to one mechanism for LV dysfunction, through hypertension and its resulting effects on the heart. There are a number of factors that can contribute to LV dysfunction both genetic and environmental. Chemotherapy treatment, for example, carries a risk of killing cardiac myocytes via apoptosis; this loss of cells puts more workload on the remaining cells. The ventricle chamber expands and dilates in an effort to normalize stresses, however ultimately normal cardiac output cannot be restored (Figure 2.4 panel A) and the patient develops heart failure. There are a number of additional factors contributing to development of heart failure such as sustained activation of neurohumoral systems that ultimately induce maladaptive changes in individual cardiac myocytes, inflammatory factors, myofibroblast differentiation/fibrosis, and changes in cardiac metabolism [Minguell ER 2004; Fan D et al. 2012; Kolwicz SC et al. 2013]. In the case of myocardial infarction or chemotherapy induced toxicity, the initial loss of myocytes initiates a cascade of maladaptive remodeling that ultimately results in heart failure. Heart failure is usually classified by the severity of the patient’s symptoms. Figure 2.4 (panel B) shows the New York Heart Association’s (NYHA) heart failure classification system:

The overall incidence of all-grade and high-grade chronic heart failure associated with anti-angiogenic (VEGFR) TKIs was 3.2 % (95 % CI 1.8–5.8) and 1.4 % (95 % CI 0.9–2.3) [Shah RR et al. 2015], respectively. A meta-analysis of randomized phase II and III clinical trials of patients with solid tumors receiving sunitinib, axitinib, cediranib, or
regorafenib reported a relative risk of all-grade cardiac dysfunction to be 2.36 (95% CI 0.95–5.87; p = 0.06). [Shah RR et al. 2015]

2.1.2.4 Distinguishing Direct and Indirect Mediators of TKI Induced Cardiotoxicity

The previous sections discussed the various ways in which cardiotoxicity from chemotherapy treatment can present itself (arrhythmias, hypertension, and LV dysfunction). What isn’t discussed or studied as frequently is whether cardiotoxicity is due to: 1) intrinsic toxicity of the chemotherapy agent, or 2) indirect effects, such as toxicity to a different organ system. In the case of TKIs, many induce hypertension due to their anti-angiogenic properties, and, as I discussed above, hypertension can cause LV dysfunction. However, depending on the drug’s RTK specificity, the drug could also be directly toxic to cardiac myocytes by disrupting their own RTK dependent pathways, which can result in LV dysfunction. So a major question arises; what are the relative contributions of hypertension (i.e. increased afterload) and intrinsic toxicity to LV dysfunction? We will attempt to answer this question in Chapter 5 of this dissertation, which explicitly looks at toxicity induced by sunitinib under varying degrees of afterload.

2.1.3 Diagnosing and Monitoring Cardiotoxicity in the Clinic

Despite the prevalence of chemotherapy-induced cardiotoxicity, there are currently no specific guidelines for treating and monitoring it [Albini et al. 2010]. Nevertheless, the field of cardio-oncology has emerged in response to the complex decision making necessary to balance the benefits and risks associated with treating cancer. Cardio-oncologists rely on a battery of tests (imaging, biopsy, biomarkers in blood) in order to formally diagnose patients with chemotherapy induced cardiotoxicity. This section will review some of these methods and how they relate to diagnosis criteria.
2.1.3.1 Methods for Diagnosing Chemotherapy Induced Cardiotoxicity

Endomyocardial biopsy remains the most sensitive method for detecting cardiotoxicity, although it is also the most invasive method. With this method, small samples of heart tissues are analyzed for distortions in myocyte organization. Electrocardiograms and Echocardiograms (2D or 3D) are utilized more often in the clinic as they are far less invasive. Electrocardiograms can reveal dysfunction in the heart’s electrical activity, and thus are useful for detecting arrhythmias, which can be side effects of certain chemotherapy regimens. Echocardiograms, on the other hand, are useful for looking at diastolic (filling) and systolic (pumping) function of the heart and the morphology of the heart’s chambers and valves. Echocardiograms are frequently used to calculate and monitor the ejection fraction of the left ventricle (LVEF); which represents the fraction of blood pumped out of the heart relative to the amount that of blood in the chamber at the end of filling (systolic function). Normal LVEFs typically range from 55%-65%, and significant decreases (>10%) over time are associated with LV dysfunction. One major disadvantage of looking at LVEF is that declines often times do not occur until later stages of cardiotoxicity. Ideally we would like to identify cardiotoxicity at its earliest stages. [Pizzino F et al. 2014; Sawaya H et al. 2012].

Next, we will discuss some emerging technologies for early detection of cardiotoxicity (i.e. precedes declines in LVEF). One of these techniques is called speckle-tracking imaging. This method focuses on analyzing the 3D twisting and torsion deformations that occur during contraction by tracking natural acoustic and inference patterns called “speckles” within an ultrasonic window. The deformation between speckles within a region is calculated, obtaining a value referred to as strain. The velocity of strain is defined as strain rate. Several studies have looked at variations in strain and strain rate in patients receiving chemotherapy and found that these values are early
predictors of eventual declines in LVEF. Magnetic Resonance imaging can also be utilized for tissue characterization to detect things like early fibrosis by administering gadolinium and looking for late gadolinium enhancement. Late gadolinium enhancement is when a tissue presents slow contrast wash-out, which is usually a sign of scar tissue/fibrosis. Early alterations of late gadolinium enhancement have been described in chemotherapy-treated patients and are predictive of declines in LVEF. [Pizzino et al 2014].

The final technique we would like to discuss is the use of biomarkers. The presence of cardiac specific proteins such as cardiac troponin is indicative of cardiac damage [Yu EF et al 2016]. Elevations in troponin levels are usually used to diagnose myocardial infarction (heart attacks). Troponin levels measured after chemotherapy treatment have been shown to be predictive of later cardiotoxicity, as has myeloperoxidase, with the combination of these two biomarkers representing the highest risk for future cardiac dysfunction [Pizzino et al 2014]. However, these studies were conducted with small patient populations and larger studies are required to confirm these results. Biomarker testing is relatively non-invasive and easy to perform and analyze.

In summary, there are a variety of methods for detecting cardiotoxicity, with some new emerging technologies that may allow for detecting very early signs of cardiotoxicity, specifically those that precede LVEF decline. Ideally, cancer patients should get testing done before treatment to get baseline levels of biomarkers (troponin) and LVEF and have these quantities monitored during and after treatment [Yu EF et al. 2016]. This is especially important if the patient has a previous history of cardiovascular disease, as it has been shown that patients who experience the most adverse cardiac outcomes following chemotherapy treatment are ones who had underlying cardiovascular disease before treatment [Albini et al. 2010].
2.1.3.2 Diagnosis Criteria for Chemotherapy Induced Cardiotoxicity

The Cardiac Review and Evaluation Committee (CREC) defines chemotherapy-induced cardiotoxicity as the presence of at least one of the following elements: [Pizzino et al. 2014]

1. Cardiomyopathy characterized by a decrease in LVEF

2. Symptoms of congestive heart failure (see NYHA guidelines)

3. Associated signs of congestive heart failure such as but not limited to: tachycardia or S3 gallop.

4. Decline of LVEF of at least 5% to below 55% with accompanying signs of congestive heart failure; or a decline of LVEF of at least 10% to below 55% without accompanying symptoms.

2.1.3.3 Categories of Chemotherapy Induced Cardiotoxicity

Cardiac dysfunction due to chemotherapy can be divided into three categories: 1) acute, 2) subacute, or 3) chronic. Acute or subacute cardiotoxicity can develop any time from the start of treatment up to 2 weeks after the completion of chemotherapy. Chronic cardiotoxicity, on the other hand, might not present itself until almost a year after the completion of treatment (early cardiotoxicity) or more than one year after treatment (late cardiotoxicity). This further reinforces the notion that patients should be monitored (biomarkers, LVEF measurements), not just during treatment, but also for a significant amount of time after treatment. Additionally, methods for pre-treatment testing with predictive value may be worth developing [Albini et al. 2010].
2.2 Sunitinib Malate: A Tyrosine Kinase Inhibitor with Cardiotoxic Effects

2.2.1 Mechanism of Action

Sunitinib (SU11248) was originally developed by a small biopharmaceutical company named SUGEN, after three of their other PDGF inhibitors failed in clinical trials (1994-1999). Two acquisitions later, sunitinib became the property of Pfizer who finished phase III clinical trials for the drug. Eventually, the drug was approved in January 2006 for the treatment of renal cell carcinoma (RCC) and gastrointestinal stromal tumors (GIST) [NIH/National Cancer Institute]. Originally designed as a PDGFR inhibitor to target tumor angiogenesis, sunitinib actually exhibits inhibitory behavior across a number of RTKs associated with angiogenesis such as VEGFR1-3, PDGFR(a, b), FMS related tyrosine kinase (FLT-3), and the stem cell receptor KIT [O’Farrell et al. 2003; Faivre et al. 2007]; . Compared to similar TKIs available at the time, such as Gleevec (imatinib), sunitinib had more of a multi-targeted effect [Fabian et al 2008]. This attribute could explain why sunitinib successfully treated tumors that were resistant to Gleevec [Demetri et al. 2006]. Currently, more than 100,000 people have been treated with sunitinib, and there are over 100 open clinical trials with this drug [clinicaltrials.gov]. This fact stresses the notion that we need to better our understanding of sunitinib cardiotoxicity, as even more people will be treated with this drug in the future.

2.2.1 Clinical Incidences of Sunitinib Cardiotoxicity

Early incidences of cardiotoxicity were observed during phase I B trials with leukemia (AML) patients when researchers were trying to determine dosage and safety. In a trial of 16 patients given sunitinib (50mg or 75mg dose), the 2 patients given the 75 mg dose experienced grade 4 hypertension and one patient developed heart failure (after
suffering a myocardial infarction) and died “due to cardiac insufficiency”. The authors commented that they believe this death could have been related to sunitinib treatment. As a result of this cardiotoxicity, the authors concluded that 50mg is the maximum tolerable dose for these patients. [Fiedler et al. 2005]

Cardiovascular toxicity of sunitinib was also reported during numerous Phase III clinical trials. In one study, GIST patients taking 50mg of sunitinib per day reported cases of Grade 1/2 (15 patients/8%) and Grade 3 hypertension (6 patients/ 3%). There were no reported cases of heart failure with 50mg dose [Demetri et al. 2006]. However in two separate phase III trials, there were reports of LV dysfunction. Telli and colleagues reported that 7 out 48 enrolled patients experienced grade 3 or 4 symptomatic LV dysfunction anywhere from 22 to 435 days after treatment. Three of those patients continued to experience heart failure even after sunitinib was removed and they began treatment for heart failure. The authors noted that a history of cardiovascular disease was a major factor in this result [Telli et al 2008]. Along these lines, Hall and colleagues published results from a phase III trial with sunitinib where they defined the number of patients with either hypertension or LV dysfunction, both before and after treatment. These results are plotted below in Figure 2.5. [Hall et al. 2013].
Figure 2.5: Hypertension and LV Dysfunction following treatment with sunitinib.
Number of patients with various grades (0-4) of hypertension (left) and/or LV Dysfunction (right) before (blue) and after (red) treatment with sunitinib. Figures were created using data from Table 2 of G. DiLorenzo et al. Ann. Oncol. 2009.

This figure shows a general trend towards worsening degrees of both hypertension and LV dysfunction following sunitinib treatment. These studies as a whole not only demonstrate the potential for adverse cardiovascular outcomes with sunitinib, they also serve as an example of how factors like medical history can play a role in whether or not a patient will develop cardiotoxicity. These confounding factors highlight the need for more standardized screening methods for drug toxicity in human cells. Chapter 4 discusses the creation of a preclinical tissue culture platform that can be combined with human cells to assess the toxicity of candidate drugs.

2.2.1 Insights into the Molecular Mechanisms of Sunitinib Cardiotoxicity

2.2.1.1 Animal and In Vitro Cell Culture Study Design

Following reports of cardiotoxicity during human clinical trials, researchers aimed to gain a better understanding of 1) sunitinib toxicity at the cardiomyocyte level and 2) the underlying mechanisms governing this toxicity. One obvious cause of cardiotoxicity
could be off-target inhibition of angiogenesis in the heart. As mentioned earlier, sunitinib can inhibit a variety of TKI’s associated with angiogenesis (VEGFR1-3, PDGFR-A/B, FLT-3, and the stem cell receptor KIT). It is widely recognized that many, if not all, of these receptors are critical for maintaining cardiovascular homeostasis and/or protecting the heart during times of increased stress. In this next section I will discuss studies that aimed to elucidate effects of sunitinib on cardiomyocytes and identify some key signaling pathways involved in this response.

The majority of these studies employed either adult mice or primary rat cardiomyocyte cell cultures. One study I will discuss utilized human atrial muscle strips. The doses of sunitinib typically utilized in these studies ranged from 0.1-250µM (mostly 1-10µM range) from cell culture studies and 20mg/kg -100 mg/kg per day for animal studies. To put these doses into perspective, a 50 mg/day treatment regimen for a patient results in average sunitinib concentrations in the blood (plasma) at around 0.1-0.2µM with peak plasma concentrations at 2µM sunitinib [Faivre et al. 2005; Harvey and Leinwand 2015]. In view of this, some experiments appear to utilize doses that are outside the physiological range. Some of the metrics used for characterizing sunitinib toxicity in these include:

1. **Apoptosis**: Caspase 3/7, 9 activation, TUNEL, cytochrome C release

2. **Cell Viability**: LDH release, mitochondrial morphology

3. **Metabolism and Energetics**: ATP levels, lipid drop formation

4. **Functional**: EF, blood pressure, calcium transients, contractile reserve

Alterations in cell signaling were also examined in many of these studies. Those findings will be discussed later in this section.
2.2.1.2 Results from Animal Studies: Insights into Afterload as a Possible Mediator of Sunitinib Cardiotoxicity

In studies using adult mice treated with sunitinib, mitochondrial morphology changes (swelling) could be observed with as little as 10 mg/kg per day of sunitinib [Chu et al. 2007]. Cardiac myocyte hypertrophy was observed with 25 mg/kg per day sunitinib, however neither hypertension nor LV dysfunction were observed at this dose. However, if phenylephrine (PE) was administered concurrently with sunitinib (25 mg/kg), there were signs of cardiomyocyte apoptosis, measured by caspase activation [Chu et al. 2007; Kerkela et al. 2007]. This is an interesting finding as PE can increase blood pressure, which would increase the amount of afterload on the heart. This result suggests that sunitinib toxicity might be dependent on mechanical loading conditions in the heart. However, PE has also been shown to act directly on cardiac myocytes by inducing myocyte hypertrophy [Clerk et al. 1998]. Therefore, while PE isn’t the best choice for regulating afterload, nevertheless these results are interesting. Finally, doses of sunitinib exceeding 40 mg/kg per day have been shown to induce LV dysfunction [Khakoo et al. 2013]. The authors observed decreases in EF as well as contractile reserve. Additionally, hearts treated with sunitinib suffered greater dysfunction after undergoing TAC (trans-aortic constriction), an intervention that greatly increases the amount of afterload experienced by the heart (referred to as “pressure overload”) [Khakoo et al. 2007]. Again, we see signs that sunitinib toxicity may be regulated by afterload. Like PE administration, TAC has its disadvantages as a method for increasing afterload because it is pathologic independent of sunitinib treatment. In Chapter 5, we will discuss how we can use our microtissue platform to assess the contribution of afterload to sunitinib cardiotoxicity.
2.2.1.3 Results from Primary Rat Cell Culture Studies

In studies that utilized doses of sunitinib around 0.1-10µM, cytochrome-C and caspase 3/7 activation were observed indicating rapid apoptosis of neonatal rat cardiac myocytes. Maayah and colleagues report decreases in cell viability of 35%, 50%, and 70% in cultures treated with 25µM, 50µM, and 100µM sunitinib respectively [Maayah et al. 2014]. There were also reported decreases in ATP levels as well as mitochondria membrane potential [Maayah et al. 2014]. The effect on cell energetics prompted Kerkela and colleagues to investigate dysregulation of AMPK (5' AMP-activated protein kinase) as the culprit. In general, AMPK is activated during times of energy depletion and helps maintain ATP levels by restricting energy utilization and increasing energy production. Kerkela et al. discovered that sunitinib can directly inhibit the activity of AMPK (i.e. phosphorylation of acetyl CoA carboxylase, p-ACC). This could contribute to decreased ATP levels in cardiac myocytes treated with sunitinib [Kerkela et al. 2009].

