Paving the Route to Plasma miR-208a-3p as an Acute Cardiac Injury Biomarker: Preclinical Rat Data Supports Its Use in Drug Safety Assessment

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ABSTRACT

Drug-induced cardiac injury (DICI) detection remains a major safety issue in drug development. While circulating microRNAs (miRs) have emerged as promising translational biomarkers, novel early detection biomarkers of cardiotoxicity are needed. This work aims at evaluating whether a panel of putative cardiac injury plasma miRs could serve as early DICI biomarkers in a 4-day rat preclinical model. Out of a panel of 68 selected targets, we identified plasma miR-208a-3p as being significantly upregulated after single administration with either isoproterenol (ISO) or allylamine (AAM). This provides the first evidence of miR-208a-3p detection after AAM administration. Moreover, similarly to cardiac troponins (cTn), plasma miR-208a-3p expression profile appears to be compound-specific with most significant early changes occurring in ISO-treated rats. Overall, miR-208a-3p performance in detecting the severity of myocardial injury, as well as the magnitude of miR-208a-3p increase after ISO or AAM administration, were comparable to that of cTn. Our results highlight the importance of assessing the whole time-dependent profiles of miR expression. Hence, time course evaluation revealed plasma miR candidates whose expression was not stable across the duration of the study in the vehicle group, restricting their utility as cardiac injury-specific biomarkers. In light of these findings, miR-208a-3p has a potential to complement the existing biomarkers of cardiac injury specifically in the context of evaluating toxicity in a time-dependant manner. Assessment of miR-208a-3p in other DICI settings would strengthen its robustness as an early detection biomarker leading to a warranted extensive and rigorous validation.

Key words: cardiotoxicity; preclinical; biomarkers; miR-208a-3p; time-dependent profiling

Since their first discovery in Caenorhabditis elegans in 1993 (Lee et al., 1993), knowledge in microRNAs (miRs) has been exponentially growing with the number of mature human miR sequences identified increasing from 152 in 2004 to 2588 in the latest miRBase (Griffiths-Jones, 2004) release (v21, June 2014).

miRs are small endogenous non-coding RNA sequences of about 22 nucleotides that regulate gene expression through messenger RNA degradation or translational repression (Bartel, 2004) and are involved in several developmental processes (Amiel et al., 2012) and various disease pathogenesis (Ardekani and Naeini, 2010).

In the setting of drug-induced tissue injuries, miRs, and more especially circulating miRs, have emerged as promising safety biomarkers. Compared to other biomarkers, miRs have potential advantages. These are highly conserved between species increasing their potential as translatable biomarkers (Ambros, 2004) and, in several species including human, rat and...
mouse, some are tissue-specific (Landgraf et al., 2007; Liang et al., 2007). Interestingly, miRs are remarkably stable over time in body fluids including plasma and serum (Mitchell et al., 2008; Turchinovich et al., 2011), urine (Dimov et al., 2009), and saliva (Michael et al., 2010). In addition, miR levels can be assessed by different sensitive methods such as RT-quantitative real-time PCR (RT-qPCR) (Rang et al., 2012) and next-generation sequencing (Fritchard et al., 2012).

Detection of drug-induced cardiac injury (DICI) remains a major safety issue in drug development. Despite significant investments by the pharmaceutical industry over the last decade, cardiovascular toxicity represents one of the leading causes of drug attrition during preclinical and clinical development (Laverty et al., 2011). As it was described recently, cardiovascular liabilities account for withdrawal of up to 45% of marketed drugs, compared to 32% for the hepatic system (Stevens and Baker, 2009). Ideally, these liabilities, which pertain both cardiovascular- and non-cardiovascular drugs, should be addressed before entering clinical development (Ferri et al., 2013).

Regarding myocardial necrosis detection, blood troponins represent the current gold standard and are used in both preclinical and clinical studies (Reagan, 2010). However, these biomarkers show several drawbacks. For instance, cardiac troponins cTnT are rapidly cleared (O’Brien, 2008) while their kinetic profile in the blood appears to be compound-specific (Tonomura et al., 2012). Also, plasma cTnT levels may be increased in patients with renal failure, even in absence of cardiac symptoms (Ahmadi et al., 2014; Kanderian and Francis, 2006; Ziebig et al., 2003). Hence, there is a crucial need for developing new non-invasive and cost-effective translational early biomarkers of drug-induced myocardial injury. Meanwhile, a number of studies have shown the potential of miRs as sensitive biomarkers for cardiovascular diseases, which has been the subject of several reviews (Bronze-da-Rocha, 2014; Kukreja et al., 2011; Small et al., 2010). Among these, plasma miR-1, miR-133a/b, miR-499, and miR-208a have been identified as associated with acute myocardial injury. After evaluation in a rat model of acute myocardial infarction (AMI) (Wang et al., 2010), plasma miR-208a appeared to be the most reliable, confirming previous results obtained in isoproterenol (ISO)-treated rats (Ji et al., 2009). Nevertheless, our knowledge regarding miRs use as safety biomarkers in preclinical development is still limited (Mikaelian et al., 2013) and few preclinical cardiotoxicity studies have been conducted to date. More precisely, data assessing miR time-dependent profiles in the setting of DICI studies (Sandhu and Maddock, 2014) is scarce. Moreover, results derive often from different study designs limiting robust qualification of these potential novel biomarkers. Methods utilized in these studies included microarray technology with subsequent validation by RT-qPCR where data generation and validation is time-consuming and cost is non-negligible (Moldovan et al., 2014).

