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ADVANCING PRECLINICAL ASSESSMENT OF HIDDEN CARDIOTOXICITY: MECHANISTIC INSIGHTS AND TRANSLATIONAL APPLICATIONS IN DRUG DEVELOPMENT

PhD thesis

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List of abbreviations

3Rs principle	Replacement, Reduction and Refinement
AAR	Area at risk
Akap6	A-kinase anchor protein 6
APPROVe	Adenomatous Polyp Prevention on Vioxx
BAM	Binary alignment map
BARI 2D trial	Bypass Angioplasty Revascularization Investigation 2 Diabetes trial
Camk2a	Calcium/Calmodulin-Dependent Protein Kinase II Alpha
CK1a	Casein Kinase 1 Alpha 1
COX-2	Cyclooxygenase-2
CPM	Counts per million
Cx43	Connexin 43 (Gja1)
DDA	Data-dependent acquisition
ECG	Electrocardiogram
ECL	Enhanced chemiluminescence
Eif4h	Eukaryotic translation initiation factor 4H
EMA	European Medicines Agency
FDA	Food and Drug Administration
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
Gsk3b	Glycogen synthase kinase-3 beta
HED	Human equivalent dose
hERG channel	Voltage-gated potassium channel Kv11.1
HRP	Horseradish peroxidase
human Km	Human correction factor
ICH	International Council for Harmonisation
IPC	Ischemic preconditioning
I/R	Ischemia-reperfusion
LAD	Left anterior descending (coronary artery)
LC-MS/MS	Liquid chromatography–tandem mass spectrometry
LFQ / MBR	Label-free quantification / Match-between-runs
MAP	Mean arterial pressure

miRNA	MicroRNA
mRNA	Messenger RNA
NCBI	National Center for Biotechnology Information
NES	Normalised enrichment scores
OECD	Organisation for Economic Co-operation and Development
PASEF	Parallel accumulation–serial fragmentation
Phosphosite	Phosphorylation site
PKP2	Plakophilin-2
PMSF	Phenylmethylsulfonyl fluoride
PPAR γ	Peroxisome proliferator-activated receptor gamma
rat Km	Rat correction factor
RECORD trial	Rosiglitazone Evaluated for Cardiac Outcomes and Regulation of Glycemia in Diabetes trial
REMS	Risk Evaluation and Mitigation Strategy
RIPA buffer	Radioimmunoprecipitation assay buffer
Ryr2	Ryanodine receptor-2
SAM	Sequence alignment map
Slc8a1	Na ⁺ /Ca ²⁺ exchanger
Stat3	Signal Transducer and Activator of Transcription-3
STRING	Search Tool for the Retrieval of Interacting Genes
TBS-T	Tris-buffered saline–Tween
TIMS	Trapped ion mobility spectrometry
TPM	Transcripts per million
TTC solution	Triphenyltetrazolium chloride solution
VADT	Veterans Affairs Diabetes Trial
VIGOR	Vioxx Gastrointestinal Outcomes Research

1 Introduction: drug withdrawal and the “socio-economic burden”

Despite significant advancements in preclinical safety testing, drug-induced toxicities remain one of the most common causes for discontinuation of clinical trials or post-marketing withdrawals of drugs. Between 1953 and 2013, 462 medicinal products were withdrawn from the market due to adverse drug reactions either worldwide or in one country only (1). The drug withdrawals were accounted for by unexpected deaths, hepatic, nervous system and cardiac toxicities. Interestingly, it was estimated in 2008 that 197.000 deaths occur due to adverse drug reactions in the European Union per year, which accumulated to a public health burden of €79 billion (2). Cardiotoxicity ranked among the leading reasons for drug withdrawal (3). The detection of such toxicities in late phases of drug development and post-marketing observations bears an inherent risk because of the increased vulnerability of the comorbid patient population. Next to patient safety, the costs and time required for development are gradually increasing with each phase of drug development (Figure 1). Therefore, early detection of drug-induced toxicities is necessary to increase patient safety and to reduce the sunk cost of drug development (4).

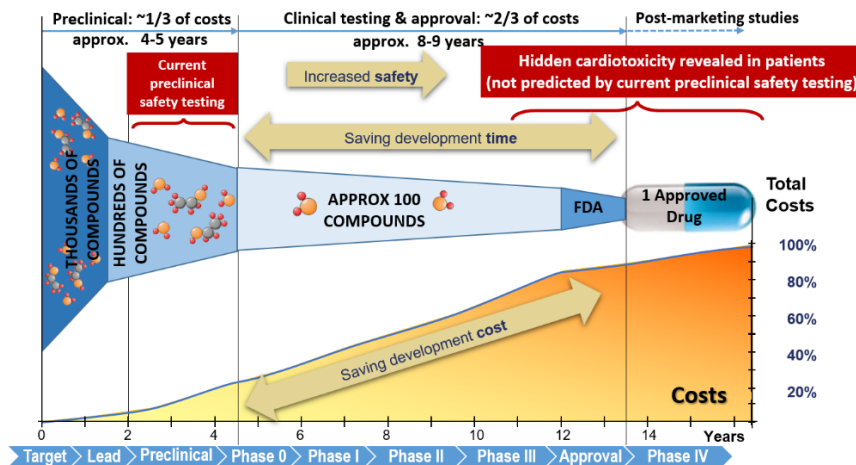


Figure 1. The stages of drug development. In the drug development process, the costs and time invested increase drastically over time. Late-stage attrition of drugs due to hidden toxicities poses a major risk to the developers and patient safety. The detection of such toxicities in early phases of drug development during the preclinical testing would increase success rates of drug development, and most importantly, increase patient safety. Obtained from Ferdinandy et. al (4) (license number: 6190800193641). (FDA: Food and Drug Administration)

1.1 Hidden cardiotoxicity manifests in the diseased heart

Adverse cardiac effects often remain undetected during early phases of drug development, due to their focus on direct toxicity and adverse drug reactions in healthy subjects (5, 6). Thus, safety testing only done in healthy hearts may hide drug toxicities that may become obvious under pathological conditions. This manifestation of drug-induced cardiac damage or dysfunction in the diseased heart is defined as ‘hidden cardiotoxicity’ (4). Especially, I/R injury and/or its major risk factors, including hypertension, hyperlipidemia, diabetes, insulin resistance, heart failure, altered coronary circulation, ageing and their co-medications, may influence cardiac cell signalling (7, 8). I/R injury refers to tissue damage caused both by the initial restriction of blood flow and by the subsequent restoration of perfusion. Mechanistically, hidden cardiotoxicity could develop by (i) exacerbating detrimental signalling pathways active during cardiac stress, or (ii) inhibiting protective survival mechanisms (Figure 2). Ultimately, both may increase cell death, pro-arrhythmic effects, and contractile dysfunction (4). However, the exact molecular pathways affected by hidden cardiotoxicity are not always known.

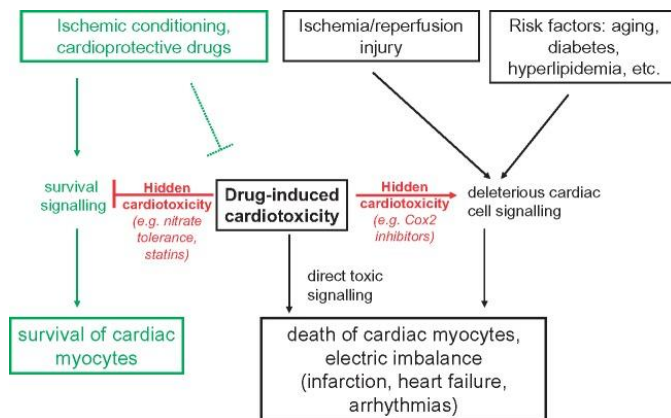


Figure 2. Influence of ischemia/reperfusion injury or cardiovascular risk factors on hidden cardiotoxicity. Hidden cardiotoxicity arises from drugs that suppress survival signalling or enhance disease-related harmful pathways. Obtained from Ferdinandy et. al (4) (license number: 6190800193641).

1.2 Limitations of current preclinical drug safety testing

Preclinical cardiotoxicity testing platforms are designed to detect toxicities early in drug development, minimising late-stage attrition and ensuring patient safety (Figure 3). Current cardiac safety testing, however, primarily relies on cell-line-based, *in vitro*

systems and surrogate markers of cardiac damage in healthy animal models or animal-derived tissues (4-6, 9). For instance, the International Council for Harmonisation (ICH) guidelines for preclinical electrophysiological safety testing do not require assessment of proarrhythmic properties in arrhythmia-susceptible tissues or animals (10-12). In general, the main focus of current preclinical arrhythmia detection lies in identifying QT-interval prolongation caused by delayed repolarisation (9). This abnormality of the cardiac action potential can trigger the life-threatening ventricular arrhythmia *torsades de pointes* (13). Drug-induced QT prolongation is predominantly associated with inhibition of the voltage-gated potassium channel Kv11.1 (hERG channel) and is considered the primary mechanism of drug-induced arrhythmias (14). Consequently, preclinical safety testing has centred on detecting hERG channel blockade in order to prevent torsades de pointes (14), while other mechanisms of proarrhythmogenicity—such as calcium-mediated arrhythmias—have been comparatively neglected (15). Importantly, comorbidities, such as diabetes, heart failure, or hypercholesterolemia, can significantly alter cardiac physiology by affecting ion channel expression and activity, mitochondrial function, electromechanical coupling, and extracellular matrix composition. These changes increase susceptibility to arrhythmias, contractile dysfunction, and cardiomyocyte death (7, 8). Therefore, testing drug effects exclusively in healthy hearts may mask adverse drug reactions that manifest only under pathological conditions or in the presence of cardiovascular risk factors (16-18).

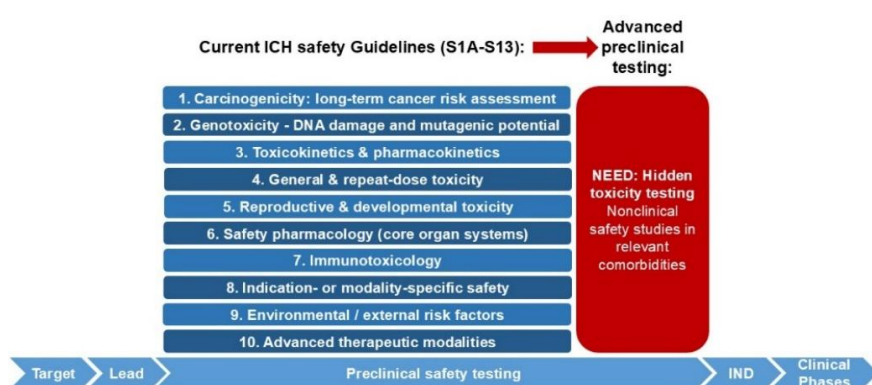


Figure 3. Current ICH safety Guidelines do not advocate testing in relevant comorbidities. ICH safety guidelines S1A-S13 are internationally harmonised nonclinical standards that guide the assessment of potential safety risks of new medicines before an IND. (ICH: International Council for Harmonisation; IND: Investigational New Drug application)

1.3 Assessing the molecular mechanism of cardiotoxicities may lead to novel tests for preclinical drug safety and the detection of biomarkers

Current preclinical cardiovascular safety assessment, as defined by ICH S7A and S7B, relies predominantly on cell line-based *in vitro* assays and the evaluation of surrogate markers of cardiac injury in healthy animals or isolated animal-derived tissues (4, 5, 9, 11, 12). While these approaches effectively identify overt functional liabilities, they are not designed to detect adverse cardiac effects that emerge only under conditions of stress or comorbidity. In contrast, disease-relevant models such as I/R injury are capable of unmasking hidden cardiotoxicity but are inherently time- and resource-intensive (16-18). In this context, investigating the molecular mechanisms of drug-induced cardiotoxicities has the potential to identify key molecular pathways and protein-level alterations that precede functional impairment, thereby enabling the development of targeted assays or predictive biomarkers aligned with regulatory safety pharmacology endpoints. Such mechanism-driven strategies could complement existing ICH S7A/S7B frameworks and provide a more efficient, cost-effective approach to early detection of hidden cardiotoxicity.

The European Society of Cardiology Working Group on Cellular Biology of the Heart has previously emphasised the critical role of *in silico*, unbiased omics-based approaches in the discovery of regulatory targets (19). Within this framework, particular attention has been given to the microRNA (miRNA)–transcriptome axis. miRNAs exert broad regulatory influence, as individual miRNAs can modulate the expression of multiple genes, while a single gene may simultaneously be under the control of several different miRNAs. Consequently, even modest alterations in miRNA expression profiles have the potential to markedly impact cardiovascular phenotypes or confer cardioprotection through their interactions with messenger RNAs (mRNAs) (19-24). The essential role of miRNAs in mediating the heart's adaptive responses to stress has also been consistently highlighted in recent studies (23, 25-27).

From a therapeutic standpoint, it is important to note that most pharmacological agents ultimately act on proteins, which function as the key executors of biochemical processes within cells (28). Protein activity is tightly regulated not only by expression levels but also by posttranslational modifications such as phosphorylation. Therefore, analytical strategies capable of characterising both protein abundance and their regulatory

modifications under pathological perturbations could provide critical insights into mechanisms and biomarker detection (29-31).

An integrated application of high-throughput transcriptomic, proteomic, and related omics technologies provides a systematic means to disentangle the complex molecular mechanisms underlying cardiovascular diseases (32-35). Such combined approaches may also reveal additive or synergistic effects of multitarget therapeutic interventions (36). We hypothesise that drug-induced hidden cardiotoxicity arises from molecular alterations that extend beyond the established mechanisms of action and are optimally captured using unbiased multi-omics approaches. Identification of these molecular perturbations may provide a rational basis for the development of targeted assays and predictive biomarkers, thereby enabling earlier and more sensitive detection of hidden cardiotoxicity in preclinical safety testing.

1.4 Post-marketing cardiotoxicity safety concerns of rosiglitazone

Rosiglitazone, a thiazolidinedione-type antidiabetic, is a potent selective agonist of the transcription factor PPAR γ (37). Post-marketing concerns regarding its cardiovascular safety emerged in 2007, when two meta-analyses including 14,291 and 27,847 participants, respectively, suggested an increased risk of myocardial infarction compared with placebo or its active control (38, 39). One of these studies further reported a possible elevation in cardiovascular mortality (38). However, the updated meta-analysis by Nissen *et al.* (2010) did not confirm an association with increased cardiovascular death (40). Accumulating safety concerns nevertheless led to the withdrawal of rosiglitazone from the European market and the imposition of strict marketing restrictions by the FDA (41, 42). Subsequently, the FDA commissioned an independent re-evaluation of the RECORD trial (Rosiglitazone Evaluated for Cardiac Outcomes and Regulation of Glycemia in Diabetes), which originally reported a positive risk-benefit ratio. The RECORD trial was an open-label study including 4,447 patients with type 2 diabetes and a median follow-up of 5.5 years. This re-analysis confirmed the initial findings: while rosiglitazone was associated with an increased risk of heart failure, it did not increase the incidence of myocardial infarction or cardiovascular death compared to combination therapy with metformin and sulfonylurea (43). Based on these results, the FDA subsequently lifted most of the marketing restrictions, though the contraindication in

patients with heart failure remained in place (42, 44). Additional evidence came from a cross-sectional analysis of the Veterans Affairs Diabetes Trial (VADT), which compared rosiglitazone with standard-of-care treatment (metformin plus sulfonylurea) in 1,791 participants followed for a median of 5.6 years. This study supported the absence of excess risk for myocardial infarction or cardiovascular death (45). Similarly, a meta-analysis of nine randomised controlled trials (24–26 weeks, 1,916 patients) comparing rosiglitazone add-on therapy with insulin monotherapy did not detect an increased risk of adverse cardiovascular outcomes (46). More recently, an umbrella review of 232 meta-analyses evaluating ten classes of diabetes drugs concluded that rosiglitazone was associated with an increased risk of myocardial infarction compared with active control but not when compared to placebo (47). These inconsistencies may partly reflect methodological limitations, including concerns about the reporting and conduct of clinical trials, particularly the RECORD trial, which has been criticised for potential bias (48, 49).

Currently, rosiglitazone remains withdrawn from the European market (41). In the United States, the FDA did not formally withdraw Avandia but progressively lifted prescribing restrictions, including the removal of the Risk Evaluation and Mitigation Strategy (REMS) in 2015 (42, 44). Nevertheless, according to current drug availability databases, all brand-name formulations of Avandia are now classified as discontinued (50). The observation that rosiglitazone's cardiotoxic effects only became apparent in post-marketing studies underscores its value as a model drug for hidden cardiotoxicity and highlights the need to develop preclinical safety testing strategies capable of detecting hidden toxicities using models that incorporate clinically relevant comorbidities. Because hidden cardiotoxicities often manifest as ischemia-related cardiac deaths, I/R-induced arrhythmias, or cardiac dysfunction, we utilised our previously validated I/R in vivo model (17), aiming to detect the clinically observed cardiotoxicity of rosiglitazone.

1.5 Rofecoxib withdrawal and cardiotoxicity: insights from clinical and preclinical detection

The cyclooxygenase-2 (COX-2) inhibitor rofecoxib was withdrawn from the market in 2004 after post-marketing studies revealed an increased risk of cardiovascular events, most prominently in the VIGOR (Vioxx Gastrointestinal Outcomes Research) and

APPROVe (Adenomatous Polyp Prevention on Vioxx) trials (51, 52). The VIGOR trial was a double-blind, randomised study that enrolled 8,076 patients with rheumatoid arthritis and had a median follow-up of nine months. Patients received either rofecoxib at 50 mg daily or naproxen at 500 mg twice daily. The results demonstrated a significantly higher incidence of myocardial infarction in the rofecoxib arm than in the naproxen arm. At the time, this difference was attributed to the fact that patients in the rofecoxib-treated group who developed myocardial infarction were at high risk of developing thromboembolic events and therefore should have been treated with low-dose aspirin (51). The observed difference was therefore interpreted as reflecting a potential aspirin-like protective effect of naproxen on platelet aggregation (53), rather than a cardiotoxic effect of rofecoxib itself (51). In contrast, the subsequently conducted placebo-controlled APPROVe trial provided evidence against this assumption. APPROVe was a multicenter, double-blind, placebo-controlled trial including 2,586 patients with a history of colorectal adenomas, randomised to receive either rofecoxib 25 mg daily or placebo. The study reported a significantly higher incidence of thrombotic events, including myocardial infarction and stroke, in the rofecoxib group compared to the placebo group (52). These results refuted the protective-naproxen hypothesis suggested by VIGOR and instead pointed directly to rofecoxib as the causal factor. Furthermore, a cumulative meta-analysis later demonstrated that signs of increased myocardial infarction risk associated with rofecoxib were already detectable before its withdrawal in September 2004 (54). Expanding on this, a comprehensive meta-analysis comprising 116,094 participants confirmed a significant association between rofecoxib and an elevated risk of arrhythmias (55). While initial explanations centred on prothrombotic effects of selective COX-2 inhibition, these adverse cardiovascular outcomes could not be comprehensively linked solely to COX-2 inhibition-induced thromboembolic mechanisms, indicating that additional off-target effects may have contributed (56-58). This highlights that there was a need to have detected the hidden cardiotoxicity of rofecoxib as early as in preclinical safety tests to prevent serious adverse events in vulnerable patients and reduce sunk costs.

