

Serum miR-208 as a biomarker of drug-induced myocardial injury in rats: A histopathology-referenced threshold analysis

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Abstract

Early identification of drug-induced cardiotoxicity (DICT) is crucial for efficient safety assessment in pharmaceutical development. While histopathology serves as the standard for evaluating structural injury, biomarkers capable of detecting toxicity at an early stage are highly desirable. This study evaluated the utility of circulating miR-208 as a highly specific biomarker for cardiotoxicity screening and aimed to establish a quantitative preclinical diagnostic criterion. First, analysis of a rat miRNA expression atlas confirmed that miR-208 is exclusively enriched in the heart, lacking the skeletal muscle background expression that frequently confounds conventional markers. Subsequently, Sprague-Dawley rats were administered five diverse cardiotoxic drugs (isoproterenol, carbofuran, allylamine, doxorubicin, and Compound A) to profile serum miR-208 responses against histopathological findings. Receiver Operating Characteristic (ROC) analysis across these models demonstrated high diagnostic accuracy (AUC = 0.91), establishing a robust cutoff value that provides 95% specificity for preclinical evaluation. Notably, in doxorubicin-treated rats, an upward trend in serum miR-208 levels was observed as early as 24 h post-administration. This early molecular response preceded the onset of structural cell death detectable by histopathology and any macroscopic functional decline measurable by echocardiography. These findings demonstrate that miR-208 is a highly specific and accurate screening tool capable of capturing early cardiotoxic signals, thereby providing an objective and powerful diagnostic advantage in preclinical safety assessments.

Introduction

Drug-induced cardiotoxicity (DICT) is one of the most critical concerns in pharmaceutical development (Ferri N et al 2013, Mamoshina P et al 2021, Ferdinandy P et al 2019). It is classified as a severe adverse event and acts as a major safety signal that frequently leads to the attrition of drug candidates during research and development, potentially causing irreversible damage (Hirakawa B et al 2008). Well-known examples of approved drugs reported to induce cardiotoxicity include doxorubicin and trastuzumab (Mamoshina P et al 2021, Lyon AR et al 2022, Leong DP et al 2024). Since the cardiovascular system is a vital organ system, drug-induced cardiotoxicity (DICT) constitutes a major development risk (Mamoshina P et al 2021, Ferdinandy P et al 2019); thus, detecting DICT as early as possible is a crucial challenge for ensuring patient safety and minimizing development costs (Gintant G et al 2016). Conventionally, histopathological examination using animal models in preclinical studies has been the gold standard for evaluating cardiotoxicity (Hirakawa B et al 2008). However, histopathological examination provides direct evidence of myocardial injury only at the time of tissue collection and therefore does not permit longitudinal monitoring in the same animal. In addition, conventional echocardiographic indices such as LVEF may remain largely preserved despite subclinical myocardial injury. In rat models of doxorubicin-induced myocardial injury, myocardial strain and ultrasonic tissue-characterization indices revealed abnormalities when LVEF showed little or no reduction, and these measures were associated with serum cardiac troponin I concentrations, histopathological cardiomyopathy scores, or myocardial collagen deposition (Romano et al 2012, Rodrigues PG et al 2019). Although advanced echocardiographic techniques can detect such subclinical changes, their application in small animals requires anesthesia, specialized equipment, and operator expertise, which may limit their use in high-throughput screening. Accordingly, minimally invasive circulating biomarkers that can be measured serially are needed to complement histopathology and cardiac imaging in routine preclinical safety studies.

In recent years, circulating microRNAs (miRNAs) have garnered significant attention as novel biomarker candidates for non-invasive liquid biopsies (Oda S et al 2021, Schofield AL et al 2021). miRNAs are single-stranded, non-coding RNAs approximately 22 nucleotides in length that regulate post-transcriptional gene expression through the degradation or translational repression of target mRNAs via the RNA interference pathway (Bartel DP 2004). Because they are often abundantly expressed in specific tissues, remain stable in circulating blood, and exhibit high sequence conservation across species, they are considered highly promising biomarkers for organ-specific toxicity in preclinical research. In the context of myocardial injury, miR-1, miR-133a, miR-208, and miR-499 have been investigated as primary biomarker candidates (Wang GK et al 2010, Nishimura Y et al 2015). However, miR-1, miR-133a, and miR-499 are highly expressed not only in the myocardium but also in skeletal muscle, their circulating levels can increase due to stress-induced or

concurrent skeletal muscle injury, posing a significant risk of false positives in cardiotoxicity evaluations (Wang GK et al 2010, Nishimura Y et al 2015). While miR-208 has been investigated as a promising biomarker candidate for myocardial injury, crucial gaps remain for its practical implementation in routine drug screening. Specifically, its selectivity for myocardial injury relative to skeletal muscle injury has not been fully evaluated, quantitative diagnostic cutoff values mathematically derived from histopathological outcomes are lacking, and its temporal relationship to echocardiographic functional decline has not been fully characterized in diverse toxicant models. Against this background, the present study aimed to evaluate the myocardial selectivity, statistical diagnostic performance, and predictive performance of miR-208 as a biomarker for the early detection of cardiotoxicity. This study also sought to establish an evaluation index that can capture the very early stages of cardiotoxicity while minimizing the risk of false positives, thereby facilitating its application to routine preclinical toxicity studies.

