

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

PhD thesis

Bennet Weber

Semmelweis University Doctoral College
Cardiovascular Medicine and Research Division



Supervisor:

Zoltán Giricz, D.Sc

Anikó Görbe, Ph.D

Official reviewers:

Sayour Alex Ali, Ph.D

Priksz Dániel, Ph.D

Head of the Complex Examination Committee:

Miklós Kellermayer, D.Sc

Members of the Complex Examination Committee:

Péter Andréka, Ph.D

László Cervenak, Ph.D

Budapest

2026

1. Introduction

Drug-induced toxicities remain a major challenge in pharmaceutical development and are a leading cause of clinical trial failure and post-marketing drug withdrawal. Between 1953 and 2013, 462 drugs were withdrawn due to adverse drug reactions, frequently involving cardiovascular complications (Onakpoya, Heneghan, & Aronson, 2016a, 2016b). In the European Union, such reactions are estimated to cause approximately 197,000 deaths annually, representing a socio-economic burden of nearly €79 billion (EuropeanCommission, 2008). These figures underscore the need for improved strategies to detect toxicities early and reduce both patient risk and development costs.

Cardiotoxicity is particularly critical due to its severity and high prevalence among withdrawn drugs (Onakpoya et al., 2016b). A key limitation of current safety assessment is that adverse cardiac effects are often detected only in late-stage trials or after market approval, when drugs are administered to large populations with comorbidities such

as diabetes, hypertension, or heart failure (Ferdinandy et al., 2019). These conditions can increase susceptibility to adverse effects, thereby revealing toxicities that are not evident in earlier testing. The concept of “hidden cardiotoxicity” describes this phenomenon and refers to adverse cardiac effects that become apparent only under pathological or stress conditions (Ferdinandy et al., 2019).

Conventional preclinical safety testing relies primarily on healthy models that do not reflect the complex comorbidities of the patient population receiving the treatment (Ferdinandy et al., 2019; Food & Drug Administration, 2010; Gintant, Sager, & Stockbridge, 2016; "Guideline on repeated dose toxicity,"). Current preclinical cardiotoxicity testing largely focuses on detecting QT interval prolongation associated with inhibition of the voltage-gated potassium channel (hERG-channel) (Gintant et al., 2016). While effective for identifying certain arrhythmogenic drugs, this approach overlooks other mechanisms, including calcium dysregulation and disease-related changes in cardiac physiology (Pasquie & Richard, 2009). Consequently,

cardiotoxic drug effects that depend on pathological conditions may remain undetected.

Especially important comorbidities affecting cardiac cell signalling are ischemia/reperfusion (I/R) injury and/or its common risk factors such as hypertension, hyperlipidemia, diabetes, insulin resistance, heart failure, altered coronary circulation, ageing and their co-mediations (Ferdinandy et al., 2023; Ferdinandy, Hausenloy, Heusch, Baxter, & Schulz, 2014). Mechanistically, hidden cardiotoxicity may result from enhanced detrimental signalling or impaired protective pathways, leading to arrhythmias, cardiomyocyte death, and contractile dysfunction (Ferdinandy et al., 2019). However, often the exact molecular mechanisms affected by hidden cardiotoxic drugs remain unknown.

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. To this end, multi-omics technologies that integrate transcriptomic and proteomic analyses enable systematic means to disentangle the complex molecular mechanisms underlying cardiovascular diseases (Chilukoti et al., 2018; Reilly & Bornfeldt, 2021; Varga et al., 2015; Zielinski, Luke, Guglietta, & Krieg, 2021).

The importance of detecting hidden cardiotoxicity is illustrated by drugs such as rosiglitazone and rofecoxib, both of which showed cardiovascular risks only in post-marketing studies.

Because rosiglitazone's adverse effects became apparent only after widespread use (Nissen & Wolski, 2007, 2010), we selected it as a model drug for detecting its hidden cardiotoxicity in a preclinical model. To this end, we utilised our previously validated (Brenner et al., 2020) I/R in vivo model, because hidden cardiotoxicities often manifest as I/R-induced cardiac deaths, arrhythmias, or cardiac dysfunction.

