

Elevations of cardiac troponin-I quantified with the Erenna[®] cTnI immunoassay are an early indicator of heart muscle injury in studies of cardiotoxicity.

Purpose

This application note describes the utility of the Erenna Immunoassay System and the Erenna cTnI Assay towards early assessment of cardiotoxicity in rats. Due to its unique high-definition technology, elevated levels of cTnI in serum can be detected earlier with the Erenna Immunoassay System (**Table 1**). This is primarily due to the fact that one has the ability to monitor very low basal cTnI levels (**Table 2**) against which small cTnI elevations can be evaluated, resulting in early recognition of toxicity. In this particular study, the effects of various chemical compound classes on cTnI levels were investigated. Two previously known cardiotoxins (isoproterenol and hydralazine), as well as two drug classes with controversial safety profiles – tyrosine kinase inhibitors (TKI's, imatinib) and peroxisome proliferator-activated receptor- gamma (PPAR γ) agonists (rosiglitazone) – were tested, all of which demonstrated significant cTnI elevations, thus demonstrating the utility of the Erenna cTnI Assay and Immunoassay System for evaluating the safety profile of candidate drugs.

Introduction

The measurement of serum cardiac troponins (cTnI and cTnT) are the gold standard for diagnosis of acute myocardial events in man and have gained increasing recognition as a new tool for the assessment of cardiotoxicity in safety studies.

The value of troponins I and T in assessment of acute myocardial damage resides in the unique functional characteristics of these proteins that, along with cardiac troponin C, form a complex bound to the myofibril of striated (skeletal and cardiac) muscles (**Figure 1**). In addition to this myofibril-bound pool there is a small cytoplasmic pool that most probably serves as a precursor pool for the cTn complex.

Table 1. Summary of cardiotoxic compounds detected in rat serum samples with the Erenna cTnI Assay.

Compound	Class	Dose	Time of Detection	Toxicity Expected?	Toxicity Detected?
Isoproterenol	β 1- and β 2-adrenoceptor agonist	0.5 mg/kg	15 min	Y	Y
Hydralazine	vasodilator	25 mg/kg	6 hours	Y	Y
Imatinib	TKI	50 mg/kg/day	14 days	N	Y
Rosiglitazone	PPAR γ agonist	80 mg/kg/day	7 days	N	Y

Table 2. Mean & range of baseline Cardiac Troponin-I (cTnI) in rats, humans, dogs and monkeys. (Schultze et al, 2009)

Animal Model	Mean Baseline [cTnI], pg/mL	Healthy Range of [cTnI], pg/mL
Rat	5	2 – 15
Human	2	1 – 12
Dog	2.5	1 – 4
Monkey	4.4 (female) 5.3 (male)	4 - 5

After myocardial damage, the cytoplasmic pool is hypothesized to be rapidly released while myofibril-bound cTnI is gradually degraded through proteolysis and released as fragments and portions of the original complex.

As increases in cTnI levels often precede observable cardiopathology, it is inherently important to be able to detect serum cTnI level increases above endogenous levels as early as possible. Currently, Troponin I in toxicology studies is measured on systems developed for use in hospital settings. These systems lack the requisite sensitivity, having lower limits of quantification (LLOQ ≥ 30 pg/mL) that are not capable of detecting the healthy range of baseline cTnI values in rats (Table 2).

Recently, there has been an expansion of highly sensitive troponin assays. These assays have increased sensitivity compared to traditional assays, and have reduced the limit of detection (LOD) in some cases to the sub-picogram level.

At the forefront is the high-definition Erenna Immunoassay System, which utilizes single-molecule counting technology to provide extreme sensitivity. The Erenna cTnI assay is the first high-definition assay for cardiac troponin-I, with an LoD of 0.2 pg/mL and an LLoQ of 0.8 pg/mL, that are orders of magnitude more sensitive than prior troponin assays.

This 30-100 fold increase in sensitivity over previously employed systems has enabled identification of accurate and robust baseline cTnI values for a variety of species including human, dog, monkey, rat (Table 2) and guinea pig. Additional studies have determined baseline reference ranges (Figure 2A) and biological variability (Figure 2B) of cTnI in healthy control rats.