In this work, we conducted an evaluation of a panel of selected miRs reported in the literature to be associated with cardiac injury (human and rodent data) in a 4-day DICI rat toxicity study. Animals were dosed once with either isoproterenol (ISO), allylamine (AAM), or the related vehicle, both compounds being known as specific cardiotoxic drugs inducing myocardial necrosis (Clements et al., 2010; Toraison et al., 1989). Plasma miRs sequential detection was achieved with the high-throughput profiling technology from Firefly BioWorks that assays the expression of up to 68 selected targets in a limited sample volume (40 μl). Conventional cardiac biomarker concentrations, including plasma cTnT levels, were also sequentially collected and treatment-related histopathological findings were assessed at 24, 48, and 72 h post-drug administration. This work highlights the need for studies to evaluate miR expression according to changes induced by treatment amidst the consideration of baseline, pre-dosing and vehicle control normalisation to account for potential selection of non-cardiac injury specific miRs. Our results also confirm miR-208a-3p as a biomarker of myocardial injury in a 4-day preclinical rat model, providing, to the best of our knowledge, the first evidence of miR-208a-3p detection after AAM exposure.

**MATERIALS AND METHODS**

**Animals.** Male Hanover Wistar rats, 6- to 7-week-old (206 ± 14 g, mean ± SD) were purchased from Charles River Laboratories (Germany). Rats (n = 72) were acclimated for 1 week prior use. Animals were group-housed in standard cages (Makrolon Eurostandard type IV overlooked cages, Tecniplast, Italy) and provided with aspen bedding (Tecniplast, Italy). Rats were kept under a 12-h light/12-h dark cycle, with controlled temperature and humidity. Standard rodent food pellets (S.D.S, France) and water were available ad libitum. All animals were observed daily for abnormal signs during the course of the study. All procedures described in this study have been approved by the European Directive 2010/63/EU on the protection of animals used for scientific purpose and with the Belgian legislation on the use of laboratory animals.

**Chemicals.** Isoproterenol (ISO) (CAS 51-30-9) and Allylamine (AAM) (CAS 10017-11-5) were purchased from Sigma-Aldrich (St Louis, Missouri).

**Experimental design.** In this study, we selected ISO and AAM as compounds known to induce myocardial injury (Clements et al., 2010; Toraison et al., 1989). ISO was dissolved in 0.9% NaCl while AAM was suspended in 0.5% methylcellulose (MC); both solutions were prepared at the required concentration just prior use. The dose and administration route were selected based on the results available in the literature showing that mild to moderate histopathological changes were observed in animals treated with ISO or AAM at their respective concentrations (Tonomura et al., 2012). As it was described recently, cardiovascular miRs’ expression can be affected by renal toxins (Ahmadi et al., 2009). Hence, our knowledge regarding miRs use as safety biomarkers in preclinical development is still limited (Mikaelian et al., 2013) and few preclinical cardiotoxicity studies have been conducted to date. More precisely, data assessing miR time-dependent profiles in the setting of DICI studies (Sandhu and Maddock, 2014) is scarce. Moreover, results derive often from different study designs limiting robust qualification of these potential novel biomarkers. Methods utilized in these studies included microarray technology with subsequent validation by RT-qPCR where data generation and validation is time-consuming and cost is non-negligible (Moldovan et al., 2014).