Hence, we previously selected rofecoxib as a model drug and demonstrated that the hidden cardiotoxicity of rofecoxib could have been shown in a rat model of myocardial I/R injury (17). Throughout the 28-day administration period, rofecoxib treatment did not produce overt signs of toxicity. However, when the animals were

subsequently subjected to I/R injury, a marked increase in acute mortality was observed. This outcome was linked to enhanced arrhythmia susceptibility in rofecoxib-treated hearts, as confirmed both in vivo and ex vivo (17). Importantly, ischemia was induced on day 29, approximately 24 hours after the final rofecoxib dose. Given the pharmacokinetic profile of rofecoxib, no active compound was expected to remain at this time point, making a direct acute drug effect unlikely (59). These findings suggest that rofecoxib may alter cardiac gene expression already under baseline conditions, thereby predisposing the myocardium to heightened vulnerability during subsequent ischemic stress. Such drug-induced molecular changes may remain silent under normal conditions but become pathogenic upon additional insults (I/R) and may cause the manifestation of hidden cardiotoxicity. Thus, we hypothesise that analysing drug-induced alterations in molecular pathways under baseline conditions may uncover novel mechanisms of hidden cardiotoxicity and identify predictive biomarkers for early safety assessment. Such advancements would improve preclinical drug safety testing and, therefore, reduce sunk costs associated with late-stage drug attrition and enhance patient safety.

2 Objectives

This thesis is based on the hypothesis that cardiac adverse drug reactions frequently manifest only in diseased hearts, thereby escaping detection in routine preclinical drug safety testing. The delayed recognition of cardiotoxic effects of rosiglitazone and rofecoxib until post-marketing surveillance highlights the urgent need for improved safety testing protocols capable of identifying hidden cardiotoxicities early in the drug development. Accordingly, the objectives of this thesis are:

- 1. To test the hidden cardiotoxicity of rosiglitazone in an I/R injury model with and without cardioprotection by ischemic preconditioning (IPC)**
- 2. To identify molecular mechanisms of hidden cardiotoxicity exemplified by rofecoxib that present prior to comorbidities**

3 Methods

3.1 Rosiglitazone ischemia/reperfusion rat model

3.1.1 *In vivo* surgical protocol and animal handling

The animal- and dose-selection were as we described (16): “*For the in vivo experiments, one-month-old male Wistar rats weighing 72–124 g were treated with 0.8 mg kg⁻¹ day⁻¹ rosiglitazone or vehicle (1% hydroxyethylcellulose) by oral gavage for 28 ± 1 days. The animals arrived at the animal facility one week before the treatment started to allow them to get acclimatized with their surroundings. Cage-side visual observation for potential discomfort of the animals was routinely performed by trained animal keepers. According to the formula described by Reagan-Shaw et al. (60), the maximum human dose (8 mg daily), showing cardiovascular side effects in clinical studies (38) was converted to the applied rosiglitazone dose:*

$$HED (mg \times kg^{-1}) = Animal\ dose (mg \times kg^{-1}) \times (rat\ Km)/(human\ Km)$$

To calculate the animal dose, the HED (human equivalent dose, 8 mg 60 kg⁻¹ for an average 60 kg adult) was divided by the ratio of the average rat correction factor (rat Km = 6) and the average human correction factor (human Km = 37).”

To further justify our dose, we reviewed the literature for studies investigating peak plasma concentrations in humans and averaged the findings. The resulting averaged peak plasma concentration range for the two applied human doses (4 mg and 8 mg) is 290.86 ng/ml to 558.943 ng/ml (61-70). In rats, the average peak plasma concentration for 1 mg/kg is 2935 ng/ml (71, 72). This dose is comparable to the dose we applied, as calculated using the formula described by Reagan-Shaw *et al.* (60). Studies showing the peak plasma concentration in rats with higher doses like 5 mg/kg and 4 mg/kg resulted in 10000 ng/ml to 12676 ng/ml (73, 74). The most applied dose in chronic rosiglitazone experiments is 3 mg/kg/day or higher (75-83). We can conclude that our applied dose of 0.8 mg/kg/day resembles the actual human conditions more accurately. Too high concentrations mimic acute overdose but do not reflect the effects of chronic treatment. Potential loss due to pharmacokinetics is negligible, with rosiglitazone having a bioavailability of 99% (84). We also realised that most studies administered the last dose 1 hour before surgery or did not detail the timing of the last dose (77, 81, 83). Therefore, it cannot be differentiated between the chronically altered phenotype resulting from

rosiglitazone treatment and the acute effect of the drug. Therefore, we applied the last dose approximately 24 hours before the start of the surgery protocol. Thus, we focused on the chronically altered phenotype observed upon repeated treatment with rosiglitazone, given its half-life of 3 to 4 hours (84).

The I/R injury model and animal care were conducted as we described (16): *“During the chronic treatment period, the animals were housed in a humidity-controlled room under a 12 h light/dark cycle with temperatures of 22 ± 2 °C. Laboratory chow and drinking water were available to the animals ad libitum. After 28 ± 1 days, the groups of animals treated with rosiglitazone or vehicle weighing 235–372 g received an intraperitoneal injection with 60 mg kg^{-1} pentobarbital as induction of anesthesia. Half-dose pentobarbital injections were repeated if the pedal reflex reoccurred to ensure deep surgical anesthesia. The body surface electrocardiogram (ECG), the heart rate and the mean arterial blood pressure (MAP) were monitored throughout the experiments using standard limb leads and the cannulated right carotid artery, respectively. Arterial access was also used for fluid supplementation with saline containing 10 IU kg^{-1} heparin. To maintain the core body temperature at a physiological level, the animals were placed on a heating pad. Rats were ventilated with 6.2 mL kg^{-1} room air at $69 \pm 3 \text{ breath min}^{-1}$ using a rodent ventilator. The 0 min of the experiment was appointed after successfully placing a 5-0 prolene suture around the left anterior descending (LAD) coronary artery. Intraperitoneal heparin injections of 100 IU kg^{-1} were administered at 35, 65, and 185 min of the experiments.*

The experimental design and surgery protocol are depicted in Figure 4. The rosiglitazone-treated and the vehicle-treated group each comprised 34 animals. On the day of surgery, both treatment groups were divided into two surgery protocols comprising I/R with or without IPC. To assign more animals to the higher-mortality groups, directed randomization was used: I/R + vehicle group ($n = 14$), I/R + rosiglitazone group ($n = 15$), IPC + vehicle group ($n = 20$), IPC + rosiglitazone group ($n = 19$). For the in vivo experiments, the total number of animals was 68. IPC was elicited by three cycles of brief 5 min LAD occlusion and 5 min reperfusion before I/R, whereas the I/R group was subjected only to 30 min of LAD occlusion. All animals were exposed to 120 min of reperfusion following the ischemic period (85, 86). Successful LAD occlusion was

confirmed by ST-segment elevation or depression, the appearance of arrhythmias, and pallor of myocardial regions distal to the occlusion site.”

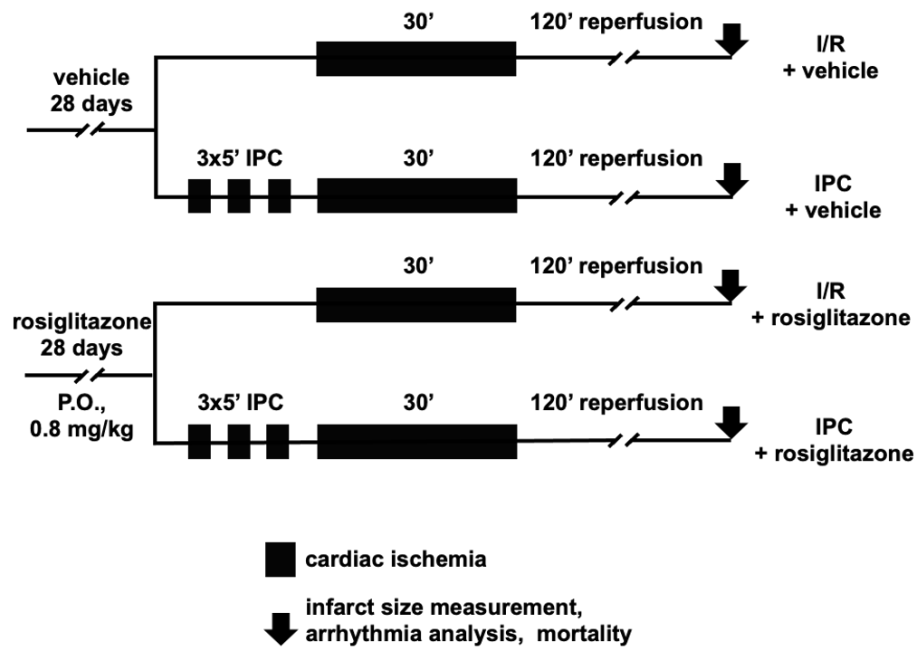


Figure 4. Protocol for *in vivo* cardiac ischemia/reperfusion injury and ischemic preconditioning model. The left anterior descending coronary artery was occluded by placing a suture around it. (IPC: ischemic preconditioning; I/R: ischemia/reperfusion; P.O.: per os). Obtained from Weber *et al.* (16).

3.1.2 Measuring infarct size

Following 120 minutes of reperfusion, hearts were excised and perfused in Langendorff mode for 2 minutes with oxygenated Krebs–Henseleit buffer (composition in mM: NaCl 118, KCl 4.7, MgSO₄ 1.2, CaCl₂ 1.25, KH₂PO₄ 1.2, NaHCO₃ 25, glucose 11) at 37 °C to remove residual blood. The LAD was then re-occluded, and non-ischemic regions were stained with Evans blue dye infused via the ascending aorta to delineate the area at risk (AAR). Hearts were sectioned transversely into 2 mm slices and incubated in 1% TTC solution (triphenyltetrazolium chloride) at 37 °C for 14 minutes to distinguish viable from infarcted myocardium. Slices were weighed, scanned, and analysed by two blinded investigators using InfarctSize 2.4b software. Infarct size was expressed as the percentage of AAR relative to the total left ventricular area (defined as 100%). Area ratios were normalised to the weight of each slice; the weight-adjusted areas were summed, divided by the total heart weight, and multiplied by 100 (16).

3.1.3 Assessment of arrhythmias

Arrhythmias occurring during the 30 minutes of ischemia and the first 15 minutes of reperfusion were evaluated by blinded investigators. The assessment followed the Lambeth Conventions, and arrhythmias were quantified using the scoring system described by Curtis and Walker (score A) (16, 87, 88).

3.1.4 Mortality analysis

In the mortality analysis, included animals died either due to irreversible ventricular fibrillation, pulseless electrical activity, or bradycardia (<150 BPM), accompanied by hypotension (MAP <15 mmHg). All iatrogenic causes of death (6 animals), as well as animals that died because of IPC-induced arrhythmias (11 animals) were excluded from the mortality analysis (16).

3.1.5 Literature search methodology

A systematic literature search was conducted to identify studies investigating the cardiotoxic or cardioprotective effects of rosiglitazone under ischemic conditions in humans, *in vivo* and in cells. On February 20, 2022, we used the search string: “((Rosiglitazone OR Avandia) AND (cardi* OR myocardi* OR heart OR cardiac OR cardio OR cardiology) AND ((Ischemia) OR (Reperfusion) OR (heart attack) OR (myocardial infarction) OR (Infarct))) NOT (review [Publication Type])”, which yielded 289 publications. After screening, we excluded 184 articles for irrelevance (e.g., studies focusing on pioglitazone, unrelated topics, reviews, comments, or lacking an abstract). The remaining clinical studies were evaluated to identify evidence supporting or opposing the presence of cardiotoxic effects of rosiglitazone in humans. *In vitro* and *in vivo* studies with different experimental endpoints were reviewed for potential associations with our findings. Studies with comparable designs were specifically analysed to identify consistencies or discrepancies related to our results (16).

3.2 Molecular analysis of rofecoxib-induced cardiotoxicity

3.2.1 *In vivo* study protocol and animal handling

The *in vivo* treatment model and animal care were conducted in the same facility using the same equipment and adhering to the same standards of animal welfare as

described in paragraph 3.1.1. The *in vivo* model was also described by Weber *et al.* (89): “For *in vivo* experiments, 2-month-old male Wistar rats of 189–287 g body weight were randomised and treated daily for 28 ± 2 days by oral gavage with 5.12 mg kg^{-1} rofecoxib or with its vehicle (1% hydroxyethyl cellulose). Male rats were used in accordance with our previous study, where we demonstrated the proarrhythmic effect of rofecoxib. The treatment duration was chosen in accordance with the OECD (Organisation for Economic Co-operation and Development) guidelines for chronic toxicity studies and testing chemicals by repeated dose 28-day oral toxicity studies in rodents (90, 91). Because the 28-day treatment duration was sufficient to demonstrate hidden cardiotoxicity, we refrained from longer toxicity studies to adhere to the principle of the 3 Rs. The last dose was administered the day before the termination of the animals. Thus, we investigated the phenotype upon 28-day-administered COX-2 inhibitor, where the acute effect is negligible because the half-life ($t_{1/2}$) is 4.3 hours in rats after one-time dosing with 5 mg kg^{-1} per os (92). The dosage of rofecoxib used in the study was derived by extrapolating dosages reported in the clinical studies demonstrating cardiovascular side effects in humans at a daily dose of 50 mg (51). This extrapolation was performed using the formula described by Reagan-Shaw *et al.* (60):

$$HED (\text{mg} \times \text{kg}^{-1}) = \text{Animal dose} (\text{mg} \times \text{kg}^{-1}) \times (\text{rat Km})/(\text{human Km})$$

The influence of pharmacokinetics on potential loss is minimal, owing to the bioavailability of rofecoxib at 93% (59). The same dose was used in our previous study where we demonstrated the hidden cardiotoxic effect in male rats. The illustration of the experimental design is in Figure 5. The treatment did not show any adverse events. In our previous studies, where we demonstrated the hidden cardiotoxicity of rofecoxib, we exposed the animals after the 28-day treatment to I/R by ligating the LAD. The animals in the current study were treated in parallel to ensure equal animal handling conditions (17). However, in this study, all animals were sham-operated, and a 5–0 Prolene suture from Ethicon was loosely looped around the LAD coronary artery. After the previously listed procedures, the 0 min of the experiment was appointed. A total of 16 animals were included in the *in vivo* experiments, with an equal distribution of eight animals in each treatment group. In the phosphorylation site (phosphosite) data set analysis, one animal needed to be excluded from each treatment group. Because of the complexity of the applied statistical approaches, precise power calculation for an appropriate n-number is

especially difficult for multi-omics studies like the current one and has only been tackled recently in some preliminary work (93). Recently published multi-omics studies performed with smaller sample sizes, between 3–8 per group, succeeded in identifying significant and relevant changes (94–97). Therefore, taking into account the 3Rs principle (Replacement, Reduction and Refinement), a sample size of $n = 8$ per group was considered reasonable and well-powered.”

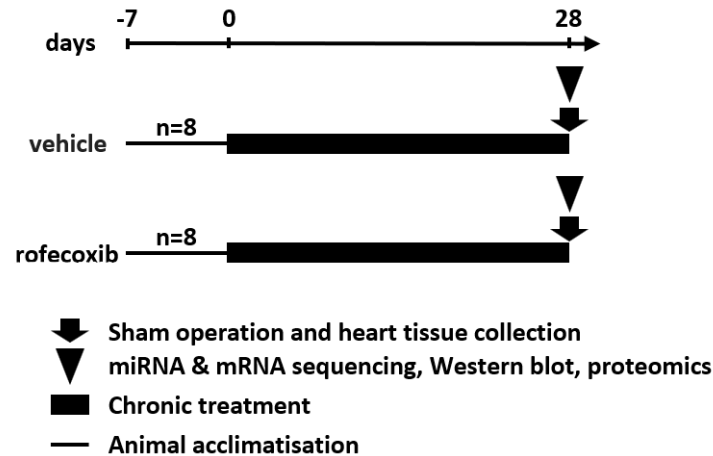


Figure 5. Protocol for *in vivo* 28-day rofecoxib treatment and tissue harvesting. The 4-week treatment of male Wistar rats with 5.12 mg kg⁻¹ day⁻¹ rofecoxib or 1% hydroxyethyl cellulose was carried out by oral gavage. The sham operation was performed by opening their chest cavity and placing a loose ligation around their left anterior descending artery. Lastly, the excised heart was retrogradely perfused in the Langendorff system to wash out the blood. (miRNA: microRNA; mRNA: messenger RNA). Obtained from Weber *et al.* (89).

3.2.2 Preparation of heart tissue for downstream analysis

Following the sham operation, rat hearts were removed and processed as described earlier (89): “The heart was removed and subjected to Langendorff perfusion for 2 min at 37 °C. During this perfusion, the hearts were flushed with oxygenated Krebs–Henseleit solution (in millimolar [mM]: NaCl 118, KCl 4.7, MgSO₄ 1.2, CaCl₂ 1.25, KH₂PO₄ 1.2, NaHCO₃ 25, and glucose 11) to remove any remaining blood from the tissue. The left ventricular tissue was excised. Afterwards, the samples were snap-frozen in liquid nitrogen and stored at –80 °C. The snap-frozen tissue samples were ground to a fine powder with meticulous care and under liquid nitrogen to avoid unnecessary thawing. The amounts of fine powder needed for the proteomics, transcriptomics and western blot analysis were measured in Eppendorf tubes. Before the independent analysis, the powdered tissues of each sample were homogenised, and RNA concentration or protein

concentration was measured to ensure comparability between the samples. Further processing is illustrated in Figure 6 and detailed in the following paragraphs.”

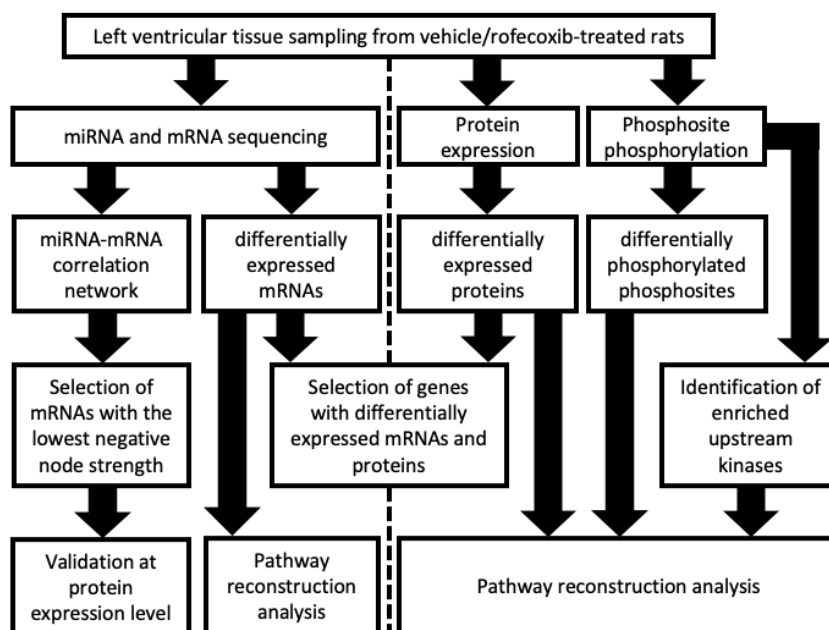


Figure 6. Workflow for *in silico* left ventricular tissue analysis. The excised left ventricular heart tissue was snap-frozen in liquid nitrogen. The snap frozen tissue samples were pulverised under liquid nitrogen. The required weight for the independent measurements (transcriptomics, proteomics, western blot) was allocated to each experiment from the pulverised tissue. (miRNA: microRNA; mRNA: messenger RNA). Obtained from Weber *et al.* (89).