In addition to affecting viability and energetics, changes in myocyte function were associated with sunitinib treatment. Rainer and colleagues reported that 1.87µM sunitinib was sufficient to negatively affect sarcomere shortening and reduce calcium transients in isolated adult rat cells [Rainer et al. 2012]. As a whole these studies suggest that sunitinib negatively affects cardiomyocyte energetics via inhibition of AMPK, viability, and function. However many studies were performed using sunitinib levels outside the range of concentrations that are clinically relevant.
2.2.1.4 Results from Human Muscle Strip Study

In addition to examining sunitinib toxicity in single adult mouse myocytes, Rainer and colleagues also assessed toxicity in muscle strips from the right atrium of patients. This part of their study stands out because it’s the only set of in vitro experiments utilizing adult human heart tissue. Muscle strips are difficult to obtain and have very high metabolic demands and unfortunately most experiments can only last for 24hr before muscle strips fail. Hence muscle strips do not serve as strong pre-clinical model for drug toxicity. Nevertheless, this study serves as model of relevant human biology and its results guided the design of our own functional experiments in rat cells. Less than 30 min of treatment with 1.87µM and 18.7µM sunitinib was sufficient to affect active force generation (decreased by 8% and 15% respectively) by muscle strips with no effect on diastolic forces. This finding helps to validate observations of LV dysfunction in human patients treated with sunitinib. [Rainer et al. 2012]

2.2.1.5 Summary and Study Limitations

In summary, the literature provides evidence that sunitinib can lead to LV dysfunction and cellular apoptosis, as well as depletion of ATP stores and mitochondrial dysfunction. However, the results presented here are highly dependent on the dose and duration of sunitinib treatment. There exists a need for more studies aimed specifically at characterizing the effects of sunitinib dose and duration on cardiotoxicity. Additionally, animal studies reveal a possible contribution of afterload to sunitinib cardiotoxicity, however better methods for controlling afterload are needed as current methods, such as PE and TAC, can have off-target effects on myocytes. Finally, there is only a single study examining sunitinib cardiotoxicity in isolated human tissues. Moreover, this study is
limited by the use of atrial (rather than ventricular) tissue and by relatively high doses of sunitinib treatment. This thesis will aim to address both of these shortcomings in the literature.

2.3 Utilizing Human Pluripotent Derived Cardiomyocytes and Tissue Engineering Methods as Models for Detecting Sunitinib Induced Cardiotoxicity

2.3.1 Establishing Standardized, Relevant Screening Methods for Predicting Cardiotoxicity

The previous sections have revealed significant shortcomings in our 1) ability to predict or identify sunitinib induced cardiotoxicity its early stages and 2) biologic understanding of mechanisms behind sunitinib’s cardiotoxic effects. Current preclinical models are not well equipped to address these concerns [Astashkina et al. 2012]. CHO cells transfected with hERG channels are poor reductionist models of cardiac myocytes due to the lack of any contractile apparatus and complex ion channel network [FDA CDER 2005]. Animal models suffer from interspecies differences in cardiac biology, which are well documented and cannot be ignored [Seok et al 2013; Houser et al. 2012]. Developing robust preclinical models of sunitinib cardiotoxicity would allow us to begin addressing both issues. A good preclinical model would: 1) utilize human cell inputs; 2) permit control over cellular, biochemical, extracellular matrix (ECM), and mechanical cues and 3) provide functional readouts that can be used as metrics for toxicity. This section will outline how we can derive suitable human cardiac cell sources and utilize current tissue engineering methods as an in vitro testing platform.
2.3.2 The Rise of Human Cardiac Cell Sources from Pluripotent Stem Cells

2.3.2.1 Advances in Cardiac Differentiation from Human Pluripotent Stem Cells

Given the complexity of the human heart, differentiating human pluripotent stem cells (hPSCs), such as induced pluripotent (iPS) or embryonic stem (ES) cells, into cardiac cell types presented major challenges for researchers (see Figure 2.6 for an overview). Cardiac development is a complex interplay of various signaling pathways that are activated at specific time points during development [LaFlamme et al. 2011]. Researchers Gordon Keller and Charles E. Murry were among the first to integrate this idea into their research. They identified Bone Morphogenic proteins (BMPs), specifically BMP-4, and Activin-A (nodal TGF-Beta pathway) as pivotal molecules for mesoderm determination, a critical step in cardiogenesis. [Kattman et al. 2011; Lian et al. 2013]

![Figure 2.6: Complexity of Cardiac Differentiation during development](Image)

Figure 2.6: Complexity of Cardiac Differentiation during development. Various signaling pathways involved in the differentiation of pluripotent stem cells to cardiac myocytes. [Adapted by permission from Macmillan Publishers Ltd on behalf of Cancer Research UK: Nature, Michael A LaFlamme and Charles E Murry, “Heart Regeneration” Vol. 473 Issue 7347, Copyright 2011: License no. 3984420749070]
Murry and Keller’s differentiation protocols often times called for numerous growth factors supplemented in expensive basal media such as StemPro®. Thus their differentiation strategies were not cost-effective when it came to scaling up production of hPS-CMs [Kattman et al. 2011]. The Wu lab at Stanford developed a more cost effective strategy for creating a chemically defined media that produced highly pure (>80%) cultures of human cardiomyocytes that is increasingly used today. The protocol calls for fewer growth factors, as well as a more cost effective basal media (RPMI 1640). Furthermore, this differentiation protocol was successful on a number of iPS cell lines, despite genetic differences between the cell lines. Many of the human experiments conducted in this work utilized this differentiation method and will be discussed in more detail in later sections. [Burridge et al. 2014]

2.3.2.1 Differences Between hPS-CMs and Adult Human Cardiomyocytes

To utilize hPS-CMs for drug screening, ideally we would like these cells to exhibit a phenotype resembling that of an adult cardiomyocyte. However, despite advances in CM differentiation and maturation methods, hPS-CMs do not exhibit many of the characteristics of an adult myocyte. These cardiomyocytes are postulated to behave more like fetal cardiomyocytes. Yang et al. highlighted many of these differences in a recent review article; and the many differences are highlighted below (Table 2-2). [Yang et al. 2014]
Table 2-2: Differences between adult cardiomyocytes and hPS-CMs. Immature Cardiomyocytes, such as hPS-CMs, do not display the same behaviors as adult human cardiomyocytes. [Adapted by permission from Wolters Kluwer: Circulation Research, Xiulan Yang, Lil Pabon, Charles E. Murry, “Engineering Adolescence” Vol. 114 Issue 3, Copyright 2014; License no. 3984421278291]

While there are various differences amongst fetal and human cardiomyocytes, hPS-CMs still hold great potential for studying human cardiac diseases and for drug screening. However, investigators must realize that certain biological questions are not suitable to answer with hPS-CMs because they don’t exhibit that particular adult phenotype. Some examples include: changes in metabolism with heart failure and studying inotropic responses; this also must be kept in mind for determining metrics for drug induced cardiotoxicity. However, hPS-CMs remain useful to for determining whether a drug is intrinsically toxic (i.e. induces apoptosis, necrosis) [Burridge et al 2016; Clements et al. 2015; Gilchrist et al 2015]. It is for that reason we sought to continue to utilize hPS-CMs in some of our experiments presented in Chapter 6.
2.3.2.2 Creating Patient-Specific hPS-CMs for Predictive Toxicology

hPS-CMs not only serve as a reliable human cardiac cell source, they also have the potential to allow for an “individualized medicine” approach to predict cardiotoxicity, specifically, creating patient-specific hPS-CMs and screening them with compounds to help predict cardiotoxic responses. This is especially useful for patients with a known genetic mutation. We can re-program their somatic cells into an iPS cell line, differentiate these cells into hPS-CMs, and study their responses in vitro (Figure 2.7 panel A). Carvajal-Vergara and colleagues were able to create hPS-CMs with patients with LEOPARD syndrome, a mutation in the Ras-MAPK pathway, which is known to cause hypertrophic cardiomyopathy. The authors compared wild-type to LEOPARD hPS-CMs and found that the latter cell type had characteristics of hypertrophy such as increased cell size and increased NFAT4 (Nuclear factor of activated T-cells 4) localization to the nucleus [Carvajal-Vergara et al. 2010]. In a separate study, Hinson and colleagues examined the role of Titin mutations with hPS-CMs derived with patients with a specific mutation. They found that the cells with the mutation exhibited lower twitch force compared to wild-type hPS-CMs [Hinson et al. 2015]. Thus the ability to create hPS-CMs from patients with an altered genetic background can provide helpful insights into consequences of certain mutations on cardiomyocyte function. This idea can be extended to include studying how certain mutations will affect how a patient responds to a certain drug.

In addition to creating hPS-CMs from patients with altered genetic backgrounds, we have also gained the ability to genetically edit iPS cells using newly developed Cas9 technology and create hPS-CMs from these cells [Musunuru 2013; Strong et al. 2017]. Genome editing allows us to either create new mutations or correct existing mutations and examine phenotypic responses (Figure 2.7 panel B). [Musunuru 2013]
Figure 2.7: Comparison of two methods for disease modeling using hPS-CMs. A) Reprograming somatic cells from healthy and diseased patients into iPSCs and differentiating them into hPS-CMs. B) Genome editing of wildtype iPS or iPS with mutation to correct or mutate genes. [Figure adapted under the terms of the Creative Commons Attribution License: Disease Models & Mechanisms, Kiran Musunuru, “Genome editing of human pluripotent stem cells to generate human cellular disease models.” Vol 6: pgs. 896-904: Copyright 2013]

Therefore, we can compare two cell lines that are genetically identical except for the presence or absence of a mutation. Hinson and colleagues also performed such experiments in their study; they created multiple iPS cell lines with different Titin mutations. They found that different mutations led to varying degrees of decreased force generation by cardiomyocytes created from these lines [Hinson et al. 2015]. Similar to creating iPS cells using patients with a genetic background, genome editing is a valuable tool for gaining insights into functional consequences of mutations and has the potential to provide us with more “informed” pre-clinical models for drug testing.
2.3.2.3 Studying Sunitinib Toxicology using hPS-CMs

A limited number of studies have begun assessing the cardiotoxicity of sunitinib using hPS-CMs. Many of these studies utilized a commercially available hPS-CM source manufactured by Cellular Dynamics Inc. In one study Cohen and colleagues found that 31µM sunitinib completely depleted ATP levels, and induced LDH release and caspase activation [Cohen et al. 2011]. In a separate study, Doherty and colleagues found that as little as 625nM sunitinib can cause disruptions in normalized beating rate, and 10µM sunitinib leads to a complete arrest of beating [Doherty et al. 2013]. Some major limitations with these studies are: 1) the use of non-physiological concentrations of sunitinib, specifically in the Cohen study; 2) no assessment of effect of afterload on toxicity; and 3) experiments were performed in flat, 2D cultures. Regarding the latter, culturing cells on rigid, flat substrates is clearly structurally and mechanically non-physiologic. 2D culture is known to cause morphologic changes such as distortions of sarcomere organization and impose a non-physiologic amount of preload and afterload on cells, which will inherently negatively affect their function [Soares et al. 2012]. In the next section, we will go over alternate methods for culturing cells using current tissue engineering methods, and argue that these tissue culture models serve as better platforms for pre-clinical drug screening on cells such as hPS-CMs.
2.3.3 Utilizing Tissue Engineering Models as Cell Culture Platforms for Drug Screening

2.3.3.1 Design Criteria for Tissue Culture Models and Limitations with Conventional Methods

Even with the right type of cells, a suitable tissue culture platform is a necessity for improving current drug screening methods. An ideal platform would permit control over cellular, biochemical, and mechanical inputs so we can assess the relative contributions of each parameter [Ma et al. 2016; Ogle et al. 2016; Zhao et al. 2016]. Additionally, the platform would allow for making multiple measurements of tissue function. In the case of screening for cardiotoxicity, some examples of important functional assessments would include force generation and action potential peaks and kinetics. Current tissue culture platforms such as 2D flat cultures and animal models are inherently limited in their ability to control one of more of the design criteria listed above, and 2D culture platforms in particular are limited in their ability to provide functional data, such as force generation. Advances in the field of cardiac tissue engineering have resulted in improved strategies that combine the advantages of 2D in vitro culture and animal models to create a platform that fits the design criteria above. In this section we will outline some of these advances and discuss how they can be utilized for improving drug screening.

2.3.3.2 Engineered Tissues Display Hallmarks of Cardiac Physiology

Engineered tissues consist of cells combined with some kind of natural or synthetic ECM that are seeded on millimeter scale or micron scale platforms. For example, cells can self-assemble on a set of pillars/cantilevers or have their adhesion guided by engineering material properties (micropatterning, 3D printing, etc.) to control
cellular alignment (Figure 2.8). [Cashman TJ et al. 2016; Boudou et al. 2012; Lind et al. 2016]

**Figure 2.8: Generating Engineered Cardiac Tissue.** A) Human engineered heart tissue formed between two pillars. [Adapted by permission from Creative Commons Attribution license; Original Article: Cashman TJ et al. “Human Engineered Cardiac Tissue Using Induced Pluripotent Stem Cells Reveal Functional Characteristics of BRAF-mediated Hypertrophic Cardiomyopathy.” PLOS One 2016; 11:e0146697.] B) Microgrooves guide cardiomyocyte alignment using topographical features. [Reprinted by permission from Macmillan Publishers Ltd: Nature Materials, Johan U. Lind, Travis A. Busbee, Alexander D. Valentine, Francesco S. Pasqualini, Hongyan Yuan, Moran Yadid, “Instrumented cardiac microphysiological devices via multi-material three-dimensional printing” Advance Online Publication, Copyright 2016: License no. 3984441121068]

Engineered tissues can be electrically stimulated to give readouts of force and action potential waveforms (Figure 2.9 panels A, D). These tissues can also respond appropriately to the calcium blocker verapamil and beta-adrenergic agonist isoproterenol (Figure 2.9 panel B) [Lind et al. 2016]. Additionally, various authors have argued that the cells themselves can mature within these platforms [Tiburcy et al 2011]. Figure 2.9 (panel C) demonstrates how NRCMs can adopt a more adult-like morphology when being cultured in engineered heart tissue (EHT).
Figure 2.9: Engineered Tissues Recapitulate Aspects of Cardiac Physiology.  
A) Neonatal rat cardiomyocytes in engineered tissues decrease their generated stresses (y-axis on plots) in response to increasing concentrations of the calcium channel blocker verapamil.  
C) NVRMs cultured in EHTs undergo advanced maturation, adopting morphologies resembling the adult phenotype. [Adapted by permission from Wolters Kluwer: Circulation Research, Malte Tiburcy et. al “Terminal Differentiation, Advanced Organotypic Maturation, and Modeling of Hypertrophic Growth in Engineered Heart Tissue” Vol. 109 Issue 10, Copyright 2011: License no. 3984450925448].  
D) Action Potentials generated by EHTs composed of hES-CMs. [Figure adapted under Creative Commons Attribution license: PLOS ONE, Sebastian Shaaf et. al, “Human Engineered Heart Tissue as a Versatile Tool in Basic Research and Preclinical Toxicology” 2011.}
2.3.3.3 Using Engineered Tissues to Study the Contribution of Cellular, biochemical, and Mechanical Microenvironments on Tissue Function

One major design criterion highlighted above was that an ideal drug screening platform should permit control over cellular, biochemical, and mechanical inputs. Understanding how a cell’s response to a drug is influenced by these factors will important for maximizing the effectiveness and safety of new candidate drugs [Ma et al. 2016]. Many engineered tissue models permit control over one or more of the factors listed. This section will discuss some of these attributes and explain their importance.