Materials & Methods

2.1. Reagents

To induce cardiac toxicity, the following commercially available compounds were used. Isoproterenol, allylamine hydrochloride, cyclophosphamide, 2,3,5,6-tetramethyl-1,4-phenylenediamine (TMPD), and bromobenzene were from Tokyo Chemical Industry (Tokyo, Japan). Doxorubicin hydrochloride (DOX), cisplatin, puromycin aminonucleoside, and methotrexate were from Fujifilm Wako Pure Chemical Corporation (Osaka, Japan). Carbofuran and acetaminophen were from Sigma-Aldrich (St. Louis, MO, USA), and Compound A, a proprietary compound, was synthesized by Nippon Shinyaku Co., Ltd. (Kyoto, Japan).

2.2. Animals

Male Sprague-Dawley rats were purchased at 5 weeks of age from Jackson Laboratory Japan (Kanagawa, Japan). The animals were housed in flat-bottom cages, 3 or 4 animals to a cage, with controlled temperature (20–26°C), humidity (35–75%), and illumination (15–300 lx) with a 12-h light/dark cycle. All animals were allowed free access to tap water and food (CE-2; CLEA Japan, Tokyo, Japan). The animals were 6 or 7 weeks of age at the start of administration. All animal experiments were approved by the Laboratory Animal Care and Use Committee of Nippon Shinyaku Co., Ltd, and performed in compliance with the Laboratory Animal Policy of Nippon Shinyaku Co., Ltd.

2.3. Study design

An overview of the study design is shown in Table 1. At the beginning of the study, male rats were allocated to groups of 3 to 6 based on their body weights measured just before starting treatment. The following compounds were administered via intraperitoneal (i.p.), intravenous (i.v.), or oral (p.o.) routes. For i.p. administration, isoproterenol was given at 0.5 and 1.5 mg/kg, carbofuran at 1.5 mg/kg, cyclophosphamide at 150 mg/kg, and methotrexate at 10 mg/kg. For i.v. administration, DOX was injected at 10, 20, or 30 mg/kg as a single dose, or at 6 mg/kg for 3 consecutive days. Cisplatin (0.5 and 1.5 mg/kg), puromycin aminonucleoside (100 mg/kg), and Compound A (200 mg/kg) were also administered intravenously. For oral administration, allylamine (100 mg/kg), acetaminophen (1000 mg/kg), TMPD (20 mg/kg), and bromobenzene (20 mg/kg) were given. Control animals in each group received the vehicle. At the scheduled necropsy time points (Day 1, 2, 3, 4 or 9 after the start of treatment as specified in Table 1), the animals were anesthetized by inhalation of 3.0% isoflurane. Blood (about 5 ml) was collected from the abdominal aorta into tubes containing a clot activator. After centrifugation at 3000 × g for 5 min at 4°C, the serum obtained was stored at – 65°C or below until required for experiments. The animals were euthanized by exsanguination from the abdominal aorta under deep anesthesia, and hearts were removed for histopathological examination.

Table 1
Overview of study design

Compound	Route	Dose (mg/kg)	Vehicle	Treatment period	Necropsy (days after dosing)	No. of animals per group (dead)
Isoproterenol	i.p.	0.5, 1.5	Saline	1	1	3
Carbofuran	i.p.	1.5	Saline	1	1	3
Allylamine	p.o.	100	0.5% MC	1	1	3
Doxorubicin	i.v.	6	Saline	3	1	3
		30	Saline	1	1	3(1)
		10, 20	Saline	1	3	3–6
Cyclophosphamide	i.p.	150	Saline	1	3	3
Acetaminophen	p.o.	1000	0.5% MC	1	1	3
TMPD	p.o.	20	0.5% MC	1	1	3
Cisplatin	i.v.	0.5, 1.5	Saline	1	4	3
Puromycin aminonucleoside	i.v.	100	Saline	1	4	3
Methotrexate	i.p.	10	Saline	1	4	3
Bromobenzene	p.o.	20	Corn oil	1	2	3
Compound A	i.v.	200	Saline	1	9	5(1)
i.p., intraperitoneal; i.v., intravenous; p.o., per os; MC, methylcellulose						