3.2.3 Proteomics sample preparation: tissue lysis, trypsin digestion and phosphopeptide enrichment

Pulverised heart tissue samples were transported on dry ice to the UCD Conway Institute Mass Spectrometry Resource for proteomic analysis using liquid chromatography-tandem mass spectrometry (LC-MS/MS). Here, sample preparation followed protocols previously established (89): “Pulverised heart tissue samples were resuspended in 500 μ l of ice-cold 8 M urea 50 mM–1 Tris HCl with phosphatase and protease inhibitors. Each sample was sonicated for 2×9 s at a power setting of 25% to disrupt the cell pellet. Each sample was clarified by centrifugation at 8000 rpm for 10 min and then quantified using a NanoDrop 2000 at 280 nm before normalisation to 400 μ g in 2- ml Eppendorf tubes. Each sample was reduced by adding 8 mM dithiothreitol (dtt) and mixing (thermomixer 1000 rpm, 30 °C) for 60 min and carboxylated by adding 20 mM iodoacetamide and mixing (thermomixer 1000 rpm, 30 °C) for 30 min in the dark.

The solution was diluted with 50 mM Tris HCl to bring the urea concentration down to 1.6 M. Lyophilized trypsin was resuspended with 50 mM Tris HCl at a concentration of 0.5 µg µl⁻¹ and added to each solution. The samples were digested overnight with trypsin in a 1:50 enzyme-to-protein ratio with gentle shaking (thermomixer 850 rpm, 37 °C). The digestion was terminated by adding trifluoroacetic acid to 1% final concentration and cleaned up using solid phase extraction (HyperSep SpinTip P-20, BioBasic C18). Peptides were eluted in 100 µl of 60% acetonitrile and 0.1% trifluoroacetic acid, of which 10 µl for proteomic analysis was vacuum-centrifuged and resuspended in 0.1% trifluoroacetic acid. The remaining 90 µl were kept for phospho-peptide analysis.

Phospho-peptide enrichment was carried out with TiO₂-based enrichment using Titansphere Phos-TiO Bulk 10 µm, according to an adapted method previously described (98). In summary, each sample was incubated with TiO₂ beads for 30 min by end-over-end rotation in 80% acetonitrile 6% trifluoroacetic acid (suitable for HPLC), 5 mM monopotassium phosphate, 20 mg ml⁻¹ 2,5- dihydroxybenzoic acid and water. The beads were washed in 1 ml of 80% acetonitrile/1% trifluoroacetic acid four times by end-over-end rotation. Phospho-peptides were then removed from the TiO₂ beads by shaking, using a thermomixer at 1500 rpm for 10 min, at room temperature with 75 µl of 50% acetonitrile and 7% ammonium hydroxide. Each sample was centrifuged, and the supernatant was removed to a 1.5-ml Eppendorf tube. The workflow was carried out twice, and the supernatant from each sample was then pooled and dried down.”

3.2.4 Measuring proteomics by liquid chromatography coupled with tandem mass spectrometry

The liquid chromatography coupled with tandem mass spectrometry measurements were carried out by the UCD Conway Institute Mass Spectrometry Resource as we described earlier (89): “Samples were run on a Bruker timsTOF Pro mass spectrometer connected to an Evosep One liquid chromatography system. Tryptic peptides were resuspended in 0.1% trifluoroacetic acid, and each sample was loaded onto an Evosep tip. The Evosep tips were placed in position on the Evosep One, in a 96-tip box. The autosampler is configured to pick up each tip, elute and separate the peptides by using 30 samples a day standardised Evosep method. The details include a 44-min gradient with a total run (cycle) time of 48 min. Initially, the analytical column (EV1137

Performance column) was equilibrated at 1500 nl min⁻¹, after which the gradient flow was reduced to 500 nl min⁻¹ and then increased to 1500 nl min⁻¹ for washing (99). The following chromatography buffers were used: Buffer B: 99.9% acetonitrile, 0.1% formic acid. Buffer A: 99.9% water, 0.1% formic acid. The mass spectrometer was operated in positive ion mode with a capillary voltage of 1700 V, dry gas flow of 3 l min⁻¹ and a dry temperature of 180 °C. All data were acquired with the instrument operating in trapped ion mobility spectrometry (TIMS) mode. Trapped ions were selected for MS/MS by using parallel accumulation serial fragmentation (PASEF). A scan range of (100–1700 m/z) was performed at a rate of 5 PASEF MS/MS frames to 1 MS scan with a cycle time of 1.03 s (100).” The liquid chromatography coupled with tandem mass spectrometry data is openly available in the ProteomeXchange Consortium via the PRIDE (101) partner repository with the dataset identifier PXD050504.

3.2.5 Processing of proteomic/phosphoproteomic raw data

The analysis of the liquid chromatography coupled with tandem mass spectrometry raw data was performed as previously described (89): “The raw data were searched against the *Rattus norvegicus* subset of the UniProt database (Swiss prot/TrEMBL) using the Fragpipe proteomics pipeline (version 19.1) with specific parameters for trapped ion mobility spectra data-dependent acquisition (TIMS DDA). In the case of proteomics analysis, the LFQ-MBR workflow was selected. Detail includes MS Fragger parameters: precursor mass tolerance ± 20 ppm, fragment mass tolerance ± 20 ppm, strict trypsin and fixed modifications oxidation of methionine and N-terminal acetylation. Phosphoproteomic analysis was processed using the LFQ-phospho workflow, which includes phosphorylation as a fixed modification on serine, threonine, and tyrosine residues (102). Results were analysed using Perseus software (103) and R programming language (104) according to Tyanova and Cox (105), similar to an earlier study of our group (106). The results of the duplicated measurements (two technical repeats) were included in the further analysis. Contaminants were filtered out by selecting proteins identified in the organism *Rattus norvegicus*. For proteomics and phosphosite datasets, if less than 75% of measurements were valid (non-missing value) in both groups, the specific protein or phosphosite was excluded from the analysis. Furthermore, those phosphosites where the corresponding protein was not detected in the proteomics dataset also were excluded in the phosphosite dataset. Then, data were

log2 transformed, and missing values were imputed around the detection limit with a normal distribution (distribution width of 0.5 and 1.8 downshift) (103). After imputation, both replicates of two samples were excluded from phosphoproteomics analysis because of potential technical errors. Those samples had markedly fewer valid values than other samples, and a bimodal distribution of log2 transformed intensities owing to the high number of imputations. For statistical analysis, log2 transformed data were used with $n = 8 \times 2$ technical replicates per group in the proteomics data set and $n = 7 \times 2$ technical replicates per group in the phosphosite dataset. If a phosphosite showed a significant difference, however, the corresponding protein also exhibited a significant change in the same direction; we declared this phosphosite non-significant. Normal scale data were used to calculate fold changes and percentile ranks. PhosphoSitePlus®v6.7.4 was used to gather information about the detected phosphosites (107)."

3.2.6 Analysis of upstream kinase enrichment

Kinase enrichment analysis was performed using the fgsea package (version 1.26.0) in R (104). Experimentally validated kinase-substrate relationships were obtained from the PhosphositePlus® v6.7.4 database (<https://www.phosphosite.org/staticDownloads.action>) (107). The analysis was based on a pre-ranked list of phosphosites, ordered according to t-statistics derived from two-sided t-tests (108). Kinases were considered significantly enriched if the permutation-based P-value was below 0.05 (89).

3.2.7 Pathway reconstruction analysis

To investigate shared biological functions and clusters of interacting genes in our set of changing proteins, phosphosites and mRNAs, we employed the STRING (Search Tool for the Retrieval of Interacting Genes) database (<https://string-db.org/>, version 12.0, released 26 July 2023, accessed on 29 June 2024) because of its high number of interactions and proteins compared to other databases (109, 110). The database incorporates 27,541,372,833 interactions from 59,309,604 proteins in 12,535 organisms. The analysis in STRING was performed as previously described (89): *"The interactions were filtered for high confidence (interaction score >0.7), because this is the default recommended value (111). Gene ontology enrichment analysis was carried out in STRING. For the gene ontology analysis, the maximum false discovery rate was set to*

0.05 and the strength to a minimum of 0.3, with a minimum count of four proteins in the network. Relevant gene ontology terms were colour-coded for the network representing the 84 differentially expressed proteins (Figure 10). Next, we extended the protein–protein interaction network with COX-2 as the known target of rofecoxib, the identified active upstream kinases and proteins with at least one differentially phosphorylated phosphosite. We performed k-means clustering to identify the sets of proteins with well-established, shared biological functions. K-means clustering requires the parameter k to be specified, which corresponds to the number of introduced centroids defining the number of resulting clusters. The number of introduced centroids was adjusted to 11 to accomplish the desired separation of biological functions in representable clusters, for example, ribosomal proteins. Further gene ontology enrichment analysis was performed in STRING for the six most populated clusters, with the network itself selected as the background for the enrichment to reveal their individual functional roles inside the network (112, 113). Lastly, the 480 differentially expressed mRNAs were investigated in the STRING, and a gene ontology enrichment analysis was performed.”

3.2.8 Transcriptomics sample preparation and measurement: total RNA isolation and small RNA or mRNA sequencing

The total RNA was isolated from pulverized heart tissue and sequenced as described earlier (89): “The total RNA of the left ventricle tissue samples ($n = 8$ per group) was extracted by employing the RNeasy RT kit according to the manufacturer's instructions. RNA concentrations of all samples were quantified using spectrophotometry (NanoDrop) and RNA HS Assay Kit with Qubit 3.0 Fluorometer. The RNA integrity numbers were determined by 2100 Bioanalyzer Instrument and agarose gel electrophoresis.

We employed the NEBNext Multiplex Small RNA Library Prep Set for Illumina for small RNA library construction according to the manufacturer's protocol. Libraries were quantified and qualified using High Sensitivity DNA1000 ScreenTape system with 2,200 TapeStation and dsDNA HS assay kit with Qubit 3.0 fluorometer. Libraries were pooled and diluted to 1.8 pM for 2×43 bp paired-end sequencing on the NextSeq 550 Sequencing System at Xenovae Ltd., using the 75-cycle High Output v2 kit, according to the manufacturer's protocol. For Gene Expression Profiling (Gex) library construction, QuantSeq 3' mRNA-Seq Library Prep Kit FWD for Illumina was applied according to the

manufacturer's protocol. The quality and quantity of the library were determined by using High Sensitivity DNA1000 ScreenTape system with 2200 TapeStation and dsDNA HS assay kit with Qubit 3.0 fluorometer, respectively. Pooled libraries were diluted to 1.8 pM for 1 × 86 bp single-end sequencing with 75-cycle High Output v2 kit on the NextSeq 550 Sequencing System at the Xenovea Ltd, according to the manufacturer's protocol.”

The raw mRNA sequencing data is openly available in ArrayExpress collection in the Biostudies (114) repository (<http://www.ebi.ac.uk/biostudies>) with the dataset identifier E-MTAB-13913 (accessible via: <https://www.ebi.ac.uk/biostudies/arrayexpress/studies/E-MTAB-13913?key=9a4a1cd1-2192-4aae-ba21-a19e6c73925d>). The raw miRNA sequencing data is openly available in ArrayExpress collection in the Biostudies (114) repository (<http://www.ebi.ac.uk/biostudies>) with the dataset identifier E-MTAB-13909 (accessible via: <https://www.ebi.ac.uk/biostudies/arrayexpress/studies/E-MTAB-13909?key=9fc0f53e-9cfb-4d9c-972e-fad4e3dcaa08>).

3.2.9 Bioinformatic analysis of small RNA and mRNA sequencing datasets

The analysis of the miRNA and mRNA sequencing raw data was performed as previously described (89): *“After trimming of adapters, small RNA sequencing reads with a length of less than 10 nucleotides and with an average Phred quality score less than or equal to 30 were excluded from further analysis by Illumina FastQ Toolkit (version v2.2.0). The reads were aligned by Bowtie2 (version 2.2.3) (115) and annotated by featureCounts (version 2.0.0) (116) using the NCBI Rnor_6.0 Rattus norvegicus reference genome assembly and the miRBase (release 22.1) reference annotation (117, 118). During the preprocessing of mRNA reads, adapters, poly(A) tails, and bases with a Phred score less than or equal to 30 were trimmed, and reads with a length below 19 nucleotides were filtered out by Cutadapt software (119). For alignment and annotation, HISAT2 (version 2.0.4) and featureCounts software were used, respectively, with Rnor_6.0 NCBI Rattus norvegicus reference genome assembly and the corresponding reference annotation (120, 121).*

The quality of raw reads of small RNA and mRNA sequencing was checked by FastQC (version 0.11.8) and MultiQC (version 1.7) (122). SAM (sequence alignment map) files were converted to BAM (binary alignment map) files by Samtools (version 1.9) (123, 124). Differential expression analysis by DESeq2 (version 1.10.1) (125) package

was followed by principal component analysis and correlation network analysis conducted in R environment (version 3.2.3) (104). To characterise the abundance of miRNAs and the genes, raw counts were normalised to get CPM (counts per million) and TPM (transcript per million), respectively, and median values were calculated in the case of all miRNAs and genes. miRNAs and genes not expressed (median CPM or median TPM = 0) were excluded, and the percentile ranks were calculated for the median CPM of miRNAs and median TPM of genes. In parallel, differential expression analysis of mRNA sequencing data was also carried out by the Tophat2 (version 2.1.1)—Cufflinks (version 2.2.1) workflow with the sole aim of enhancing the accuracy of the results by introducing an additional filtering criterion for the construction of the correlation network (126, 127). The differential regulation thresholds were set as $P < 0.05$, and $\log_2$ fold change > 0.2 or < -0.2 (Wald test, $n = 8$ per group) (128).

To investigate the potential miRNA-target interactions, miRNAs and genes showing a differential expression according to DESeq2 analysis (Wald test, $P < 0.05$, $n = 8$ per group) were selected and, in the case of the numerous differentially expressed mRNA genes, an additional filtering criterion was applied (Tophat2-Cufflinks adjusted P -value < 0.05). Spearman's rank correlation coefficients (ρ values) were calculated between the selected miRNAs and target genes ($n = 8$ per group), and miRNA-target pairs with a significant negative correlation were kept for the construction of a miRNA-target correlation network. Complementarity between miRNA seed regions and the mRNA transcripts was investigated by a Python (version 3.5.2) script with the use of BioPython (version 1.76) module (129) and the reference genome specified earlier. The node strength of each node was calculated by summing the Spearman's ρ values of the edges incident to the node. This correlation network was visualised by EntOptLayout (version 2.1) plug-in for the Cytoscape software platform (version 3.10.1) (130, 131). ”

3.2.10 Western blots

To validate changes seen in transcription, we investigated selective targets on protein level by western blot as described earlier (89): “Snap-frozen heart samples were homogenised in radioimmunoprecipitation assay buffer (1xRIPA) containing 1 mM PMSF (phenylmethylsulfonyl fluoride), 0.1 mM sodium fluoride, and complete HALT protease inhibitor and phosphatase inhibitor cocktail. Protein concentration was measured with a bicinchoninic acid assay in a plate reader (Thermo Scientific Multiskan

GO). Equal amounts of protein were mixed with one fourth volume of Laemmli buffer containing β -mercaptoethanol. A total of 10 μ l solution containing 40 ug protein sample was loaded in each lane of Tris-glycine sodium dodecyl sulphate-polyacrylamide gels. The separation was performed in 4–20% and fixed 12% Criterion TGX Gels. PageRuler™ Plus Prestained Protein Ladder or Spectra™ multicolour broad range protein ladder was employed as a molecular marker. To test for the most suitable positive control of each antibody, we performed western blots with a variety of organs from a male Wistar rat (testis, adrenal gland, lung, heart, liver, kidney, spleen, and skeletal muscle). The rat organ demonstrating the highest protein of interest expression was loaded as a positive control for antibody detection. The selection of the organ for use as a positive control was validated through experimental means. Proteins were transferred onto a polyvinylidene difluoride membrane with Trans-Blot® Turbo™ Transfer System or conventional transfer. Membranes were blocked in 5% milk in Tris-buffered saline containing 0.05% Tween-20 for 2 h at room temperature. Membranes were probed with primary antibodies, and after three washes in TBS-T (Tris-buffered saline-Tween), membranes were incubated with corresponding HRP-conjugated secondary antibodies for 2 h and washed in TBS-T. For the antibody details of primary and secondary antibodies, see Tables S11 and S12 (shown by Weber *et al.* in Supporting Information (89)). After incubating the membranes with a Clarity enhanced chemiluminescence (ECL) kit western blotting substrate or 1:1 mix of Clarity Max and Clarity ECL western blotting substrate, membranes were imaged with ChemiDoc XRS + System. Band intensities were analysed in the Image Lab software and normalised to the loading control (GAPDH; Table S12). A maximum of one sample (loaded well) per treatment group needed to be excluded because of incorrect loading or background noise on membranes (data not shown here, see Weber *et al.* in Supporting Information (89)).”

3.3 Statistical analysis

For the rosiglitazone *in vivo* study, the Chi-Square test was applied to identify differences in the mortality percentages. The arrhythmia scores are expressed as the median with individual data points. The infarct size percentages are expressed as the mean with standard error. The absence of normal distribution was shown by Shapiro–Wilk and Kolmogorov–Smirnov tests, and consequently, the Kruskal–Wallis test with Dunn’s post

hoc tests for multiple comparisons (arrhythmia - and infarct size analysis) was used to match the need of non-parametric datasets. The significance level was set to 5 % (16).

In each experiment of the multi-omics analysis of rofecoxib-treated hearts, the change caused by rofecoxib was compared to its control (vehicle-treated animals), and therefore, Rofecoxib/Control is the direction of the comparison. The intensity of the target protein bands was normalised to the corresponding GAPDH loading control to correct for potential variations in sample loading. Subsequently, statistical comparisons were made using unpaired, two-tailed Student's t-tests (P-value <0.05; n = 7–8 animals per group). Protein expression levels and phosphosite intensities obtained from mass spectrometry were log₂-transformed prior to analysis. For proteomic data, statistical differences between treatment groups were also assessed using unpaired, two-tailed Student's t-tests (P <0.05; n = 7–8 animals × 2 technical replicates per group). Thresholds for differential expression were defined as a P-value <0.05 combined with a log₂ fold change greater >0.2 or <−0.2 (89, 128). Statistical analysis and graphical representation were carried out in GraphPad Prism 8 (GraphPad Software Inc.) and in R environment (104). Investigators were blinded towards the animals' treatment groups during the analysis. The data and statistical analysis are in accordance with recommendations on experimental design and analysis in pharmacology (132).

3.4 Ethical considerations

All animal procedures were performed in accordance with the principles of the 3Rs. The study complied with the Guide for the Care and Use of Laboratory Animals (US National Institutes of Health, NIH Publication No. 85-23, revised 1996), as well as the European Union Directive 2010/63/EU on the protection of animals used for scientific purposes. Ethical approvals were granted by the Animal Ethics Committee of Semmelweis University (Budapest, Hungary) and the National Scientific Ethical Committee on Animal Experimentation (Budapest, Hungary). Additional authorisation was obtained from the Food Chain Safety and Animal Health Directorate of the Government Office for Pest County, Hungary (permit number: PE/EA/1784-7/2017) (16, 89).