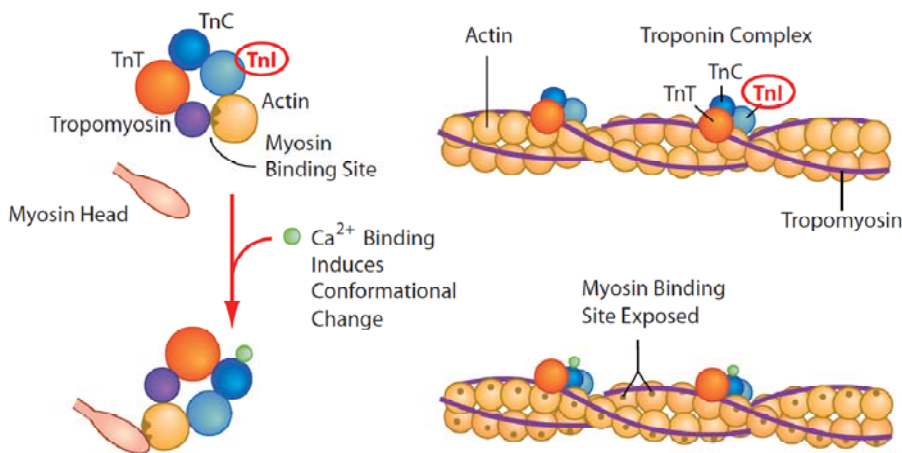


Figure 1. The troponin complex consists of Troponin- I (TnI), Troponin-T (TnT) and Troponin-C (TnC), which as a complex binds to tropomyosin and actin myofibrils. The troponin complex regulates muscle contraction via conformational changes that are induced after calcium binding.

Study Objectives

- To investigate pre-clinical cardiotoxicity of several therapeutic compound classes utilizing the high-definition Erenna cTnI immunoassay.
- To validate the utility of the Erenna cTnI assay for improving the safety profile of candidate drugs by detecting minute release of cTnI from cardiomyocytes at earlier study time points or lower therapeutic doses and before physiological changes can be detected.

These values are surprisingly consistent with studies in humans, with regard to both the healthy reference range values (Apple et al, 2010; **Figure 3A**) and biological variability in human clinical studies (Wu et al, 2009; **Figure 3B**). While it has been generally accepted that troponin increases identified by the earlier less sensitive assays are indicative of cellular cardiac damage, the exact biological significance of previously undetectable cTnI increases above baseline is currently a hot topic of clinical investigation.

Recently, PPAR γ agonists (e.g. Rosiglitazone, Avandia) have been reported to elicit adverse cardiac events in a subset of patients. Other classes of therapeutic compounds, like tyrosine kinase inhibitors (TKI's) and statins, may also need more sensitive evaluations for cardiotoxicity. For these classes of compounds, more sensitive methods for detecting low grade damage to cardiomyocytes have potential to guide decision making during clinical and pre-clinical drug development.