2.4. Quantification of miRNAs by RT-qPCR

miRNAs in serum were measured using the quantitative reverse transcription polymerase chain reaction (RT-qPCR). Total RNA, including small RNAs, was extracted from 200 μ L of serum using the Maxwell® RSC miRNA Plasma and Serum Kit (Promega Corporation, Wisconsin, US) in accordance with the manufacturer's protocol. Nucleic acid concentrations were determined by the absorbance at 260 nm using a NanoDrop One spectrophotometer (Thermo Fisher Scientific, Waltham, MA), after which RNase-free water was added to adjust the RNA concentration to 6 ng/ μ L. Extracted RNA (30 ng) was reverse-transcribed into cDNA in a reaction volume of 5 μ L with the TaqMan MicroRNA Reverse Transcription Kit (Thermo Fisher Scientific) and the reverse transcription primer Rno-miR-208a-3p, which was included in the kit. The cDNA was amplified using TaqMan Universal Master Mix II, no UNG (Thermo Fisher Scientific) and a specific PCR primer. Serum miRNA levels were quantified using the StepOnePlus Real-Time PCR System (Thermo Fisher Scientific). The PCR conditions were 95°C for 10 min followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. The resulting cycle threshold (Ct) value was analyzed by the $\Delta\Delta$ Ct method. The Ct values for the samples were normalized to that of rno-miR-16-5p as an internal control (Davoren et al 2008, Sharapova et al 2016). A Ct value of 40 cycles or more was regarded as being below the limit of quantification. To calculate $\Delta\Delta$ Ct, the Δ Ct of the vehicle control from the same test was used.

2.5. Histopathology

At necropsy, the heart was removed and fixed in 10% neutral buffered formalin. The fixed tissues were processed by the routine method, embedded in paraffin, cut into 4- μ m sections and stained with hematoxylin and eosin (HE). Microscopic tissue evaluation was performed for all control and treated animals. Histopathological findings were graded as – (findings absent), $\pm$ (minimal), 1+ (mild), 2+ (moderate), 3+ (marked), and 4+ (severe).

2.6. Echocardiography

Echocardiography was performed on saline- or DOX-treated rats using a high-resolution ultrasound system (Vevo 3100; Fujifilm VisualSonics) equipped with an MX250 linear array transducer. Briefly, the rats were anesthetized with 3.0% isoflurane and placed on an imaging platform. Heart rate was continuously monitored. Two-dimensional B-mode images were obtained in the parasternal short-axis view at the level of the papillary muscles to guide the M-mode recordings. From the M-mode tracings, the left ventricular anterior wall thickness (LVAW), left ventricular posterior wall thickness (LVPW), and left ventricular internal dimension (LVID) were measured during both diastole (d) and systole (s). Left ventricular fractional shortening (LVFS) and Left ventricular ejection fraction (LVEF) were calculated using the standard formulas provided by the system's analysis software (Vevo LAB 5.8.2; Fujifilm VisualSonics). All measurements were averaged from at least three consecutive cardiac cycles.

2.7. Statistical analysis

Statistical analyses were performed using SciPy (version 1.13.1) for Welch's t-tests and scikit-learn (version 1.6.1) for receiver operating characteristic (ROC) analysis. Individual animals were considered the experimental unit. The number of animals analyzed (n) is reported in Table 1 and in the relevant figure and table legends. Unless otherwise stated, n represents the number of individual animals. Data are presented as individual values and as mean $\pm$ standard deviation (SD), as indicated. Comparisons between two independent groups were performed using a two-tailed Welch's t-test, which does not assume equal variances. ROC analysis was performed using pooled individual-animal data across all compounds to evaluate the ability of serum miR-208 to discriminate between animals with and without myocardial histopathological findings. The area under the ROC curve (AUC) was calculated, and the diagnostic cutoff was selected to provide 95% specificity; the corresponding sensitivity was then determined. Serum miR-208 values were expressed as fold changes relative to the mean of the matched vehicle-control group. Microarray expression intensities were $\log_{10}$ -transformed for visualization. All hypothesis tests were two-sided, and significance is expressed with P values: *, $P < 0.05$, ***, $P < 0.005$.

Results

Database-Based Analysis for Exploring Heart-Specific miRNAs as Biomarker Candidates

To identify miRNAs with high myocardial tissue specificity, we analyzed the rat miRNA expression atlas (GSE52754) reported by Minami et al. (2014). First, to identify miRNAs with sufficient detection sensitivity in myocardial tissue, screening was conducted using an expression intensity threshold of 50 in the raw microarray data. Heatmap analysis revealed miRNAs that were highly enriched in myocardial tissue among all miRNAs, identifying rno-miR-208, -499, -1, -133a, and -490 (Fig. 1a).

Next, to verify the tissue specificity of these candidates as biomarkers, their expression levels were compared with those in skeletal muscle tissue. Conventional markers, such as miR-1 and miR-133a, exhibited extremely high expression not only in the myocardium but also in skeletal muscle, suggesting a potential risk of false positives resulting from skeletal muscle injury. Furthermore, although miR-499 and miR-490 were enriched in the myocardium, their myocardial specificity was inferior to that of miR-208. In contrast, while miR-208 showed high expression in the myocardium, its expression in skeletal muscle was at background levels (Fig. 1b). Based on these results, miR-208 was selected as the biomarker candidate with the highest myocardial specificity and the greatest suitability for cardiotoxicity evaluation.