In this work, we conducted an evaluation of a panel of selected miRs reported in the literature to be associated with cardiac injury (human and rodent data) in a 4-day DICI rat toxicity study. Animals were dosed once with either isoproterenol (ISO), allylamine (AAM), or the related vehicle, both compounds being known as specific cardiotoxic drugs inducing myocardial necrosis (Clements et al., 2010; Toraison et al., 1989). Plasma miRs sequential detection was achieved with the high-throughput profiling technology from Firefly BioWorks that assays the expression of up to 68 selected targets in a limited sample volume (40 μl). Conventional cardiac biomarker concentrations, including plasma cTnT levels, were also sequentially collected and treatment-related histopathological findings were assessed at 24, 48, and 72 h post-drug administration. This work highlights the need for studies to evaluate miR expression according to changes induced by treatment amidst the consideration of baseline, pre-dosing and vehicle control normalisation to account for potential selection of non-cardiac injury specific miRs. Our results also confirm miR-208a-3p as a biomarker of myocardial injury in a 4-day preclinical rat model, providing, to the best of our knowledge, the first evidence of miR-208a-3p detection after AAM exposure.

Pathological examination. Animals were humanely killed by exsanguination under deep anesthesia (isoflurane inhalation). Before killing, blood was obtained from the posterior vena cava as part of the sequential blood collection over the whole-time course study. Heart tissue was then collected from all rats (n = 6 animals per group and per time point) and fixed in 10% neutral buffered formalin. The fixed heart samples were trimmed, processed, and embedded in paraffin wax. Sections were cut at a thickness of 2–4 μm and stained with hematoxylin and eosin (transversal section: heart apex and base; 1 slide per animal). The sections were examined by light microscopy and the histopathological findings were recorded by a certified pathologist.
The severity of the main treatment-related lesions was graded using the following score system: grade 1: minimal; grade 2: slight; grade 3: moderate; grade 4: marked.

miRs profiling with the Firefly circulating miRNA assay. Samples were processed by Firefly BioWorks (www.fireflybio.com) using the Firefly Circulating miRNA Assay according to the manufacturer’s protocol. In brief, 40 μl of plasma was digested to release miRs and inactivate proteases. The digest was then incubated with Firefly Particles in order to hybridize miRs with target-specific probes embedded in the barcoded particles (Chapin et al., 2011). After hybridization, universal adapters bearing PCR priming sites were ligated to each end of the bound miRs. Adapted miRs were eluted from the particles and amplified by PCR using a single primer set modified with a Cy5 fluorophore. The PCR product was then incubated with Firefly Particles for a secondary hybridization. Particles were scanned on a Guava easyCyte 8HT flow cytometer (Millipore), and the output was analyzed using the Firefly Analysis Workbench software.

Measurement of plasma cTnT, cTnI, Myl3, and FABP3 concentrations. Concentrations of cardiac troponin T (cTnT) and cardiac troponin I (cTnI), myosin light chain 3 (Myl3), and fatty acid binding protein 3 (FABP3) were assessed in plasma samples using the Cardiac Injury Panel 3 (rat) assay kit from Meso Scale Discovery (Gaithersburg, Maryland) as per the manufacturer’s instructions.

Statistical analyses. SAS 9.1.2 (SAS institute Inc, Cary, North Carolina) was used for the statistical analyses of the data. Mean comparisons were performed with the nonparametric Kruskal-Wallis and Wilcoxon tests. Variations in plasma cTnT, cTnI concentrations, and in plasma miR levels in response to the treatment (ISO or AAM) were expressed as effect sizes (Cohen’s d) and the relationship between the biomarker circulatory levels and the histopathological lesions was investigated by calculating the nonparametric coefficient of correlation of Spearman r. The collective ability of the biomarkers to classify the animals as cardiac injured or not (based on the histopathological score) was explored by discriminant analysis. Probabilities of less than 0.05 were considered significant.

RESULTS

Isoproterenol or Allylamine Single Administration Induces Moderate Cardiac Injury in a 4-Day Preclinical Rat Model

Histopathological evaluation of the heart tissue after a single administration of ISO (1.5 mg/kg, i.p.) or AAM (100 mg/kg, per os) at 24, 48, and 72-h post-drug administration revealed findings in the myocardium of the lower part of the left ventricular wall and interventricular septum. Lesions consisted of moderate necrosis of the myocardial cells, slight to moderate inflammation (mainly mononuclear) of the myocardium, and at 72-h post-drug administration, minimal to slight fibroplasia in some animals (Fig. 1). No lesions were detected in the heart tissue of the control animals (saline- or MC-water-treated rats).

Identification of Plasma miRs as Potential Biomarker Candidates for Acute Cardiac Injury in Rats with the Firefly Circulating miRNA Profiling Assay

Plasma miR detection was achieved with the high throughput miR-profiling technology from Firefly BioWorks which assays the expression of a panel of 68 selected targets in a limited sample volume (40 μl of crude plasma per animal; as described in the Methods). Circulating miRs identified in the literature as associated with cardiac injury as well as three potential normalizers and two hemolysis markers were included in the 68-plex panel (Supplementary Table 2).