4 Results

4.1 Rosiglitazone does not show major hidden cardiotoxicity in preclinical models of ischemia/reperfusion

First, we aimed to test the clinically reported hidden cardiotoxicity of rosiglitazone by testing its effect on I/R. To this end, rats were treated for four weeks, with the last dose being administered 24 h prior to the surgery. On the day of surgery, animals underwent 30 min of coronary occlusion followed by 120 min of reperfusion (Figure 4). During ischemia and early reperfusion, arrhythmias and mortality were monitored, and infarct size was determined after reperfusion as the hard endpoint for ischemic injury.

4.1.1 Twenty-eight-day rosiglitazone treatment did not aggravate I/R injury

The infarct size was assessed as a ratio to the total tissue exposed to ischemia (i.e., risk area) to evaluate the effect of rosiglitazone on I/R injury. The AAR corresponds to the portion of the myocardium following coronary occlusion that reflects the maximal potential extent of myocardial infarction. Figure 7A shows that infarct size following I/R was unaffected by rosiglitazone treatment compared to the I/R vehicle group.

During ischemia and early reperfusion, cardiac arrhythmias were scored to reflect their severity and accumulation based on the Lambeth conventions. The individual arrhythmia scores of animals are presented in Figure 7B. During I/R, the score did not increase significantly with rosiglitazone treatment compared with the vehicle-treated group.

To assess hidden cardiotoxicity, overt mortality during I/R injury was evaluated. Statistical analysis using the chi-square test revealed no significant differences among the I/R vehicle and I/R rosiglitazone group. The corresponding mortality rates are displayed as percentages in Figure 7C. Two animals in the I/R vehicle group and one animal in the rosiglitazone I/R group died during ischemia due to irreversible ventricular fibrillation. Another animal in the rosiglitazone I/R group died due to bradycardia during reperfusion. These results show that rosiglitazone does not aggravate acute mortality, arrhythmia susceptibility or infarct size in this model of I/R injury.

4.1.2 Twenty-eight-day rosiglitazone treatment abolished the antiarrhythmic effects of IPC

To investigate whether rosiglitazone interferes with cardioprotection and thereby contributes to hidden cardiotoxicity, we included both vehicle- and rosiglitazone-treated groups that underwent a robust cardioprotective intervention induced by short, repetitive periods of ischemia (IPC) prior to I/R.

IPC-induced cardioprotection successfully reduced infarct size in both rosiglitazone- and vehicle-treated animals (Figure 7A). Additionally, rosiglitazone, IPC or their combination had no significant influence on the AAR (expressed as % of the left ventricle; I/R vehicle: $43.6 \pm 4.7\%$; I/R rosiglitazone: $48.3 \pm 2.3\%$; IPC vehicle: $37.7 \pm 2.9\%$; IPC rosiglitazone $41.3 \pm 2\%$).

The analysis of acute mortality (expressed as percentages) revealed no significant differences among the four treatment groups (Figure 7C), with similarly low mortality rates observed across all groups. Therefore, no beneficial effect of IPC was detected. Animals that did not survive the brief recurrent ischemia/reperfusion cycles during IPC were excluded from further analysis and graphical representation (IPC vehicle: six exclusions; IPC rosiglitazone: five exclusions). In the IPC vehicle group, all remaining animals survived the full protocol. One rat in the IPC rosiglitazone group experienced asystole and died during the reperfusion phase.

The anti-arrhythmic effects elicited by IPC effectively reduced arrhythmia development during I/R in the vehicle-treated animals. Comparing the two IPC-treated groups, the arrhythmia score did not differ significantly. However, because no significant difference was detected between the rosiglitazone-treated groups with and without IPC, rosiglitazone may negate the antiarrhythmic benefits of IPC (Figure 7B).

In summary, rosiglitazone abolishes the antiarrhythmic benefits of IPC without impairing its infarct-size-limiting effects.

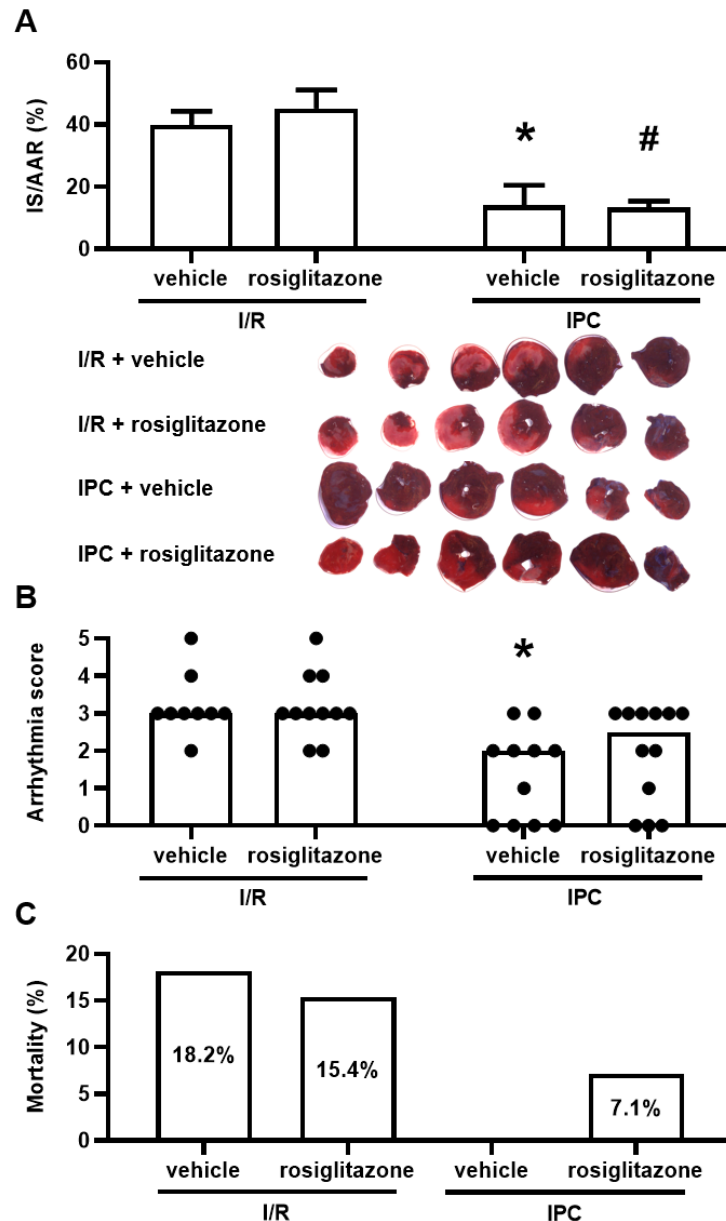


Figure 7. Rosiglitazone does not cause major hidden cardiotoxicity in an ischemia/reperfusion model **A.) Myocardial infarct sizes as a ratio of the area at risk.** Results are presented as mean with standard error. Rosiglitazone neither aggravates infarct size of ischemia/reperfusion injury nor interferes with cardioprotection by ischemic preconditioning (Kruskal–Wallis, Dunn’s post hoc, * $p < 0.05$ vs. I/R + vehicle, # $p < 0.05$ vs. I/R + rosiglitazone, $n = 9–10$). **B.) Arrhythmia scores assessed during myocardial ischemia and the first 15 min of reperfusion.** Results are presented as the median of the individual data points. Rosiglitazone does not raise the arrhythmia score during ischemia/reperfusion but abolishes the antiarrhythmic effects of ischemic preconditioning (Kruskal–Wallis, Dunn’s post hoc, * $p < 0.05$ vs. I/R + vehicle, $n = 11–12$). **C.) Mortality analysis.** Rosiglitazone and IPC do not influence mortality during ischemia/reperfusion injury (Percentage; chi-square test, $n = 11–14$). (IPC: ischemic preconditioning; I/R: ischemia/reperfusion, IS/AAR: infarct size/area at risk). Obtained from Weber *et al.* (16).

4.2 Hidden cardiotoxicity induced by rofecoxib is mediated by altered cardiac calcium handling, contributing to increased arrhythmia susceptibility.

To investigate the molecular mechanisms underlying hidden cardiotoxicity induced by rofecoxib, we employed a 28-day treatment model in rats, administering rofecoxib at a daily dose of 5.12 mg kg⁻¹. Twenty-four hours after the final dose, left ventricular tissue was harvested for comprehensive molecular analysis (Figures 5 and 6). We performed an integrative approach, including protein expression, phosphoproteomics, mRNA and miRNA expression analyses. This multi-omics approach aimed to identify transcriptional, posttranscriptional, translational, and post-translational regulatory changes that may contribute to the development of hidden cardiotoxicity, with a particular focus on pathways potentially predisposing the myocardium to arrhythmia and ischemic injury.

4.2.1 Cardiac protein expression is affected by hidden cardiotoxicity induced by rofecoxib

In the proteomic analysis, we found that the principal component analysis did not show a clear separation of the treatment groups (Figure 8A), which suggests that the baseline expression in the heart is not drastically changed by the treatment. However, to investigate if there were differences in the protein levels of treated and untreated animals a statistical analysis was performed. The differential expression profile is displayed in a volcano plot (Figure 8B). The results showed that 4.8% (84) of the detected proteins changed significantly (Figure 9). Additional information on the significantly changing proteins is shown in Weber *et al.* 2025 in Supporting Information Data S3 (89).

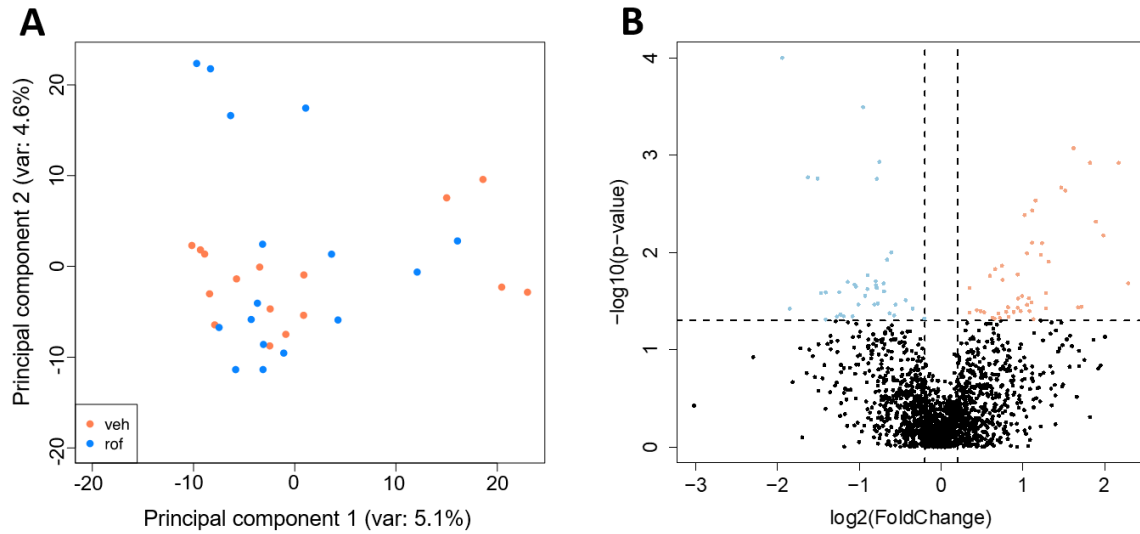


Figure 8. A: Principal component analysis representing the proteomic dataset of rofecoxib-treated hearts. Principal component analysis of the global protein expression profile is shown. The proportion of variance explained (var) by the first and second principal components is demonstrated in parentheses on the respective axes. **B: Differential protein expression profile of rofecoxib-treated hearts.** This volcano plot displays the log₂ fold change (x-axis) and -log₁₀(P-value) (y-axis) for protein expression differences between treatment groups ($n = 8 \times 2$ technical replicates per group). Statistical comparisons were performed using a two-tailed, unpaired t-test. The horizontal line denotes the P-value threshold of 0.05, and the vertical lines indicate log₂ fold-change cutoffs of -0.2 and 0.2. Downregulated proteins are shown in blue, and upregulated proteins are shown in red. Obtained from Weber *et al.* (89).

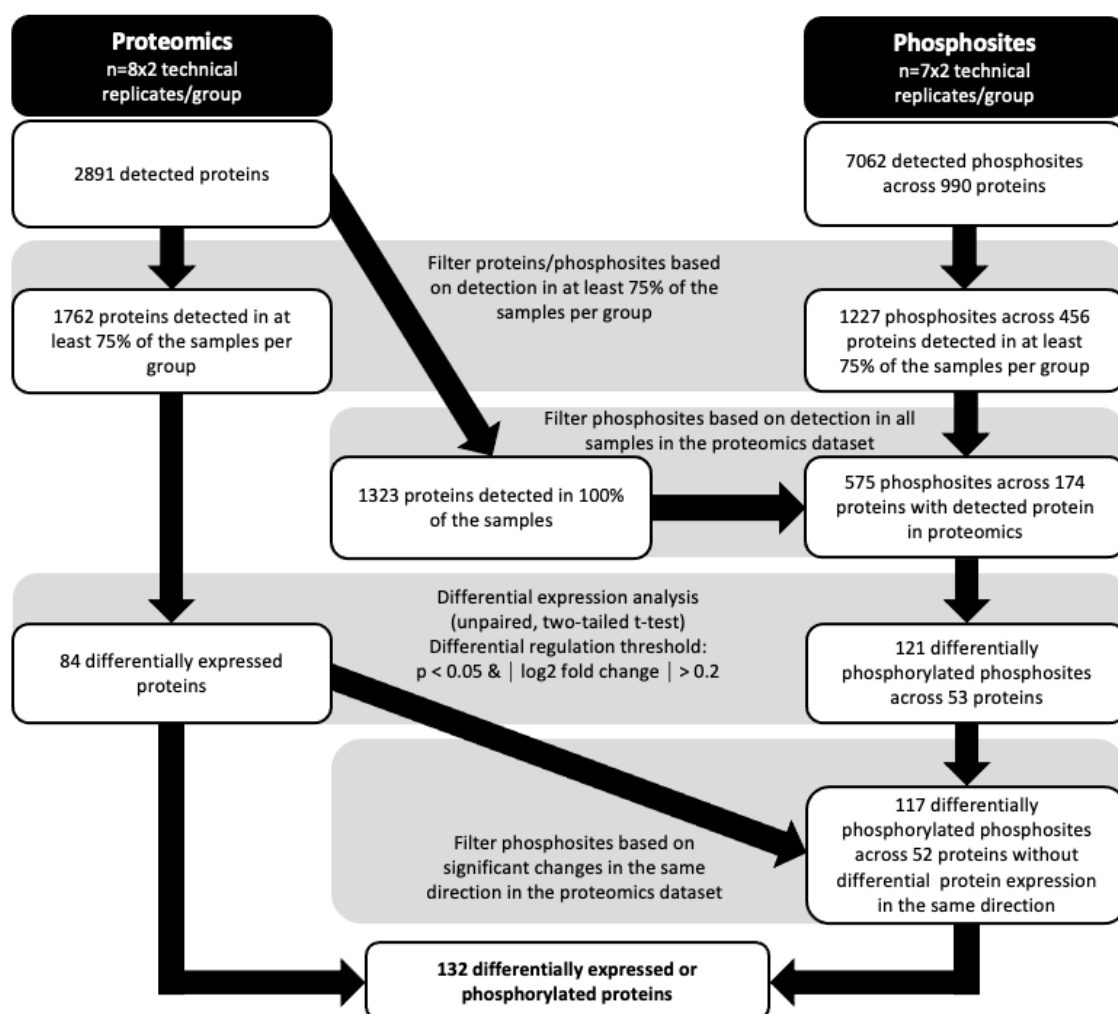


Figure 9. Proteomics and phosphosite *in silico* workflow of rofecoxib-treated hearts. For the phosphoproteomics analysis both replicates of two samples were excluded, because of potential technical errors. Those samples showed a bimodal distribution of log₂ transformed intensities owing to the markedly fewer valid values than other samples and the resulting high number of imputations. Obtained from Weber *et al.* (89).

4.2.2 Alterations in cardiac protein expression may contribute to hidden cardiotoxicity by modulating translation and degradation pathways

A protein–protein interaction network was generated to investigate the biological processes associated with proteins exhibiting altered expression following rofecoxib treatment (Figure 10A). The STRING enrichment value assesses the degree of connectivity within the network by comparing the observed number of interactions to those expected in a random protein set of equal size. In this case, the calculated P-value of 0.0582 indicates limited connectivity among the identified proteins. Nevertheless, gene ontology overrepresentation analysis revealed significant enrichment in pathways related

to protein ubiquitination, translation, the tricarboxylic acid cycle, and branched-chain amino acid metabolism (Figure 10B). These findings suggest that rofecoxib-induced hidden cardiotoxicity may involve alterations in protein expression mediated through dysregulation of protein translation and degradation processes.

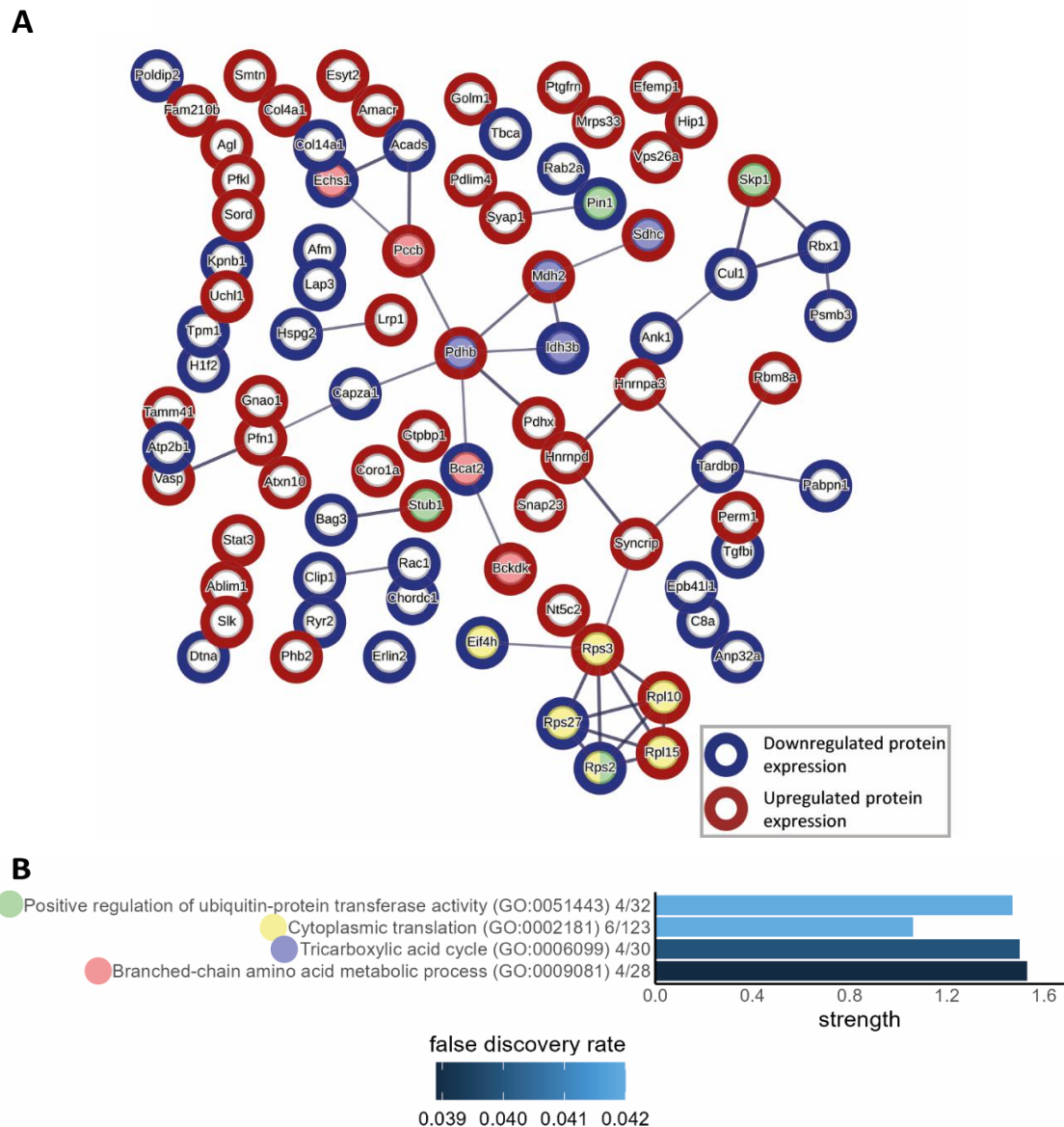


Figure 10. Protein–protein interaction network analysis visualizing differentially expressed proteins in rofecoxib-treated hearts alongside overrepresented gene ontology terms. A: The interaction network was constructed using the STRING database (string-db.org), incorporating proteins identified as differentially expressed in the proteomic dataset (P-value <0.05). Edge thickness corresponds to the interaction confidence, with only high-confidence interactions (interaction score >0.7) included. Node outline colours reflect the respective log2 fold change direction of the proteins. **B:** Significantly enriched gene ontology terms are displayed, ranked according to their enrichment strength (log10[observed/expected]). Node fill colours correspond to the gene ontology terms represented within the protein network. Obtained from Weber *et al.* (89).