2.3.3.3.1 Cellular Inputs

Heart tissue is not just composed of cardiac myocytes; there are also non-myocytes such as fibroblasts and vascular cells (endothelial and smooth muscle) present. Previous works have demonstrated the important role these non-myocytes play in maintaining cardiac homeostasis [Tian et al. 2012]. Hence, tissue engineers must decide what non-CM cell types to incorporate and at what ratio. Early work with EHT utilizing neonatal rat heart isolations found that tissues made from the “native” mix of heart cells generated higher diastolic and systolic forces and were more sensitive to calcium compared to EHTs composed of CM enriched cultures [Naito et al. 2006]. In a separate study using neonatal rat cells, Radisic and colleagues found that not only the presence of fibroblasts impacted tissue function, but also the order in which cells are seeded [Radisic et al. 2008]. The authors found that pre-seeding tissues with fibroblasts and seeding CMs later created tissues with better tissue shortening (analogous to force generation), better elongation of CMs, and more electrical sensitivity. In a study utilizing human ES-CMs, Zhang and colleagues found that within the range of 45%-90% CMs, the
active force generated per CM actually decreased as purity increased, suggesting that the non-myocyte fraction played an important role in hES-CM maturation within tissues [Zhang et al. 2013]. In summary, cellular factors are important determinates of tissue function, with non-myocytes being key players that should be included in engineered tissues.

2.3.3.3.2 Biochemical Inputs

Biochemical inputs clearly modulate cardiac structure and function. An advantage of in vitro systems is the ability to selectively adjust these inputs. In contrast, animal models suffer from a relative lack of control over biochemical factors. Specifically, the target tissue will also be influenced by factors secreted by other organ systems, which could confound results. The in vitro nature of engineered tissues allows the user to control biochemical factors introduced to the tissue through the addition of culture medium. Many researchers are even switching from serum containing medium to serum-free, chemically defined medium in order to better assess the effects of different chemical factors. In one study utilizing EHT, the authors found that neonatal rat EHTs grown in serum-free media without any growth factor supplementation did not produce active contractions. Growth factors such as IGF-1, EGF, bFGF, endothelin-1, angiotensin II, cardiotrophin-1, and TGFB-1 were crucial for active force generation [Naito et al 2006]. Thus, biochemical factors play a major role in tissue function and may affect responses to drug compounds.
2.3.3.3 Mechanical Factors

Mechanical stimulation is especially important when trying to model cardiac tissue, as cardiac cells are constantly exposed to some degree of mechanical loading during all stages of the cardiac cycle. Therefore any engineered tissues should aim to include some degree of mechanical loading. In models where cells self-assemble on flexible cantilevers, tissues are allowed to undergo shortening isotonic contractions, which corresponds with the in vivo heart [Boudou et al. 2012]. This setup would be favorable for daily maintenance of tissues. However, for assessment of contractile performance, it would be ideal to have the tissue contract at a fixed length or adjustable lengths because resting tissue length has a profound effect on active force generation [Brady et al. 1991]. Early EHT models also incorporated cyclic strain as a source of mechanical loading [Zimmermann et al. 2000]. While the heart does beat cyclically, the application of cyclic strain may lead to lengthening contractions (tissue elongated during contraction). Such contractions, termed eccentric contractions, have been shown to induce cellular apoptosis in muscle strip models [Liaoa et al 2003]. Thus the application of cyclic strain to engineered tissues may not be well suited for inquiries focused on in vitro toxicity.

One mechanical parameter particularly relevant to sunitinib cardiotoxicity is afterload. In models where tissues develop on cantilevers, the “spring constant” of the cantilever serves as the major determinate of afterload. The spring constant will be a function of the cantilever geometry and material properties. In some EHT experiments, the authors reinforced their silicone posts with metal braces during culture to make them completely rigid [Hirt et al. 2012]. They found that this increase in afterload increased expression of hypertrophic genes such as ANP.
(atrial naturetic peptide) and BNP (brain naturetic peptide), increased tissues cross sectional area, and increased glucose consumption [Hirt et al. 2012]. The authors did not look into long term effects on generated force to see if force generation would ultimately decline in response to the chronic afterload increase. Additionally, making the rods completely rigid represents a non-physiologic increase in afterload, which hurts the relevance of their findings. Chapter 5 of this thesis will be aimed at examining how sunitinib’s toxicity is influenced by the degree of afterload.

Along those lines, another important class of mechanical forces to consider are electro-mechanical forces, specifically field stimulating engineered tissues. The Radisic Lab has helped elucidate the role of electrical stimulation on the maturation of engineered cardiac tissues for the past decade. In one of their earliest works using neonatal rat engineered tissues, they reported that continuous stimulation (pulse: rectangular, 5V/cm, 2ms duration, 1Hz) led to a high degree of ultrastructural organization within tissues, where cardiomyocytes would align in the direction of electrical stimulation [Radisic et al. 2004]. In more recent work utilizing hPS-CMs (cardiac microwires), they report that continuous stimulation increased gene expression of developmental genes (myosin light chains BNP) and increased conduction velocities so that are within the range of healthy adult human hearts [Thavandirana et al. 2015]. Thus, electrical stimulation represents another important factor to consider incorporating into an engineered tissue model.
2.3.4 Concluding Remarks

Advances in human pluripotent stem cell technology and bioengineering have the potential for creating robust human cardiac tissue models that allow for user-control over cellular, biochemical, and mechanical inputs, all of which have been shown to influence cell function. It’s important to start considering these factors earlier on in drug development to create drugs that are safer and more effective. Future advances in high throughput screening of such tissues would make them a very attractive model system to adopt in pharmaceutical industry.
Chapter 3: Experimental Methods and Procedures

3.1 Cell Culture Maintenance and Derivation

3.1.1 Primary Culture of Neonatal Rat Cardiomyocytes (NRCMs)

NRCMs were isolated from day 0-1 Sprague Dawley rat pups. Pups were
decapitated and a median sternotomy was immediately performed to excise hearts. Hearts
were placed in fresh cold Hanks Balanced Salt Solution (HBSS; Gibco, Life
Technologies, cat no 14170-112 ) with 1% Penicillin/Streptomycin/Glutamine (P/S/G;
Gibco, cat no 10378-016), trimmed free of atria and other blood vessels, and put in fresh
HBSS+P/S/G. Hearts were minced in a 6-well plate with ethanol-cleaned scissors and
washed twice in fresh HBSS+P/S/G. Minced hearts were incubated with 10mL of trypsin
(Worthington Biochemical, cat no LS003703) solution (0.1%-0.2% trypsin in
HBSS+1%P/S/G) for 15min at 37⁰C. The resulting supernatant solution consisting of red
blood cells and debris was removed and 10ml of fresh trypsin solution was added.
Solution was incubated for 15 min at 37⁰C while shaking at 200 rpm. The supernatant
was discarded again, 10ml of trypsin solution was added, and the preparation was
incubated for an additional 15 min at 37⁰C with shaking. This time the supernatant was
collected and filtered through a 70µm nylon mesh filter (Beckman Dickinson, cat no.
352350) into a 50ml conical tube containing 10ml of ice cold culture media consisting of
10% Horse Serum (Gibco, cat no 26050-088), 5% Fetal Bovine Serum (HyClone, cat no
SH30071.03), 10mM HEPES (Gibco, cat no 1563-0080), 1% P/S/G in 3:1 ratio of
Dulbecco’s Modified Eagle Medium (DMEM; Gibco cat no 11965-084) and Medium 199
(M199 1x; Gibco cat no 11043-023) on ice to stop trypsin reaction. The remaining pellet
was subjected to additional rounds of trypsin digestion to release more cells. Released
cells were collected by centrifuging filtered permeate at 80xg for 5 min at 4°C (brake off).

Cells were plated on tissue culture plastic for 1 hr at 37°C and 5% CO₂ and supernatant was collected to enrich for cardiomyocytes. This CM enriched fraction was plated on fibronectin (Sigma Aldrich, cat no. F1141) coated dishes overnight. These cells were used the next day for microtissue seeding.

3.1.2 Human Pluripotent Stem Cell Culture and Cardiac Differentiation

The human iPS cell line SV20 was kindly provided by iPS Core lab of Penn Cardiovascular Institute (http://web.expasy.org/cellosaurus/CVCL_EL23). Undifferentiated colonies were maintained on Matrigel™ (Beckman Dickinson, cat no. 35427) coated plates (0.5 mg Matrigel™ per 6-well plate) in mTESR™ medium (Stem Cell Technologies, cat no. 05850) at 37°C, 5% CO₂, and 21% O₂. Colonies were passaged every 3-5 days using accutase (Sigma Aldrich, cat no A6964).

For cardiac differentiation, iPS cells were allowed to grow for 4 days or until they reach ~75% confluency.

- **Day -2:** Cells were dissociated with accutase and re-plated in Matrigel™ coated 12-well plates (2 mg per 12-well plate) at a density of 10⁶ cells/well in mTeSR media supplemented with 5μM Rock Inhibitor (Y27632; Enzo, cat no ALX-270-333-M005) overnight.

- **Day 0:** Media was switched to RPMI 1640 (Gibco, cat no 11875-085) + B27 supplement (minus insulin; Gibco, cat no A1895601) +1μM Chiron 99021 (LC Laboratories, cat no. C-6556)
• **Day 2**: Media was switched to RPMI 1640 + B27 supplement (minus insulin) + 2μM Wnt-C59 (Tocris Bioscience, cat no. 5148)

• **Day 4**: Media was switched to RPMI+ B27 (minus insulin).

• **Day 6**: Media was switched to RPMI +B27 (plus insulin; Gibco, cat no 17504-044).

From this point on, cells were maintained in RPMI+B27 (plus insulin) media. Initial beating was observed starting at day 7-12. Metabolic selection (glucose starving) was performed to enrich for cardiac lineages after day 10. In brief, media was exchanged to RPMI (without glucose; Gibco, cat no 11879-020) +5mM sodium DL-lactate (Sigma Aldrich cat no L4263) for 6-10 days with media changes every 2 days. Cells were given several days to recover after metabolic selection before performing any experiments. After metabolic selection, CM purity was in the range of 80-90%.

3.1.3 Human Mesenchymal Stem Cell Culture

Human Mesenchymal Stem Cells (hMSCs) were purchased from Lonza. hMSCs were maintained in alpha MEM media (Gibco, cat no 12561-056) supplemented with 2mM (1%) Glutamax (Gibco, cat no 3505-061), and 100U/mL Penicillin / Streptomycin solution (P/S; Gibco, cat no. 15141-122). Cells were passaged every 3-4 days with 0.05% Trypsin EDTA (Gibco, cat no. 25300-054) up until passage 10 where cells became senescent. Cells from passages 7-10 were used for experiments involving human CMTs to minimize hMSC proliferation within microtissues.
3.2 Fabrication of µTUG Arrays

3.2.1 Microfabrication of Silicon Masters

Multilayer templates were created by photo-patterning SU-8 (all from Microchem) photoresist onto silicon wafers (Single-sided polished, Prime grade) using successive spin coats (CEE 200X, Brewer Science, Inc.), alignment (AMB 3000 HR Mask Aligner, AMB Inc.) and UV exposure (360nm), and baking steps. Figure 9 details this process. The base cantilever structure was created by spin coating SU8 2002 to create ~2.4µm layer and exposing this layer to 100mJ of UV (Figure 9, Step 2). To fill in the interstitial layer (the well), SU8 2100 was spin coated to create a ~200µm layer, followed by spin coating SU8 2010 + 30% S1813 to create a small ~2.4µm layer, and finally, exposing a bottom layer photomask to 700mJ of UV (Figure 9, Steps 3-4). The top lithography was created by spin coating SU8 2050 to create another thin ~2.4µm layer and exposing a top layer photomask to 95mJ of UV (Figure 9, Steps 5-6). These masters were then developed in a single step in SU-8 developer (Microchem) which was stopped by submersion in isopropyl alcohol (IPA, Sigma cat no I9516), followed by a hard bake (Figure 3.1, Step 7).
Figure 3.1: Fabrication of Silicon Masters with Soft Lithography. Templates were created by photo-patterning SU-8 photoresist onto wafers using successive spin coat, alignment and UV exposure, and baking steps. Figure Credit: Elise A. Corbin, PhD.
3.2.2 Fabricating µTUG arrays from Silicon Masters

“Stamps” (negatives of silicon wafer), were cast by pouring a pre-polymer (10:1 base to curing agent ratio) of poly-dimethyl siloxane (PDMS, Sylgard 184; Dow Corning, cat no 2120925) onto wafers, degassing molds, baking overnight at 60⁰C, and peeling off with ethanol. These stamps could then be used multiple times to generate µTUG arrays. Stamps were plasma cleaned under air for 1 min on high using a Harrick expanded plasma cleaner (Sigma Aldrich, cat no Z561657) and silanized with trichloro-(1H, 1H, 2H, 2H-perfluoroctyl) silane (Sigma Aldrich, cat no 448931) under vacuum overnight. For active force measurements, fluorescent beads (Fluoresbrite® YG Carboxylate Microspheres 3.00µm; Polysciences, Inc.) diluted 1:3000 in ethanol were centrifuged into silanized stamps to embed beads in final device. PDMS pre-polymer (5:1 to 15:1 base to curing agent ratio) was then poured onto silanized stamps and stamps were then inverted onto a hardened layer of PDMS in a 35 mm dish (Corning, cat no 430165). These substrates were baked at 60⁰C for 24hr. Stamps were removed from the molds carefully using ethanol as a lubricant to avoid damaging tops of cantilevers. Molds were washed in IPA and dried before seeding cells.
3.3 Preparation of Cardiac Microtissues

3.3.1 Neonatal Rat Cardiac Microtissue Seeding Procedure

Neonatal rat microtissues were prepared in the same manner as described in Boudou et al. 2012 with a few exceptions. Figure 3.2 (panel A) gives an overview of the microtissue seeding process. Briefly, µTUG arrays were sterilized with 70% ethanol and left to dry for 30min (Figure 3.2 panel A Step 1-2). To prevent cell attachment against the walls of the wells, the molds were treated with 0.02% w/v Pluronic F127 (Sigma Aldrich, cat no P2443) for 3hr (Figure 3.2 panel A Step 3) on the surface and 30min inside the wells by centrifuging pluronic solution at 1600xg for 2min (Figure 3.2 panel A Step 4). Pluronics were washed out with Dulbecco’s Phosphate Buffered Saline solution (DPBS no Ca++/Mg++; Gibco cat no 14040-117) followed by dH₂O (Figure 3.2 panel A Step 5). At this time, NRCMs were detached from tissue culture plates with 0.05% Trypsin EDTA for 4 min. 10⁶-1.2x10⁶ NRCMs were used to seed each array. A Collagen I gel (rat tail; Corning, cat no. 354236) was used as an ECM in which to create microtissues. A liquid neutralized collagen I solution (1 mg/ml) was created by mixing collagen I, fibrinogen (0.5 mg/mL final concentration; Sigma Aldrich, cat no F8630), HEPES (10mM final conc.), NaHCO₃ (4.17mM final conc.; Sigma cat no S5761), 1M NaOH (neutralization ratio: 0.5; Sigma, cat no S8045), M199 (10x) (1x final conc.; Sigma Aldrich, cat no. M0650) in sterile dH₂O on ice. A base layer of collagen (1ml per array) was added and degassed to pull collagen into the wells (Figure 3.2 panel A Step 6). Cells were then resuspended into collagen solution, added to arrays, and centrifuged twice at 350xg for 90 seconds (arrays rotated 90 degrees in between spins) (Figure 3.2 panel A Step 7-8). Excess collagen in between wells was removed using a vacuum aspirator (Figure 3.2 panel A Step 9). Arrays were inverted and centrifuged at 50xg for 17 seconds to move
cells down the pillars. Collagen was polymerized inside an incubator at 37°C, 5% CO₂, 21% O₂ for 15min (Figure 3.2 panel A Step 10). Culture media consisting of DMEM high glucose + 10% Horse Serum + 2% Chicken Embryo Extract (Charles River Laboratories, cat no 10100330) + 1% Antibiotic/Anti-mycotic solution (Gibco, cat no 15420-062) was added to arrays and arrays were put back into the incubator (Figure 3.2 panel A Step 11). Cells compacted ECM and tissues formed over the course of 24hr (Figure 3.2 panel B).

