Protein Biomarkers of Drug Induced Cardiotoxicity in the Isolated Heart: Building a Multi-scale Approach

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ABSTRACT #0146
POSTER #0104

ABSTRACT

Analysis of blood samples for specific protein biomarkers is a common diagnostic approach for assessing cardiac injury in human. Aside from myocardial infarctions, protein biomarkers can also be used to detect drug-induced cardiotoxicity during preclinical assessment. Currently, industry cannot efficiently predict low-frequency adverse cardiac events resulting in FDA Phase 4 failure and withdrawal of some compounds. This study is the first in a multi-phase approach to combine assessment of cardiac function and biomarker analysis to develop a comprehensive early-stage cardiotoxicity screening assay. We used the Langendorff isolated rat heart model to investigate drug-induced changes in cardiac function (left ventricular contractility and ECG) and specific protein biomarkers in response to dose escalation. Benefits of using the isolated heart model compared to an *in-vivo* model include reduced biomarker kinetic clearance and the ability to integrate cardiac function with biomarker responses e.g., proteins released from damaged myocytes. Using known cardiac effectors to induce degenerative structural changes in the heart, we quantified the changes in concentrations of cardiac troponin I, troponin T, and TNFα found in perfusate collected from the heart at designated intervals during exposure to establish a correlation between changes in protein biomarker concentration and functional endpoints. We found that a dose response was present in both function and protein biomarkers for many of the compounds tested. These results suggest that integrating multiple modalities into a single comprehensive assay allows for better prediction of toxicity, a finding that will translate into a useful preclinical screening tool to detect drug-induced cardiotoxicity.

INTRODUCTION

Cardiotoxicity is the leading cause of drug removal from the market, responsible for approximately 30% of drug withdrawals (Laverty et al., 2011). Additionally, the number of drugs submitted to regulators and regulatory approvals have decreased and the total cost of bringing a drug to market has increased (Mol et al., 2012; Lasser et al., 2002; Fung et al., 2001). There is an industry need to engage in sensitive, translatable, and predictive assays of acute and latent cardiotoxicity. Cancer therapeutics such as anthracyclines and tyrosine-kinase inhibitors pose added complications to the drug development process due to the incidence of latent cardiotoxicity that is often not observed in pre-clinical screening (Ewer and Lenihan 2009). Increasingly, using multiple assays such as protein biomarkers in combination with contractility assessment is being considered as a better approach to increase the sensitivity of cardiotoxicity screening.

The term biomarker is most commonly associated with biological compounds found in blood and/or urine but the term actually describes indicators of any biological state. Common cardiac contractility biomarkers such as developed pressure, dP/dt, and heart rate, are routinely used to assess cardiac dysfunction and drug induced cardiotoxicity. Similarly, proteins that are released from the heart during injury can be collected and used as biomarkers to represent cardiac injury. Common protein biomarkers used to detect cardiac injury are the cardiac troponins I and T. These are two of the subunits used to regulate the contractile mechanism of cardiac tissue with troponin I being a highly specific indicator of cardiac muscle necrosis and cellular damage. Another common biomarker known as TNFα (tumor necrosis factor alpha) is a pro-inflammatory, cytokine that has been implicated in tumor regression and is a marker of immune mediated stress response (Fransen et al., 1985; Kreigler et al. ,1988). Though these protein and contractile biomarkers are valid indicators of dysfunction, the relationship between the concentrations of injury related proteins released from the heart, and the amount of contractile dysfunction, is not well profiled in the isolated heart. The aim of this study was to observe the effect of known cardiac effectors on select protein concentrations concurrently with contractility measurements. We hypothesized that protein biomarkers are a more sensitive indicator of cardiotoxicity and there would be increases in protein biomarker concentrations released from the heart prior to changes in contractility parameters.

METHODS

This study was conducted in an Association for Assessment and Accreditation of Laboratory Animal Care International (AAALAC)-accredited facility in compliance with the National Research Council (NRC) Guide for Care and Use of Laboratory Animals after the approval of the Institutional Animal Care and Use Committee of Battelle.

Isolated Heart Preparation

42 male Sprague Dawley rats were anesthetized (80mg/kg sodium pentobarbital via IP injection) after 0.3mL of sodium heparin (1000 USP units/mL via IP injection). Surgical plane of anesthesia was verified and rats were intubated and ventilated externally via small animal ventilator. A clam shell incision and pericardiotomy was performed. Hearts were quickly removed and submerged in 50mL chilled cardioplegic solution. Hearts were secured to the cannula of the emka© isolated heart apparatus via suture and then reanimated (after a 10 minute arrested state) by retrograde perfusion of modified Krebs solution at 37°C. A fluid-filled balloon attached to a pressure transducer was inserted into the left ventricle and slowly inflated to 8-10mmHg end diastolic pressure. Surface ECG electrodes were placed in contact with the epicardium. Hearts were equilibrated to the Krebs solution perfusion for 20 minutes and then Krebs containing 0.1% DMSO (solvent for test articles) for an additional 20 minutes.