4.2.3 Hidden cardiotoxicity induced by rofecoxib involves alterations in cardiac protein phosphorylation

To explore posttranslational regulation associated with hidden cardiotoxicity, phosphoproteomic analysis was performed to assess changes in serine, threonine, and tyrosine phosphorylation between rofecoxib-treated and untreated animals. In total, 575 phosphosites corresponding to proteins identified in the proteomic dataset were detected in at least 75% of samples per treatment group (Figure 9). As with the global proteomic analysis, principal component analysis did not reveal a distinct separation between the treatment groups (Figure 11A). Nonetheless, a statistical analysis was conducted to detect if there were differences in phosphosite phosphorylation of treated and untreated animals, showing that 20.3% (117) of the detected phosphosites differed significantly (Figure 3; Details of significantly altered phosphosites are shown in Weber *et al.* 2025 in Supporting Information Data S3 (89). The differential phosphorylation profile is visualised in the volcano plot (Figure 11B). The 117 phosphosites belong to 52 proteins, that exhibited altered phosphorylation at one or more phosphosites. Four proteins showed changes both in expression level and phosphorylation. In total, 132 proteins exhibited expression changes and/or altered phosphorylation on at least one of their phosphosites due to hidden cardiotoxicity induced by rofecoxib.

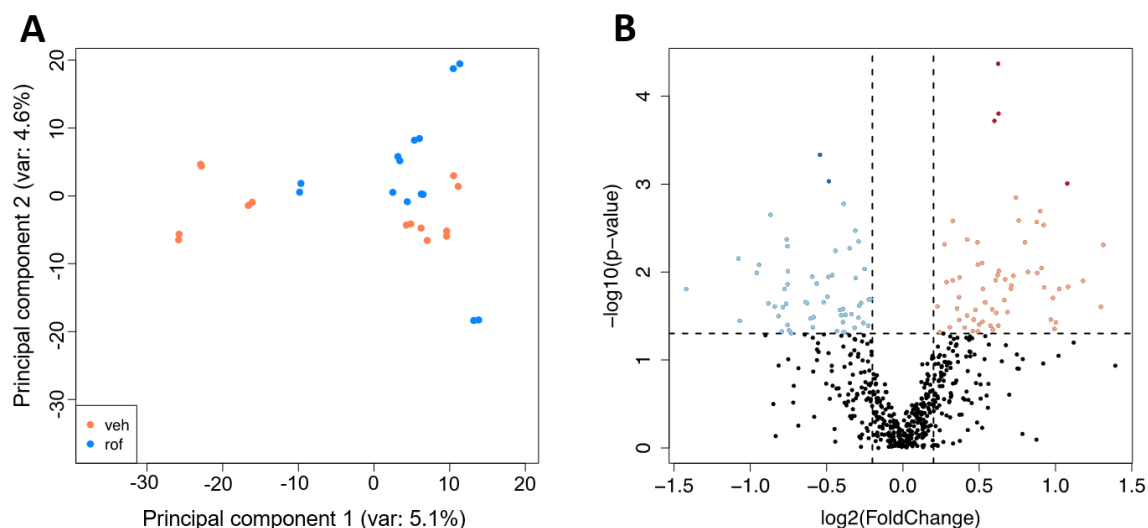


Figure 11. A: Principal component analysis representing the phosphorylation site dataset of rofecoxib-treated hearts. Principal component analysis of the global phosphorylation site profile is shown. The proportion of variance explained (var) by the first and second principal components is demonstrated in parentheses on the respective axes. **B: Differential phosphorylation site phosphorylation profile of rofecoxib-treated hearts.** This volcano plot displays the log₂ fold change (x-axis) and $-\log_{10}(\text{P-value})$ (y-axis) for phosphorylation differences between treatment groups ($n = 8 \times 2$ technical replicates per group). Statistical comparisons were performed using a two-tailed, unpaired t-test. The horizontal line denotes the P-value threshold of 0.05, and the vertical lines indicate log₂ fold-change cutoffs of -0.2 and 0.2 . Downregulated phosphorylation sites are shown in blue, and upregulated phosphorylation sites are shown in red. Obtained from Weber *et al.* (89).

4.2.4 The phosphorylation of Connexin 43 by Camk2a and Ck1a may contribute to hidden cardiotoxicity

To further extend our systematic investigation, we next sought to identify kinases potentially responsible for the phosphorylation changes observed in the phosphoproteomic analysis. For this purpose, we examined the upstream kinases predicted to regulate the detected phosphosites using the PhosphositePlus® v6.7.4 database (107). As described in Section 3.2.6, the applied bioinformatic approach enabled the identification of kinases based on known or predicted target-phosphosite associations and provided enrichment scores indicating the strength of these associations. We found that connexin 43 (Cx43, Gja1) displayed phosphorylation at multiple sites previously reported as targets of Calcium/Calmodulin-Dependent Protein Kinase II Alpha (Camk2a) and Casein Kinase 1 Alpha 1 (Ck1a), both showing significant normalised enrichment scores (NES) (Table 1). Although neither Camk2a nor Ck1a were detected in the

proteomic or phosphoproteomic datasets, the observed phosphorylation pattern of Cx43 suggests their potential involvement. Alterations in Cx43 phosphorylation mediated by these kinases may affect gap junction channel open probability, thereby contributing to the hidden cardiotoxic effects observed after rofecoxib administration.

Table 1. Enrichment analysis of upstream kinase activity

Note: Significant kinase activity score (NES) (P-value <0.05; permutation test). Obtained from Weber *et al.* (89).

Upstream kinase	P-value	Normalised enrichment score	Detected phosphosites	UniProt IDs of leading edges	Target gene abbreviation
Camk2a	3.973E-04	1.985	9	P08050_S330, P08050_S297, P08050_S328, P08050_S296, P08050_S325	Gja1
Ck1a	0.002	1.641	3	P08050_S330, P08050_S328, P08050_S325	Gja1

4.2.5 Clusters of significantly interacting proteins exhibit shared biological processes potentially contributing to hidden cardiotoxicity

As previously demonstrated, although the differentially expressed proteins were not extensively interconnected, they were associated with several relevant biological processes. To gain deeper insights into the molecular mechanisms potentially contributing to hidden cardiotoxicity—and to examine the interaction between proteins with altered expression and those exhibiting differential phosphorylation—a comprehensive protein–protein interaction network was constructed. This network incorporated both the differentially expressed proteins and those with significantly altered phosphosites, alongside the identified upstream kinases that may play a role in hidden cardiotoxicity (Figure 12). Given the central role of COX-2 in rofecoxib’s mechanism of action, we further explored potential links by incorporating COX-2 as a node within the generated network using the STRING bioinformatics platform. Upon centring the network around COX-2, only a single known interaction was identified, connecting COX-2 with the transcription factor Signal Transducer and Activator of Transcription 3 (Stat3) (109). STRING analysis also provided numerical information on the connectivity of the proteins (nodes) in the network. The constructed network demonstrated a significant protein–protein interaction enrichment (see description in Section 3.2.7) with a P-value of 4.66e-15. Additionally, individual nodes can be described by a local clustering coefficient, which is a value ranging from 0 to 1 and quantifies how close the node is to

forming a fully connected subset with its immediate neighbours. In case of a network, this value is simply the average of the local clustering coefficient of each node, which is given by the ratio of the number of edges between its neighbours to the total possible number of edges. The herein presented network exhibits a local clustering coefficient of 0.366 that signals a high interconnectivity of nodes and, therefore, a tightly interlinked network. Subsequent cluster analysis identified distinct groups of proteins sharing both a high degree of interaction and common biological functions, including actin cytoskeleton organisation, mRNA processing, regulation of proteolysis, cytoplasmic translation, tricarboxylic acid cycle metabolism, calcium ion signalling, and electrical conduction (one Cluster is in Figure 13A, the other clusters are not shown here). Notably, in agreement with the network of differentially expressed proteins (Figure 10), two of the clusters play a role in proteolysis and translation. However, these clusters encompass additional related proteins, thereby reinforcing the significance of these pathways in the context of hidden cardiotoxicity. The cluster represented in Figure 13A displayed a significant protein–protein interaction enrichment P-value of $1.46\text{e-}7$ and a local clustering coefficient of 0.674, which emphasises a very high connectivity within the cluster and a tightly interlinked subset of proteins. Remarkably, the gene ontology analysis revealed that the proteins within this cluster (Tables 2 and 3) are central regulators of calcium ion homeostasis and cardiac conduction (Figure 13B). Collectively, these findings suggest that the hidden cardiotoxicity induced by rofecoxib, manifesting only upon additional cardiac stress such as I/R injury, can be attributed to cardiac protein expression changes and posttranslational modifications of a diverse set of cardiac proteins already at baseline.

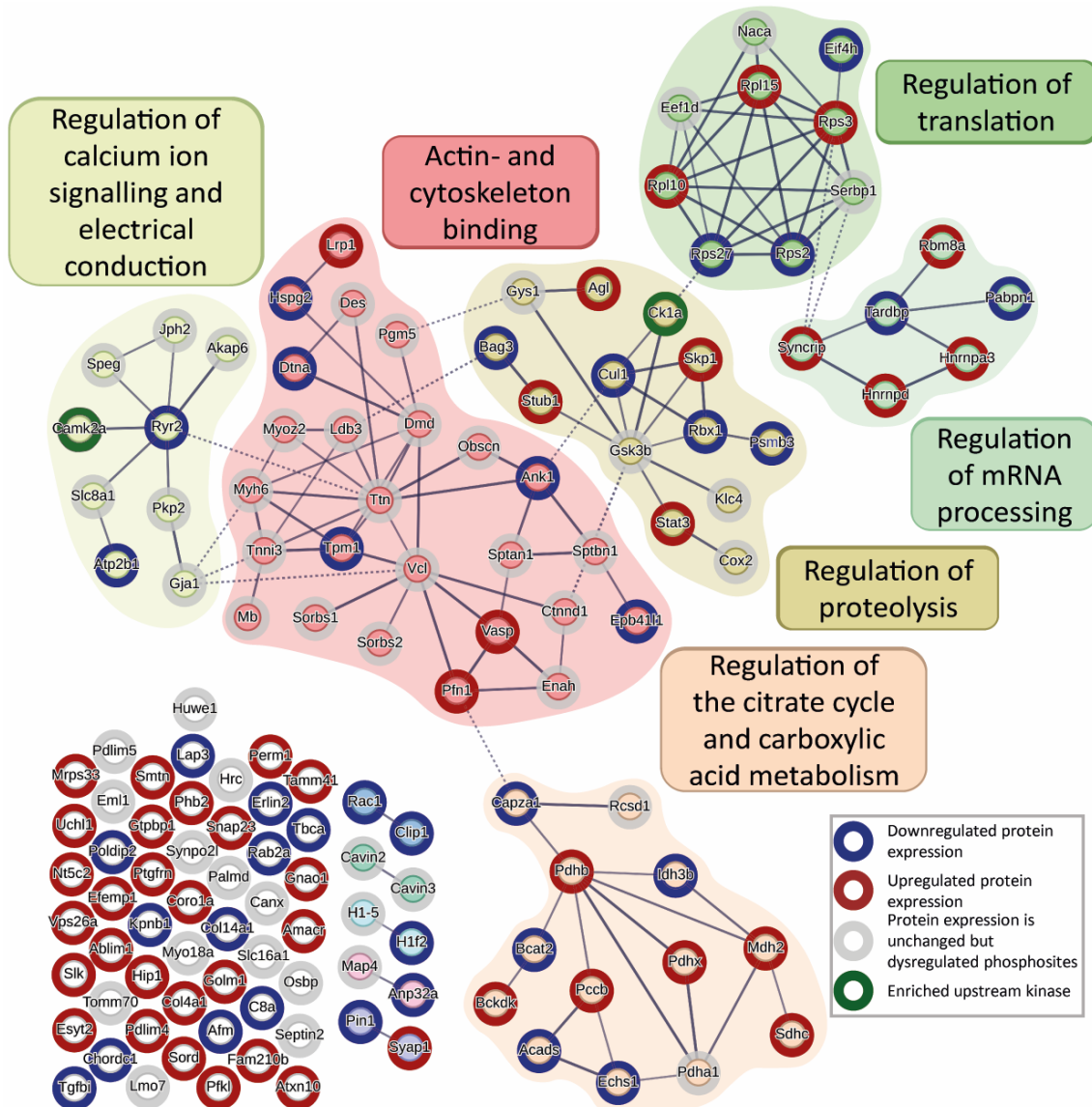


Figure 12. Protein-protein interaction network in rofecoxib-treated hearts incorporating differentially expressed proteins, proteins with altered phosphorylation sites, enriched upstream kinases, and COX-2. The protein-protein interaction network was constructed using the STRING database and illustrates the interactions among differentially expressed proteins (P-value <0.05), proteins with dysregulated phosphosites (P-value <0.05; filtered for duplicates), significantly enriched upstream kinases (q-value <0.05) and COX-2. The thickness of the edges reflects the confidence level of the protein-protein interactions, with dotted lines indicating inter-cluster connections. Interactions were filtered for high confidence using a minimum interaction score threshold of 0.7. Node outline colours correspond to the log2 fold change in protein expression. K-means clustering was applied to the network, with the number of centroids set to 11. Node fill colours indicate cluster membership, and the six largest clusters are associated with distinct biological functions. Obtained from Weber *et al.* (89).

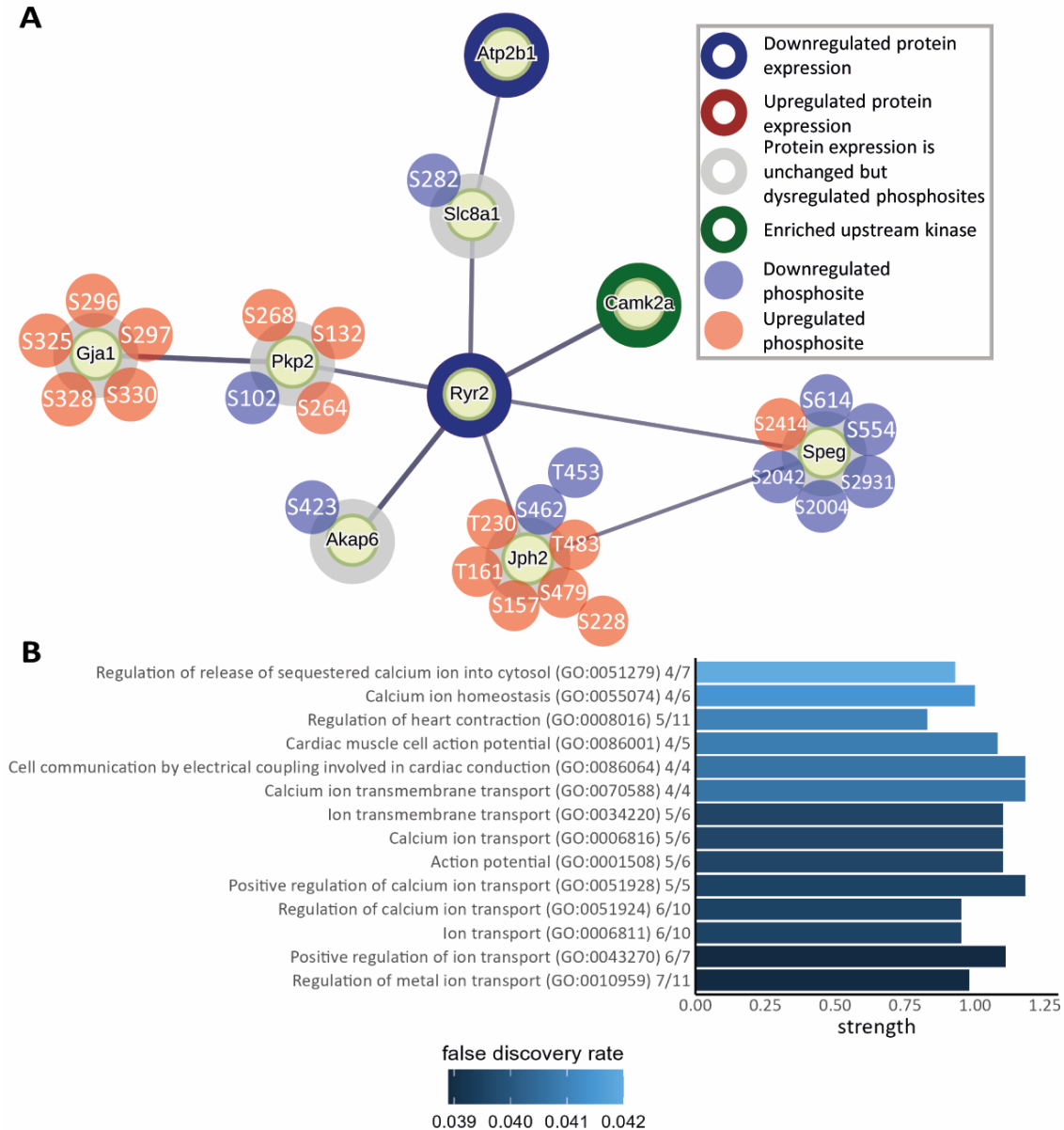


Figure 13. Cluster associated with calcium ion signalling and cardiac electrical conduction. **A:** Using the STRING database (string-db.org), k-means clustering was applied to the protein–protein interaction network shown in Figure 12, resulting in the shown cluster. The thickness of the edges reflects the confidence level of the protein–protein interactions. Interactions were restricted to high-confidence associations (interaction score >0.7). Node outline colours indicate the log2 fold change in protein expression, while the surrounding circles represent differentially phosphorylated phosphosites, with their colours denoting the direction of phosphorylation changes. Tables 2 and 3 show the data of the mass spectrometry analysis. **B:** Relevant overrepresented gene ontology terms were plotted according to their strength ($\log_{10}[\text{observed/expected}]$). Obtained from Weber *et al.* (89).

Table 2. Differentially phosphorylated phosphosites from the protein–protein interaction cluster shown in Figure 13.