Compound Profile and Pathological Findings

To induce cardiotoxicity using compounds with diverse mechanisms of action, an *in vivo* study was conducted in which Sprague-Dawley (SD) rats were administered the following compounds: isoproterenol (Zhang J et al 2008), carbofuran (Gupta RC et al 1991, Mori Y et al 2010, Jaiswal SK et al 2017), allylamine (Boor PJ et al 1979, Sklar JL et al 1991), doxorubicin (Christidi E et al 2021, Wu YZ et al 2019, Zhang YY et al 2017), and Compound A (details regarding its chemical structure and target are not disclosed).

The histopathological findings of the heart are shown in Table 2. In the isoproterenol-treated group, degeneration/necrosis of cardiomyocytes and mononuclear cell infiltration were observed at minimal to moderate severity, along with minimal edema (Fig. 2a). In the carbofuran-treated group, degeneration/necrosis of cardiomyocytes and mononuclear cell infiltration were observed at a minimal severity. In the DOX-treated group, degeneration/necrosis of cardiomyocytes, as well as inflammatory and mononuclear cell infiltration, were all observed at a minimal to moderate severity (Fig. 2b). In the Compound A-treated group, degeneration/necrosis of cardiomyocytes and inflammatory cell infiltration were observed ranging from minimal to marked severity (Fig. 2c). The other compounds, unexpectedly including allylamine, did not induce any significant structural changes detectable by histopathological examination.

Evaluation of Circulating miR-208 Levels and ROC-AUC Analysis

Changes in serum miR-208 levels in each treatment group at 24 h after the final administration are illustrated in Fig. 3a. Serum miR-208 was markedly elevated in all animals treated with isoproterenol, doxorubicin, and Compound A. In contrast, miR-208 levels in the allylamine and carbofuran groups remained low. Notably, although the carbofuran-treated group exhibited minimal histopathological changes, it did not elicit a corresponding elevation in serum miR-208. To evaluate the overall correlation between serum miR-208 levels and histopathological findings, the data from all compounds were aggregated and plotted according to the presence or absence of myocardial lesions (Fig. 3b). Subsequently, receiver operating characteristic (ROC) analysis was performed to establish a diagnostic criterion (Fig. 3c). The cutoff value was calculated to provide a specificity of 95% based on the fold change relative to the mean of each matched control group, corresponding to a 5% false-positive rate among histopathology-negative animals (indicated in Fig. 3b). In the diagnosis of cardiotoxicity, the area under the ROC curve (AUC) for miR-208 was 0.91 (sensitivity, 0.57; cutoff value, 52.4). The DOX-treated group showed very high miR-208 expression, even in animals without histopathological findings.

Early Detection of Doxorubicin-Induced Cardiotoxicity Preceding Functional and Structural Change

The DOX-treated group exhibited a marked increase in serum miR-208 with minimal or no concurrent histopathological findings. To further investigate the detailed pathophysiological background of the myocardium in this model, serum miR-208 measurement and echocardiography were conducted at 72 h post-dose. In the group administered 20 mg/kg DOX, a statistically significant decrease in heart rate was observed. While no significant changes were detected in other echocardiographic parameters compared with the control group, trends toward lower left ventricular ejection fraction (LVEF) and fractional shortening (LVFS), early signs of DOX-induced cardiotoxicity, were noted (Table 3). At this point, a marked elevation in miR-208 was observed in all animals. Although minimal myocardial degeneration/necrosis was observed in some animals, there was no clear correlation between serum miR-208 levels and the presence of histopathological findings (Table 4). These results suggest that early cardiotoxic changes are already occurring in the DOX-treated group before decreases in contractility or structural alterations reach levels detectable by histopathological examination.

To further verify the temporal kinetics of miR-208, an independent time-course experiment was conducted using the exact same DOX administration design. In this cohort, serial blood sampling was performed to solely track serum miR-208 levels at 24 and 72 h post-administration. The results confirmed that an upward trend in serum miR-208 levels began as early as 24 h after DOX administration, prior to becoming statistically significant at 72 h (Fig. 4). For the time-course blood sampling in this study, blood was collected from the jugular vein through muscles of the chest. A separate validation study confirmed that the blood collection procedure via jugular vein puncture did not cause any fluctuation in miR-208 levels (Fig. S1).

Table 2: Individual histopathological findings in the heart after treatment with various compounds.