Figure 3.2: Constructing Cardiac Microtissues. A) Process flow diagram for microtissue seeding procedure on 120 well arrays. B) Cells self-assemble and compact collagen matrix over 24hr to create a tissue bound to pillars. Figure adapted from: Legant WR, Pathak A, Yang MT, et al. “Microfabricated tissue gauges to measure and manipulate forces from 3D microtissues”. PNAS. 2009; 106:10097-10102; Copyright 2009 National Academy of Sciences.
3.3.2 Human Cardiac Microtissue Seeding Procedure

Human CMTs were prepared in the same manner as rat CMTs with a few exceptions: arrays were treated with 0.1% Pluronic F127 to allow for tissue release from the sides of the well. Monolayers of beating iPS-CMs (day 16-day 30) were detached from culture plates using TrypLE Express (Gibco, cat no 12605-010) for 7-10min at 37°C/5%CO₂. Human MSCs were detached from culture plates with 0.05% Trypsin EDTA for 5min at 37°C/5%CO₂. iPS-CMs and hMSCs were mixed to create tissues composed of 93% CMs /7% hMSCs. Tissues were maintained in RPMI+ 20% FBS+100U/mL P/S+ 5µM Y27632 media for the first 24hr and then switched to RPMI + B27 (plus insulin) + 1% P/S media, which was exchanged every 2 days. Beating would often times not be visible during the first 2 days in culture.

3.4 Drug Studies

Stock solutions (10mM) of Sunitinib malate (LC Laboratories, cat no S-8803) were prepared in DMSO (Life Technologies, cat no.D12345) and were re-made every 6 months to ensure biological activity. Stock solutions were diluted at least 1:1000 in media for experiments to avoid any DMSO induced toxicity. Samples treated with DMSO at the same %v/v were used as controls (vehicle). Microtissues treated with 1µM Staurosporine (Sigma Aldrich, cat no S6942) were used as a positive control for apoptosis. Carbonyl cyanide m-chlorophenyl hydrazone (CCCP; Sigma Aldrich, cat no C2759) was used a positive control for mitochondrial membrane potential disruption at a concentration of 50µM and was incubated with cells for 30min. In a subset of experiments, 5-Aminoimidazole-4-carboxamide 1-β-D-ribofuranoside, Acadesine (AICAR; Sigma
Aldrich, cat no A9978) was administered concurrently with sunitinib at a concentration of 1mM.

### 3.5 Analytical Methods

3.5.1 Detecting Cell Death in Cardiac Microtissues

3.5.1.1 Measuring Apoptosis via Caspase 3/7 Activation

One of our primary metrics for assessing cardiotoxicity in CMTs is the effect of sunitinib on cell viability, and specifically the induction of apoptosis. Caspase activation is one of the earliest signals of apoptosis in cells and involves the proteolytic cleavage of pro-caspase molecules into activated caspase molecules that either activate other caspases (initiator caspases) or are directly involved in carrying out apoptosis (executioner). Caspases 3 and 7 are executioner caspases expressed in nearly all tissues, and were a key readout for toxicity in most experiments. Because caspases are activated post-translationally, the assay we choose must be able to selectively label the active form of the caspase. Additionally, because our microtissues only contain roughly 500 cells per tissue, we also need to utilize an assay that is sensitive enough to get a signal from only a few thousand cells. In light of this observation, we decided to proceed with a luminescence based assay as they tend to be more sensitive than calorimetric ones. Specifically we chose to utilize the Caspase-Glo® 3/7 assay (Promega Corporation, cat no PRG8090). The Caspase-Glo® assay contains a 2-part reagent consisting of 1) a cell lysis buffer and 2) a DEVD tetrapeptide conjugated to a luminogenic substrate. Upon cell lysis, activated caspases 3 and 7 will cleave the DEVD peptide, releasing the luminogenic substrate allowing it to react with luciferase and produce light that can be quantified (Figure 3.3 panel A).
Initial experiments with the Caspase-Glo® assay suffered from background issues, most likely from the serum-containing media that CMTs are cultured in. Although the assay is supposedly compatible with serum containing culture media, our observations suggested otherwise after comparing signal-to-noise ratios for samples in a) serum containing media, b) DPBS with residual media (~10%), or c) samples washed thoroughly in DPBS. The results shown in Figure 3.3 (panel B) demonstrate the importance of removing any residual media in order to maximize signal-to-noise ratios.

![Figure 3.3](image)

**Figure 3.3: Measuring Activated Caspase 3/7 in CMTs with Caspase-Glo®3/7.**

A) Cleavage of luminogenic substrate containing DEVD sequence by activated caspases induces luciferase rxn. Picture used with permission from Promega Corporation. B) Signal-to-noise ratios for samples treated with either 1% DMSO or 1µM sunitinib in various media. C) Testing the linear region of Caspase-Glo® assay using cells treated with 0.1% DMSO (blue line) or 1µM Staurosporine (red line); n=3 technical replicates.
We also took care to identify the linear operating range of this assay that was estimated by the manufacturer to be 50-20,000 cells. This allowed us to accurately report fold changes in caspase between different samples. We measured activated caspase 3/7 levels in vehicle- and Staurosporine- (1uM; Sigma Aldrich cat no S6942) treated 2D cultures with cell counts of $10^3$, $5\times10^3$, and $10^4$ cells per well to verify that assay was linearly dependent in this region (Figure 3.3 panel C). This experiment confirmed that we can measure caspase levels using as few as 6 microtissues per experimental sample (roughly 1000 cell per well for 3 technical replicates). We also confirmed that that total protein content was did not significantly vary between experimental samples due to factors such as differences in tissue size (CV=0.084; n=4 independent experiments). Only morphologically intact CMTs were used for this and other assays, and this required manual selection of CMTs.

3.5.1.1.1 Final Protocol for Caspase 3/7 Measurements

Tissues were treated for 4-12hr with either DMSO or sunitinib to induce apoptosis. Arrays were washed with DPBS (w/o Ca$^{++}$, Mg$^{++}$) to remove residual media. At least 6 tissues (preferably 12 tissues) were collected per experimental sample and placed into a 1.5 mL Eppendorf tub containing ~20µl DPBS. Tissues were then re-suspended in 366µl DPBS per tube. An equal volume (366µl) of Caspase-Glo® reagent was added per tube and samples were pipetted and vortexed to completely lyse tissues. 200µl of sample was added to a single well of a white-walled 96-well plate (Greiner bio-one, cat no. 655083. Plates were incubated 1hr at room temperature in the dark and then analyzed on BioTek Synergy H1 Multi-Mode plate reader equipped with Gen5.0 software (Settings: quick shake, read luminescence, Gain 135, Integration Time 0.01s).
3.5.1.2 *Trypan Blue Exclusion to Detect Late Apoptotic and Necrotic Cells*

Measuring caspase 3/7 activation provides us insight into what is occurring during the early apoptotic phase. We also wish to determine whether these cells complete apoptosis and/or enter into a necrotic phase. Trypan Blue exclusion is a quick, cost-effective method for identifying such cells as the dye will permeate into cells with damaged membranes much quicker than for cells with intact membranes, staining them blue, thus making them easily identified under a microscope. CMTs were treated for longer durations (~24hr) during this experiment to allow progression into post-apoptotic phase. Whole arrays or individual tissues were digested in a 1mg/mL collagenase IV (Gibco, cat no. 17104-019) solution in DPBS (with Ca\(^{++}\), Mg\(^{++}\)) with 10% FBS for 15min to digest collagen I. Tissues were further broken down into single cells by dissociating with 0.05% Trypsin EDTA at 37\(^{0}\)C with shaking at 200rpm. Cells were centrifuged at 200xg for 5min and re-suspended in CMT culture medium at a concentration of approximately 5x10^6 cells per ml. Trypan blue exclusion was performed by mixing 0.7mL of media with 0.2mL of 0.4% trypan blue (Sigma Aldrich, cat no T-0776) in DPBS and 5µL of cell suspension. The solution was incubated for 1-2 min at room temperature to allow for labeling of dead cells. The number of blue and white cells was counted (at least 100 counts in total) and the percentage of dead cells was calculated using the following equation 1:

\[
\%\ Dead\ Cells = \left( \frac{\text{blue cells}}{\text{blue cells} + \text{white cells}} \right) \times 100 \quad (Eq. 1)
\]
3.5.2 Characterizing Changes in Mitochondria Function and ATP Levels with Sunitinib Treatment

In light of sunitinib’s effects on cellular energy sensing machinery (e.g. AMPK), we also desired to characterize any metabolic changes. Based on previous findings discussed in chapter 2, we chose to focus on changes in mitochondria and ATP levels. These experiments had to be performed in flat 2D cultures, as the assays we utilized were not adaptable to microtissue culture (see further discussion below).

3.5.2.1 Measuring Changes in Cardiomyocyte Mitochondrial Membrane Potential

Abnormalities in mitochondrial morphology have been cited in the literature, and there have been some preliminary experiments conducted suggesting that sunitinib can diminish mitochondrial membrane potential. We adapted the cardiomyocyte cell sorting protocol originally published by Hattori and colleagues to purify out cardiomyocytes based on mitochondria membrane potential via FACS [Hattori et al. 2010]. Because cardiomyocytes are more metabolically active than non-myocytes they express higher levels of mitochondria. Hattori and colleagues used a lipophilic cell permeable dye to selectively label the inner membrane of mitochondria, Tetramethylrhodamine, Methyl Ester Perchlorate (TMRM; Life Technologies, cat no T668). Because this dye labels in the inner mitochondria, it is also a good marker for mitochondria membrane potential, as it cannot enter the inner membrane without an intact membrane potential (Figure 3.4 panel A).

We discovered that Hattori’s protocol suffered from limitations such as poor cell viability during and after cell sorting, which could confound results when a cardiotoxic agent such as sunitinib was being analyzed (Figure 3.4 panel C). We found that culturing NRCMs for 24-48hr before FACS and performing FACS in serum-free media, as TMRM
labeling is not compatible with lipophilic molecules such as serum, significantly improved viability with negligible effects on CM purity as measured by cardiac troponin I (cTn-I) staining (Figure 3.4 panels B-C). These improvements helped to ensure that we would not observe any unwanted decreases in mitochondrial membrane potential during analysis.

To demonstrate that our protocol could be used to specifically analyze mitochondrial membrane potential, we conducted experiments with cells treated with a known disruptor of mitochondria membrane potential, Carbonyl cyanide m-chlorophenyl hydrazone (CCCP; Sigma-Aldrich, cat no C2759). Of the three TMRM populations we observe on our histogram plot, we see that CCCP affects primarily the TMRM high population, cardiomyocytes with high levels of mitochondrial activity. We chose to analyze changes in this population to characterize changes in mitochondrial membrane potential for our studies with sunitinib.
**Figure 3.4: Measuring Changes in Mitochondria Membrane Potential via FACS.**

A) Density dot plot of neonatal rat heart cells labeled with TMRM (580nm/30) plotted against autofluorescence (513nm/17). Population P3 represents TMRM high population consisting of CMs with abundant mitochondrial activity.

B) Post-FACS immunolabeling of cTnl positive cells (red) and nuclear staining (green) to identify all cells to validate FACS protocol.

C) Culturing neonatal rat heart cells post-isolation is critical for maintaining viability (red bar) post sorting and does not diminish CM purity (blue bar).

D) **Left:** Characterizing changes in mitochondrial membrane potential using TMRM labeled cells treated with CCCP, a known disruptor of mitochondrial membrane potential. **Right:** This method can be used to identify changes in mitochondria membrane potential in cells treated with sunitinib.
3.5.2.1.1 Final Protocol Analyzing Mitochondrial Membrane Potential

NRCMs were cultured in 24-well plates for 24-48hr post isolation before any drug treatments. Cells were treated with 1µM sunitinib for 30min-24hr. We found that using concentrations in excess of 1µM lead to background interference due to sunitinib auto-fluorescence. Cells were washed thoroughly with DPBS (no Ca++, Mg++), and labeled with 10nM TMRM in serum free media (3:1 DMEM: M199 (1x), 10mM HEPES, 2mM Glutax, 1% Insulin-Transferrin-Selenium Supplement (Gibco, cat no. 51500-056), 100U/mL P/S) at 37°C and 5% CO₂ for 30min. After the incubation period, cells were washed again with DPBS (no Ca++, Mg++) and incubated with 0.05% Trypsin EDTA for 3 min at 37°C and 5% CO₂. We found it critical to only treat with trypsin for <3min as any membrane damage will cause the TMRM to leak out. Microtissues require too much enzymatic digestion to obtain single cells; hence they were not used in these experiments. The trypsin solution was inactivated with a defined trypsin inhibitor solution (Gibco, cat no R007-100). Cells were collected in serum free media supplemented with 0.1% BSA (Sigma-Aldrich, cat no. A1470) at concentration of 2.5x10⁶ cells/mL and filtered through a 70µM mesh filter. Samples were analyzed on a Beckman Dickinson LSRII Flow Cytometer equipped with a 488nm (blue laser for excitation and 575/26 emission filter to measure TMRM.

Post-experimental analysis of data was performed using Flow Jo V10 software (FlowJo LLC). Cellular debris was filtered out using side scatter (SSC) and forward scatter (FSC) plots (SSC-A vs. FSC-A, Figure 3.5 panel A). Cell doublets were filtered out using SSC-H vs. SSC-A or SSC-A vs. SSC-W plots (Figure 3.5 panel B). The TMRM positive population was identified using a 575/26 histogram plot (Figure 3.5 panel C).
Finally, within the TMRM positive plot, the TMRM high and TMRM low populations were identified (Figure 3.5 panel D).

