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miR-21-5p as a potential biomarker of inflammatory infiltration in the heart upon acute drug-induced cardiac injury in rats

Vitalina Gryshkova[a], Alisha Fleming[a], Portia McGhan[b], Pierrette De Ron[a], Renaud Fleurance[a], Jean-Pierre Valentin[a], Andre Nogueira da Costa[c,*]

[a] Investigative Toxicology, Non-Clinical Development, UCB Biopharma SPRL, Belgium
[b] Faculty of Biological Sciences, LS2 9JT, Leeds, UK
[c] Experimental Medicine and Diagnostics, Global Exploratory Development, UCB Biopharma SPRL, Belgium

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ABSTRACT

Investigation of genomic changes in cardiotoxicity can provide novel biomarkers and insights into molecular mechanisms of drug-induced cardiac injury (DICI). The main objective of this study was to identify and characterize dysregulated microRNAs (miRNAs) in the heart associated with cardiotoxicity. Wistar rats were dosed once with either isoproterenol (1.5 mg/kg, i.p), allylamine (100 mg/kg, p.o.) or the respective vehicle controls. Heart tissue was collected at 24 h, 48 h and 72 h post-drug administration and used for histopathological assessment, miRNA profiling, immunohistochemical analysis and *in situ* hybridization. Multiplex analysis of 68 miRNAs in the heart revealed a significant upregulation of several miRNAs (miR-19a-3p, miR-142-3p, miR-155-5p, miR-208b-3p, miR-21-5p) after isoproterenol and one miRNA (miR-21-5p) after allylamine administration. Localization of miR-21-5p was specific to inflammatory cell infiltrates in the heart after both treatments. Immunohistochemical analysis of Stat3, a known miR-21-5p regulator, also confirmed its upregulation in cardiomyocytes and inflammatory cell infiltrates. The toxicity signatures based on miRNA networks, identified *in vivo*, can potentially be used as mechanistic biomarkers as well as to study cardiotoxicity *in vitro* in order to develop sensitive tools for early hazard identification and risk assessment.

1. Introduction

Cardiovascular toxicity has accounted for 14% of drug attritions worldwide since 1950 (Onakpoya et al., 2016). Prediction of some functional cardiotoxicity can be successfully achieved by using electrophysiological tests, *e.g.* hERG and QTc data (Ewart et al., 2014; Wallis, 2010). The early detection of structural cardiotoxicity and identification of the corresponding molecular mechanisms has proven to be challenging (Laverty et al., 2011; Mellor et al., 2011). Novel mechanistic biomarkers are required for better understanding of structural cardiotoxicity and establishing *in vitro* systems to predict cardiotoxic side effects of new drug candidates.

Biofluid biomarkers offer a convenient alternative to monitor organ toxicity, both in preclinical and clinical settings. Cardiac troponin T (cTnT) and troponin I (cTnI), "gold standards" of cardiac injury, are very sensitive plasma biomarkers of myocardial infarction (MI) (Kociol et al., 2010; Mair et al., 1996; Pierson et al., 2013). Troponins were also shown to be sensitive biomarkers of drug-induced cardiac injury (DICI) in animal models with elevated levels detected as early as 2 h for cTnI and 8 h for cTnT after cardiotoxicant administration (Tonomura et al., 2009; Zhang et al., 2008; Reagan, 2010). However, troponins are released after heart tissue damage has occurred and their half-life in plasma is very short (Tonomura et al., 2009). Using troponins in *in vitro* models, such as stem cell cardiomyocytes, has proven to be challenging due to their late release and detection limits (Chaudhari et al., 2016; Holmgren et al., 2015). This prompts a need for identification of more stable and sensitive biomarkers indicative of early events in cardiac injury.

microRNAs (miRNAs) have emerged as promising biomarkers to study normal or pathological conditions as well as toxic responses to drugs (Etheridge et al., 2011). miRNAs are small (19–25 nucleotides) regulatory RNAs that bind to a complementary region on messenger RNA (mRNA) to inhibit their translation and promote mRNA degradation (Ambros, 2004; Bartel, 2004). Non-canonical ways of positive regulation of gene expression by miRNAs are described in detail by Cipolla A. (Cipolla, 2014). miRNAs are implicated in a range of diseases, including cancer, autoimmune diseases, neurobiological disease and cardiovascular pathologies (Tufekci et al., 2014). Involvement of

* Corresponding author.
E-mail address: Andre.DaCosta@ucb.com (A. Nogueira da Costa).