Test No	Compound	Dose (mg/kg)	Histopathological findings [#]			
			Degeneration/Necrosis, cardiomyocyte	Focal hemorrhage	Infiltration, mononuclear cell	Edema
1	Isoproterenol	0.5	±(1/3), 1+(1/3)	–	±(1/3), 1+(1/3)	±(1/3)
	Isoproterenol	1.5	±(1/3), 1+(1/3), 2+(1/3)	–	±(1/3), 1+(1/3), 2+(1/3)	±(1/3) 1+(1/3)
	Carbofuran	1.5	±(2/3)	–	±(2/3)	–
	Doxorubicin	30	2+(1/2)	±(1/2)	1+(1/2)	1+(1/2)
	Doxorubicin	6	–	–	–	±(1/3)
2	Isoproterenol	0.5	2+(3/3)	–	2+(3/3)	–
	Isoproterenol	1.5	2+(2/2)	–	2+(2/2)	–
	Allylamine	100	–	–	–	–
	Doxorubicin	10	–	–	–	–
	Doxorubicin	30	±(2/3)	–	±(1/3)	–
	Cyclophosphamide	150	–	–	–	–
	Acetaminophen	1000	–	–	–	–
	TMPD	20	–	–	–	–
3	Cisplatin	0.5	–	–	–	–
	Cisplatin	1.5	–	–	–	–
	Puromycin aminonucleoside	100	–	–	–	–
	Methotrexate	10	–	–	–	–
	Bromobenzene	20	–	–	–	–
4	Doxorubicin	20	±(3/6)	–	–	–
	Compound A	200	±(1/5), 1+(1/5), 2+(3/5)	–	1+(1/5), 2+(1/5), 3+(2/5)	–

–, findings absent; ±, minimal; 1+, mild; 2+, moderate; +3 marked

[#]: Histopathological findings show the grade and number of animals.

Table 3: Summary of serum miR-208 expression in cardiotoxicity models.

Group	Saline (N = 3)	Doxorubicin (N = 5)
Dose (mg/kg)	-	20
LVIDd (mm)	6.19 ±0.27	5.93 ±0.76
LVIDs (mm)	2.59 ±0.88	3.43 ±0.71
LVPWd (mm)	1.92 ±0.44	2.05 ±0.78
LVPWs (mm)	3.30 ±0.34	3.05 ±0.77
LVAWd (mm)	1.92 ±0.37	1.50 ±0.24
LVAWs (mm)	3.20 ±0.45	2.42 ±0.28
LVEF (%)	86.09±10.93	71.97±8.80
LVFS (%)	58.33±13.07	42.37±7.82
Heart rate (bpm)	374±10.15	287±18.04 ***
miR-208 (fold change)	1.18 ±0.86	18.01 ±4.14 ***

Values are means ± SD; N = number of rats. LVID, left ventricular (LV) internal diameter; LVPW, LV posterior wall thickness; LVAW, LV anterior wall thickness; LVEF, LV ejection fraction; and LVFS, LV fractional shortening were calculated from the M-mode tracing. d, diastole; s, systole. ***; P < 0.005 compared with the saline group.

Table 4: Histopathological findings, echocardiography and miR-208 expression in the heart after treatment with doxorubicin in test no. 4.

Test No	Compound	Dose (mg/kg)	Individual No	Histopathological findings [#]	Echocardiography		miR-208 expression
				Degeneration/Necrosis, cardiomyocyte	LVEF (%)	LVFS (%)	
5	Saline	0	01M01	–	91.29	64.01	0.56
			01M02	–	73.52	43.38	0.82
			01M03	–	93.45	67.59	2.17
	Doxorubicin	20	02M01	±	78.11	47.68	16.6
			02M02	–	69.94	39.96	20.0
			02M03	±	62.97	34.40	21.3
			02M04	–	83.75	53.10	11.4
			02M05	±	65.06	36.70	20.8

–, findings absent; ±, minimal.

#: Histopathological findings show the grade.

Discussion

While the high myocardial specificity and the baseline potential of circulating miR-208 as a biomarker for drug-induced cardiotoxicity (DICT) have been well documented (Ji X et al 2009, Wang GK et al 2010, Calvano J et al 2016), the present study makes two distinct, highly practical contributions to preclinical toxicology. First, rather than simply confirming the elevation of miR-208, we established a concrete, data-driven diagnostic cut-off value for rat models (AUC = 0.91, 52.40-fold change) by integrating *in vivo* data across a diverse array of toxicant models. By incorporating compounds with distinct mechanisms of action ranging from sympathomimetic overstimulation to direct mitochondrial toxicity, this threshold was designed to be robust and universally applicable across different modes of injury, achieving a stringent 95% specificity that tightly controls the false-positive rate. Second, we explicitly demonstrated the temporal superiority of miR-208 over conventional diagnostic modalities. In the DOX-induced cardiotoxicity model, an upward trend in serum miR-208 levels was clearly detectable as early as 24 h post-administration. Crucially, this molecular elevation occurred well before the onset of structural alterations, which remained largely absent or minimal even at 72 h, and preceded any macroscopic functional decline measurable by echocardiography.

The most significant implication of these findings is that they bridge the gap between the theoretical, molecular utility of miR-208 and its practical, day-to-day application in preclinical safety assessments. Preclinical drug development is frequently confounded by borderline or minimal histopathological findings, creating uncertainty that complicates decisions regarding further development. By providing a specific, quantitative cut-off value optimized precisely for rat models, this study equips toxicologists with an objective, definitive tool to evaluate cardiotoxic liabilities. This approach significantly reduces the industry's reliance on subjective pathological grading and allows for the prevention of unwarranted compound attrition due to false positives, which are common with less specific muscle markers.