The identification process of miRs as potential biomarkers is described in Figure 2. Briefly, the selection was conducted through a pilot study focusing on four different time points: BD, +4, +8, and +24 h after ISO or AAM exposure (n = 6 rats per time point and per group). Analysis of the 68 miR expression profiling data revealed 11 miRs as dysregulated after a single administration with ISO or AAM (Kruskal-Wallis test; global treatment effect: ISO or AAM vs vehicle; P < 0.05) (Table 1, Supplementary Fig. 1). Within this set of data, rno-miR-103-3p, rno-mir-92a-3p, and rno-miR-106b-5p were identified as the most appropriate normalizers (genorm-like algorithm) while no significant variation was observed in the hemolysis marker expression profiles (rno-miR-451 and rno-miR-486) within each group of animals (ISO-, AAM-, and vehicle-treated rats, respectively) (data not shown). From the 11 miRs found dysregulated, 4 fulfilled the additional selection criteria of (1) homogeneity between groups before dosing and (2) no time variation among the vehicle group. These miRs were miR-208a-3p, miR-210-3p, miR-29b-3p, and miR-328-3p, with miR-208a-3p being the sole miR dysregulated in ISO- and AAM-treated rats (Supplementary Fig. 2). Interestingly, plasma miR-208a-3p could not be detected before dosing or in the vehicle-treated rats throughout the duration of the study (data not shown). Noteworthy, two different probes (based on the mouse and rat sequences available in miRBase v.20) were used in order to detect miR-208a-3p with both sets of data collected correlating well (Spearman r = 0.7, P < 0.001). We therefore chose mmu-miR-208a-3p for further characterization as a potential biomarker for detecting ISO- and AAM-induced myocardial injury.

Plasma miR-208a-3: a Strong Biomarker of Myocardial Damage Whose Kinetic Profile Appears to Be Compound-Specific

In order to confirm mmu-miR-208a-3p’s performance as a biomarker for detection of myocardial injury after ISO and AAM treatment, plasma levels of mmu-miR-208a-3p were measured in whole extent of the 4-day DICI study using the Firefly Circulating miRNA Profiling Assay. In addition of mmu-miR-208a-3p, three additional miRs identified in the pilot study as the most appropriate normalizers (rno-miR-103-3p, rno-mir-92a-3p, and rno-miR-106b-5p) were included in the analysis. As shown in Figure 3A, we confirmed miR-208a-3p release after ISO- and AAM-single administration. A single administration of ISO at 1.5 mg/kg i.p. leads to a significant increase of miR-208a-3p that peaks at +4 h (P < 0.001) and remains detectable until 24-h post-drug administration (P < 0.001), whereas a single administration of AAM at 100 mg/kg per os shows a late and transient increase of miR-208a-3p at 24 h after dosing (P < 0.001). Of note, plasma concentrations of miR-208a-3p were undetectable before dosing in all groups, as well as in the vehicle animals throughout the whole duration of the study (data not shown). Hence, plasma miR-208a-3p displays a kinetic pattern that is compound-specific where most significant early changes appear in ISO-treated rats (as early as at +1 h, P < 0.001). When analyzing time points 4 and 24-h post-drug administration with ISO and AAM, respectively (pilot study data), and comparing miR expression levels in treated to vehicle rats, miRs 126a-3p
and miR-34c-5p are dysregulated at 4-h post-ISO treatment, while miR-133a-3p and miR-133b-3p are altered at 24-h after post-AAM treatment ($P < .05$; ISO or AAM vs vehicle). Nevertheless, time course evaluation of these miRs revealed that these miR potential candidates are either unstable over time in vehicle-treated rats and/or are not significantly altered in the treated rats through the duration of the study (Supplementary Fig. 3).

FIG. 1. Isoproterenol or Allylamine Single Administration Induces Slight to Moderate Myocardial Injury. A, Heart histopathology findings were scored as described in the Methods; shown are the means ± SEM, $n = 6$ rats per time point. B–H, Histopathological changes in the heart as detected with hematoxylin and eosin staining. Normal heart tissue is evident from rats treated with appropriate vehicle (original magnification, 40×) (B). Representative sections of ISO-treated rats (C, D) and AAM-treated rats (E, F) at 24 and 48-h post-drug administration respectively: inflammatory cell infiltrate surrounding necrotic cardiomyocytes (black arrows)—original magnification 10× (C, E) and 40× (D, F). G, H, At 72 h after treatment, inflammatory cell infiltrate and beginning fibroplasia were observed in areas with necrotic myofibers (black arrows); representative sections of AAM-treated rats—original magnification 10× (G) and 40× (H); normal cardiomyocytes (white arrow). Abbreviations: AAM, allylamine; ISO, isoproterenol.
Similar to miR-208a-3p Release, Changes in Cardiac Troponin I and Cardiac Troponin T Occur Earlier in ISO-treated rats