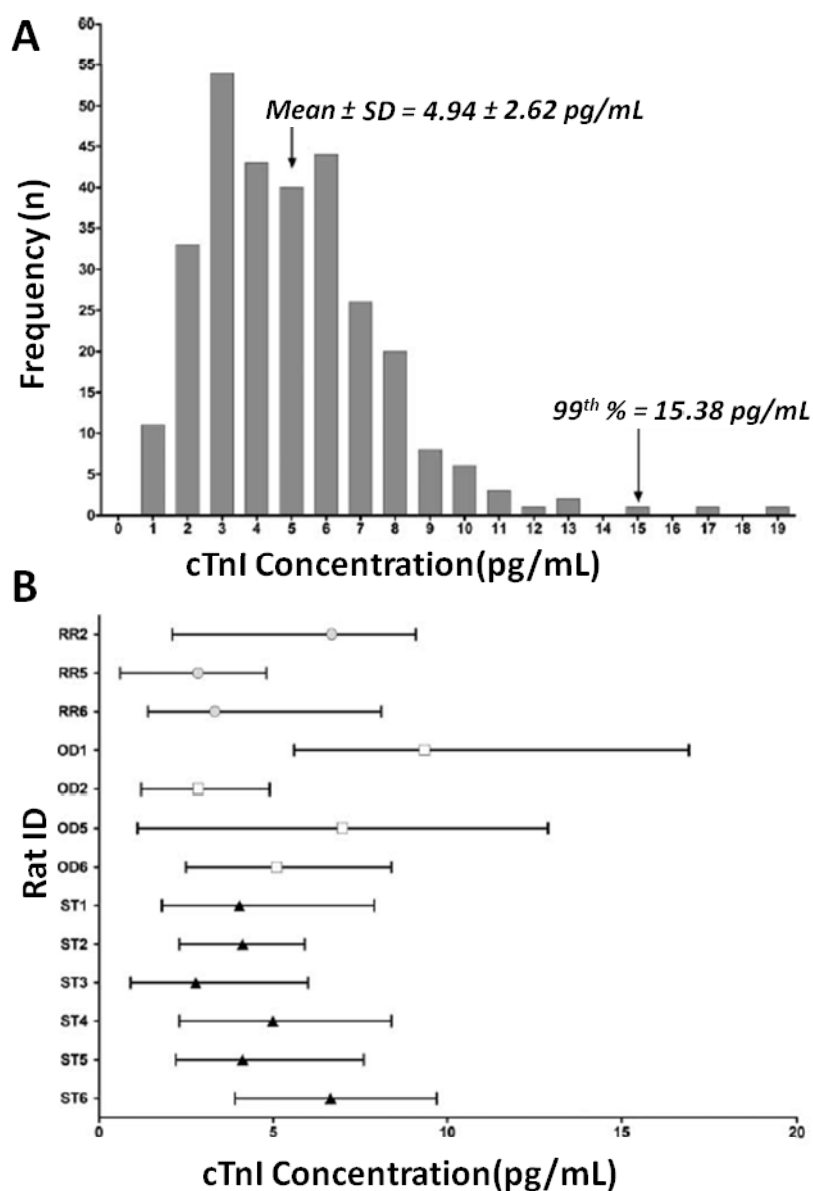


Figure 2. Baseline distribution and biological variability of cTnI in SD rats. (A) Distribution of cTnI, mean \pm SD and 99th%. (B) Biological variability of cTnI over 24 hours, mean and absolute range.

Abbrev: RR, resting rats; OD, oral dose saline; ST, simulated transport.

(adapted from Schultze et al., 2009)

Methods

cTnl Assays: Cardiac troponin-I was measured using the Singulex high-definition Erenna® Immunoassay System (Singulex, Alameda, CA). In some cases (isoproterenol and rosiglitazone studies), samples were also tested using the AccuTNI™ Access® 2 Immunoassay System (Beckman Coulter, Fullerton, CA) for comparison.

Isoproterenol: Male Fischer 344 rats (F344/NHsd) aged 5 to 6 weeks were divided into groups (n=5 rats each) and given single subcutaneous injections of vehicle or isoproterenol (0.5 or 8 mg/kg). Rats were sampled at 0.25, 0.5, 1, 2, 4, 8, or 24 hours post-dose. Rats were anesthetized, bled, humanely euthanized, and tissues were processed for histopathologic examination.

Hydralazine: Male Han Wistar (CrI:WI(Han)) aged 8 weeks were given intravenous injections of hydralazine (25 mg/kg). Single doses (1x) were administered and rats were sampled at 6, 24 and 48 hrs after dosing. Double doses (2x) were administered 24 hours apart, and rats were sampled at 6 and 24 hrs after the second dose.

Rosiglitazone: Male Han Wistar rats (CrI:WI(Han)) were aged 9 weeks (10/group) and sampled at four time-points: 6 hours, 24 hours, 7 days and 14 days. Dosed rats were treated with an oral dosing regimen of vehicle control, 10 mg/kg or 80 mg/kg per day of Rosiglitazone.