Furthermore, the clear demonstration of miR-208 elevation preceding both functional and morphological deterioration provides profound mechanistic and diagnostic insights. Conventional assessments typically capture late-stage, irreversible events; histopathology visualizes the endpoint of necrosis, while echocardiography detects functional loss only after the heart's compensatory reserve is exhausted. The upward trend of miR-208 at 24 h may suggest that it acts as an ultra-early, dynamic warning signal reflecting pre-pathological molecular perturbations. Mechanistically, under DOX-induced stress, which is characterized by the acute generation of reactive oxygen species (ROS) and transcriptomic

alterations (Linders AN et al 2024), this early leakage may be driven by transient alterations in cell membrane permeability (Minotti G et al 2004, Akao M et al 2003) or stress-induced active exosomal secretion (Hessvik NP et al 2018). Because this release occurs prior to overt structural disruption, miR-208 opens a diagnostic window, allowing researchers to monitor cardiotoxicity in its true nascent stage and potentially implement earlier interventions or dose adjustments in toxicological screening.

Despite the utility of the established preclinical criteria, several limitations in the present study must be acknowledged. First, although we demonstrated that miR-208 rises prior to evident pathological and echocardiographic changes, this study did not include a direct, chronological comparison with current gold-standard cardiac biomarkers, specifically cardiac troponin I and T (cTnI/cTnT) (Adamcova M et al 2005, Wallace KB et al 2004, O'Brien PJ 2008). While miR-208 possesses undeniable advantages in tissue specificity, establishing whether it exhibits an earlier, more proportionate, or more sensitive release profile than cTnI/cTnT under sub-lethal stress requires rigorous, head-to-head evaluations using tightly controlled time-course models. Second, the temporal dynamics of miR-208 in this study were evaluated primarily during the acute to sub-acute phase (up to 72 h post-administration). Because anthracycline-induced cardiotoxicity typically manifests clinically as a cumulative, dose-dependent, and delayed-onset cardiomyopathy (Von Hoff DD et al 1979, Lyon AR et al 2022), the predictive value, stability, and persistence of circulating miR-208 in chronic, low-dose toxicity settings remain to be fully elucidated. It will be crucial to determine whether miR-208 levels reflect cumulative damage over weeks or months. Finally, while the specific 52.40-fold cutoff value proposed here provides a robust standard for rat models, there are significant interspecies differences. Variations in baseline miRNA expression, clearance rates, metabolic kinetics, and heart rates between rodents and larger mammals necessitate careful recalibration (Schofield AL et al 2021). These preclinical criteria must be thoroughly validated in non-human primates and ultimately in human clinical cohorts before this framework can be directly translated to human diagnostic applications.

In conclusion, these findings demonstrate that miR-208 is a highly specific and accurate screening tool capable of capturing early cardiotoxic signals, thereby providing an objective and powerful diagnostic advantage in the preclinical safety assessment of cardiac drugs.

Abbreviations

AUC: Area under the curve

DICT: Drug-Induced cardiotoxicity

DOX: Doxorubicin

HE: Hematoxylin and eosin

LVAW: left ventricular anterior wall thickness

LVEF: Left ventricle ejection fraction

LVFS: Left ventricular fractional shortening

LVID: Left ventricular internal dimension

LVPW: left ventricular posterior wall thickness

ROC: Receiver operating characteristic

ROS: Reactive oxygen species

RT-qPCR: reverse transcription PCR

SD: Standard deviation

SE: Standard error of the mean

TMPD: 2,3,5,6-tetramethyl-1,4-phenylenediamine

Declarations

Data Availability

Data are available upon reasonable request to the corresponding author.

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Contributions

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Kimika Hayashi: investigation, data curation

Yutaka Tonomura: conceptualization, supervision, methodology

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Ethics declarations

Competing Interests

The authors declare no competing interests.

Ethical Approval

This study was approved by the Laboratory Animal Care and Use Committee of Nippon Shinyaku Co., Ltd, and performed in compliance with the Laboratory Animal Policy of Nippon Shinyaku Co., Ltd.