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miRNAs in various pathological processes in the heart, like cardiomyopathies, cardiac remodeling, heart failure, arrhythmia and ischemia/reperfusion makes them attractive as potential diagnostic and prognostic tools (Song et al., 2015).

Certain miRNAs (miR-1-3p, miR-208a-3p, miR-499-5p) are enriched in the myocardium across species and can be detected in biofluids upon acute cardiac injury (Devaux et al., 2012; Thompson et al., 2016; Vacchi-Suzzi et al., 2013). Circulating miRNAs are stable, conserved across species and changes in biofluidic miRNAs have been associated with troponin levels in acute cardiac injury (Thompson et al., 2016). To date, involvement of miRNAs in pathogenesis of MI is well described in *in vitro/in vivo* models and clinical setting (Bostjancic and Glavac, 2014). Several miRNAs were shown to be associated with different biological processes in MI, *i.e.*, apoptosis (miR-1, miR-206, miR-21); angiogenesis (miR-92a, miR-15b, miR24) and fibrosis (miR-29, miR-24, miR-711) (Bostjancic and Glavac, 2014). However, the reports on tissular miRNAs and their regulation in DICI are limited. A panel of anti-hypertrophic (miR-1, miR-21 and miR-27b) and pro-hypertrophic (miR-22, miR-24, miR-199a, miR-212, miR-214) miRNAs was found to be upregulated in rat hearts after isoproterenol administration (Creemers et al., 2012). Another study showed that tissular miRNAs could be valuable biomarkers for detection of chronic doxorubicin-induced cardiac toxicity before histopathological lesions had occurred (Vacchi-Suzzi et al., 2012).

We have previously identified miRNAs dysregulated in the plasma of rats after either isoproterenol (ISO) or allylamine (AAM) administration (Glineur et al., 2016). The present work aimed to identify and characterize tissular miRNAs as well as to establish any possible correlation with plasma miRNA in the same model of DICI. We demonstrated upregulation of several miRNAs (miR-19a-3p, miR-142-3p, miR-155-5p, miR-208b-3p, miR-21-5p), where miR-21-5p was upregulated after administration of either ISO or AAM. Localization of miR-21-5p appeared to be specific to inflammatory cell infiltrates in the heart. Analysis of transcription factor Stat3, a miR-21-5p regulator, showed its specific upregulation in cardiomyocytes and inflammatory cells for both treatments. Our results suggest a link between miR-21-5p, Stat3 and inflammation in DICI and a possibility of using miR-21-5p as a biomarker of inflammation in cardiotoxicity.

2. Materials and methods

2.1. Animals and study design

Animal studies were conducted as previously described by Glineur et al. (2016). Briefly, male Hanover Wistar rats, 6–7 week-old were randomly assigned to one of four treatment groups (n = 18 rats/treatment), and were administered with either 1.5 mg/kg of isoproterenol (ISO, intraperitoneally, i.p, in 0.9% NaCl), 100 mg/kg allylamine (AAM, orally, p.o, in 0.5% methylcellulose (MC)) or the respective vehicle controls (VC1, 0.9% NaCl, intraperitoneally, i.p; VC2, orally, p.o, MC). ISO (CAS 51-30-9) and AAM (CAS 10017-11-5) were purchased from Sigma-Aldrich (St. Louis, Missouri). All experimental procedures were approved by the ethical committee for animal experimentation at UCB Biopharma SPRL and were in accordance with 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.