The kinetic patterns of the conventional cardiac biomarkers cardiac troponin I (cTnI), cardiac troponin T (cTnT), myosin light chain 3 (Myl3), and fatty acid binding protein 3 (FABP3) were assessed in the plasma of ISO- and AAM-treated rats. As shown in Figures 3B and C, most significant early changes in cTnI occurred in ISO-treated rats: cTnI highest increase is observed at 2-h post-ISO single administration (ISO vs vehicle; \( P < .001 \)) while cTnT peaks at 8-h post-drug administration (ISO vs vehicle; \( P < .001 \)) although a slight increase is observed at earlier time points (ISO vs vehicle; \( P < .001 \)). In rats treated with AAM, cTnI and cTnT kinetic profiles display a late and transient peak at 24-h post-drug administration (\( P < .001 \)). Of note, cTnI and cTnT could not be detected in related vehicle-treated rats due to the assay lower limit of detection and quantitation (data not shown). With regard to the sequential profiles of Myl3 and FABP3, Myl3 performs better as an early biomarker in ISO-treated rats (Supplementary Figs. 4A–B) while FABP3 lacks specificity, especially in the context of AAM exposure (Supplementary Figs. 4C–D). From these four conventional biomarkers, two (the cTnT and cTnI) fulfill the biomarker selection criteria as defined in Figure 2.

Performance of miR-208a-3p in Comparison to Cardiac Troponins T and I

In order to assess the robustness of miR-208a-3p in comparison with the cTn, we evaluated how these biomarkers performed together in the present 4-day DICI rat preclinical model. Evaluation of cTnI, cTnT, and miR-208a-3p levels at the most relevant time points showed that, in the setting of ISO-induced cardiac injury, Spearman’s correlation between the three biomarkers is significant, especially at 4-h post-drug administration (\( r > 0.65, P < .05 \) (Table 2)). In AAM-treated rats, miR-208a-3p expression profile is late and transient, similar to the cTnI and cTnT release, which is attested by Spearman’s correlation that is highly significant at 24-h post-drug administration (\( r = 0.74 \) and \( r = 0.73 \), respectively, \( P < .01 \) for each biomarker pairwise comparison) (Table 2). To further characterize the performance of these
AAM administration induces a sharp increase in each biomarker histopathology scoring (Fig. 1A) and the expression level of biomarkers. We correlated the severity of the lesion based on the value of zero ng/ml. Data are presented as the means ± SEM; nonparametric Wilcoxon test used to investigate differences due to treatment (ISO or AAM vs vehicle, by time). **P < .01. Abbreviations: AAM, allylamine; AU, arbitrary units; BD, before dosing; cTnI, cardiac troponin I; cTnT, cardiac troponin T; ISO, isoproterenol; mmu-miR-208a-3p, Mus musculus microRNA-208a-3p.

DISCUSSION

DICI is one of the main causes of drug attrition in preclinical and clinical settings with cardiovascular liabilities accounting for ~27% of drug failure at early stages of drug development (Laverty et al., 2011). Cardiac biomarkers that are routinely used for assessing myocardial injury include creatine kinase-MB isoenzymes, cardiac myoglobin, and cTn. Nevertheless, many of these biomarkers lack sensitivity and/or specificity (Osaki et al., 2014). Even the cTn, robust biomarkers of myocardial damage, display some drawbacks including being rapidly cleared (Wallace et al., 2004) while cell cardiac injury that does not lead to altered cardiac cell permeability may not be associated with increases in cTn (Sandhu and Maddock, 2014). There is thus an unmet challenge of developing novel early translational biomarkers which, alone or in association with previously identified biomarkers, would improve DICI detection. Circulating miRs have emerged as promising safety biomarkers and are currently extensively described in this context. miRs combine several attributes as attractive biomarkers: highly conserved between species, stable in body fluids (plasma, urine, saliva, ...), tissue and/or disease-specific, and can be measured by sensitive techniques such as RT-qPCR (Kroh et al., 2010). However, few preclinical studies aiming at profiling miRs in the setting of DICI are available while generated data can be contradictory, especially when utilizing doxorubicin as a cardiac injury model (Desai et al., 2014; Horie et al., 2010; Nishimura et al., 2015; Vacchi-Suzzi et al., 2012; Wang et al., 2015).