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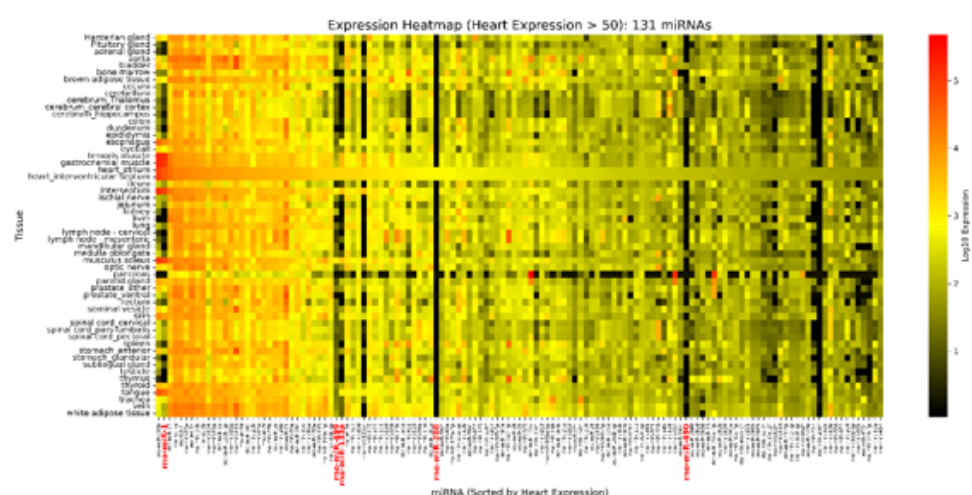
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Figures

(a)



(b)



Figure 1

Screening of myocardium-specific miRNAs using a rat miRNA expression atlas.

(a) Expression heatmap of miRNAs across multiple rat tissues based on microarray data (GSE52754). The color scale indicates $\log_{10}$ -transformed expression intensity. Five candidate miRNAs highly enriched in the myocardium are highlighted in red on the x-axis.

(b) Scatter plot comparing miRNA expression levels between the heart and skeletal muscle ($\log_{10}$ scale). The dashed diagonal line represents equal expression in both tissues. Colored circles indicate the five specific candidate miRNAs, while gray circles represent the remaining miRNAs.

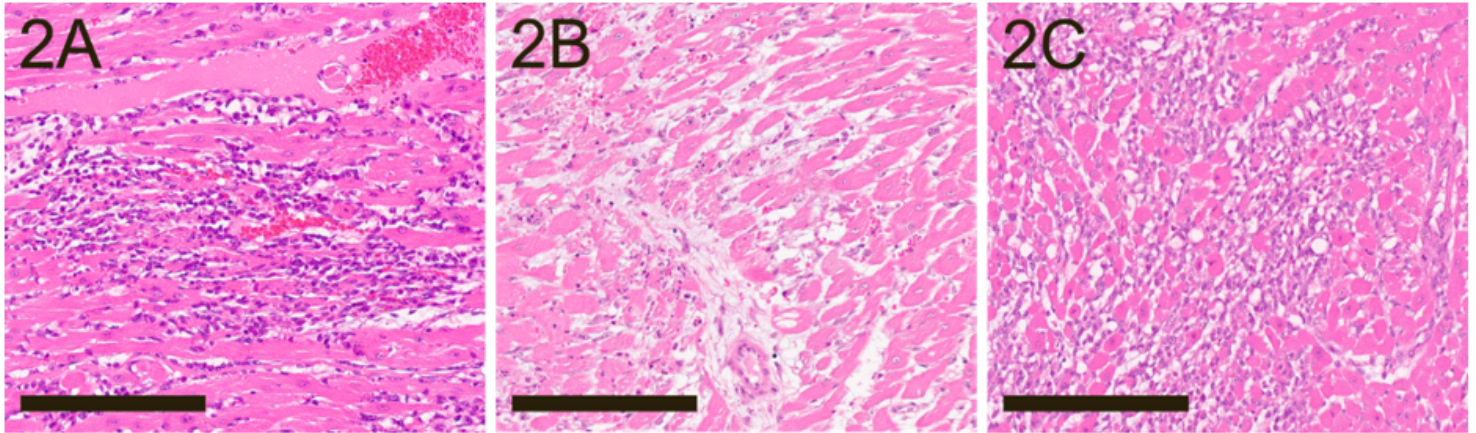


Figure 2

Histopathology of hearts.

(a) Moderate degeneration/necrosis of cardiomyocytes (primarily in the left ventricle) and mononuclear cell infiltration were observed. Test no. 2: isoproterenol (0.5 mg/kg) group. Hematoxylin and eosin (HE) stain. Original magnification, 20X; Scale bar = 200 μm . (b) Moderate degeneration/necrosis of cardiomyocytes, mild edema, minimal mononuclear cell infiltration, and minimal focal hemorrhage were observed. Test no. 1: doxorubicin (30 mg/kg) group. HE stain. Original magnification: 20X; Scale bar = 200 μm . (c) Moderate degeneration/necrosis of cardiomyocytes and marked mononuclear cell infiltration were observed. Test no. 4: Compound A (200 mg/kg) group. HE stain. Original magnification: 20X; Scale bar = 200 μm .