**Figure 3.5: Determining Changes in TMRM High Population.** A) Density dot plot of neonatal rat heart cells SSC-A vs. FSC-A to remove cell debris from analysis. Numbers displayed on plot represent % cells falling within gate B) Using SSC-H vs. SSC-A dot plots to remove doublets from analysis. C) Using TMRM (576/26) histogram to identify TMRM positive cells. D) Identifying TMRM high population within TMRM positive parent population.

3.5.2.2 Measuring Changes in Cellular ATP levels

Another method we chose for characterizing the effects of sunitinib on energetics was to examine changes in cellular ATP levels. This assay is especially important as sunitinib has been shown to inhibit AMPK activity in vitro, which could lead to depletion
of ATP levels during times of stress, such as a hypertensive state. Similar to our choice for a caspase assay, we decided to utilize a luminescence based assay that can be applied to a few thousand cells. We chose the ATP assay from Calbiochem (EMD Millipore, cat no 119107) which measures ATP levels from as few as 1000 cells. The assay utilizes luciferase to catalyze the formation of light from ATP and luciferin, and the light can be measured using a luminometer (Figure 3.6 panel A).

We first identified the linear working range of the assay using purified ATP provided by the manufacturer. We found that we can measure as little as 1 picogram of ATP and stay above background and that the assay performs linearly in the range of 1-10\(^4\) picograms of ATP (Figure 3.6 panel A). However, we ran into difficulties when trying to perform this assay with microtissues. Even if we used tissues from the entire 140 well array, we couldn’t get our luminescence readings (RLU) to fall on the standard curve (Figure 3.6 panel B). We then switched to performing experiments in 2D, as we wouldn’t be limited by cell number in such experiments. We assayed ATP levels from vehicle (DMSO) and treated (1\(\mu\)M sunitinib) cells plated at densities of 10\(^5\), 3x10\(^5\), 6x10\(^5\) cells per well. We found that 10\(^5\) cells is sufficient to obtain luminesce readings that fell on the standard curve. However, with increasing cell densities, the nucleotide releasing buffer (i.e. lysis buffer) doesn’t seem capable of breaking down this much cellular material, as our readings didn’t linearly increase as we expected (Figure 3.6 panel C). Hence we performed all subsequent experiments in 2D at a cell density of 10\(^5\) cells per well.
Figure 3.6: Measuring Changes in ATP Levels. A) Standard Curve obtained from using ATP standards to determine linear region of assay. B) Performing ATP experiments in CMTs does not yield sufficient signal to fall on standard curve and may not allow us to distinguish differences between vehicle and sunitinib treated samples. C) Using NRCMs cultured in 2D flat culture at higher cell densities allow us to see changes in ATP levels between vehicle (1% DMSO) and treated (1µM sunitinib) cells.

3.5.2.2.1 Final Protocol for Measuring ATP Levels

Based on the considerations above, our final protocol for assaying ATP levels in 2D NRCM cultures is as follows: NRCMs were cultured in 24-well plates and treated with sunitinib for 24hr. Cells were detached with 0.05% Trypsin-EDTA for 4min at 37°C, 5% CO2. Cells were collected and counted and 3x10^5 cells per experimental sample (3 technical replicates) were aliquoted into 1.5 mL Eppendorf tubes. Cells were centrifuged at 200xg for 4min to pellet cells and were re-suspended into 30µl DPBS (No Ca^{++}/Mg^{++}).

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Nucleotide releasing buffer (300µl) was added to each tube to lyse cells. Samples were vortexed or sonicated (Bioruptor® Plus, Diagenode, Inc.) to ensure complete breakdown of cellular products. ATP monitoring enzyme (3µl) was added to each tube to induce luciferase reaction. Samples were aliquoted into a white-walled 96-well plate, taking care to leave at least 1 column and 1 row in between samples. ATP standards in nucleotide releasing buffer (1-10⁴ picograms) were also aliquoted onto 96-well plates, and ATP monitoring enzyme was added to these samples as well. Plates were shaken gently for 5 min to ensure mixing and analyzed on a BioTek Synergy H1 Multi-Mode plate reader equipped with Gen5.0 software (Settings: 10s shake, read luminescence, Gain 100, Integration Time 0.01s) (BioTek Instruments Inc.).

3.5.3 Characterizing Changes in Microtissue Function following Sunitinib Treatment

3.5.3.1 Measuring Changes in Microtissue Electrophysiology

Our final metric for characterizing microtissue responses to sunitinib was how tissue functionality is affected. One critical aspect of cardiac biology is the generation of action potentials, which are required for systolic force generation. Cardiac microtissues should be able to “capture” an electrical potential and beat synchronously with the signal provided its frequency is within physiological range (neonatal rat 4-6Hz). Microtissue arrays were field stimulated by placing 2 carbon electrodes into either side of the array via drilling holes into the lid of a 35mm dish. Platinum wire attached to the electrodes connected to anode and cathode leads of stimulator (Muscle Research System, World Precision Instruments). Electrical stimulation parameters were: biphasic, rectangular pulse, 15V/cm, 1ms duration, 1ms delay, and 1Hz frequency. Microtissues were analyzed with respect to spontaneous beating rate (number of contractions counted within 60 sec interval), excitation threshold (ET, minimum stimulation voltage required for
synchronous contractions), and maximum capture rate (MCR, maximum beating frequency). These parameters were assessed starting on microtissue day 2, before and after treatment with sunitinib using a Nikon TE2000U inverted microscope (Nikon Corporation) equipped with temperature and CO₂ control (37⁰C, 5% CO₂).

3.5.3.2 Measuring Changes in Microtissue Force Generation

We also examined changes in force generation due to sunitinib treatment. Microtissues exert two types of forces: static (diastolic) and active (systolic). Static tension refers to forces generated in the absence of beating, and is the result of actin-myosin cytoskeletal forces that are generated by all cells, mainly fibroblasts. These forces control tissue compaction and are thought to be a major contributor to overall tissue integrity. Static tension was inferred by measuring the displacement of the pillars. Images were acquired using a 10x Plan Fluor objective on a Nikon TE2000U inverted microscope equipped with QImaging Exi Blue camera (QImaging) and NIS BR Elements software (Nikon Corporation). Images were taken from the bottom, middle, and top of the micro-wells (Figure). The width of a single cap (φ), as well as the separation between caps (S) and bases of pillars (B) was measured in ImageJ (NIH, imagej.net) in units of pixels (Figure 3.7 panel A). We derived the displacement (µm) of the pillar using the formula provided in equation 2, where 0.1706 is the conversion factor between pixels and microns for the objective used in these experiments.

\[
\text{Displacement } \delta \ (\mu m) = \frac{(B - (S + \varphi))}{0.1706 \frac{\text{pixels}}{\mu m}} \quad \text{(Eq2)}
\]

We then calculated the force using Hooke’s law \( F = k\delta \), where \( \delta \) is the displacement we calculated in equation 2 and \( k \) is the spring constant of the pillar (Figure 3.7 panel B). The spring constant is a function of the material properties of the pillar and its geometry.
We directly measured the spring constants of pillars using a Hysitron TI 950 TriboIndenter nanoindenter equipped with a Berkovich tip attached to a force transducer (Hysitron Inc.). The probe tip is calibrated against a hard surface to determine its elastic properties. The sample of interest is then loaded (PDMS array) and the probe performs a “scratch test” where it displaces a single cantilever on the array 14 microns in a horizontal direction (x-direction). The pillar is displaced four times in both the +x and –x directions. Based on measured forces exerted on probe from these displacements, as well as knowing the mechanical properties of the probe, we can directly calculate the spring constant of the pillar using Hooke’s law.

Figure 3.7: Measuring Forces Generated by CMTs. A) Top: Image of the top of a microwell allows us to measure cap width (φ) and distance between caps (S). Bottom: Image of bottom well permits measurement of distance between pillar bases (B). Scale bar represents 100 microns in both photos. B) Calculating generated forces using displacement (δ) and pillar spring constants derived from material properties and pillar geometry.
For active force measurements, microtissues were electrically paced at 1Hz and fluorescence (435nm/20 excitation, 480nm/30 emission) video was captured (3 sec, 13 frames per sec, gain 4, exposure time 50ms) using an Exi Blue camera and NIS Elements BR software to visualize active displacement of beads embedded in pillars. Displacement of individual beads (in pixels) was calculated using ImageJ software. At least two beads, on opposite pillars, were analyzed per microtissue and averaged.

3.5.4 Other Analytical Methods
3.5.4.1 Immunocytochemistry

To check the purity of CMs obtained post FACS, cells were labeled with the cardiac marker cardiac troponin-I (cTn-I, rabbit polyclonal H-170; Santa Cruz Biotechnology, cat no sc-15368). Briefly, cells obtained from sorting were re-suspended in CMT media and plated into fibronectin-coated LabTek™ well chamber slides (Thermo Fisher, cat no 154534) at a density of 0.3x10⁶ cells per well and allowed to attach overnight. The next day, cells were fixed with 2% formaldehyde (Thermo Fisher, cat no 28906) for 15min at room temperature, followed by 3 washes with DPBS (no Ca⁺⁺, Mg⁺⁺). Samples were permeabilized with 0.01% Triton X-100 (Sigma Aldrich, cat no T9284) in DPBS (no Ca⁺⁺, Mg⁺⁺) for 10min at room temperature. To prevent non-specific binding of secondary antibodies, samples were blocked with 5% normal goat serum (Cell Signaling Technology, cat no. 5425) in DPBS (no Ca⁺⁺, Mg⁺⁺) for 2hr at room temperature. A polyclonal rabbit cardiac troponin-I antibody (H-170, sc-15368; Santa Cruz Biotechnology) was used at 1:2000 ratio in Dako Antibody Diluent (Agilent Technologies, cat no S0890) and incubated with samples overnight at 4°C with gentle shaking. Samples were then stained with goat anti-rabbit IgG (H+L) rhodamine (TRITC) conjugate (1:1000 in blocking solution; Jackson Immunoresearch Laboratories, cat no
111-025-144) for 2hr at room temperature, in the dark. To identify all cells, CMs+ non-myocytes, samples were counterstained with 1µg/mL 4', 6-diamidino-2-phenylindole (DAPI; Life Technologies, cat no D21490) for 10min in the dark at room temperature to label nuclei. Samples were imaged using Leica TCS SP8 laser-scanning confocal microscope under 20X magnification. An entire well was scanned and individual images were stitched together. The percentage of cTnI+ cells was determined by manually counting the number of double stained (TRITC and DAPI positive) and single stained (DAPI positive only) cells. At least 500 cells were assayed from each well on chamber slide. At least 2 biological replicates (2 chamber slide wells) were assayed per experiment.

3.5.4.2 Protein Quantification

While optimizing Caspase-Glo® assay, we had to confirm that that total protein content was did not significantly vary between experimental samples. We performed BCA protein assays (Pierce, Thermo Fisher Scientific, cat no 23227) according to manufacturer’s instructions. Absorbance was measured using a SpectraMax M5 spectrophotometer (Molecular Devices) equipped with Softmax Pro 4.8 software. Absorbance readings were normalized to the experimental sample with the lowest reading to create a linear correction factor. We found the correction factor to be unnecessary as CV of all protein readings across experiments was <0.0833. For ATP assay, protein readings were performed using Qubit® system (Thermo Fisher Scientific cat no Q33211) according to manufacturer’s instructions. We generated standard linear correction factors in the same manner as stated above and used them to normalize calculations for ATP (pg).
3.6 Statistical Analysis

All graphs were plotted as mean ± standard deviation (SD); except for box-and-whisker plots where error bars extend from median to min/max value. Individual experiments measuring caspase 3/7 and ATP levels were conducted on 96-well plates with 3 technical replicates per experimental sample. In necrosis experiments utilizing trypan blue exclusion, at least 70 cells were counted per measurement and both chambers of hemocytometer were assayed to give 2 technical replicates per experimental group. At least 2 experiments, utilizing different batches of NRCMs, were performed.

Measurements of microtissue function, such as electrical and force generation, were assayed on individual tissues, which were treated as biological replicates rather than technical replicates because we found that the variability between tissues within a single array was far greater than the variability between different arrays. Table 3-1 compares coefficients of variation (CV) of individual tissues from a single array versus CV between at least 2 separate arrays for several functional readouts. At least 10-15 tissues were assayed per array for functional measurements. Additionally, due to inter-tissue variability, we chose to track the functional changes in individual tissues instead of doing a bulk average before and after treatment. Accordingly, these data are presented as percent (%) change from baseline measurements conducted before treatment.

For experiments utilizing flow cytometry, at least 20,000 cells were analyzed per experimental sample, and the percentage of TMRM high cells was calculated from this population as described in section 3.4.2.1.1. Statistics were performed from n=3 experimental replicates.

Unpaired two-tail t-tests were performed when appropriate (GraphPad QuickCalcs, GraphPad Software Inc.). Significance levels were determined between
sunitinib and vehicle treated samples and were set at *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, and ****$p < 0.0001$ or as otherwise indicated.

<table>
<thead>
<tr>
<th>Coefficient of Variation (CV)</th>
<th>Force Generation</th>
<th>Spontaneous Beating Frequency</th>
<th>Excitation Threshold</th>
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<td>Within Single Microtissue Array</td>
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<td>0.39-0.49</td>
<td>0.13-0.28</td>
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<tr>
<td>Between Microtissue Arrays</td>
<td>0.023</td>
<td>0.134</td>
<td>0.027</td>
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Table 3-1 Sources of variability in measurements of microtissue function
Chapter 4: Creating an In Vitro Model of Human Sunitinib Cardiotoxicity Using the Rat Cardiac Microtissue Platform

4.1 Rationale

The high prevalence of chemotherapy induced cardiotoxicity observed in patients can be partly attributed to a lack of preclinical models that can 1) detect early signs of cardiotoxicity, such as cardiomyocyte apoptosis and decreases in force generation and 2) identify pivotal mechanisms of sunitinib cardiotoxicity. Identifying pivotal mechanisms of sunitinib cardiotoxicity using in vivo human clinical trials would not be cost effective and may raise ethical concerns depending on the hypothesis being tested. Current in vitro cell culture and animal models suffer from limitations that minimize their usefulness for modeling human sunitinib cardiotoxicity, such as the ability to incorporate or adequately control biomechanical loading conditions [Astashkina et al. 2012; Ma et al. 2016; Ogle et al. 2016; Zhao et al. 2016], an essential facet of cardiac biology. As such, there is a critical need to develop robust preclinical models that can accurately detect cardiotoxicity and serve as tools for enhancing our understanding of the mechanisms behind sunitinib cardiotoxicity.

Therefore, in this chapter we describe the development of an in vitro preclinical model of human sunitinib cardiotoxicity using a previously established cardiac microtissue platform. We created 3D cardiac microtissues (CMT) from neonatal rat cardiomyocytes that self-assemble on silicone (PDMS) cantilevers [Boudou et al. 2012]. We used this system to characterize sunitinib cardiotoxicity using metrics for cell viability (apoptosis and necrosis) and cardiac function (force generation and electrophysiology), and examined how these characteristics are impacted by sunitinib dose and treatment duration.
4.2 Experimental Procedures

4.2.1 Creating rat cardiac microtissues

Neonatal rat cardiac myocytes were isolated as described in section 3.1.1. Cells were pre-plated for 1hr at 37°C to enrich for cardiomyocytes, and cultured overnight on fibronectin coated dishes. The next day, cells were washed with PBS (no Ca++ or Mg++) and media was exchanged to get rid of dead cells leftover from the isolation. Microtissues were seeded 3hr later according to methods described in section 3.3.1.