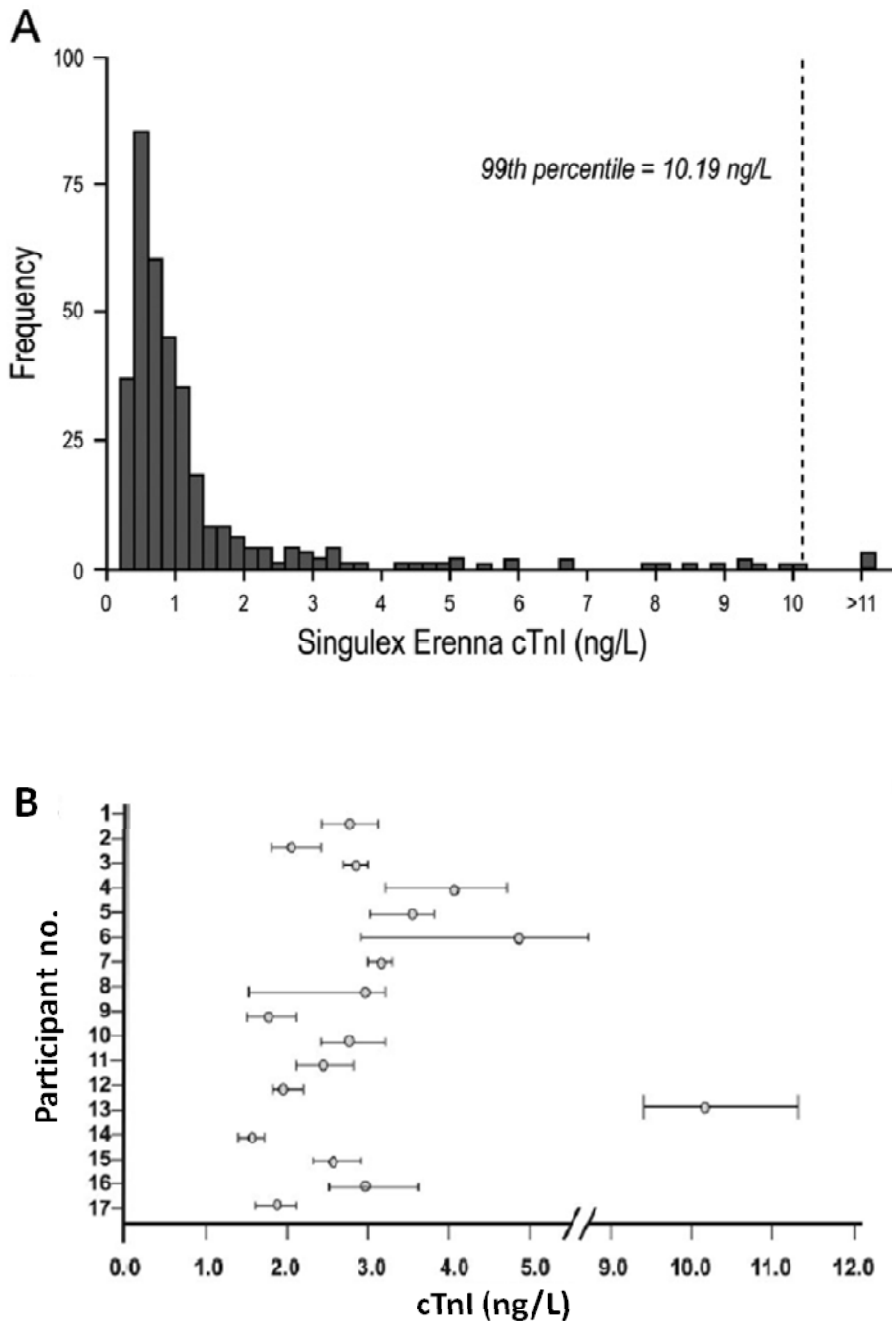


Figure 3. Baseline distribution and biological variability of cTnl in human clinical studies. (A) Baseline references ranges of healthy blood donors measured with the high-definition Erenna cTnl immunoassays (adapted from Apple et al, 2010). (B) Long term biological variability of cTnl in baseline subjects measured with the Erenna cTnl immunoassay over 8 days (adapted from Wu et al, 2009).

Samples were frozen at -70°C for 2 years, and re-evaluated for cTnI with the Singulex Erenna® cTnI assay.

Imatinib (IMB): Groups of adult male Sprague Dawley (SD) or spontaneously hypertensive rats (SHR) were treated with 50 mg/kg

(n=5) or 100 mg/kg (n=10) IMB or water (n=10) daily (p.o.) for 14 days. For comparison, a dose of 100 mg/kg is approximately 2x the recommended human dose of 600 mg/m². Tissues and blood samples were collected 24 hrs after last dosing.

Results and Discussion

To first establish the utility of the Erenna cTnI Assay and Immunoassay System for cardiotoxicity testing, a direct comparison was made versus the Access AccuTNI™ offered by Beckman Coulter. The Access AccuTNI™ is currently being used in hospital based settings as well as for cardiotoxicity testing in clinical and pre-clinical applications, and thus was used as a concordance method. For this application, cTnI levels were measured after administration of isoproterenol and hydralazine, two known cardiotoxic compounds.