2.2. Heart collection

Animals were killed by exsanguination under deep anesthesia (isoflurane inhalation) at 24 h (D1), 48 h (D2) and 72 h (D3) post-drug administration (n = 6 rats/time point/group). Hearts were collected from all rats. For histopathological examination, immunohistochemistry (IHC) and *in situ* hybridization (ISH), heart tissue (transversal section: heart apex and base) was fixed in 10% neutral-buffered formalin. The fixed heart samples were trimmed, processed and embedded in paraffin wax. The rest of the heart (section of approximately 3 mm between the apex and the base of the heart) were stored in RNAlater (Sigma-Aldrich, St. Louis, Missouri) at −80 °C to be used for total RNA extraction.

2.3. RNA extraction

Heart tissue was homogenized in TRIzol reagent (Invitrogen, Carlsbad, Ca, USA) and total RNA was extracted according to the manufacturer's recommendations. miRvana columns (Ambion, TX, USA) were used to clean up total RNA. Concentration and purity of the isolated RNA was evaluated using a Nanodrop spectrophotometer (ND-8000, Thermo Fisher, Langenselbold, Germany). The mean value of Abs260/280 was 2,1 (min–max 1,5–2,5). RNA integrity was confirmed by Bioanalyzer 2100 (Aligent Technologies, France) with mean RNA integrity number (RIN) 8,4 (min–max - 6,2–10).

2.4. miRNA multiplexing

68 miRNAs were previously selected based on their implication in cardiovascular diseases (Table S1). miRNA multiplexing was performed on total RNA (n = 18 rats/treatment) according to Firefly miRNA Assay manufacturer's recommendations (Abcam, Cambridge, UK). Briefly, 5 ng of RNA was hybridized with Firefly particles at 37° C for 60 min. After washing (2×), universal oligonucleotide adapters were ligated to the 3′ end of the hybridized RNA to serve as priming sites for PCR. Next, labeled miRNAs were dehybridized from particles and underwent one-step RT-qPCR. The PCR product was then incubated with Firefly™ particles for a secondary hybridization. Finally hybridized particles were scanned on a Guava easyCyte 8HT flow cytometer (Millipore, Germany).

2.5. Quantitative real-time PCR (qRT-PCR)

miRNA cDNA was synthesized using the TaqMan Advanced miRNA cDNA synthesis kit and assay protocol, including the miRNA-amplification step (Applied Biosystems, Foster City, CA, USA). RT-qPCR using TaqMan probes (miR-21-5p, miR-26a-5p, miR-29a-3p and miR-103-3p) was carried out in a ViiA7 instrument (Applied Biosystems, Foster City, CA, USA). Each sample was run in duplicate. Variation in expression levels of the miR-21-5p were corrected by normalizing to the mean Ct of miR-26a-5p, miR-29a-3p and miR-103-3p and calculated as ΔCt values. ΔΔCt values were calculated for each sample compared to the mean ΔCt of the vehicle group, and subsequently converted to a fold change (FC) by applying the formula $2^{-\Delta\Delta Ct}$.

2.6. In situ hybridization (ISH)

ISH was performed on formalin-fixed paraffin-embedded (FFPE) tissue sections (n = 9 rats/treatment) in a Discovery Ultra instrument (Ventana, 750-601). Hybridization with 5 nM of digoxigenin (DIG)-labeled miRNA probes for miR-21-5p and scrambled negative control (Exiqon, Vedbaek, Denmark) was done at 52 °C during one hour. After incubation with anti-DIG-HRP antibody (Ventana, 760-4822), DISCOVERY Amp BF Kit (Ventana, 760-226) in conjunction with DISCOVERY anti-BF HRP (Ventana, 760-4828) was used to amplify the signal. The staining was developed with Silver kit (RUO), DISCOVERY (Ventana, 760-227) and Haematoxylin II (Ventana, 790-2208) was utilized for counterstaining. Slides were scanned using a Nano-Zoomer XR scanner (Hamamatsu, Japan) at 20× magnification for whole slide imaging and analyzed using NDP View 2 software (Hamamatsu, Japan). Presence of the miRNAs was assessed by the appearance of a dark precipitates.