4.2.2 Assessment of cell viability in microtissues treated with sunitinib

Microtissues were treated with sunitinib (or DMSO vehicle) 48hr post seeding to allow for complete tissue compaction and induction of synchronous beating. Activated caspase 3/7 levels were used as a metric for apoptosis, and were measured using Caspase-Glo® 3/7 assay described in section 3.5.1.1. Tissues were treated with drugs 4-12hr before assaying for apoptosis. To assess necrosis, tissues were enzymatically dissociated and stained with trypan blue to determine the percentage of dead cells in microtissues, according to methods described in section 3.5.1.2. Microtissues treated with 1µM staurosporine served as positive controls for apoptosis (6hr) and necrosis (24hr).

4.2.3 Assessment of cardiac function in microtissues treated with sunitinib

Baseline measurements of passive and active forces were conducted prior to treating with sunitinib, 48hr following microtissue seeding, according to methods in section 3.5.3.2. Post-treatment measurements were taken after 24hr of sunitinib treatment. Similarly, baseline measurements of electrophysiology (spontaneous beating rate, excitation threshold, and maximum capture rate) were taken prior to drug treatment, 24 and 48hr post microtissue seeding, according to methods described in 3.5.3.1.
4.3 Results

4.3.1 Rat CMTs recapitulate decreases in cell viability following sunitinib treatment

In cardiac microtissues derived from neonatal rat ventricular myocytes, we first examined time- and dose-dependent effects of in vitro sunitinib exposure on apoptosis as indicated by caspase 3/7 activation. We employed staurosporine, a potent apoptosis inducer, as a positive control. We found that a clinically relevant concentration of sunitinib (1µM) induced significant activation of caspases 3, 7 though to a lesser degree than staurosporine (Fig 4.2 panel A). Next, we evaluated how caspase activation is influenced by sunitinib dose and treatment duration. We found that caspase activation reaches a maximum level at 8 hours in CMTs treated with 1µM and 10µM sunitinib (Fig 4.2 panel B), so all future experiments examined caspase 3/7 activation at 8 hours regardless of dose.
Figure 4.2: Detecting apoptosis in cell viability with sunitinib treatment using a rat cardiac microtissue model- A) Activated caspase 3/7 levels in response to treatment with 0.1% DMSO (Vehicle), 1µM sunitinib, or 1µM staurosporine for 8hr (DMSO and sunitinib) or 6hr (staurosporine); (****p<0.0001 relative to vehicle n=3 experiments; (***) p<0.001 1µM sunitinib vs. staurosporine n=3 experiments). B) Time dependent changes in caspase 3/7 activation. Fold changes (relative to vehicle) in caspase levels were determined for CMTs treated with 1µM or 10µM sunitinib for 4hr, 8hr, or 12hr [**p<0.01 1µM sunitinib 4hr vs 8hr; *p<0.05 1µM sunitinib 4hr, 8hr vs. 12hr time point; n=2 experiments (8hr) n=2 experiments (4hr, 12hr); ‡‡p<0.01 10µM sunitinib 4hr vs. 8hr; ‡ p<0.05 10µM sunitinib 4hr, 8hr vs 12hr; n=2 experiments for all time points]. C) Dose dependent changes in caspase activation in CMTs treated with 50nM, 200nM, 1µM, 10µM sunitinib for 8hr [*p<0.05 50nM vs. 200nM sunitinib n=2 experiments; *p<0.05 200nM vs. 1µM sunitinib n=2 experiments (200nM) n=4 experiments (1uM); **p<0.01 1µM, vs.10µM sunitinib n=4 experiments (1uM), n=2 (10uM) experiments]. D) Non-linear curve fitting of panel C results yielded a log₂ function (gray line), R²=0.993.
When examining how caspase activation is influenced by sunitinib dose, we determined that 50nM is the threshold concentration for caspase activation for our model; and observed dose dependent increases in caspase 3/7 activation in the range 50nM to 10µM sunitinib (Fig 4.2 panel C). Doses greater than 10µM were not deemed physiologically relevant to humans given observed concentrations of sunitinib in human blood are typically 0.1-1.89µM. Interestingly, we found that the degree of caspase activation correlated strongly with sunitinib dose. Using non-linear curve fitting methods, we discovered that our results fit strongly with a logarithmic ($\log_2$) function that produces an $R^2$ value $>0.99$ (Fig 4.2 panel D). Finally, we found increased numbers of late apoptotic/necrotic cells in CMTs treated with 10µM sunitinib as compared to vehicle (DMSO) treated cells after dissociating CMTs and staining with trypan blue (Fig 4.3). Collectively, these results demonstrate the ability of the rat microtissue model to detect decreases in viability due to sunitinib treatment that is dependent on sunitinib dose and treatment duration, and established dose and treatment duration for subsequent experiments.
Figure 4.3: Late apoptosis and necrosis in rat cardiac microtissues treated with sunitinib. Detecting necrotic/late apoptotic cells. CMTs were dissociated following 24hr treatment with 10µM sunitinib or 1µM staurosporine and the percentage of cells stained with trypan blue was manually measured and normalized to vehicle levels to express as a fold change [**p<0.01 10µM sunitinib vs. 1µM staurosporine (n=2 experiments); ***p<0.001 vehicle vs. 10µM sunitinib n=2 experiments (sunitinib) n=3 experiments (vehicle)].

4.3.2 CMTs reveal changes in cardiac function following sunitinib treatment

LV dysfunction remains an important toxicity of sunitinib treatment; however there are a limited number of studies characterizing changes in myocyte force generation. Accordingly, in this part of our study, we modeled changes in microtissue force generation and electrophysiology using the microtissue model. We found that 1µM sunitinib was sufficient to decrease the spontaneous beating rates of microtissues, and beating rates continued to decline in a dose dependent manner (Fig 4.4 panel A), with complete arrest at 10µM sunitinib. Static (diastolic) forces generated by microtissues also decreased in a dose dependent manner (Fig 4.4 panel B). We found that active (systolic) force generation increased during the 24hr treatment period, which we attribute to tissues
maturing over time. Microtissues treated with 10µM sunitinib showed significantly smaller increases in force generation (Fig 4.4 panel B). When microtissues were subjected to field stimulation we found no significant differences in excitation threshold nor maximum capture rate between vehicle and sunitinib treated tissues (Fig 4.4 panel c-d). Results from figures 4.2-4.4 suggest that the rat cardiac microtissue model may serve as a robust tool for early detection of drug induced cardiotoxicity, as it can detect changes in cell viability and cardiac function.
Figure 4.4: Modeling variations in cardiac function following administration of sunitinib in rat CMTs - A) Dose dependent decreases in spontaneous beating rates following 24hr of treatment with 0.1% DMSO (vehicle n=10 tissues), 1µM sunitinib (n=11 tissues), or 10µM sunitinib (n=11 tissues) (**** p<0.0001 vs. vehicle; *p<0.05 1µM sunitinib vs 10µM sunitinib). B) Dose dependent decreases in static (diastolic) tension generated by CMTs treated for 24hr with 0.1% DMSO (n=12 tissues), 1µM sunitinib (n=20 tissues), or 10µM (n=10 tissues) sunitinib (**p<0.01 vehicle vs 1µM sunitinib, 1µM sunitinib vs. 10µM sunitinib; ****p<0.0001 vehicle vs 10µM sunitinib). Decrease in active (systolic) tension with 10µM sunitinib treatment [****p<0.0001 vehicle (n=4 tissues) or 1µM sunitinib (n=5 tissues) vs. 10µM sunitinib (n=9 tissues)]. We did not observe any changes in C) Excitation Threshold or D) Maximum Capture Rate following 24hr treatment with 0.1% DMSO or 10µM sunitinib. Data in panels A and C are plotted as box-and-whisker plots; Median-1st Quartile are plotted in red, 3rd Quartile-Median are plotted in green, error bars extent from median to min/max.
4.4 Discussion

Currently, more than 100,000 people have been treated with sunitinib, and there are over 100 open clinical trials with this drug (clinicaltrials.org); a fact that stresses the notion that we need to improve our understanding of sunitinib cardiotoxicity. Many basic and translational studies in cardio-oncology are constrained by inadequate preclinical models. Animal models do not allow for precise control over biomechanical and biochemical factors [Houser et al. 2012]. Likewise, the use of 2-dimensional (2D) cell cultures for the study of sunitinib cardiotoxicity also suffers from serious shortcomings. Most important is the inability to incorporate essential features of cardiac physiology such as the application of mechanical preload and afterload [Ma et al 2016]. Taking advantage of recent advances in tissue engineering, we created in 3-dimensional (3D) CMTs that better mimic in vivo conditions without sacrificing control over cellular, biochemical, and mechanical inputs [Legant et al. 2008; Boudou et al. 2012]. Using the CMT platform, we successfully created a preclinical model for characterizing sunitinib toxicity. In the next chapter we will be using this model to gain insights into the mechanisms of sunitinib cardiotoxicity while establishing a template for broader preclinical analysis of cardiotoxicity for the thousands of tyrosine kinase inhibitors currently in development.

We first confirmed that our microtissue model could recapitulate previously observed increases in cell death with sunitinib treatment. Our model was able to detect significant increases in caspase 3/7 activation following treatment of physiological doses of sunitinib, which is consistent with reports by Hassinoff et al. and Dohertry et al. [Hassinoff et al. 2008; Doherty et al. 2013]. We are the first to report that the degree of caspase 3/7 activation correlates logarithmically (log2) with sunitinib dose. Additionally we found that caspase activation was time dependent, a finding that also observed by
Hassinoff and colleagues [Hassinoff et al. 2008]. Our results also demonstrated that many of these early apoptotic cells go on to become non-viable, leading to cardiomyocyte loss, which is consistent with studies reporting elevated troponin levels in patients [Chu et al. 2007], CK-MB elevations in rodent studies [Maayah et al. 2014], and increases in TUNEL positive cells in *in vitro* NVRM studies [Chu et al. 2007]. In summary, our microtissue model detects activation of apoptosis pathways and cell death with sunitinib treatment and our results are in-line with existing studies in the literature, while still providing some novel findings regarding the relationship between sunitinib dose and activation of apoptosis.

Our microtissue model detected dose dependent decreases in spontaneous beating rate and full cardiac arrest at 10µM sunitinib, which is consistent with findings by Doherty and colleagues [Doherty et al. 2013]. These dose-dependent decreases in beating may have been connected to findings that sunitinib blocks hERG channels at physiological doses (see section 2.1.2.1 for more details). Neonatal rodent cells do possess a small hERG current and blocking it has been shown to lead to arrhythmias [Lin et al. 2010; Gilchrist et al. 2015]. Because we did not measure action potential waveforms in this experiment, we cannot be certain that our findings are due to hERG channel blockage. Interestingly, we found no difference in electrophysiology parameters such as excitation threshold and maximum capture rate, both of which are measured under field stimulation. These results suggest that in the presence of an outside current, sunitinib’s effects on electrophysiology are limited. Furthermore, sunitinib has been primarily shown to induce atrial fibrillation, and CMTs we created are primarily of ventricular phenotype [Tamargo et al. 2015], so that may explain why we didn’t observe more dramatic effects on some electrophysiology parameters.

In addition to changes in electrophysiology, we also observed dose dependent decreases in static (diastolic) tension generated by microtissues. Static tension is a
measure of forces exerted by tissue at rest and would be influenced by cardiomyocyte loss. We cannot ascertain whether myocyte loss is solely responsible for observed decreases or if other mechanisms contribute to reduced force generation. Our findings for tension partially differ from those of Rainer and colleagues who reported decreases in systolic but not diastolic stresses in short term (30min) sunitinib experiments (doses ≥1.87µM) performed on human atrial muscle strips, but many differences in experimental preparation may have contributed to the differences observed [Rainer et al. 2012].

4.5 Limitations and Conclusions

4.5.1 Study Limitations

One limitation in this study is the use of neonatal rat cells to model adult human cardiotoxicity. In the absence of an adult human cardiomyocyte cell line, NRCMs have been utilized for years and are able to recapitulate many aspects of human cardiac biology. Nevertheless, there are valid concerns about potential inter-species differences. Though cardiac myocytes derived from human pluripotent derived cardiac stem cells mitigate the species difference, these cells are functionally immature. Another limitation in our work is the inability to distinguish between dead cells and “sick” cells in our functional data. We cannot discern whether cells are becoming “sick” due to sunitinib and can’t generate as much force per cell, or whether there are fewer live cells contributing to force generation. However, our results demonstrating that sunitinib decreases spontaneous beating rate may give us some insight into this issue. In the case of spontaneous beating rate, we are only looking at the function of live cells, as the dead cells cannot beat. Our results clearly demonstrate a decrease in the beating rates of live cells in CMTs treated with sunitinib. This result suggests that sunitinib is making cells “sick” and less
functional. Hence it may be possible that sunitinib is impacting myocyte force generation independent of myocyte attrition due to apoptosis. Another limitation with the microtissue model is the inability measure systolic tension at a fixed tissue length. Because of the decrease in diastolic tension we were concerned that sarcomere lengths were also changed, which in itself would affect systolic tension due to the Frank-Starling relationship [Brady et al. 1991]. Therefore, the effects of sunitinib on active force generation can be confounded by any changes in static tension. For example, if sunitinib causes decreases in static tension that lead to increases in sarcomere length, that sarcomere change alone will tend to increase active force generation. If sunitinib is actually directly decreasing active force generation, we may not be able to observe this decrease because of the increase in sarcomere length favoring increased active force generation. Ideally, to separate out length-dependent changes of active tension, these measurements must be performed on tissues of a fixed length. We currently do not have the capability to manipulate microtissues in this manner.

4.5.2 Conclusions

As tyrosine kinase inhibitors such as sunitinib continue to be widely used in the treatment of cancer, better preclinical models for identifying the risks and mechanisms of cardiotoxicity will be very important. We have created an in vitro model of sunitinib cardiotoxicity that allows us to identify effects on cell viability and cardiac function. We were able to recapitulate decreases in myocyte viability and function that were described in previous studies and we were the first group to fully characterize the effects of sunitinib dose and duration on cardiomyocyte loss and function. The model described in this study may also serve as robust tools for disease modeling.
Chapter 5: Identifying Pivotal Mechanisms of Sunitinib

Cardiotoxicity: Contributions of Afterload and Mitochondria Dysfunction

5.1 Rationale

In humans, sunitinib causes increases in blood pressure, and induces hypertension in up to 47% of treated individuals [Bono et al. 2011; Demetri et al. 2006; Di Lorenzo et al. 2011]. A small cohort of these patients goes on to develop LV dysfunction (<10%) [Shah RR et al 2015; Telli et al. 2008; Hall et al. 2013]. However, the direct interaction between load and cardiac dysfunction in the setting of sunitinib remains completely unknown. Hypertension induces the heart to generate greater forces, and it is well documented that increased afterload is risk factor for eventual LV dysfunction [Ozkan et al. 2011; Fernandes-Silva et al. 2016]. The question of whether the development of hypertension merely coincides with sunitinib toxicity or if increased afterload directly potentiates the risk of cardiac toxicity for any specific dose/duration has important clinical relevance. We hypothesized that increased afterload will augment the cardiotoxic effects of sunitinib.