Rofecoxib illustrates the most prominent example of hidden cardiotoxicity, with clinical and preclinical evidence (Bombardier et al., 2000; Brenner et al., 2020; Bresalier et al., 2005). The latter was demonstrated by Brenner et al. in a rat model of myocardial I/R injury. Rofecoxib showed no overt toxicity during the 28-day treatment period. However, rofecoxib increased acute mortality and arrhythmia susceptibility following I/R injury, demonstrating its hidden cardiotoxicity (Brenner et al., 2020). These findings suggest that rofecoxib may alter cardiac gene expression under baseline conditions, prompting us to investigate drug-induced molecular alterations to uncover novel mechanisms of hidden cardiotoxicity and identify predictive biomarkers for early safety assessment.

In conclusion, current preclinical safety strategies are insufficient to detect hidden cardiotoxicity. Integrating disease-relevant models with molecular profiling approaches may enable earlier detection, reduce late-stage drug attrition, and ultimately improve patient safety while lowering the socio-economic burden of drug withdrawal.

2. Objectives

This thesis hypothesises that cardiac adverse drug reactions often manifest only in diseased hearts, escaping routine preclinical detection. The delayed recognition of rosiglitazone and rofecoxib cardiotoxicity underscores the need for improved early safety testing. Accordingly, this work aims to:

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

Rosiglitazone ischemia/reperfusion rat model and analysis

Male Wistar rats were treated with rosiglitazone (0.8 mg/kg/day) or vehicle for 28 days. The selected dose was extrapolated from the human dose that has caused toxicity using established body-surface-area-based conversion methods (Reagan-Shaw, Nihal, & Ahmad, 2008). The final dose was administered 24 hours before surgery to avoid acute drug effects. After treatment, rats underwent an I/R injury protocol with or without ischemic preconditioning (IPC), under controlled physiological and anaesthetic conditions. Animals were randomised into four groups (I/R $\pm$ IPC, with or without rosiglitazone), and haemodynamic and physiological parameters were continuously monitored. Successful induction of ischemia was confirmed by ECG changes, arrhythmias, and visible myocardial pallor.

After 120 minutes of reperfusion, hearts were excised, perfused, and stained with Evans blue and TTC (triphenyltetrazolium chloride) to delineate the area at risk and infarcted tissue. Infarct size was expressed as a

percentage of the area at risk relative to the left ventricle, with weight-adjusted calculations. Arrhythmias during ischemia and early reperfusion were assessed according to the Lambeth Conventions using a standardised scoring system for ventricular extrasystoles, ventricular tachycardias and ventricular fibrillations. Mortality was also analysed, excluding all iatrogenic causes of death (Weber et al., 2022).

Molecular analysis of rofecoxib-induced cardiotoxicity

Using the same experimental conditions and animal care as in the rosiglitazone model, this in vivo study applied rofecoxib (5.12 mg/kg/day) or vehicle for 28 days to male Wistar rats. The dosing was derived from human doses associated with toxicity using established body surface area-based conversion methods (Reagan-Shaw et al., 2008). Unlike the previous study investigating an I/R injury model to assess functional hidden cardiotoxicity (Brenner et al., 2020), animals in this study were sham-operated to focus on baseline molecular alterations without ischemic stress. No overt toxicity was observed during the treatment period.

Following the sham operation, rat hearts were excised, perfused to remove blood, and left ventricular tissue was isolated, snap-frozen, and stored at -80°C . The tissue was then processed into powder and prepared for proteomic, transcriptomic, and Western blot analyses. Pulverised heart tissue was further processed for liquid chromatography–tandem mass spectrometry–based proteomic and phosphoproteomic analysis. The resulting data were analysed using established pipelines (e.g., FragPipe, Perseus, and R) with stringent filtering, normalisation, and statistical testing to identify significantly regulated proteins and phosphorylation sites. Furthermore, upstream kinases of dysregulated phosphorylation sites were identified using bioinformatic approaches.

Subsequent pathway reconstruction to investigate shared biological functions and clusters of interacting genes among differentially regulated proteins and phosphosites was performed using the STRING (Search Tool for the Retrieval of Interacting Genes) database.