In this work, we evaluated the performance of a panel of reported cardiac injury plasma miRs (human and rodent literature data; Supplementary Table 2) in an acute DICI rat preclinical model. Out of 68 selected targets, we showed that 11 different miRs were significantly dysregulated after ISO or AAM single administration within 24-h post-drug administration (Table 1). When applying our filtering criteria of homogeneity between groups before dosing and no time variation among the vehicle group, a single miR was found to be specifically upregulated in both ISO and AAM-treated rats, i.e., miR-208a-3p (Supplementary Fig. 1 and Fig. 3A). Our study stresses the importance of designing experiments that enable time course evaluation comparisons and that include predosing measurements in all groups. Indeed, as we elegantly showed in this work, out of the 10 different plasma miRs whose expression was modified in treated rats (Table 1), 6 were not stable in the vehicle group, restricting their utility as cardiac injury-specific biomarkers. Of note, conventional plasma biomarkers Myl3 and FABP3 encounter the same limitations, especially FABP3.

Biomarkers, we correlated the severity of the lesion based on the histopathological scoring (Fig. 1A) and the expression level of each biomarker. cTn and miR-208a-3p performed equally for detecting the severity of myocardial damage induced by ISO, especially at 2- and 4-h post-drug administration (r values ranging from 0.69 to 0.8; P < .05) (Table 3 and Supplementary Figs. 5A–C). Only at time point 8-h post-ISO administration, does cTnT significantly outperform miR-208a-3p (r of 0.66 vs 0.27, P < .01). When AAM administration induces a sharp increase in each biomarker expression, i.e., at 24-h post-drug administration, the correlation between the histopathology scoring and the biomarker levels is low to moderate (0.45 < r < 0.50) (Table 3 and Supplementary Figs. 5D–F). Effect sizes (ES) were calculated to assess the magnitude of each biomarker increase in response to the treatment compared to vehicle levels at the various time points. As shown in Table 3, all three biomarkers show large ES values (all ES values above 0.9) in the setting of ISO-induced cardiac injuries, with cTnI being the best performer at earliest time points (CTnI ES = 2.5 and 2.2, at 1 and 2-h post-ISO administration, respectively). In contrast, at 24-h post-AAM administration, ES values of all three biomarkers are moderate (<0.65) (Table 3), reflecting the high variability observed for each biomarker at this time point in AAM-treated rats (Fig. 3), especially with the cTn. Lastly, miR-208a-3p discriminant ability to detect ISO- or AAM-injured rats compared to non-injured rats is comparable to that of the cTn (90–95% of accurate classification).

FIG. 3. Kinetic patterns of plasma miR-208a-3p cardiac troponin I (cTnI) and T (cTnT) after single administration withISO and AAM in rats. Out of the 68 miR targets profiled through a pilot study with the Firefly Circulating miRNA Assay, miR-208a-3p was selected as a biomarker candidate for acute cardiac injury. This was confirmed when evaluating, with the same technology, plasma miR-208a-3p expression profile through the full time course study. Plasma miR-208a-3p release is induced in ISO and AAM-treated rats (A). Time courses of plasma cTnI (B) and cTnT (C) concentrations after single administration of ISO (1.5 mg/kg, i.p.) or AAM (100 mg/kg, per os). Most early significant changes in cTnI and cTnT occur in ISO-treated rats. Of note, undetected levels of cTn were assigned a value of zero ng/ml. Data are presented as the means ± SEM; nonparametric Wilcoxon test used to investigate differences due to treatment (ISO or AAM vs vehicle, by time). **P < .01. Abbreviations: AAM, allylamine, AU, arbitrary units; BD, before dosing; cTnI, cardiac troponin I; cTnT, cardiac troponin T; ISO, isoproterenol; mmu-miR-208a-3p, Mus musculus microRNA-208a-3p.
Table 2. Spearman’s Correlation Coefficient (r) Between miR-208a-3p and cTnI or cTnT Levels After Single Dosing with ISO or AAM at Selected Time Points Based on Biomarker Significant Increase

<table>
<thead>
<tr>
<th>Cardiotoxic Treatment</th>
<th>Collection Time points (h)</th>
<th>Spearman’s correlation coefficient (r) between miR-208a-3p and cTnI</th>
<th>Spearman’s correlation coefficient (r) between miR-208a-3p and cTnT</th>
</tr>
</thead>
<tbody>
<tr>
<td>ISO</td>
<td>1</td>
<td>0.55</td>
<td>0.44</td>
</tr>
<tr>
<td>ISO</td>
<td>2</td>
<td>0.50</td>
<td>0.52</td>
</tr>
<tr>
<td>ISO</td>
<td>4</td>
<td>0.66*</td>
<td>0.70*</td>
</tr>
<tr>
<td>ISO</td>
<td>8</td>
<td>0.39</td>
<td>0.27</td>
</tr>
<tr>
<td>AAM</td>
<td>24</td>
<td>0.74**</td>
<td>0.73**</td>
</tr>
</tbody>
</table>

Abbreviations: AAM, allylamine; ISO, isoproterenol.