In addition to afterload, mitochondrial dysfunction is thought to be another mechanism for sunitinib cardiotoxicity. Endomyocardial biopsies from patients being treated with sunitinib have revealed swollen mitochondria, and in vitro studies with sunitinib have revealed qualitative changes in mitochondrial membrane potential [Chu et al. 2007; Kerkela et al. 2009]. However, mitochondria dysfunction as it relates to sunitinib has not been fully characterized.
Therefore, in this chapter we assessed the contributions of afterload and mitochondria dysfunction in the context of sunitinib cardiotoxicity. We were able to create cantilevers with different stiffnesses to vary the amount of afterload experienced by microtissues. We used apoptosis, via caspase 3/7 activation, as a metric for characterizing the cardiotoxic effects of afterload. In the second part of this chapter, we quantitatively characterized mitochondria dysfunction with sunitinib treatment using our own advances in mitochondria labeling and flow cytometry. We evaluated mitochondrial function changes with sunitinib treatment duration. We then looked at the impact of mitochondrial dysfunction on cell energetics by measuring cellular ATP levels. Finally, we evaluated whether upstream activation of AMPK, using molecule AICAR, could rescue sunitinib induced caspase 3/7 activation.

5.2 Experimental Methods

5.2.1 Characterizing mitochondrial dysfunction

In these experiments, NRCMs were cultured in flat culture in 24-well plates for 48hr post isolation. Cells were treated with 1µM sunitinib for 30min-24hr and subsequently labeled with TMRM to identify quantitative changes in mitochondria membrane potential as described in section 3.4.2.1.1. Cells treated with 50µM CCCP were used as a positive control for membrane potential disruption in these experiments. ATP levels were measured after 24hr treatment with sunitinib as described in section 3.5.2.2.1. We also explored the ability of the AMPK activator AICAR to reduce sunitinib induced caspase activity as described in section 4.2.2.
5.2.2 Creating microtissues under varying degrees of afterload

Devices were fabricated using either a 5:1 or 15:1 base-to-curing ratio to create arrays with stiff or soft pillars respectively. The resulting spring constants from these pillar configurations were 1.16157±0.09821 N/m (28 pillars tested) for stiff pillars and 0.55982±0.17612 N/m for soft pillars (20 pillars tested), as measured by nanoindentation as described in section 3.5.3.2. Rat cardiac microtissues were seeded in the same manner as described in section 4.2.1.

5.2.3 Assessing the Contribution of Afterload

We chose to characterize the effect of afterload on sunitinib cardiotoxicity in terms of apoptosis, specifically caspase 3/7 activation. Activated caspase 3/7 was measured using Caspase-Glo® 3/7 assay as described in sections 3.5.1.1 and 4.2.2.

5.3 Results

5.3.1 Sunitinib induces decreases in mitochondrial membrane potential and cellular ATP levels

Recognizing that mitochondrial dysfunction is a proposed mechanism of sunitinib cardiotoxicity, we assayed mitochondrial membrane potential in NVRMs at various time points following treatment with 1µM sunitinib using TMRM and a flow cytometric analysis. When mitochondria membrane potential is impaired, TMRM will not be able to label the inner membrane of mitochondria. We used (CCCP), a known disruptor of mitochondrial membrane potential, as a positive control for these experiments. Figure 5.1 (panel A) shows a typical histogram of TMRM levels in vehicle, sunitinib (1µM), and CCCP (50µM) treated NVRMs. The plot demonstrates modest decreases in mitochondrial membrane potential with 1uM sunitinib treatment; albeit this decrease is not as significant
as one observed with CCCP treated cells. We hypothesized that like caspase activation, mitochondrial membrane potential may also exhibit time dependent changes, therefore we quantified differences in mitochondrial membrane potential between vehicle and sunitinib treated cells across various time points (Fig 5.1 panel B). We found significant decreases in mitochondria membrane potential at 4 hour and 8 hour treatment durations, but none before or after this time interval. Interestingly, these time points also correspond to when we observed caspase 3/7 activation. We also observed modest decreases in cellular ATP levels in NVRMs treated with 1uM sunitinib for 24hr (Fig 5.1 panel C).

Previous studies have identified the inhibition of AMPK activity as an off target effect of sunitinib, which is another possible explanation for decreased ATP levels. We hypothesized that treating CMTs with an AMPK activator, AICAR, could reverse some of the observed cardiotoxic effects of sunitinib, specifically caspase 3/7 activation. However, upstream activation of AMPK with AICAR was not sufficient to attenuate caspase 3/7 activation in sunitinib treated cells (Fig 5.1 panel D). Our results reveal time dependent changes in mitochondrial membrane potential with sunitinib treatment, which may contribute to observed decreases in cellular ATP levels.
Figure 5.1: Characterizing changes in mitochondrial function and cell energetics with sunitinib treatment—A) Flow cytometry histogram showing levels of TMRM, a dye used to measure mitochondrial membrane potential. NVRMs grown in flat culture were treated with 0.1% DMSO (vehicle), 1µM sunitinib, or 50µM CCCP (known disruptor of mitochondria membrane potential) for 30min (CCCP) or 4hr (DMSO and sunitinib) and then were labeled with 10nM TMRM. B) Time dependent decreases in mitochondria membrane potential following treatment with 1µM sunitinib. NVRMs grown in flat culture were treated with 0.1% DMSO or 1µM Sunitinib for 30min-24hr and labeled with TMRM to assess mitochondria membrane potential (*p<0.05 n=3 experiments). C) Decreases in ATP levels in NVRMs following 24hr treatment with 1µM sunitinib (*p<0.05 n=3 experiments). D) Upstream activation of AMPK with molecule AICAR did not reverse sunitinib induced caspase activation in rat CMTs (n=2 experiments).
5.3.2 Cardiotoxic effects of sunitinib are augmented by increased afterload in CMT model

Though hypertension frequently accompanies sunitinib treatment and most patients with sunitinib-induced cardiotoxicity have hypertension, it remains unclear whether hypertension is a marker or a mediator of cardiac dysfunction. Recognizing that the stiffness of the cantilever is the primary load constraining active shortening of the tissue (i.e. afterload), we cultured CMTs for 48hr on stiff (5:1 base-to-curing ratio) and soft (15:1 base-to-curing ratio) pillars and treated them with either 0.1% DMSO (vehicle) or 1µM sunitinib for 8 hours. Figure 5.2 (panel A) shows caspase 3/7 activation for this experiment. In vehicle treated CMTs, we see greater caspase activation in soft pillars versus stiff pillars. However, in sunitinib treated tissues, we observed greater caspase activation in stiff versus soft pillars. When this data is graphed as a fold change (Fig 5.2 panel B), we see that increased afterload augments sunitinib-induced caspase 3/7 activation. These results suggest that increased afterload augments the myocardial toxicity associated with a given dose and duration of sunitinib.
Figure 5.2: Using CMTs to assess the contribution of afterload to observed cardiotoxic effects of sunitinib - A) rat CMTs cultured on stiff (5:1) or soft (15:1) pillars were treated with 0.1% DMSO (vehicle) or 1µM sunitinib for 8hr. Differences in active caspase 3/7 levels were observed between soft and stiff vehicle treated CMTs (** p<0.01; n=3 experiments) and sunitinib treated CMTs (*** p<0.001; n=3 experiments). B) Results in part A) plotted as fold changes in active caspases 3/7 relative to vehicle treated CMTs (**p<0.01 n=3 experiments).
5.4 Discussion

In addition to creating a preclinical model for sunitinib we also wanted to gain mechanistic insights to sunitinib cardiotoxicity. Our findings of modest, yet significant, decreases in mitochondrial membrane potential with sunitinib treatment complements patient biopsies showing swollen mitochondria and in vitro studies showing qualitative decreases in mitochondrial membrane potential at a single time point [Kerkela et al. 2009; Chu et al. 2007; French et al. 2010]. To our knowledge, we are the first group to define the time-dependence of changes in mitochondrial membrane potential following sunitinib. Interestingly, decreases in mitochondrial membrane potential corresponded to same time points when we observed caspase activation. It has been previously shown that activated caspases 3 and 7 are key mediators of mitochondrial apoptosis events, such as decreases in membrane potential [French et al. 2010; Lakanhi et al. 2006; Safiulina et al. 2006]. Mitochondrial swelling has been shown to accompany loss in membrane potential. Future work would need to done to separate out the direct effects of sunitinib on mitochondrial function vs. indirect effects on mitochondria due to sunitinib-induced apoptosis. One experiment that may be useful to distinguish these two mechanisms would be to treat NRCMs with a caspase 3/7 inhibitor prior to administration of sunitinib. If mitochondria function is still impaired in the absence of caspase activation, then sunitinib is more likely to be directly impacting mitochondrial function, as opposed to indirectly via caspase 3/7 activation.

We further observed modest decreases in ATP levels, similar to those reported by Kerkela and colleagues [Kerkela et al. 2009], and these may be consequent to the changes in mitochondrial membrane integrity. Another possible explanation for decreases in ATP levels is AMPK inhibition by sunitinib (IC$_{50}$=0.062±0.029µM for rat [Laderoute et al. 2010]). Cohen and colleagues reported that sunitinib cardiotoxicity could be rescued by
the addition of an upstream AMPK activator, AICAR [Cohen et al. 2011]. In our experiments with AICAR treatment, we found no significant change in caspase 3/7 activation. Interestingly our results appear to support findings by Kerkela and colleagues [Kerkela et al. 2009], who report that sunitinib affects intrinsic kinase activity. Perhaps upstream activation of AMPK by AICAR is not properly targeting the mode of sunitinib’s inhibition of AMPK, and a different AMPK activator should instead be used. In summary, our characterization of mitochondrial dysfunction in NRCMs ties together our caspase results from microtissues as well as previous findings of swollen mitochondria and decreased ATP levels.

The CMT model is particularly well-suited for examining whether increases in afterload augment cardiotoxic effects of sunitinib. We varied afterload by altering the stiffness of the pillars on which CMTs are tethered. We observed an increased magnitude of sunitinib-associated caspase activation with pillars. When CMTs are cultured on stiff pillars in the absence of sunitinib, we actually observed somewhat lower caspase activation, further supporting an interaction between sunitinib and increased afterload. More specifically, the switch in increased apoptosis from soft to stiff pillars following sunitinib treatment suggests that sunitinib may be impairing normal adaptive responses to increased afterload. Increased mechanical forces, such as afterload, have been shown to promote the formation and maturation of focal adhesions, which are adhesions between cellular integrins and ECM molecules. Tyrosine kinases, Src and focal adhesion kinases (FAK) are recruited to these focal adhesions and are activated via phosphorylation. Activated Src and FAK can inhibit apoptosis via MAPK/ERK pathway [Aikawa et al. 1997]. This could explain why we saw lower caspase activation on stiff pillars: the increased afterload may have resulted in greater levels of activated Src and FAK [Franchini et al. 2000]. It appears that sunitinib can inhibit these tyrosine kinases, though to a lesser degree than its effects on RTKs. For example, in a model of glioblastoma,
treatment with 10µM sunitinib led to decreased levels of activated FAK and Src [de Boürd et al. 2007]. Perhaps inhibition of the anti-apoptotic effects of Src and FAK by sunitinib can explain why we observed greater caspase activation on stiff pillars following sunitinib treatment even though caspase levels on stiff pillars was in fact lower in vehicle treated groups.

Our results are in line with previous rodent studies that found that concurrent administration of a vasopressor (phenylephrine) was required to observe LV dysfunction with sunitinib [Doherty et al. 2013; Khakoo et al. 2008], which suggest that afterload is a key mediator of sunitinib’s effects on LV function. It is tempting to speculate that the energetic stress caused by decreased in ATP generation (due to sunitinib) combined with an increased bioenergetics demand induced by increased afterload reach the threshold for inducing caspase activation. Even in the absence of definitive proof for this speculation, our findings support a more aggressive treatment of hypertension in patients treated with sunitinib to prevent (or limit) LV dysfunction.

5.5 Limitations and Conclusions

5.5.1 Study Limitations

One major limitation in the study was the inability to conduct experiments relating to mitochondrial membrane potential and ATP measurements with CMTs. Flow cytometry required a single cell suspension and digesting microtissues enzymatically resulted in TMRM dye leaking out. ATP measurements required an abundance of cells that we could not obtain with CMTs. Additionally, in these experiments, we found that dosing cells with concentrations >1µM sunitinib resulted in significant background fluorescence. Hence we only conducted these experiments at the 1µM sunitinib level.
Fortunately, this is a clinically relevant concentration of sunitinib. Finally, with regard to our experiments with afterload, we realize our study would hold more clinical relevance if we could induce changes in afterload after CMT formation instead of creating CMTs on different in the presence of different pillar stiffness, because secondary changes in cantilever stiffness would better mimic the secondary development of hypertension after sunitinib exposure. The ability to induce secondary changes in afterload is an active area of investigation in our research group.

5.5.2 Conclusions

Many studies cite an association between developing hypertension and the eventual development of LV dysfunction during or after sunitinib treatment. The contribution of increased afterload to sunitinib’s cardiotoxic effects has been understudied. Though it is possible that higher afterload in vivo simply unmasks LV dysfunction induced by sunitinib, our findings support the concept that increased afterload actually augments cardiotoxicity of sunitinib. Our results suggest that management of hypertension may be important for preventing eventual LV dysfunction in patients receiving sunitinib. Additionally, we are the first group to fully characterize loss of mitochondrial membrane potential with sunitinib treatment, and relate the temporal pattern of these changes to the timing of caspase activation following sunitinib exposure.
Chapter 6: Analyzing Responses of Human Cardiac Microtissues to Sunitinib

6.1 Rationale

Although neonatal rat cardiomyocytes do recapitulate most of the essential features of cardiac physiology, the clinical relevance our rat cardiac microtissue model may ultimately be limited by inter-species differences between rodents and humans [Houser et al. 2012]. In the absence of a human cardiomyocyte cell line, researchers have begun creating sources of human cardiomyocytes by differentiating pluripotent stem cells. As section 2.3.2 discussed, these pluripotent derived cardiomyocytes are immature (fetal-like) and do not recapitulate many features of adult cardiac biology [Yang et al. 2014]. Nevertheless, these cells are currently being utilized to study responses to pharmacological agents, and their ability to be incorporated into 3-dimensional constructs tends to help them mature compared with 2-dimensional culture conditions [Burridge et al. 2016; Broekel et al. 2016; Fong AH et al. 2016]. For this reason, we felt that it was important to start transitioning our cardiac microtissue model to include studies with CMTs composed of human cardiac cell sources.

Therefore, in this chapter we examined the responses of human CMTs to sunitinib using caspase 3/7 activation as our primary metric for cardiotoxicity. We compared sensitivity of human and neonatal rat CMTs. Finally, we assessed how caspase activation in response to sunitinib is impacted by afterload in human CMTs.
6.2 Experimental Methods

6.2.1 Creating human cardiac microtissues

Human cardiomyocytes were derived from pluripotent stem cells according to the differentiation protocol described in section 3.1.2. These cardiomyocytes were mixed with human mesenchymal stem cells (MSCs) to create tissues that were 93% CM / 7% MSCs. Microtissues were seeded according to methods described in section 3.3.2. Microtissues were treated with sunitinib on day 5 of microtissue culture.

6.2.2 Assessing the Contribution of Afterload

Devices were fabricated using either a 5:1 or 15:1 base-to-curing ratio to create arrays with stiff or soft pillars respectively, as described in section 5.2.1. We chose to characterize the effect of afterload on sunitinib cardiotoxicity in terms of apoptosis, specifically caspase 3/7 activation. Activated caspase 3/7 was measured using Caspase-Glo® 3/7 assay as described in sections 3.5.1.1 and 4.2.2.