Contractility Determination and Test Article Administration

Baseline parameters of ECG, contractility (dP/dt max and min, left-ventricular developed pressure, end diastolic pressure), heart rate (HR), and coronary flow parameters were collected for at least 20 minutes following equilibrium. Hearts were then perfused with up to 5 increasing concentrations of test articles in consecutive 20 minute durations. Data was collected using emka© technologies IOX Data Acquisition Software, and ECGs were analyzed with ECGAUTO software.

Protein Biomarker Determination

Protein for biomarker analysis was collected from the spent perfusate (70mL) directly from the heart during the last 5 minutes of both baseline and each subsequent test article concentration period. The perfusate was immediately transferred into Millipore© Centricon Plus-70 Centrifugal Filter Units. Quantification of protein concentrations was performed at an independent laboratory using rat Cardiovascular Disease Panel 1 (CVD1) Milliplex custom 5-plex kits (for troponin I (TnI), troponin T (TnT), B-type natriutetic peptide (BNP), Interleukin-6 (IL-6), and tumor necrosis factor alpha (TNFα)). The protein concentrations were measured on the Luminex 200™ system and analyzed using xPonent® software version 3.0.

Test Article Concentrations

Positive controls: FCCP 0.001µM, 0.01µM, 0.03µM, 0.06µM
Verapamil 1.0µM, 0.01mM, 0.03mM, 0.06mM, 0.1mM

Anthracyclines: Doxorubicin 0.001µM, 0.01µM, 0.1µM, 1.0µM, 0.01mM
Doxorubicin-ol 0.001µM, 0.01µM, 0.3µM, 0.1µM, 1.0µM

Tyrosine-Kinase Inhibitors: Erlotinib 0.001µM, 0.01µM, 0.1µM, 1.0µM, 0.01mM
Sorafenib 0.1µM, 6.0µM, 3.0µM, 1.0µM, 0.01mM

RESULTS

As indicated by Figures 1, 2, and 3, there were significant decreases in contractility (indicated by dP/dt_{max}) of the Verapamil and FCCP groups compared to each groups baseline measurements and the control group. There were significant increases in the concentrations of troponin T, troponin I, and TNFα in the FCCP group seen at the highest concentration, however, contractility began to decline prior to changes in protein levels. The Verapamil group did not show significant changes in protein concentrations, although contractility declined beginning at C2. As indicated by figure 4, 5, and 6, there were overall increases in contractility at concentration 3-5 for Dox-ol and at C4 for Doxorubicin compared to the control group. In the Doxorubicin group neither troponin T nor troponin I concentrations changed significantly while TNFα increased only at the highest dose for Dox-ol. The contractility responses varied in the tyrosine-kinase inhibitor groups as indicated by Figures 7, 8, and 9. Both Erlotinib and Sorafenib closely mimicked the gradual increase of the control group until the third drug concentration increase where the Sorafenib group decreased significantly in contractility while Erlotinib remained steady with the control group. The Erlotinib protein concentrations did not change significantly throughout the experiment. The Sorafenib treated group did not change significantly in either troponin concentrations but increased significantly in TNFα concentration beginning with the first dose and prior to a major decline in contractility.