Note: Signal intensities measured by mass spectrometry were used to derive the descriptive quantitative values presented. Qualitative annotations—including previously reported upstream kinases, downstream effects, and human site analogues—were obtained from the PhosphoSitePlus v6.7.4 database. n=7x2 technical replicates/group. Obtained from Weber *et al.* (89).

Uniprot ID	Gene abbreviation	Phosphorylation site	Human site analogue	P-value	log2 fold change	Percentile of mean intensity	Previously described upstream kinases/ downstream effects
F1M7L9-2	<i>Pkp2</i>	S102	S102	0.023	-0.882	82 nd	Upstream: Ischemia
F1M7L9-2	<i>Pkp2</i>	S132	S132	0.029	0.611	11 th	Upstream: CXCL12
F1M7L9-2	<i>Pkp2</i>	S264	S293	0.000	0.624	32 nd	No information
F1M7L9-2	<i>Pkp2</i>	S268	S297	0.003	0.876	38 th	No information
Q2PS20	<i>Jph2</i>	T453	-	0.016	-1.420	39 th	Not characterized
Q2PS20	<i>Jph2</i>	S462	S469	0.022	-0.520	54 th	No information
Q2PS20	<i>Jph2</i>	T483	T490	0.025	0.227	96 th	Upstream: BL2536, EGF
Q2PS20	<i>Jph2</i>	S479	S486	0.048	0.242	97 th	Upstream: EGF
Q2PS20	<i>Jph2</i>	S228	S235	0.011	0.621	40 th	No information
Q2PS20	<i>Jph2</i>	S157	S157	0.020	0.982	36 th	No information
Q2PS20	<i>Jph2</i>	T161	T161	0.037	1.002	37 th	No information
Q2PS20	<i>Jph2</i>	T230	T237	0.001	1.077	41 st	No information
Q63638	<i>Speg</i>	S614	S609	0.007	-1.078	20 th	No information
Q63638	<i>Speg</i>	S2931	S2931	0.032	-0.816	23 rd	No information
Q63638	<i>Speg</i>	S2042	S2037	0.025	-0.787	21 st	No information
Q63638	<i>Speg</i>	S554	S549	0.031	-0.375	15 th	Upstream: ischemia
Q63638	<i>Speg</i>	S2004	P1999	0.033	-0.291	74 th	No information
Q63638	<i>Speg</i>	S2414	S2414	0.027	0.585	62 nd	No information
Q01728	<i>Slc8a1</i>	S282	S285	0.043	-0.590	35 th	No information
Q9WVC7	<i>Akap6</i>	S423	S424	0.027	-0.414	23 rd	No information
P08050	<i>Gja1</i>	S328	S328	0.005	0.271	93 rd	Upstream: Ck1a Downstream: protein conformation
P08050	<i>Gja1</i>	S330	S330	0.003	0.327	92 nd	Upstream: Ck1a Downstream: protein conformation
P08050	<i>Gja1</i>	S325	S325	0.019	0.368	94 th	Upstream: Ck1a Downstream: protein conformation
P08050	<i>Gja1</i>	S297	S297	0.004	0.422	78 th	No information
P08050	<i>Gja1</i>	S296	S296	0.008	0.491	78 th	No information

Table 3. Differentially expressed proteins from protein-protein interaction cluster in Figure 13.

Note: Signal intensities were measured by mass spectrometry. n=8x2 technical replicates/group. Obtained from Weber *et al.* (89).

Uniprot ID	Gene abbreviation	P-value	log2 fold change	Percentile of mean intensity
B0LPN4-2	<i>Ryr2</i>	0.045	-1.194	12 th
P11505-1	<i>Atp2b1</i>	0.020	-0.803	6 th

4.2.6 Transcriptomic analysis reveals limited insights into the molecular mechanisms underlying rofecoxib-induced hidden cardiotoxicity

To assess whether transcriptomic alterations contribute to the development of hidden cardiotoxicity, we performed mRNA and miRNA expression analyses on left

ventricular tissue from rats treated with rofecoxib or vehicle. Overall, 61% of annotated mRNAs and 85% of annotated miRNAs were detected in at least one sample across both treatment groups. Additionally, approximately 36% of mRNAs and 57% of miRNAs were consistently identified in all samples from both the rofecoxib-treated and control groups (Table 4). The principal component analysis did not demonstrate a clear separation of the treatment groups (Figures 14A, 14B). This suggests that rofecoxib does not profoundly change the baseline expression in the heart. To determine whether rofecoxib treatment influenced selective gene expression, a differential expression analysis was performed on mRNA and miRNA profiles. Among transcripts detected in at least one sample, 2.7% (480) of mRNAs and 1.1% (7) of miRNAs were found to be differentially expressed between treated and control groups. The resulting differential expression profiles are illustrated in the volcano plots (Figures 14C, 14D). To explore the potential biological functions associated with the 480 mRNAs significantly altered by rofecoxib treatment, an interaction network was constructed. STRING network analysis yielded a P-value of 0.0582, indicating that these transcripts would not translate to a strongly interconnected set of proteins. Furthermore, gene ontology overrepresentation analysis revealed no significantly enriched biological processes based on the predefined criteria outlined in Section 3.2.7. These findings suggest that the differentially expressed mRNAs are unlikely to contribute substantially to the underlying mechanisms of hidden cardiotoxicity, indicating that transcriptomic analysis offers only limited insight into the cardiotoxic effects of rofecoxib.

Table 4. Quantitative overview of transcriptomic data sets and their overlap with proteomic findings in rofecoxib-treated hearts.

Note: (n = 8 per group) NA: not applicable. Obtained from Weber *et al.* (89).

Input data	criteria	messenger RNA		microRNA	
RNA sequencing	Number of RNAs detected in at least one sample	17993		639	
RNA sequencing	Number of RNAs detected in at least one sample by treatment group	vehicle: 16873	rofecoxib: 17027	vehicle: 610	rofecoxib: 620
RNA sequencing	Number of RNAs detected in all samples	10668		425	
RNA sequencing	Number of RNAs detected in all samples by treatment group	vehicle: 11019	rofecoxib: 11325	vehicle: 437	rofecoxib: 443
RNA sequencing	Identification of RNAs with P-value <0.05	597		18	
RNA sequencing	Identification of significantly differentially expressed RNAs with P-value <0.05 and log2 fold change <-0.2 or >0.2	480		7	
RNA sequencing & proteomics	Identification of differentially expressed RNAs by P-value with also differential protein expression by P-value	3		NA	
RNA sequencing	Identification of differentially expressed RNAs by P-value with significant miRNA-mRNA correlation by P-value	23		13	
RNA sequencing	Identification of differentially expressed RNAs by P-value with significant miRNA-mRNA correlation by P-value and a node strength of <-1	14		10	
RNA sequencing & proteomics	Identification of detected candidates in the proteomics data set with a node strength of <-1 in the miRNA-mRNA network	4		NA	
RNA sequencing & proteomics	Identification of differentially expressed candidates by P-value in the proteomics data set with node strength of <-1 in the miRNA-mRNA network	0		NA	

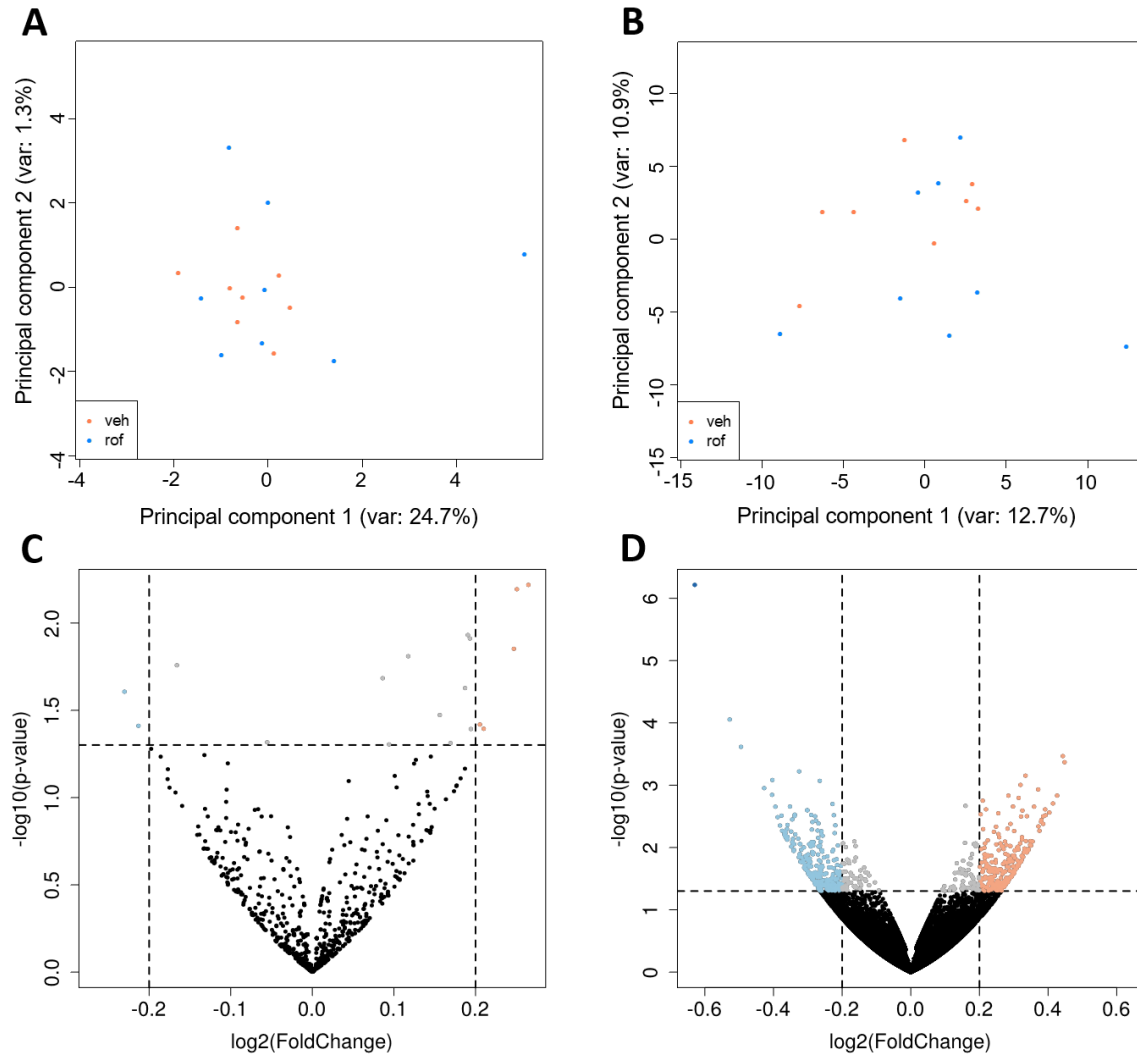


Figure 14. Principal component analysis representing the A: microRNA and B: mRNA data set of rofecoxib-treated hearts. Principal component analysis of the global microRNA expression profile is shown. The proportion of variance explained (var) by the first and second principal components is demonstrated in parentheses on the respective axes. **Differential C: microRNA D: mRNA profile of rofecoxib-treated hearts.** This volcano plot displays the log₂ fold change (x-axis) and $-\log_{10}(\text{P-value})$ (y-axis) for microRNAs between treatment groups ($n = 8 \times 2$ technical replicates per group). Statistical comparisons were performed using a two-tailed, unpaired t-test. The horizontal line denotes the P-value threshold of 0.05, and the vertical lines indicate log₂ fold-change cutoffs of -0.2 and 0.2 . Downregulated RNAs are shown in blue, and upregulated RNAs in red. mRNA: messenger RNA. Obtained from Weber *et al.* (89).

4.2.7 Rofecoxib-induced alterations in selected genes observed at both transcript and protein levels

To determine whether alterations in protein expression were associated with transcriptional changes, we examined the overlap between the 84 differentially expressed

proteins identified by mass spectrometry and the 480 differentially expressed mRNAs. Only three proteins with significant expression changes at the protein level had corresponding differential expression at the mRNA level (Figure 15, Table 5). These findings highlight a low correlation between the mRNA transcripts and differentially expressed proteins. Consequently, the majority of protein expression changes associated with hidden cardiotoxicity appear to be independent of transcriptomic alterations. Moreover, none of the overlapping proteins were associated with any relevant biological processes or clusters identified in Figures 10 or 12.

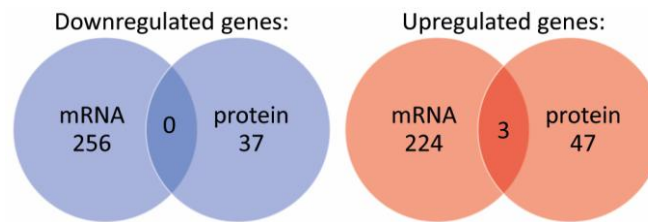


Figure 15.: Venn diagrams illustrating the overlap of differentially expressed mRNAs and proteins, filtered by a P-value <0.05 and an absolute log2 fold change >0.2 or <-0.2. (mRNA: messenger RNA). Obtained from Weber *et al.* (89).

Table 5. mRNAs that are differentially expressed (P-value <0.05) and exhibit concordant directional changes in protein expression within the proteomics dataset (P-value <0.05).

Note: Obtained from Weber *et al.* (89).

Gene abbreviation	P-value	log2 fold change	Percentile of mean vehicle TPM expr.	Protein ID	P-value	log2 fold change	Percentile of mean intensity
Upregulated expression							
<i>Pdlim4</i>	0.015	0.285	73 rd	M0R4H5	0.005	1.889	64 th
<i>Hip1</i>	0.003	0.255	94 th	A0A8I6AA26	0.046	0.824	65 th
<i>Uchl1</i>	0.009	0.249	13 th	Q00981	0.030	0.939	33 rd

4.2.8 A posttranscriptional regulation model did not indicate a regulatory impact of differentially expressed miRNAs

To explore the potential effect of posttranscriptional regulation exerted by miRNAs on mRNAs, we constructed a miRNA–mRNA correlation network. Analysis of this network (Figure 16) identified 23 mRNAs (listed in Table 6) that showed 59 significant negative correlations with 13 miRNAs (Table 7). The network focuses on negative correlations with a P-value <0.05 to emphasise the biologically significant

inhibitory function of miRNAs. Fourteen mRNAs and ten miRNAs were identified as differentially expressed based on P-value and exhibited significant miRNA–mRNA correlations with node strength values below -1 (Tables 6, 7). The mRNA exhibiting the strongest negative node strength, indicating the highest number of miRNA correlations, was C11orf96. However, its corresponding protein was undetectable by mass spectrometry and did not display a statistically significant difference when analysed by western blot (Table 6). The proteins corresponding to other mRNAs identified in the network, which were either detected by mass spectrometry or selected for validation by western blot, did not show differential expression (Table 6). These findings indicate that miRNA-mediated posttranscriptional regulation of mRNAs does not account for the observed changes in protein expression, suggesting that hidden cardiotoxicity induced by rofecoxib is unlikely to be driven by this mechanism. Furthermore, this reinforces the generally low correlation between mRNA expression and protein levels.

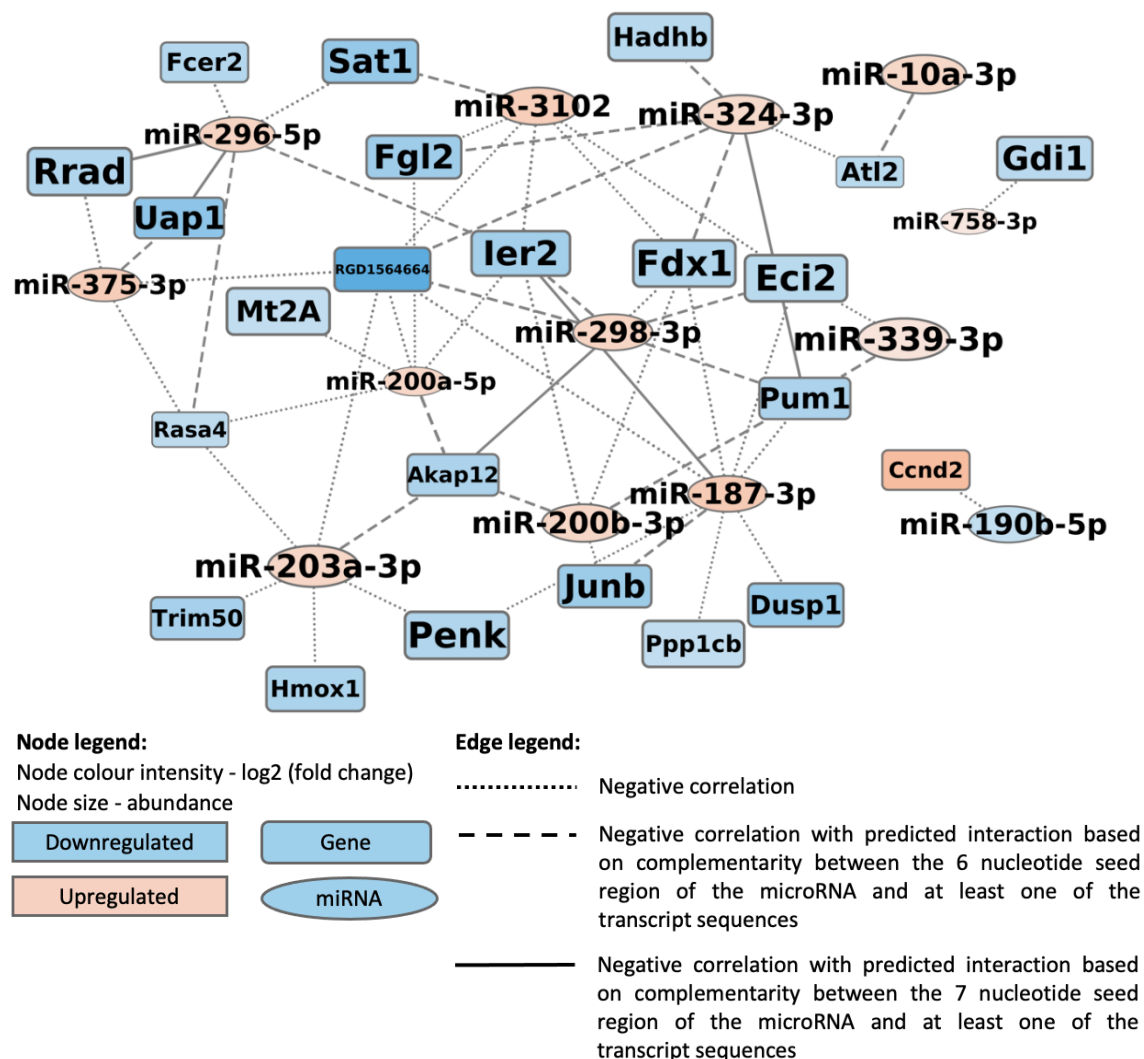


Figure 16. miRNA–mRNA network showing negative miRNA–mRNA correlations in rofecoxib-treated hearts. Spearman correlation analysis ($n = 8$ per group) was performed between 597 differentially expressed mRNAs (P -value <0.05) and 18 differentially expressed miRNAs (P -value <0.05). This analysis identified 23 mRNAs exhibiting 59 significant negative correlations (P -value <0.05) with 13 miRNAs. Data underlying this network can be found in Table 6 for mRNAs and Table 7 for miRNAs. (miRNA: microRNA; mRNA: messenger RNA). Obtained from Weber *et al.* (89).