6.3 Results

6.3.1 CMTs composed of human pluripotent stem cell derived cardiomyocytes exhibit afterload dependent caspase 3/7 activation following sunitinib treatment

To extend these observations, we created CMTs composed of human iPS derived cardiomyocytes mixed with human mesenchymal stem cells (huMSCs) and examined their responses to sunitinib. Human CMTs treated with 1µM and 10µM sunitinib for 8hr exhibited significant elevations in caspase 3/7 levels (Fig 6.1 panel A). When we compared these responses to ones we obtained with rat CMTs treated at same concentration of sunitinib, we found that human CMTs have nearly 3-fold greater caspase
activation than rat CMTs at the 10µM dose. When human CMTs are treated with a more physiologically relevant dose of sunitinib (1µM), we found that caspase activation is very similar to what we observed in rat CMTs (Fig 6.1 panel A). These results suggest that human CMTs exhibit robust responses to sunitinib treatment.

Next we sought to address our hypothesis that increased afterload augments sunitinib cardiotoxicity in human CMTs. Human CMTs were cultured on stiff (5:1 PDMS) or soft pillars (15:1) for 5 days before being treated with 1µM sunitinib for 8 hours. We found that human CMTs cultured on stiff pillars exhibited a nearly 3-fold increase in caspase 3/7 activation following sunitinib treatment compared to human CMTs cultured on soft pillars (Fig 6.1 panel B). In fact, caspase 3/7 levels were minimally increased (1.15±0.078 fold change) following sunitinib treatment in human CMTs cultured on soft pillars. This finding varies from what we observed in rat CMTs, where caspase 3/7 levels did increase following sunitinib treatment (1.74±0.064 fold change). In summary, increase afterload augments sunitinib induced caspase 3/7 activation in human CMTs.
Figure 6.1: Sunitinib cardiotoxicity in CMTs composed of human pluripotent derived cardiomyocytes (iPS-CMs) - Differentiation day 20-30 iPS-CMs derived from SV20 iPS cell line were combined with 7% huMSCs to create human CMTs. A) Fold changes in activated caspase 3/7 levels of human CMTs on stiff pillars treated 8hr with 0.1% DMSO (vehicle) or 1µM, 10µM sunitinib for 8 hours compared to neonatal rat CMTs [**p=0.0116 10µM sunitinib human CMTs (n=2 experiments) vs. neonatal rat CMTs (n=2 experiments)]. B) Human CMTs were cultured on stiff (5:1) or soft pillars (15:1) and treated with 1µM sunitinib for 8hr. Plotted are fold changes (relative to vehicle) in caspase 3/7 levels in human and rat CMTs [**p<0.01 human CMTs on stiff vs. soft pillars (n=2 experiments); **p<0.001 human CMTs (n=2 experiments) vs. neonatal rat CMTs (n=3 experiments) cultured on soft pillars].
6.4 Discussion

The final part of our study was aimed at examining responses of human pluripotent derived cardiomyocytes to sunitinib. We found the human CMTs exhibited significant activation of caspase 3/7 in response to 10µM sunitinib, more than 3 times what we observed in rat CMTs (Fig 6.1 panel A). It’s possible that this difference is species-related or maturation-related. Such differences could impact sensitivity to cardiotoxic agents like sunitinib. Our results are in stark contrast to other studies utilizing iPS-CMs that required much higher concentrations of sunitinib to see decreases in cell viability (caspase activation and/or LDH release) in 2D cultures. For example, Cohen and colleagues had to dose flat cultures of iPS-CMs with 23µM sunitinib to get a 2 fold activation of caspase 3/7, which is well outside physiological range of sunitinib concentrations observed in vivo, making their results less clinically relevant (Cohen et al. 2011). The human CMT model, on the other hand, displayed more than a 2-fold increase in caspase 3/7 activation with just 1µM sunitinib (on stiff pillars). These differences may be due to differences in iPS-CM derivation and/or choice of culture platform (2D vs. 3D), but it is tempting to speculate that the biomechanical loading intrinsic to the CMT model (and clinical sunitinib use) is an important regulator of sunitinib cardiac toxicity. Such speculation is further supported by our finding that increased afterload not only augments, but is required for sunitinib induced caspase 3/7 activation in human CMTs (Fig 6.1 panel B). The dependence of cardiotoxicity on afterload correlates well to clinical findings that hypertension is associated with the eventual development of LV dysfunction. Our results suggest that human CMTs with capacity for adjusting in vitro afterload represent an important advance for modeling human sunitinib cardiotoxicity.
6.5 Limitations and Conclusions

One major limitation associated with pluripotent derived cardiomyocytes is that they are fetal-like in nature and do not exhibit all of the hallmarks of adult cardiomyocyte physiology. Therefore their responses to pharmacological agents may differ from the response of an in vivo adult heart. Nevertheless, we believe that iPS-CMs are suitable to use in experiments primarily focused on examining effects of sunitinib on cell viability. Additionally, in our experiments examining the contribution of afterload to sunitinib toxicity, we would preferentially perform these experiments using secondary changes in afterload, as we discussed earlier in section 5.4. Proposed methods for inducing secondary afterload changes in CMTs will be discussed in the next chapter. Despite these limitations, our finding that human CMTs experience afterload dependent caspase activation in response to sunitinib supports clinical findings that hypertension, in many cases, is a predictor of future LV dysfunction. The fact that our CMT model recapitulates this finding makes it a useful tool for studying sunitinib induced cardiotoxicity.
Chapter 7: Conclusions and Future Work

7.1 Summary of work

Cardiotoxicity remains an important detrimental clinical consequence of chemotherapy treatment. Sunitinib, a multi-targeted oral tyrosine kinase inhibitor used to treat renal cell carcinoma and gastrointestinal stromal tumors, is associated with hypertension in up to 43% of patients and left ventricular dysfunction in up to 9.7% of patients. Current preclinical models, such as animal models and in vitro cell culture models are not well-equipped for identifying and characterizing pivotal mechanisms of sunitinib induced cardiotoxicity. Thus, our current understanding of sunitinib cardiotoxicity is limited; in particular our understanding of the role of alterations in mechanical loading and mitochondrial function is very limited. This dissertation centered on: 1) Creating a preclinical model of human sunitinib cardiotoxicity using engineered tissues; and 2) Elucidating the roles of mitochondrial dysfunction and increased mechanical loading in the context of sunitinib cardiotoxicity.

7.1.1 Creating a preclinical model of human sunitinib cardiotoxicity using engineered tissues

In Chapter 4 we described the development of an in vitro preclinical model of human sunitinib cardiotoxicity using a previously established cardiac microtissue platform where 3D cardiac microtissues (CMT) could be formed from neonatal rat cardiomyocytes. We used this system to characterize sunitinib cardiotoxicity using metrics for cell viability (apoptosis and necrosis) and cardiac function (force generation and electrophysiology), and examined how these characteristics are impacted by sunitinib dose and treatment duration.
We found that as little as 50nM sunitinib was sufficient to induce caspase 3/7 activation (1.43 ±0.10 fold increase over vehicle), which is well within the range of sunitinib concentrations found in human blood plasma [Faivre et al. 2005]. The degree of caspase 3/7 activation scaled to sunitinib dose logarithmically (log2; R²>0.99). Caspase 3/7 levels were found to peak at 8hr following treatment. We also detected increases in the proportions of necrotic cells in microtissues treated with sunitinib (1.84±0.11 fold higher than vehicle). These results suggested that our microtissue model was suitable for detecting early changes in cardiomyocyte viability with sunitinib treatment.

Next we sought to assess changes in CMT function with sunitinib treatment. We observed dose-dependent decreases in diastolic and systolic tension with sunitinib treatment. We could not discern whether observed decreases in systolic tension were due to changes in resting sarcomere length, which is unlikely given the decreases in diastolic tension we observed. Decreases in diastolic tension would tend to favor increases in tissue (and sarcomere) length which by the Frank Starling principle would cause active tension to increase. We also observed dose-dependent decreases in the spontaneous beating frequency of microtissues treated with sunitinib. However, when microtissues were subjected to field stimulation, we found no difference in electrophysiology parameters such as excitation threshold and maximum capture rate. A major limitation we cited in this group of experiments was the ability to distinguish whether the observed changes in function are directly or indirectly due to sunitinib treatment. Specifically, is sunitinib directly impacting force generation and beating frequency; or is the injury to myocytes and apoptosis responsible for the decreases in function we observed? This is an important consideration for all research groups utilizing multi-cellular preparations to assess drug toxicity and its impacts on function. Nevertheless, the cardiac microtissue model appears
to recapitulate many of the observed cardiotoxic effects of sunitinib cited in the literature and clinic.

7.1.2 Elucidating the roles of mitochondrial dysfunction and increased mechanical loading

The direct interaction between load and cardiotoxicity in the setting of sunitinib has been uncertain. We hypothesized increased afterload will augment the cardiotoxic effects of sunitinib. In addition to afterload, mitochondria dysfunction is thought to be another mechanism for sunitinib cardiotoxicity given the evidence of mitochondria swelling from patient biopsies [Chu et al. 2007]. However, mitochondria dysfunction as it relates to sunitinib has not been fully characterized.

Chapter 5 began with characterizing changes in mitochondrial dynamics with sunitinib treatment. Experiments with TMRM, an indicator of mitochondrial membrane potential, we found modest yet significant decreases in mitochondrial membrane potential in populations of cells treated with 1µM sunitinib after 4hr and 8hr of treatment. Interestingly these time points corresponded with observed activation of caspases 3 and 7. We related our findings of mitochondrial dysfunction with decreases (~30%) in cellular ATP levels. A limitation of these studies was that they had to be performed in 2D flat culture because technical factors precluded performing these experiments with cardiac microtissues. Nevertheless, these experiments suggest that mitochondrial dysfunction occur with sunitinib treatment.

The latter part of Chapter 5 focused on addressing the hypothesis that increased afterload potentiates the cardiotoxic effects of sunitinib. We were able to create tissues under varying degrees of afterload by altering the mechanical properties of the pillars on which they form. Using caspase 3/7 activation as a primary metric for cardiotoxicity, we
found that tissues grown on stiffer pillars exhibit a higher fold change in caspase 3/7 activation in response to sunitinib treatment compared to tissues grown on soft pillars (2.91±0.21 stiff vs. 1.74±0.06 soft fold increase relative to vehicle; p<0.01). These results demonstrate that afterload is a direct modulator of sunitinib induced apoptosis. Our results suggest that management of hypertension may be important for preventing eventual LV dysfunction in patients receiving sunitinib.

7.1.3 Evaluating the effects of sunitinib in CMTs composed of human cardiomyocytes

In the final part of this dissertation, Chapter 6, we examined the responses of human CMTs to sunitinib using caspase 3/7 activation as our primary metric for cardiotoxicity. We compared sensitivity of human and neonatal rat CMTs. We found that human CMTs demonstrated increased caspase 3/7 activation in response to a physiological dose sunitinib (1µM) similar to what we observed in neonatal rat CMTs. This similarity suggests that the rat CMT model can capture some aspects of sunitinib induced cardiotoxicity, such as changes in cell viability. Finally, we assessed how caspase activation in response to sunitinib is impacted by afterload in human CMTs. Similar to rat CMTs increased afterload augments sunitinib induced caspase 3/7 activation. However, in contrast to rat CMTs, human CMTs cultured on soft pillars exhibited minimal caspase activation in response to sunitinib. Our finding that increased afterload potentiates sunitinib cardiotoxicity in both rat and human CMTs strongly supports our hypothesis that increased afterload enhances sunitinib cardiotoxicity rather than simply unmasking in vivo LV dysfunction.
7.2 Clinical Implications of this Work

A key finding in this study was that afterload does not merely coincide with LV dysfunction in patients but actually exacerbates sunitinib-induced cardiotoxicity. Furthermore, our results with human CMTs demonstrate that a certain amount of afterload is actually required to observe sunitinib induced apoptosis, and suggests that management of hypertension may be important for preventing eventual LV dysfunction. These human studies serve as the “in vitro analog” to findings in patients that hypertension typically precedes or coincides with the development of LV dysfunction, and increases the clinical relevance of this work. We believe that our findings with afterload also demonstrate the need to develop more in vitro models, similar to our cardiac microtissue model, that can manipulate mechanical forces, as our research demonstrates that these forces play a role in chemotherapy induced cardiotoxicity.

Though our findings are not sufficient to change clinical guidelines for managing/preventing hypertension and LV dysfunction in patients being treated with sunitinib, we hope this work will inspire a clinical investigations specifically examining whether intensive anti-hypertensive treatment can reduced the frequency and/or severity of LV dysfunction in patients treated with sunitinib and perhaps other tyrosine kinase inhibitors as well.
7.3 Future Work

Most of our future work for the project will focus on further elucidating the role of afterload in the context of sunitinib cardiotoxicity. Future experiments will utilize human cardiac microtissues. We would like to expand our metrics for evaluating the effects of sunitinib under varying degrees of afterload. Specifically we would like to include experiments that measure force under varying degrees of afterload, in the presence or absence of sunitinib. This will require us to be able to make force measurements at a fixed tissue length, to account for any resting sarcomere length changes that may be occurring. We are actively working to develop methods to control tissue length by inserting iron particles in pillar caps and controlling pillar displacement with a magnet (Fig 7.1 panel A). Additionally, we would like to be able to induce secondary increases in afterload in our CMT model. We believe that this attribute would make our work more clinically relevant as a secondary change in afterload is a better mimic of the secondary development of hypertension in patients. Furthermore, if secondary increases in afterload can be reversed, it would give us the opportunity to begin answering the question whether sunitinib induced cardiotoxicity could be reversed by decreasing the amount of afterload on the heart. To accomplish this, we plan on using magnetic particles, except in this case the particles would be embedded in a very soft layer of PDMS at the bottom of the well. The application of a magnetic field would cause the iron particle to align, resulting in the hardening of the bottom layer, effectively shortening the pillars, thus increasing their spring constants (Fig 7.1 panel B).
Figure 7.1: Modifying the cardiac microtissue platform to study the effect of afterload on cardiac function in the context of sunitinib. A) Making force measurements at fixed tissue lengths by controlling pillar displacement with embedded iron particles and magnetics. One pillar will be pulled with a magnet to control tissue length by being, while the other pillar will report resulting forces. B) Secondary changes in afterload are accomplished by embedding iron particles into a soft layer of PDMS on the bottom of the wells. Under a magnetic field this bottom layer will harden significantly, effectively shortening pillar length, thus increasing spring constant. Figure credit: Elise A. Corbin, PhD.
Another area for future work would be to study sunitinib in human CMTs created from iPS lines from patients with different histories of cardiotoxicity from sunitinib. Penn’s Cardiovascular Institute has access to blood samples from patients undergoing chemotherapy, which could be used to generate iPSC lines (See section 2.3.2.2 for a discussion on patient specific iPS-CMs). Specifically, we would like to test whether iPSC-CM CMTs from patients who have experienced sunitinib cardiotoxicity have greater in vitro susceptibility than iPSC-CM CMTs from patients without cardiotoxicity despite higher exposures. If we are successful in those studies, than we would examine whether gene editing can mitigate sunitinib cardiotoxicity (see section 2.3.2.2 for a discussion on gene editing).
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**Human Pluripotent Stem Cell Derived Cardiomyocytes**


**Engineered Cardiac Tissue for Drug Screening**


**Methods**