* and ** Spearman’s correlation is significant at the 0.05 and 0.01 levels, respectively.

Table 3. Spearman’s Correlation Coefficient (r) Between miR-208a-3p, cTnI, cTnT levels, and the Histopathology Scores, as well as Effect Size for Each Biomarker, After Single Dosing with ISO or AAM at Selected Time Points Based on Biomarker Significant Increase

<table>
<thead>
<tr>
<th>Histopath score</th>
<th>miR-208</th>
<th>cTnI</th>
<th>cTnT</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>r</td>
<td>r</td>
<td>r</td>
</tr>
<tr>
<td>ISO 1 h</td>
<td>0.53</td>
<td>1.34</td>
<td>0.70**</td>
</tr>
<tr>
<td>ISO 2 h</td>
<td>0.80**</td>
<td>0.93</td>
<td>0.73**</td>
</tr>
<tr>
<td>ISO 4 h</td>
<td>0.80**</td>
<td>1.04</td>
<td>0.69**</td>
</tr>
<tr>
<td>ISO 8 h</td>
<td>0.27</td>
<td>1.04</td>
<td>0.57</td>
</tr>
<tr>
<td>ISO 24 h</td>
<td>0.45</td>
<td>0.57</td>
<td>0.49</td>
</tr>
<tr>
<td>AAM 24 h</td>
<td>0.45</td>
<td>0.57</td>
<td>0.49</td>
</tr>
</tbody>
</table>

Effect size were calculated as standardized differences (Cohen effect size 4) of the two modalities being compared (treatment vs vehicle), using the standard deviation of ISO- or AAM-treated rats. Abbreviations: AAM, allylamine; histopath, histopathological; ISO, isoproterenol.

* and ** Spearman’s correlation is significant at the 0.05 and 0.01 levels, respectively.

( Supplementary Fig. 4 ), which is consistent with previous results (Clements et al., 2010; Tonomura et al., 2012). Besides, FABP3 (Pritt et al., 2008) and Myl3 (Tonomura et al., 2009) distribution is not limited to the heart tissue restraining their performance as selective cardiac injury biomarkers. Furthermore, our analyses reveal that time snapshot comparisons between vehicle and treated rats, focusing on selected time points, may also lead to misleading conclusions ( Supplementary Fig. 3 ). Reflected on these findings, it is imperative to undertake studies that grasp the whole time-dependent profile of miR biomarker candidates in all groups including the vehicle. This is in line with a recent study highlighting the need of taking into account pre-profiling factors influencing serum miR levels such as the effect of hemolysis or fasting (MacLellan et al., 2014).

With regard to miR-208a-3p, the present work demonstrates that its release is induced after a single dose administration of either ISO or AAM, providing the first evidence of plasma miR-208a-3p detection in AAM-treated rats. Through the thorough evaluation of this miR as a biomarker candidate, including sequential cTn measurements and histopathology finding scoring as an endpoint, we also showed that miR-208a-3p is a strong cardiac specific biomarker whose expression profile appears to be compound-specific (Fig. 3A). Similar to cTn kinetic patterns after single administration with ISO or AAM (Figs. 3B, C), most significant early changes occur in ISO-treated rats suggesting miR-208a-3p expression profile may imply a compound-specific mechanism of action. Indeed, as a β-adrenergic agent, ISO induces cardiac pathological alterations that occur rapidly after administration (Clements et al., 2010) while AAM-induced lesions derive from its metabolite acrolein (Boor and Hysmith, 1987), which may explain the delayed release of miR-208a-3p and cTn after AAM versus ISO exposure. From a preclinical safety perspective, these results suggest that plasma miR-208a-3p, as a cardiac injury biomarker, may face the same limitations as the cTn which exhibit critical diagnostic windows (Tonomura et al., 2012). However, further studies would be warranted to address this question.

When assessing miR-208a-3p robustness in comparison with cTn through correlation analysis (Table 2), we show that plasma miR-208a-3p has an optimal performance at time points 4 and 24-h post-ISO and AAM administration, respectively. When taking into consideration the severity of the lesions as an endpoint, our analyses indicate that, overall, miR-208a-3p performance is comparable to that of cTn (Table 3 and Supplementary Fig. 5).