Table 6. mRNAs from the miRNA–mRNA correlation network (Figure 8), ranked according to node strength.

Note: All statistically significant negative correlations between miRNAs and mRNAs (P-value <0.05) were represented as edges in the network. The mRNA node strengths were calculated by summing the Spearman's rank correlation coefficients (rho values) of their connected edges. Among the 23 mRNAs included in the network, 14 with node strengths less than -1 are shown along with their mRNA sequencing data (n = 8 per group), miRNA–mRNA network data (n = 8 per group), and protein expression levels assessed by mass spectrometry (n = 8 per group) and western blot (n = 7–8 per group), above the dashed line. Box plots from the RNA sequencing, proteomics, and western blots are, as well as western blot membranes are not shown here (see Supporting Information Weber *et al.* (89)). Nine mRNAs with a negative node strength higher than -1 are presented with their mRNA sequencing data (n=8/group), miRNA-mRNA network data (n=8/group) and expression on protein data by mass spectrometry (n=8/group), below the dashed line. The miRNA data from the miRNA–mRNA correlation network are presented in Table 7 (miRNA: microRNA; mRNA: messenger RNA; ND: not detected; TPM: transcripts per million; X: predicted interaction based on complementarity between the six nucleotide seed regions of the microRNA and at least one of the transcript sequences; #: predicted interaction based on complementarity between the seven nucleotide seed regions of the microRNA and at least one of the transcript sequences; -: not tested; node str.: node strength). Obtained from Weber *et al.* (89).

mRNA sequencing and miRNA-mRNA correlation network						Proteomics				Western blot
Gene abbreviation	P-value of differential expression	log2 fold change	Percentile rank of median TPM expr.	Node str.	miRNAs with negative correlation (rho value)	Uniprot ID	P-value	log2 fold change	Percentile of mean intensity	P-value
<i>RGD1564664</i>	6.09E-07	-0.629	80 th	-4.371	miR-187-3p (-0.718) miR-375-3p (-0.650) miR-203a-3p (-0.647) miR-200a-5p (-0.615) X miR-298-3p (-0.609) X miR-324-3p (-0.571) miR-3102 (-0.562) miR-200b-3p (-0.750) miR-3102 (-0.741)	ND	ND	ND	ND	0.256
<i>Ier2</i>	0.006	-0.337	85 th	-3.809	X miR-298-3p (-0.632) miR-200a-5p (-0.615) # miR-187-3p (-0.568) X miR-296-5p (-0.503)	ND	ND	ND	ND	0.332
<i>Fdx1</i>	0.005	-0.288	92 nd	-3.174	miR-187-3p (-0.691) miR-298-3p (-0.644) miR-200b-3p (-0.638) X miR-324-3p (-0.624) miR-3102 (-0.576)	A0A8I5Y749 G3V7L0	0.204 0.498	0.246 -0.142	36 th 31 st	0.261
<i>Pum1</i>	0.003	-0.301	71 st	-2.991	miR-187-3p (-0.647) X miR-339-3p (-0.609) # miR-324-3p (-0.606) X miR-200b-3p (-0.594) X miR-298-3p (-0.535) X miR-200a-5p (-0.738)	ND	ND	ND	ND	0.789
<i>Akap12</i>	0.019	-0.299	71 st	-2.441	X miR-203a-3p (-0.588) X miR-200b-3p (-0.579) # miR-298-3p (-0.535) miR-187-3p (-0.635) miR-3102 (-0.615) X miR-298-3p (-0.544) miR-339-3p (-0.529)	Q5QD51-2	0.609	0.062	46 th	0.259
<i>Eci2</i>	0.027	-0.252	92 nd	-2.324		A0A8L2QBE2	0.507	-0.072	94 th	0.880

Table 6. continued

mRNA sequencing and miRNA-mRNA correlation network						Proteomics				Western blot
Gene abbreviation	P-value of differential expression	log2 fold change	Percentile rank of median TPM expr.	Node str.	miRNAs with negative correlation (rho value)	Uniprot ID	P-value	log2 fold change	Percentile of mean intensity	P-value
<i>Rasa4</i>	0.039	-0.232	43 rd	-2.176	miR-375-3p (-0.600) miR-203a-3p (-0.544) X miR-296-5p (-0.524) miR-200a-5p (-0.509)	ND	ND	ND	ND	0.460
<i>Fgl2</i>	0.007	-0.359	78 th	-1.576	X miR-324-3p (-0.565) miR-3102 (-0.509) miR-200a-5p (-0.503)	ND	ND	ND	ND	-
<i>Rrad</i>	0.032	-0.284	95 th	-1.341	miR-375-3p (-0.694) # miR-296-5p (-0.647)	ND	ND	ND	ND	-
<i>Sat1</i>	0.002	-0.395	79 th	-1.235	miR-296-5p (-0.659) X miR-3102 (-0.576)	ND	ND	ND	ND	0.817
<i>Atf2</i>	0.039	-0.252	26 th	-1.221	X miR-10a-3p (-0.688) miR-324-3p (-0.532)	F1LQ09	0.590	0.095	44 th	0.878
<i>Penk</i>	0.033	-0.284	95 th	-1.115	miR-203a-3p (-0.591) miR-187-3p (-0.524)	ND	ND	ND	ND	0.652
<i>Junb</i>	0.006	-0.365	75 th	-1.112	X miR-187-3p (-0.606) miR-200b-3p (-0.506)	ND	ND	ND	ND	0.597
<i>Uap1</i>	0.001	-0.427	68 th	-1.106	# miR-296-5p (-0.600) X miR-375-3p (-0.506)	ND	ND	ND	ND	-
<i>Fcer2</i>	0.043	-0.270	64 th	-0.650	miR-296-5p (-0.650)	ND	ND	ND	ND	-
<i>Ccnd2</i>	0.009	0.350	64 th	-0.618	miR-190b-5p (-0.618)	ND	ND	ND	ND	-
<i>Mt2A</i>	0.032	-0.219	93 rd	-0.579	miR-200a-5p (-0.579)	ND	ND	ND	ND	-
<i>Hadhb</i>	0.012	-0.258	95 th	-0.576	X miR-324-3p (-0.576)	Q60587	0.413	0.118	39 th	-
<i>Gdi1</i>	0.026	-0.248	79 th	-0.570	miR-758-3p (-0.570)	ND	ND	ND	ND	-
<i>Dusp1</i>	0.003	-0.388	79 th	-0.568	miR-187-3p (-0.568)	ND	ND	ND	ND	-
<i>Hmox1</i>	0.023	-0.306	84 th	-0.526	miR-203a-3p (-0.526)	ND	ND	ND	ND	-
<i>Trim50</i>	0.014	-0.326	75 th	-0.524	miR-203a-3p (-0.524)	ND	ND	ND	ND	-
<i>Ppp1cb</i>	0.026	-0.215	91 th	-0.512	miR-187-3p (-0.512)	P62142	0.840	-0.031	77 th	-

Table 7. Differentially expressed miRNAs (p <0.05) from the miRNA–mRNA correlation network (Figure 7), ranked by node strength. All significant negative miRNA–mRNA correlations (p <0.05) were included as edges in the network. miRNA node strength was calculated as the sum of Spearman’s rank correlation coefficients (rho values) for all connected edges. The table lists the 13 differentially expressed miRNAs involved in the network along with their sequencing data (n = 8/group) and correlation metrics (n = 8/group). Data for associated mRNAs can be found in Table 6. mRNA: messenger RNA; miRNA: microRNA; CPM: counts per million. Obtained from Weber *et al.* (89).

miRNA	P-value of differential expression	log2 fold change	Percentile rank of median CPM expression	Node strength	mRNAs with negative correlation
miR-187-3p	0.006	0.265	48 th	-5.468	RGD1564664, Fdx1, Pum1, Eci2, Junb, Dusp1, Ier2, Penk, Ppp1cb
miR-296-5p	0.040	0.251	42 nd	-3.582	Sat1, Fcer2, Rrad, Uap1, Rasa4, Ier2
miR-3102	0.014	0.194	54 th	-3.579	Ier2, Eci2, Sat1, Fdx1, RGD1564664, Fgl2
miR-200a-5p	0.012	0.247	17 th	-3.559	Akap12, RGD1564664, Ier2, Mt2A, Rasa4, Fgl2
miR-298-3p	0.038	0.191	49 th	-3.500	Fdx1, Ier2, RGD1564664, Eci2, Pum1, Akap12
miR-324-3p	0.049	0.193	59 th	-3.474	Fdx1, Pum1, Hadhb, RGD1564664, Fgl2, Atl2
miR-203a-3p	0.012	0.210	70 th	-3.421	RGD1564664, Penk, Akap12, Rasa4, Hmox1, Trim50
miR-200b-3p	0.040	0.205	55 th	-3.068	Ier2, Fdx1, Pum1, Akap12, Junb
miR-375-3p	0.006	0.187	39 th	-2.450	Rrad, RGD1564664, Rasa4, Uap1
miR-339-3p	0.015	0.169	75 th	-1.138	Pum1, Eci2
miR-10a-3p	0.024	0.118	53 rd	-0.688	Atl2
miR-190b-5p	0.039	-0.213	51 nd	-0.618	Cend2
miR-758-3p	0.050	0.094	6 th	-0.570	Gdi1

5 Discussion

5.1 Overview of main findings

This thesis shows that cardiac adverse drug reactions often go undetected in standard preclinical safety testing because drug-induced changes in cardiac gene expression can remain silent under healthy conditions and only manifest as toxicity with comorbidities or secondary insults, *e.g.*, I/R injury. Although rosiglitazone did not aggravate I/R injury, it eliminated the cardioprotective antiarrhythmic effects of ischemic preconditioning, indicating a subtle form of hidden cardiotoxicity. In parallel, our molecular analysis of the previously observed hidden cardiotoxicity of rofecoxib—manifesting as increased fatal arrhythmias during I/R—revealed that drug-induced alterations in protein expression and phosphorylation could prime the myocardium for more pronounced vulnerability to ischemic stress, thereby increasing arrhythmia susceptibility. A particularly striking molecular change identified in the multi-omics analyses is the dysregulation of calcium handling, which may underlie the development of calcium-mediated arrhythmias.

Together, these findings underscore the need to refine preclinical safety testing by incorporating models that better reflect comorbidities and by systematically investigating the molecular mechanisms underlying hidden cardiotoxicity.

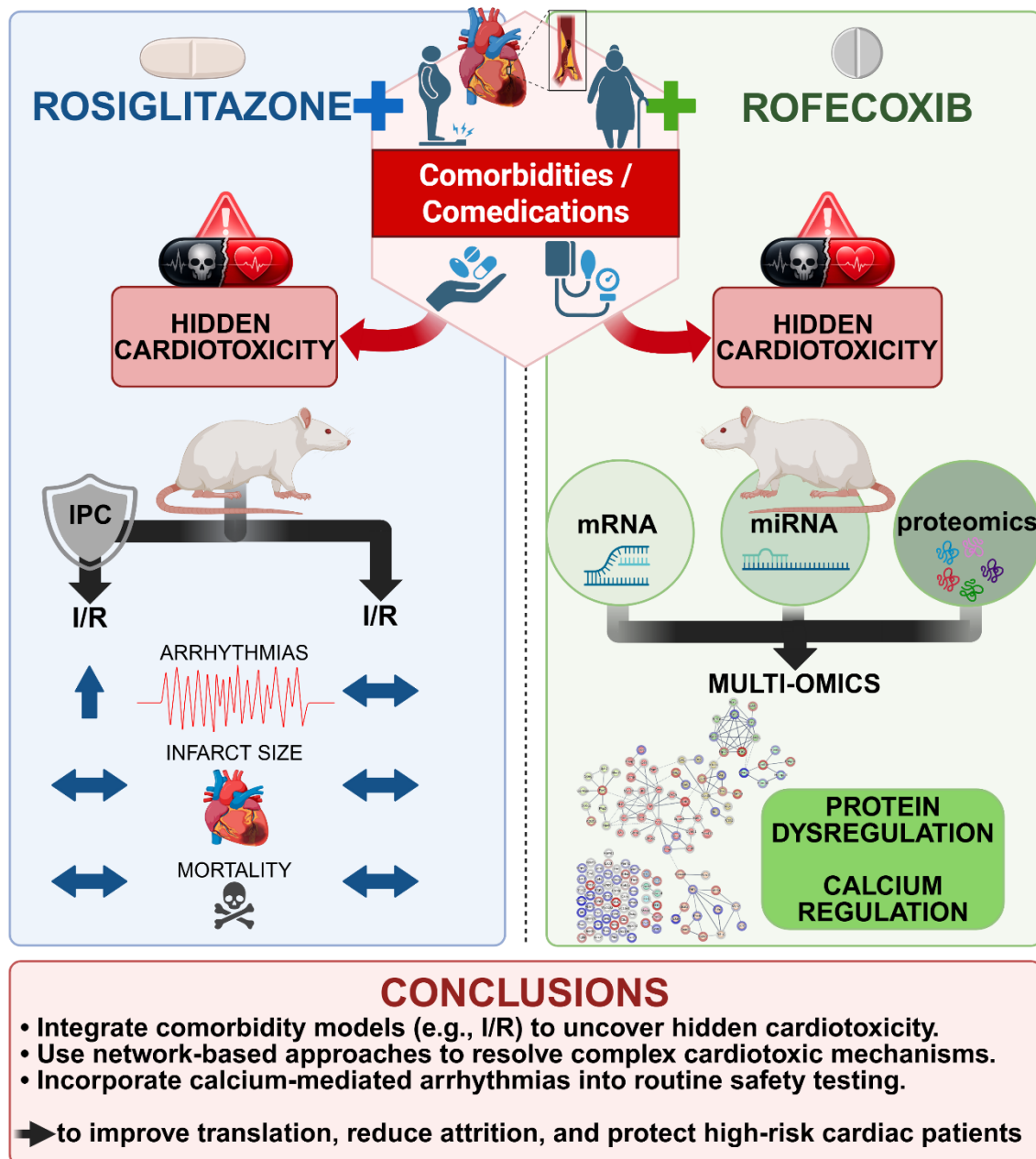


Figure 17. Advancing preclinical detection of hidden cardiotoxicity through comorbidity models and network-based mechanistic analysis. The graphical abstract was created in BioRender (Weber, B. (2026) <https://BioRender.com/c5jhbi8>). (I/R: ischemia/reperfusion; IPC: ischemic preconditioning; ⚡: protection; miRNA: microRNA; mRNA: messenger RNA).

5.2 Rosiglitazone and interference with ischemic preconditioning: detecting no major hidden cardiotoxicity

Rosiglitazone administration for 28 days did not aggravate I/R-induced infarct size. At first glance, this finding appears to be inconsistent with several earlier reports in which

shorter treatment regimens (7-14 days) with significantly higher doses of rosiglitazone (3–5 mg/kg/day) reduced myocardial infarction size in rats (77-79, 81, 83). However, the observed infarct-size-limiting effect may reflect an acute pharmacological action, as in three of those studies the final dose was administered one hour before surgery (77, 81, 83), while the remaining two papers did not specify the timing of the last administration (78, 79). This interpretation is supported by multiple reports demonstrating cardioprotection in an acute treatment model (82, 83, 133, 134). Our experiments also showed that rosiglitazone did not increase arrhythmia severity during I/R, based on ventricular premature beat, ventricular tachycardia, or ventricular fibrillation scores. Regarding intrinsic arrhythmic risk, several studies of acute rosiglitazone exposure demonstrated a shorter latency from coronary occlusion to ventricular fibrillation (133, 135-137), and Palee *et al.* reported increased arrhythmia scores and spontaneous ventricular fibrillation in both rats and pigs after I/R (133, 136). These effects, however, are attributable to acute dosing. In contrast, chronic rosiglitazone treatment has been associated with reduced arrhythmia risk in rats during I/R (77), likely influenced by active metabolites and the substantially higher doses used in this study. Expanding on our findings that chronic rosiglitazone treatment did not affect arrhythmia severity or infarct size, we further observed that long-term rosiglitazone administration did not increase mortality during I/R injury in rats. Similar findings were reported in pigs exposed to ischemia until ventricular fibrillation, where acute rosiglitazone treatment did not reduce defibrillation success rates (135). However, literature regarding mortality is mixed: acute rosiglitazone treatment increased mortality in rats during I/R (133), and 14-day treatment in rats (3 mg/kg/day) worsened 8 weeks post myocardial infarction survival (80). However, in mice, 14 days of high-dose treatment (20 mg/kg/day) improved survival rates (76). Moreover, previous studies typically used doses that produced peak plasma concentrations in rodents (73, 74) exceeding those measured in humans (61-70). Indeed, a single 1 mg/kg dose in rats generates higher peak plasma levels than those observed in humans after a single 4–8 mg dose (71, 72). Therefore, the 0.8 mg/kg/day dose used in our study better reflects clinically relevant human exposure. One study in rabbits using a comparable dose (0.5 mg/kg/day) reported reduced infarct size and suggested a reduction in I/R-induced arrhythmias (75). However, the same group also documented substantial infarction in sham-operated animals, similar to the infarct size observed in rosiglitazone-

treated rabbits subjected to I/R and failed to present supporting data regarding the proposed reduction in I/R-induced arrhythmias.

Here, we have demonstrated that 28-day rosiglitazone treatment did not interfere with the infarct-size-limiting effect of IPC; however, rosiglitazone abrogated the antiarrhythmic effects of IPC. A similar phenomenon has been reported in pigs, where rosiglitazone worsened the severity of ventricular fibrillation in animals otherwise protected by IPC (135). This aligns with clinical research exploring whether antidiabetic agents interfere with cardioprotective signalling pathways (8). For example, repaglinide abolished IPC-mediated protection in patients with coronary artery disease undergoing exercise stress testing, resulting in more pronounced ischemic ECG abnormalities and thus increased arrhythmia susceptibility (138, 139). Nonetheless, the translational value of rosiglitazone's interference with the antiarrhythmic effects elicited by IPC is limited. To date, the application of IPC in the clinics has been inconsistent (140, 141), and recent multicentre work indicates that the cardioprotective effect of ischemic preconditioning is not uniformly reproducible (142). However, IPC activates several cardioprotective pathways, many of which are shared with other cardioprotective interventions. Therefore, it is reasonable to conclude that rosiglitazone may also counteract the benefits of alternative cardioprotective strategies. Additionally, rosiglitazone increased arrhythmogenic potential in isolated ventricular myocytes from diabetic hypertensive rats, even in the absence of I/R injury (143), suggesting that the drug may exert proarrhythmic effects in diseased myocardium. Clinical pharmacovigilance data have also identified rosiglitazone to be proarrhythmic. A retrospective pharmacovigilance study identified rosiglitazone to be the second most frequently reported drug associated with ventricular fibrillation at the time (144), although increased sudden cardiac death risk has not been demonstrated (145).

This finding aligns well with several clinical studies that found no increase in cardiovascular or all-cause mortality with rosiglitazone therapy (39, 43, 46, 47, 146). Similarly, in the Bypass Angioplasty Revascularisation Investigation 2 Diabetes (BARI 2D) trial, insulin sensitisation with rosiglitazone after percutaneous coronary intervention didn't reduce mortality compared to insulin-provision over 5.3 years of follow-up among diabetic patients with coronary artery disease (146). One trial even reported a reduction in cardiovascular death (VADT) (45). Furthermore, in patients with

established ischemic cardiovascular disease, rosiglitazone increased myocardial infarction risk relative to pioglitazone but did not affect overall mortality (147). These clinical findings align with our observations, suggesting that chronic rosiglitazone does not exacerbate myocardial infarction-related mortality. Although Nissen *et al.* initially reported increased cardiovascular mortality in 2007 (38), their 2010 update no longer found statistical significance (40). Other comparative analyses have linked rosiglitazone to higher mortality relative to pioglitazone (148-150). However, when each thiazolidinedione was independently compared to the standard of care, metformin, neither was associated with increased all-cause mortality (150).