Isoproterenol: Concentrations of cTnI in serum were measured after administration of isoproterenol in rats¹. cTnI was measured using the 2.5 µL serum (Erenna) and 140 µL serum (Beckman) samples in the respective immunoassays. All samples measured with the Erenna cTnI assay were quantifiable at each time interval tested and in all animals of the study, including pre-dose baseline animals (10/10); whereas, only 3 of 10 pre-dose rats were measurable with the Access assay. As early as fifteen minutes post-administration, marked increases from baseline cTnI values, followed by decreases after 4 hours, were observed (Figure 4). Cardiac TnI values obtained for identical specimens using the Beckman Access assay are presented for comparison to the Erenna values.

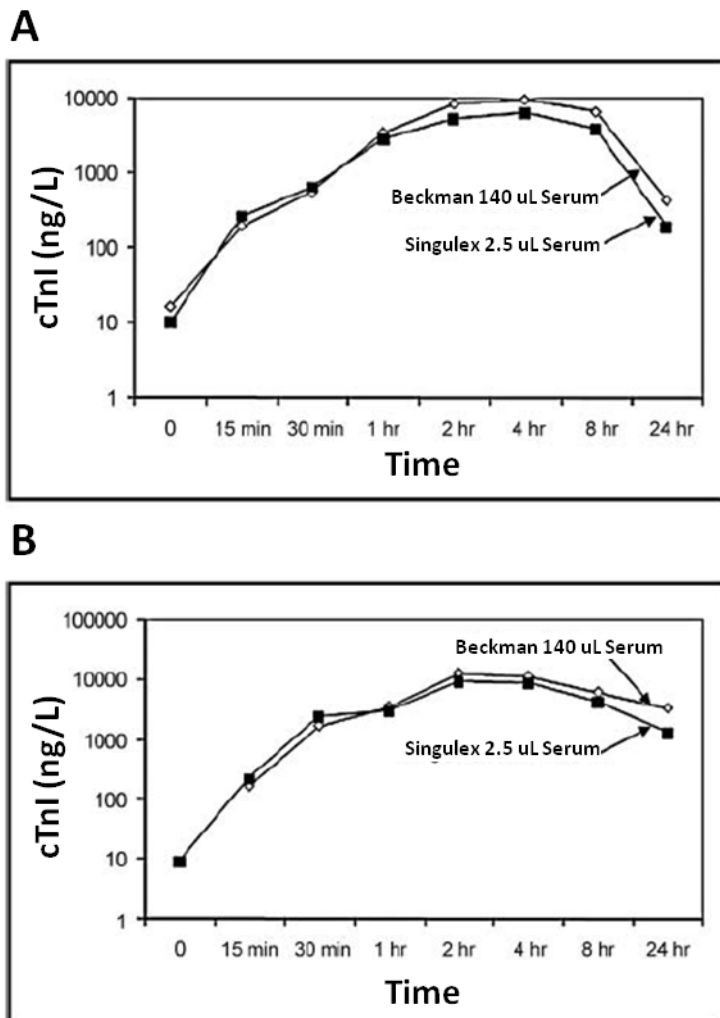


Figure 4. Rat cTnI concentrations measured by the Erenna (2.5 µL) and Access (140 µL) cTnI assays after administration of isoproterenol at (A) 0.5 mg/kg and (B) 8 mg/kg. (adapted from Schultze et al, 2008)

Hydralazine: Having established the functionality of the Erenna cTnI assay, another known cardiotoxin, hydralazine, was evaluated. However, not only serum cTnI was measured but correlate histopathology was compared as a further validation of the assay. A single dose of hydralazine caused an increase of cTnI at 6 hr post dose, followed by a decrease at 24 hr and a return to baseline values by 48 hrs (Figure 5). Elevated values of cTnI coincided with acute myocardial necrosis at histology. However, elevated cTnI levels were observed in the absence of histopathologic lesions in multiple rats, and preceded the development of histopathologic changes in the heart that matured to cardiomyopathy⁵.

We next applied the Erenna cTnI Assay and Immunoassay System for cardiotoxicity testing of compounds with controversial cardiotoxicity profiles in humans to determine if earlier cardiotoxicity could be detected in animal models using this more sensitive assay methodology. We tested two drug classes – tyrosine kinase inhibitors (TKI's, imatinib) and peroxisome proliferator-activated receptor-gamma (PPAR γ) agonists (rosiglitazone). We used the Erenna System to measure cTnI levels to demonstrate the utility of high-definition technology for earlier and more sensitive evaluations of cardiotoxicity that can improve the safety profile of candidate drugs.