Altogether, this set of data reveals that plasma miR-208a-3p performance is robust enough to be used as an early biomarker of cardiac injury detection, similarly to cTn. While these results are in agreement with previous observations (Ji et al., 2009; Nishimura et al., 2015; Wang et al., 2010), they also provide additional information, especially regarding miR-208a-3p kinetic profile. In comparison with other studies where plasma miR-208a-3p increase is sustained through 24 h after single ISO administration (Nishimura et al., 2015), the present work shows a whole time-dependent profile of plasma miR-208a-3p with a kinetic of increase and peaks at 4 and 24 h (ISO and AAM administration, respectively) and subsequent decrease to baseline values throughout the duration of the study (Fig. 3). These distinctive findings regarding miR-208a-3p kinetic profile in the setting of ISO-induced cardiac injury may be explained due to differences in route of administration and dosing regimen. Interestingly, previous reports suggesting miR-208a-3p as a useful DICI biomarker revealed that ISO administration in rats induces a transient increase of plasma miR-208a-3p (that remained significant increased up to 12 h after administration) over a period of 24-h post-drug administration (Ji et al., 2009), which is consistent with our results. To further characterize the robustness of plasma miR-208a-3p in comparison with cTn, we evaluated the magnitude of each biomarker increase compared to vehicle levels in our 4-day DICI rat model. Effect size analyses show that the magnitude of miR-208a-3p and cTn increases (Table 3) are mostly similar, which is in accordance with previous observations made in ISO-treated C57BL6/J wild-type mice (Liu et al., 2014). Noteworthy, in Liu et al. (2014) study, as well as in this work, plasma cTn concentrations were assessed using relative low sensitivity commercialized immunoassays, which may lead to underestimate cTn response magnitude.
Nevertheless, based on the present work, miR-208a-3p detection of cardiotoxicity does not appear to be superior than cTn, yet it is similarly reliable. In studies conducted in patients suffering of AMI and evaluating circulating miR profile expression at early stages of symptom onset, miR-208a-3p expression could be detected in AMI patients when compared to healthy controls (Hsu et al., 2014; Wang et al., 2010). Both in human and in rat, miR-208a-3p is encoded by the α-cardiac muscle myosin heavy chain 6 gene and its nucleotide sequence is highly conserved between the two species. Moreover, miR-208a-3p is exclusively expressed in the heart tissue (Ji et al., 2009; Wang et al., 2010) where it plays a vital role in cardiac conduction (Huang and Li, 2015). Altogether, this confirms miR-208a-3p strong potential as a translational cardiac injury biomarker.

Consequently, our recommendation would consist in monitoring plasma miR-208a-3p along with cTn as a panel of early biomarkers to assess cardiac safety in a preclinical setting. Such an approach would provide further characterization of miR-208a-3p compared to cTn while increasing our understanding of the mechanisms of action associated with miR-208a-3p and cardiac toxicity. Although some data is available as to miR-208a-3p cardiac specificity (Nishimura et al., 2015), data on half-life and clearance of circulating miRs (Mikaelian et al., 2013) is scarce. Similarly, the precise cellular release mechanisms of miRs into the blood remain to be investigated (Creemers et al., 2012).

In conclusion, the present work highlights the critical need of undertaking studies that assess the whole time-dependent profile of miR expression as a result of drug-induced tissue injury. The prioritization of specific miRs as potential biomarkers facilitates a more comprehensive and robust evaluation of miR candidates. To this extent, variations that can be observed among the vehicle and that may lead to misleading conclusions will be easily detected. Out of a panel of 68 reported cardiac injury plasma miRs, we identified a single miR candidate, i.e. plasma miR-208a-3p as being a strong cardiac injury biomarker in the setting of ISO- and AAM-single administration, providing the first evidence of miR-208a-3p detection after AAM exposure. A thorough evaluation of its performance in comparison with the current gold standard cTn revealed its even ability to detect DICI. As cost-effective, customizable, and sensitive technologies such Firefly Bioworks platform are being developed, monitoring plasma miR-208a-3p as a non-invasive, sensitive, and specific cardiac biomarker is of high feasibility. Firefly Bioworks assay works from as little as 40 μl of crude plasma while it leverages PCR sensitivity and enables the high throughput profiling of circulating miRs of interest (Flowers et al., 2015; Le et al., 2014). Moreover, characterization of miR-208a-3p compared to cTn will reinforce the evaluation of this miR as a robust biomarker. An extensive assessment, with additional compounds, of miR-208a-3p ability to early and specifically detect cardiotoxicity issues would enable its rigorous validation. Strengthening the credibility of the use of miR-208a-3p, as part of a panel of early biomarkers including cTn, combined to histopathological evaluation has indeed the potential to support thorough cardiac hazard identification and risk assessment in a translational manner.

**SUPPLEMENTARY DATA**

Supplementary data are available online at http://toxsci.oxfordjournals.org/.

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**REFERENCES**