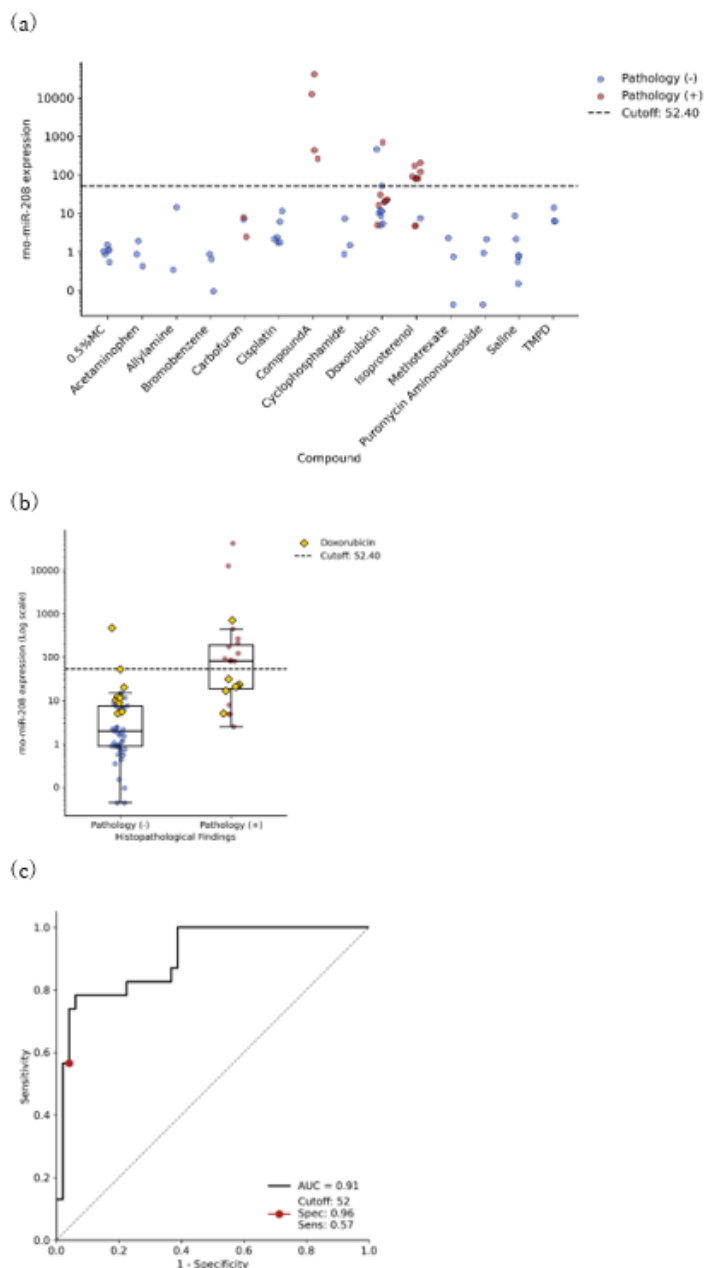


Figure 3

Evaluation of serum miR-208 levels and ROC analysis.

(a) Serum miR-208 expression levels in rats at the time of necropsy following administration of each compound. Blue and red circles represent animals without (Pathology (-)) and with (Pathology (+)) myocardial histopathological findings, respectively. The horizontal dashed line represents the cutoff value. (b) Box plot of aggregated serum miR-208 expression categorized by the presence or absence of histopathological findings (log scale). Yellow diamonds indicate individual data points from doxorubicin-treated animals. The horizontal dashed line represents the cutoff value. (c) Receiver operating characteristic (ROC) curve for the diagnosis of cardiotoxicity across all evaluated compounds. The red dot indicates the cutoff point.

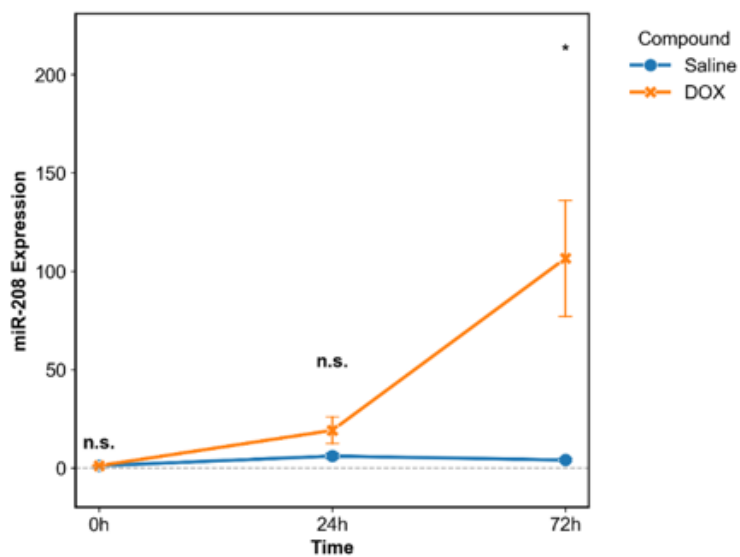


Figure 4

Time-course comparison of miR-208 expression between the saline and DOX groups.

Points and error bars represent the mean $\pm$ SE. *; $P < 0.05$ compared with the saline group. n.s., not significant.

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