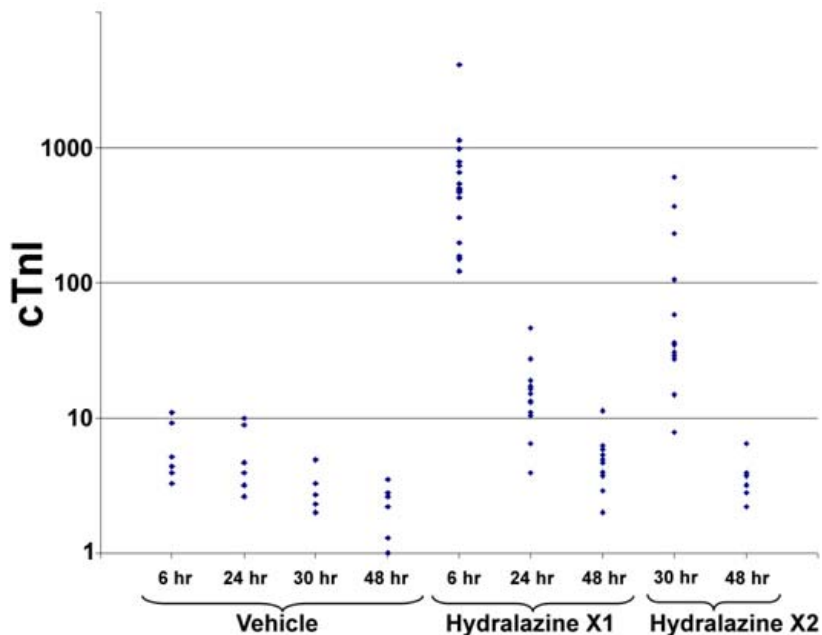


Figure 5. Individual serum cTnI concentrations in rats after vehicle control (saline) or one or two doses of or hydralazine (25 mg/kg). (from Mikaelian et al, 2009)

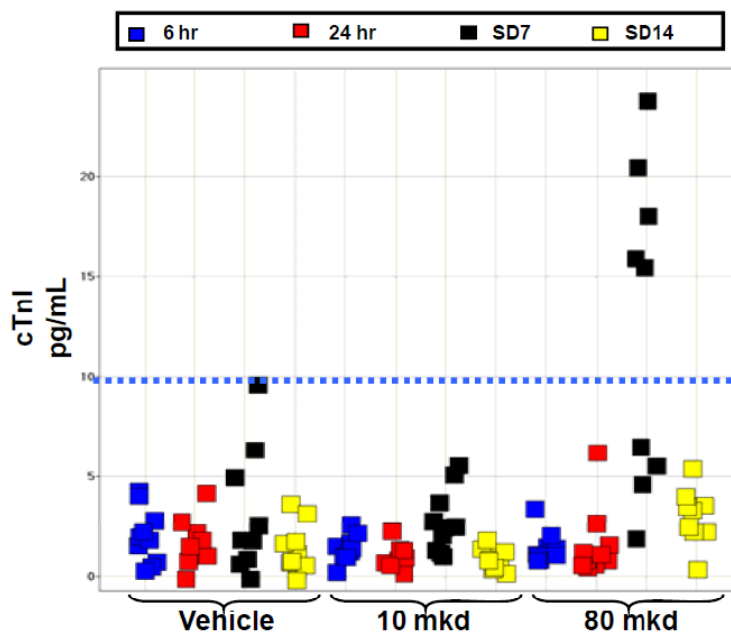


Figure 6. Rat cTnI concentrations as measured by the Singulex Erenna[®] assay increase above baseline value (dotted line; unpublished data) on SD7 in 5/9 rats given 80 mg/kg/day (mkd) rosiglitazone. (from Hirkaler et al, 2010)

Rosiglitazone: As noted above, it is important to quantify all study time points – including basal, endogenous levels – in order to elucidate early and subtle elevated cTnI levels that often fall below the nominal detection limits of current platforms and techniques. A noteworthy example is rosiglitazone, a drug in a class with a controversial safety profile. The Singulex Erenna® cTnI assay was used to analyze the sera of rats given rosiglitazone for up to 14 days⁶ (Figure 6). All samples, whether treated or not, assayed with the Beckman Coulter Access® 2 AccuTNI™ were below LLOQ and therefore could not be measured. In contrast, the Singulex Erenna® Immunoassay System identified significant drug-induced cTnI increases. At the 80 mg/kg/day dose, cTnI levels as measured by the Singulex Erenna® assay increased approximately 5 fold in 5 of 9 rats on SD7, returning to control levels on SD14 (Figure 6).