Our experiments show that 28-day treatment with rosiglitazone exerts only subtle hidden cardiotoxic effects in animal models of myocardial I/R injury by abrogating the antiarrhythmic actions of IPC. Moreover, rosiglitazone did not exacerbate the detrimental effects of I/R or cause overt mortality in rats.

5.3 Molecular mechanisms underlying rofecoxib-induced hidden cardiotoxicity and increased arrhythmia susceptibility

In contrast to the subtle hidden cardiotoxic effects of rosiglitazone, previous work established a clear hidden cardiotoxic profile for rofecoxib in the same rat I/R model. Although 28-day rofecoxib administration caused no overt toxicity under baseline conditions, rats exhibited markedly increased acute mortality during I/R, attributable to heightened arrhythmia susceptibility both *in vivo* and *ex vivo* (17). Importantly, I/R was induced ~24 h after the final dose—when no active compound remained according to rofecoxib pharmacokinetics—indicating that the drug’s harmful effects did not arise from acute pharmacological action but from drug-induced alterations in the heart at baseline (59). These observations led us to hypothesise that drug-evoked molecular reprogramming in the healthy heart may constitute a mechanistic basis for the hidden cardiotoxicity of rofecoxib.

To explore this hidden cardiotoxicity, the present study conducted the first *in silico* multi-omics analysis of rofecoxib-induced molecular alterations in rats, integrating cardiac mRNA and miRNA expression profiles, miRNA–mRNA network modelling, global proteomics, and phosphoproteomics. Prior studies have investigated celecoxib-induced miRNA changes in mouse hearts (151), mRNA alterations in coronary artery

cells (152), and differential proteomic expression in neonatal rat cardiomyocytes following rofecoxib treatment (153). We identified changes in a diverse set of cardiac protein expressions and phosphorylations, whereas transcriptomic alterations were comparatively mild. Only three differentially expressed proteins exhibited corresponding mRNA changes, and the modelled inhibitory miRNA-mediated posttranscriptional inhibition on mRNAs did not translate into detectable changes at protein level. This disconnection between modulation of RNA and protein expression indicates that neither transcriptional nor miRNA-driven regulations are likely to be mediators of the observed cardiotoxicity. Moreover, RNA expression exhibited a low correlation to protein levels in the heart. Instead, data suggest that rofecoxib-induced alterations in protein abundance and posttranslational modifications of multiple signalling pathways may prime the myocardium to be susceptible to injury and thus underlie rofecoxib's hidden cardiotoxicity.

However, we did not detect a canonical or direct relation between the dysregulated proteins and COX-2. Only a single link between COX-2 and an identified dysregulated protein, the transcription factor Stat3, was identified. Moreover, this transcription factor did not elicit meaningful transcriptional changes. Whether undetected mediators could be responsible for a COX-2-driven canonical pathway driving the detected changes remains unknown. Nonetheless, others have investigated the cardiotoxic potential of COX-2 inhibition and found no conclusive evidence to support the classical explanation of an imbalance between platelet-activating and platelet-inhibiting prostanoids resulting from COX-2 blockade (56-58). Several alternative hypotheses have therefore emerged. One proposes that the physicochemical properties of rofecoxib might promote pro-atherogenic effects independent of COX-2 inhibition (154). Another suggests that rofecoxib influences the expression of β 1-adrenergic receptors and arachidonic-acid-metabolising enzymes in cardiac and renal tissues (155). Importantly, Koshman *et al.* (56) reported that rofecoxib-related cardiotoxicity does not correlate with COX-2 selectivity or potency, implying that direct COX-2 inhibition cannot fully account for the observed adverse cardiac outcomes. This supports the interpretation that the deleterious effects of rofecoxib primarily arise through off-target effects. To this end, the current study illustrates the value of applying unbiased multi-omics approaches to reveal mechanisms that go beyond traditional hypothesis-driven strategies.

As we did not identify a connection of the dysregulated proteins to COX-2 in our own data or the literature, we concluded that the changes in expression or phosphorylation of the identified 132 proteins are likely due to off-target effects. Processes such as ubiquitination, proteolysis, and translational regulation can produce protein-level changes without corresponding differential mRNA expression. Importantly, gene ontology analysis of the network in Figure 10 and clusters in Figure 12 revealed proteins implicated in these pathways. These included multiple ribosomal proteins, Eukaryotic translation initiation factor 4H (Eif4h), Glycogen synthase kinase-3 beta (Gsk3b) and E3 ubiquitin-protein ligase complex subunits (Stub1, Cull1, Rbx1, Skp1). To our knowledge, this is the first evidence that perturbations in these biological processes may contribute to hidden cardiotoxicity exemplified by rofecoxib.

Moreover, we propose that these changes in protein regulation mediated the dysregulation of pathognomonic molecular pathways relevant to arrhythmogenesis, particularly calcium handling and electrical conduction. The interconnected cluster of proteins in Figure 13 associated with these pathways could explain previous reports that rofecoxib increases susceptibility to fatal arrhythmias during I/R (17). Earlier work on COX-2 inhibitors showed that acute celecoxib treatment, but not rofecoxib, can modulate L-type Ca^{2+} currents in vascular smooth muscle cells independent of the COX-2 enzyme (156). However, our study examined long-term (28-day) exposure in cardiac tissue, suggesting that rofecoxib does not directly block calcium channels acutely but may remodel calcium-handling pathways.

Disturbances in calcium cycling can initiate arrhythmias directly by triggering abnormal depolarisations and directly by modulating action potential time course and duration (157). In line with this, we previously observed rofecoxib-induced prolongation of the action potential in left ventricular papillary muscle under simulated I/R conditions (17). If action potentials are prolonged in special ventricular cells (M-cells), this may cause a spatial dispersion of repolarisation and therefore promote arrhythmias (158). The $\text{Na}^+/\text{Ca}^{2+}$ exchange current is suggested to be a contributor to this action potential prolongation (158). Importantly, we found the $\text{Na}^+/\text{Ca}^{2+}$ Exchanger (Slc8a1) to be dephosphorylated at Ser282. Although the functional consequences of this modification remain incompletely understood (159, 160), the $\text{Na}^+/\text{Ca}^{2+}$ Exchanger is connected to

arrhythmogenesis and therefore could be a mechanism of hidden cardiotoxicity exemplified by rofecoxib.

Beyond this, several altered proteins central to excitation–contraction coupling and action potential generation were identified in the gene ontology analysis, including Ryanodine receptor 2 (Ryr2), Plakophilin 2 (Pkp2), Cx43, and A-kinase anchor protein 6 (Akap6) (Figure 6). Pathogenic mutations in Ryr2 and Pkp2 are well-known drivers of arrhythmogenic cardiomyopathies (161-163). Moreover, Ryr2 is associated with diastolic Ca^{2+} leaks from the sarcoplasmic reticulum in heart failure, which may result in $\text{Na}^+/\text{Ca}^{2+}$ exchanger–triggered delayed afterdepolarisation and calcium-mediated arrhythmias (15, 164, 165). We found Ryr2 to be downregulated, as well as the $\text{Na}^+/\text{Ca}^{2+}$ Exchanger to be dephosphorylated, which therefore may contribute to hidden cardiotoxicity. Similarly, studies in knockout mice demonstrate that Pkp2 loss alters calcium cycling and the expression of related proteins (166, 167). We detected multiple phosphorylation changes in Pkp2 (Ser102↓, Ser1332↑, Ser264↑, Ser268↑), although their cardiac effects are not yet characterised.

Furthermore, Pkp2 forms the "connexome" with the voltage-gated Na^+ channel Nav1.5 and the gap junction protein Cx43, a physical interaction network (168). Cx43 also showed differential phosphorylation at several sites (Serine 325↑, Serine 328↑, Serine 330↑, Serine 296↑, Serine 297↑). Phosphoregulation of the connexome may (17)influence the action potential upstroke (169), which provides a plausible explanation for the slowed upstroke previously observed in rofecoxib-treated isolated papillary muscles exposed to simulated I/R (17). Others have found that ischemia causes dephosphorylation of the triplet Serine Residues 325, 328, and 330, as well as Cx43 lateralisation (170), and that dephosphorylation of Cx43 may lead to uncoupling of gap junction communication (171). This describes a potential mechanism of ischemia-induced ventricular arrhythmias (170, 171). Similarly, Himelman *et al.* showed that mimicking the phosphorylation of the triplet with glutamic acids protected against arrhythmias induced by isoproterenol (172). Although Cx43 is known to be associated with cardioprotection, the complex network and sensitive regulation of Cx43 as both a hemichannel and a gap-junction component underscore its potential to cause an imbalance in conductance that may have contributed to the hidden cardiotoxicity induced by rofecoxib (173, 174).

Here, we demonstrated for the first time that an unbiased multi-omics analysis can reveal underlying regulatory pathways that potentially contribute to hidden cardiotoxicity observed under stress conditions. The diverse protein expression and phosphorylation changes identified in this work are potential results of off-target effects and provide a mechanistic explanation for the previously observed hidden cardiotoxicity induced by the model drug, rofecoxib. The dysregulated proteins are associated with relevant biological processes, such as calcium ion handling, and act in concert to render the heart susceptible to arrhythmias under baseline conditions. When the heart encounters a secondary insult, arrhythmias may emerge, consistent with the previously described fatal arrhythmias observed during I/R (17).

5.4 Translational implications of detecting hidden cardiotoxicity and its molecular mechanisms in drug safety assessment

Both rofecoxib and rosiglitazone ultimately demonstrated hidden cardiotoxicity in clinical use, while adverse cardiac effects were not detected during standard preclinical safety testing (38, 40, 52). Our work, in conjunction with previous findings on rofecoxib, demonstrates that such hidden cardiotoxicity can indeed be unmasked in a preclinical comorbidity model, specifically myocardial I/R injury. In this setting, rofecoxib previously elicited a pronounced pathological phenotype, revealing its latent proarrhythmic potential (17). In contrast, rosiglitazone produced a comparatively weaker manifestation of hidden cardiotoxicity within the same model, suggesting that the susceptibility to adverse cardiac effects may depend on the type of comorbidity. It is possible that the adverse effects have a stronger dependence on specific patient populations that need to be reflected in the preclinical model, such as those with metabolic syndrome, diabetes, or heart failure. Collectively, these observations underscore the crucial need for comorbidity-inclusive preclinical testing strategies to identify cardiotoxic effects that remain undetected in healthy experimental systems but emerge under clinically relevant conditions (4-6, 9).

The molecular analysis of rofecoxib-induced changes in the heart suggests that drug-induced hidden cardiotoxicity may arise from calcium-dependent arrhythmias by acting on sarcoplasmic reticular and calcium-cycling networks. These mechanisms fall outside current regulatory safety protocols (4-6, 9), which focus primarily on voltage-

gated ion channels. For example, the ICH guidelines for preclinical electrophysiological safety testing do not mandate the evaluation of proarrhythmic effects in tissues or animal models that are inherently prone to arrhythmias (10-12). Current preclinical strategies focus primarily on detecting QT-interval prolongation resulting from delayed repolarisation (9), a disturbance of the cardiac action potential that can precipitate the life-threatening arrhythmia torsades de pointes (13). Drug-induced QT prolongation is largely attributed to inhibition of the hERG channel, which is considered the principal mechanism underlying many pharmacological arrhythmias (14). Consequently, safety assessments have predominantly centred on identifying hERG channel blockade to mitigate the risk of torsades de pointes (14), while other proarrhythmic mechanisms have been comparatively neglected (15). Toxicity screening, therefore, fails to identify calcium- or conductance-dependent arrhythmias, which tend to appear only in the presence of comorbidities. Comorbidities may present as a secondary insult, altering cardiac physiology by affecting ion channel expression and activity, mitochondrial function, electro-mechanical coupling, and extracellular matrix composition (7, 8).

Therefore, assessing drug effects solely in healthy hearts may overlook adverse reactions that emerge only under pathological conditions or in the presence of cardiovascular risk factors (4, 16-18). Moreover, greater emphasis should be placed on detecting the effects of drugs on calcium-handling proteins, as previously advocated by Pasquie and colleagues in the context of cardiac safety testing (15).

5.5 Future perspectives for a preclinical hidden cardiotoxicity testing platform

While the I/R comorbidity model proved sensitive for detecting the hidden cardiotoxicity of the selected model drugs, additional comorbidity models, such as metabolic syndrome, diabetes, or heart failure, may better capture patient- and population-specific risks and should be systematically established and validated. Additionally, drugs with so far unknown cardiovascular risk profiles should be tested in the I/R comorbidity model to further validate the sensitivity. Finally, to enable higher-throughput and more translational screening, these insights should be extended to human-based *in vitro* systems, such as induced pluripotent stem cell-derived cardiomyocytes engineered to mimic relevant comorbidities.

Moreover, the findings of this work exemplify the value of unbiased, multi-omics network approaches in moving beyond linear, hypothesis-driven methods to reveal previously unrecognised molecular mechanisms. This perspective aligns with the principles of network pharmacology, which challenges the traditional “one target–one drug” paradigm (175-178) and instead advocates for multi-target therapeutic designs capable of addressing the interconnected pathways (36). By uncovering the mechanisms underlying the hidden cardiotoxicity of rofecoxib, this unbiased network approach opens several important avenues for future research. First, extending the multi-omics and network-based strategy applied here to additional drug classes may help determine whether the molecular signatures identified for rofecoxib represent shared or drug-specific pathways of hidden cardiotoxicity. This would be instrumental in detecting biomarkers of hidden cardiotoxicity and developing treatment strategies against it. Second, given that calcium-dependent and conductance-dependent arrhythmias evade traditional preclinical safety testing, dedicated assays capable of detecting calcium-handling abnormalities should be developed and integrated into preclinical cardiac safety testing. Together, these future directions aim to develop a comprehensive, multimodal framework for the early detection, mechanistic understanding, and ultimately, the prevention of hidden cardiotoxicity across diverse pharmacological agents, thereby decreasing the rate of drug attrition and increasing patient safety.

6 Conclusions

This thesis demonstrates that hidden cardiotoxicity represents a critical blind spot in current preclinical drug safety assessment. Standard testing in healthy models often fails to detect adverse cardiac effects that manifest only in the presence of comorbidities or secondary stressors. This is exemplified by the post-marketing detection of the cardiotoxicity of rosiglitazone and rofecoxib. Here, we demonstrated that our preclinical I/R model identified a subtle, hidden cardiotoxicity of rosiglitazone by abolishing the antiarrhythmic effects of ischemic preconditioning. Moreover, by applying an unbiased multi-omics network analysis, this work reveals how the previously detected hidden cardiotoxicity of rofecoxib primes the myocardium at baseline through protein-level and phosphoregulatory disturbances. Importantly, calcium-handling pathways were affected, which can precipitate calcium-mediated fatal arrhythmias under stress. These findings highlight the translational relevance of studying drug effects in vulnerable, disease-relevant conditions and demonstrate the power of network approaches in uncovering mechanisms that traditional hypothesis-driven methods overlook. However, further multi-omics profiling of other drugs is necessary to identify shared or drug-specific pathways of hidden cardiotoxicity. Ultimately, this work advocates for the integration of comorbidity models and testing calcium-mediated arrhythmias into early drug development pipelines. Such advances will be essential for improving translational predictability, reducing drug attrition, and enhancing the safety of patients who are most susceptible to cardiac adverse drug reactions.

7 Summary

Current preclinical safety testing fails to detect drug-induced cardiac adverse effects that manifest only under stress or in the presence of comorbidities. If preclinical testing in healthy tissues fails to detect these effects, as demonstrated by rosiglitazone and rofecoxib, we termed this phenomenon hidden cardiotoxicity.

The objective of this thesis is to advance the preclinical safety testing to improve drug development and increase patient safety. To this end, the aims of this thesis are twofold: First, to determine whether rosiglitazone exhibits hidden cardiotoxicity in a rat model of I/R injury. Second, to elucidate the molecular mechanisms that underlie rofecoxib-induced hidden cardiotoxicity by applying an unbiased multi-omics analysis.

To address these aims, 28-day drug-treatment studies were performed in rats. For rosiglitazone, we employed an I/R model, including IPC as a cardioprotective intervention. Infarct size, arrhythmias, and acute mortality were assessed. For rofecoxib, cardiac tissue collected 24 hours after the final dose underwent transcriptomic (mRNA, miRNA) and proteomic analysis combined with network-based pathway reconstruction.

The results show that rosiglitazone did not aggravate I/R injury; however, rosiglitazone abolished the antiarrhythmic effects of IPC, indicating a subtle form of hidden cardiotoxicity. Investigating the previously demonstrated hidden cardiotoxicity of rofecoxib, which manifested as an increase in fatal arrhythmias during I/R, the unbiased multi-omics analysis revealed drug-induced alterations in cardiac protein expression and phosphorylation. Importantly, an interconnected cluster of dysregulated proteins affects calcium-handling and conduction-related pathways under baseline conditions. In contrast, transcriptomic changes were comparatively limited and poorly correlated with protein-level effects.

In conclusion, this thesis demonstrates that the subtle, hidden cardiotoxicity of rosiglitazone can be detected in a preclinical model of I/R injury, which remains undetected in conventional safety testing but manifests under cardiac stress. Moreover, the hidden cardiotoxicity induced by rofecoxib arises from baseline alterations in protein expression and phosphorylation that affect, for example, calcium handling. The findings support the incorporation of comorbidity-relevant models and calcium-mediated arrhythmia testing into preclinical safety pipelines to better protect vulnerable patient populations and improve the translational reliability of drug development.

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9 Bibliography of the candidate's publications

Publications related to the Candidate's PhD dissertation:

Weber Bennet Y, Brenner Gábor B, Váradi Barnabás, Ágg Bence, Kovácsházi Csenger, Balogh Olivér M, Egan Donagh, Wynne Kieran, Matallanas David, Schulz Rainer, Ferdinandy Péter, Giricz Zoltán, Görbe Anikó Assessment of the molecular mechanisms of drug-induced hidden cardiotoxicity by a multi-omics approach: The example of rofecoxib BRITISH JOURNAL OF PHARMACOLOGY 182: 19 pp. 4668-4692. (2025)
IF: 7.7

Weber Bennet Y¹, Brenner Gábor B¹, Kiss Bernadett, Gergely Tamás G, Sayour Nabil V, Tian Huimin, Makkos András, Görbe Anikó, Ferdinandy Péter, Giricz Zoltán Rosiglitazone Does Not Show Major Hidden Cardiotoxicity in Models of Ischemia/Reperfusion but Abolishes Ischemic Preconditioning-Induced Antiarrhythmic Effects in Rats In Vivo PHARMACEUTICALS 15: 9 Paper: 1055, 17 p. (2022)
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Publications independent of the Candidate's PhD dissertation:

Di Venanzio Ludovica, Atzemian Natalia, Weber Bennet Y, Gorica Era, Kleeberger Jan Alphard, Mohammed Shafeeq A, Costantino Sarah, Dzemali Omer, Ruschitzka Frank, Paneni Francesco Pharmacologic and Lifestyle Interventions to Modulate the Epigenome in Heart Failure HEART FAILURE CLINICS 22: 1 pp. 45-56. (2026)
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