Both BNP and IL-6 concentrations were below levels of detection with this assay

controls

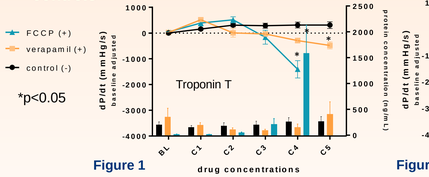

Figure 1

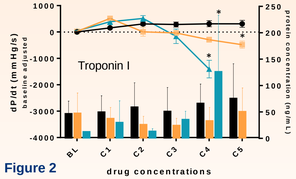

Figure 2

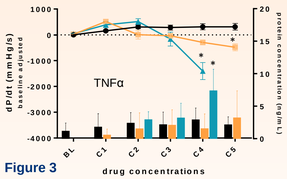

Figure 3

anthracyclines

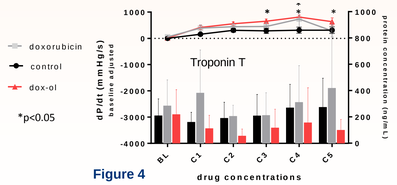

Figure 4

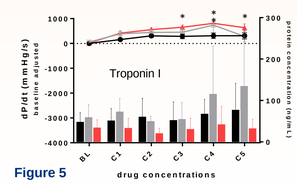

Figure 5

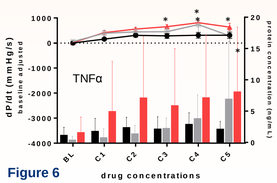

Figure 6

tryosine-kinase inhibitors

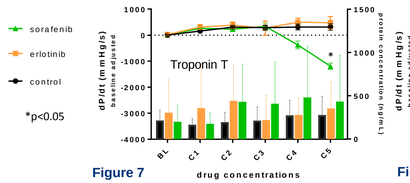

Figure 7

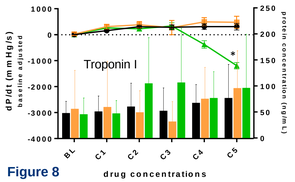

Figure 8

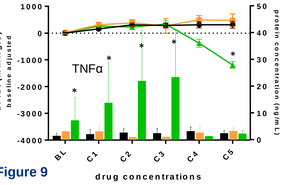

Figure 9

DISCUSSION

Our original hypothesis that protein biomarker would increase prior to changes in contractility appears to be dependent on the drug's mechanism of action. The hypothesis was true for Sorafenib, showing a significant increase in TNFα concentration prior to any contractile dysfunction. Erlotinib had little effect on the level of TNFα as well as contractility even though both drugs are tyrosine-kinase inhibitors. The difference may be that Sorafenib is unique in targeting the Raf/Mek/Erk pathway (Wilhelm et al., 2008) and is eliciting a stress response from a pathway that results in contractile dysfunction and acute cardiotoxicity. Interestingly, the highest observed concentrations of troponin I for Sorafenib were during periods C3 and C4 which was also the highest for the TNFα concentrations, possibly demonstrating an apoptotic effect on the cardiomyocytes. The dual role of TNFα as an apoptotic signaler as well as a cellular protection signaler (via NF-κB) complicates the relationship (Boelsterli 2003) and warrants further investigation.

The anthracyclines group showed no dysfunction in contractility over the dose regimen and was therefore not predictive in relationship to the protein biomarker concentrations. The Dox-ol group exhibited a significant increase in TNFα concentration in the last dose period which is close to the therapeutic concentration, possibly causing stress effects on the heart due to increased workload. Though this may not be of value as a predictor of cardiotoxicity based on acute changes in contractility, it may shed light on the mechanisms responsible for latent cardiomyopathy associated with anthracyclines treatment.

The results of the control group were also not predictive based on protein concentration but this was to be expected. Verapamil is a calcium channel blocker that will significantly reduce contractile performance prior to causing cellular toxicity as was seen with the decrease in dP/dt well before any indicators of cellular damage. Similarly with FCCP, a mitochondrial disrupter, the decrease in energy production (and therefore contractility) will be evident before any cellular damage is detectable in protein biomarkers.

Overall, the strength of this combined assay relies on the unique capabilities of the isolated heart preparation that enables collection of biomarkers directly from the cardiac tissue without the kinetic clearance effect of the intact whole animal model as well as real time cardiodynamics. This capability is important in isolating the drug effects from potential global downstream cascades and compensatory mechanisms that may conceal local tissue toxicities in the whole animal. There is still added value in the cases where the protein biomarkers did not predict the changes in contractility because they give clues as to whether toxic levels cause cell damage or inflammatory responses. The predictive value of this assay is beneficial in lead compound, go /no-go decisions at a fraction of the cost of a traditional whole animal telemetry study.

CONCLUSIONS

In summary, the current paradigm of drug induced cardiotoxicity screening is in need of technological advancement. The failures in phase four market withdrawals are not only costly to the drug developers but also risk the safety of the public. Using more precise analysis of cardiotoxicity is mutually beneficial to the industry and public interests. There are numerous advancing technologies available that will be integral in the integration of cardiotoxicity assays in order to build a multi-scale approach. By using the isolated heart model we were able to increase the sensitivity of protein biomarker detection by collecting directly from the cardiac tissue therefore reducing the washout associated with the whole animal model. The benefits of contractility analysis combined with protein biomarker analysis will help to translate into a better predictive model of acute and latent cardiotoxicity. In parallel studies we are combining cardiac energetic analysis (via NMR ^{31}P spectra) as well as novel kinetic dosing models to this combined assay to develop an even greater comprehensive cardiotoxicity screening model. This integration will also fulfill the requests of the regulators and policymakers to advance the translation from drug development to clinical therapies.

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