Imatinib (IMB): Similar to rosiglitazone, IMB has some controversy surrounding its safety profile. IMB is an interesting class due to its influence on a concomitant disease, hypertension. Notwithstanding the anti-hypertensive effects, the cardiotoxic effects of IMB in hypertensive rat strains were analyzed⁷. Increased serum levels of cTnI were detected in all IMB-treated groups (Figure 7). The overall levels were higher in SHR (31.5±24.0, 41.3±29.0 and 53.9±12.3 pg/ml) compared to SD (6.80±5.7,

25±20 and 30±25 pg/ml) at the control, 50 and 100 mg/kg doses, respectively. Observed increases in serum cTnI corresponded to the presence of dose-dependent cardiac lesions, which were characterized by cytoplasmic vacuolization, myofibrillar loss, interstitial infiltration with chronic inflammatory cells and fibrosis (proliferation of myofibroblasts). These results indicate that hypertension, as expressed in SHR, appears to be a factor that can intensify the cardiotoxic effects of IMB and that monitoring for cardiac troponin I may provide a sensitive means of detecting IMB toxicity.

Conclusions

We have described the application of the Erenna® Immunoassay System towards early assessment of cardiotoxicity. The Erenna cTnI assay is a significant improvement over traditional methods of cardiotoxicity testing, and is the only high-definition assay that can quantify minute concentrations of cTnI in serum from healthy, baseline animals. This capability is essential for detecting the minute release of cTnI molecules from cardiomyocytes in toxicity studies at earlier study time points, lower therapeutic doses and before physiological changes can be detected.

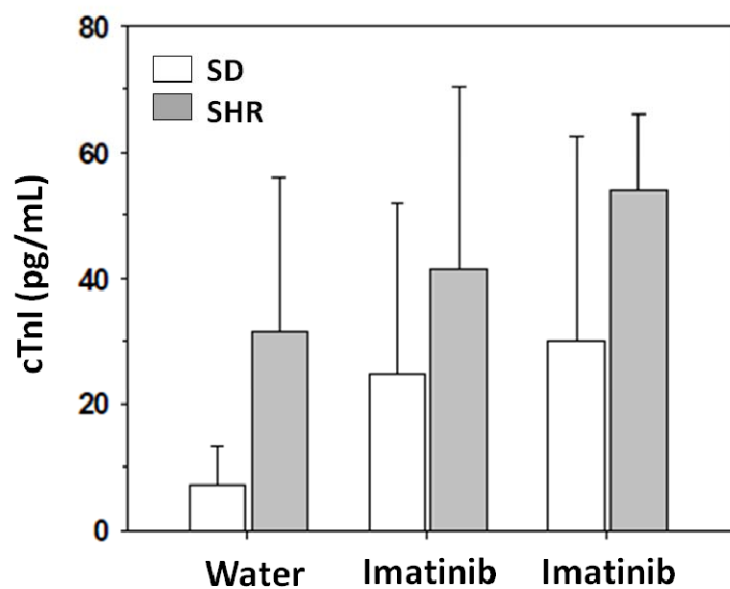


Figure 7. Serum cTnI concentrations in SD (white) and SHR (grey) rats following administration of water or imatinib (50 or 100 mg/kg) for 14 days. Results shown as mean±SD. (from Herman et al, 2010)

We demonstrate use of the Erenna cTnI Assay towards safety testing in rats of various classes of chemical compound classes, including two previously known cardiotoxins (isoproterenol, hydralazine), as well as two drug classes with controversial safety profiles – tyrosine kinase inhibitors (TKI's, imatinib) and PPAR γ agonists (rosiglitazone).

With the Erenna[®] Immunoassay System, increases in serum cTnI are detected earlier and precede observable cardiopathology. These studies indicate that serum cTnI is a more sensitive biomarker than histopathology for identifying acute myocardial damage in pre-clinical rat models of cardiotoxicity.

In conclusion, cTnI measured by the Erenna[®] Immunoassay System was more sensitive than histopathology and other clinical platforms currently available to identify acute cardiotoxicity, and has the potential to guide decision making during pre-clinical and clinical drug development, demonstrating the utility of the Erenna cTnI assay for improving the safety profile of candidate drugs.

References

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