Additionally, transcriptomic profiling (miRNA and mRNA sequencing) was conducted, followed by

differential expression analysis using DESeq2. To investigate potential miRNA–target interactions, significantly differentially expressed miRNAs and mRNAs were selected, and Spearman’s rank correlation coefficients (ρ values) were calculated between miRNAs and their potential target genes. miRNA–mRNA pairs showing significant negative correlations were used to construct a miRNA–mRNA correlation network. Finally, selected mRNAs with a high number of incident significant miRNA correlations were chosen for validation at the protein level by Western blot (Weber et al., 2025).

4. Results

Twenty-eight-day rosiglitazone treatment abolished the antiarrhythmic effects of IPC, thus showing subtle hidden cardiotoxicity

Rosiglitazone treatment for 28 days did not affect infarct size following ischemia/reperfusion (I/R) injury when compared to vehicle-treated animals. Similarly, rosiglitazone did not significantly influence arrhythmia severity during ischemia and early reperfusion. Analysis of mortality during I/R injury also revealed no significant differences between treatment groups. Deaths occurred due to ventricular fibrillation or bradycardia, but their incidence was low and comparable. Overall, rosiglitazone did not worsen infarct size, arrhythmia susceptibility, or mortality with I/R injury.

To further assess hidden cardiotoxicity, the interaction between rosiglitazone and the known cardioprotective intervention, ischemic preconditioning (IPC), was investigated. IPC significantly reduced infarct size in both vehicle- and rosiglitazone-treated animals, indicating preserved infarct-limiting effects. IPC did not significantly

affect overall mortality in this model. Additionally, IPC reduced arrhythmia severity in vehicle-treated animals, demonstrating its expected antiarrhythmic effect. This protective effect was abrogated in rosiglitazone-treated animals, suggesting that rosiglitazone abolishes the antiarrhythmic benefits of IPC.

In summary, while rosiglitazone does not exacerbate I/R injury directly, it interferes with IPC-mediated antiarrhythmic protection, indicating a subtle form of hidden cardiotoxicity.

Hidden cardiotoxicity induced by rofecoxib may be mediated by altered cardiac calcium handling, thereby increasing arrhythmia susceptibility

Proteomic, phosphoproteomic, mRNA, and miRNA datasets of rofecoxib-treated left ventricular tissue were integrated to identify molecular alterations at multiple biological levels. The results showed that 84 proteins were significantly altered. Protein–protein interaction analysis of differentially expressed proteins revealed limited overall connectivity, although gene ontology analysis

identified enrichment in pathways related to protein ubiquitination, translation, and metabolic processes.

Phosphoproteomic analysis detected 117 significantly altered phosphorylation sites, corresponding to 52 proteins. In total, 132 proteins showed changes in expression and/or phosphorylation.

Additionally, two upstream kinases showed significant enrichment based on known or predicted target-phosphorylation site associations. Specifically, we found that connexin 43 (Cx43, Gja1) was phosphorylated at multiple sites previously reported to be targets of Calcium/Calmodulin-Dependent Protein Kinase II Alpha (Camk2a) and Casein Kinase 1 Alpha 1 (Ck1a).

Integration of proteomic, phosphoproteomic, and enriched kinase data revealed a highly interconnected protein-protein interaction network with significant enrichment, suggesting coordinated regulation of affected pathways.

Network and clustering analyses identified functionally related protein groups involved in cytoskeletal organisation, translation, proteolysis, metabolism, calcium signalling, and cardiac conduction. Importantly, the calcium-handling cluster comprises proteins that are

central regulators of calcium homeostasis and cardiac conduction.

In contrast, transcriptomic analysis revealed relatively minor changes in gene expression. Only a small proportion of mRNAs and miRNAs were differentially expressed, and no significant biological pathway enrichment was identified.

Comparison of proteomic and transcriptomic data showed minimal overlap, with only three proteins exhibiting corresponding changes at the mRNA level. This highlights a low correlation between transcript and protein expression and suggests that most protein-level alterations occur independently of transcriptional regulation.

Analysis of negative miRNA–mRNA correlations identified a miRNA-mRNA correlation network; however, the proposed posttranscriptional regulation of the incident miRNAs on their mRNAs did not translate into corresponding protein changes.

Overall, these results demonstrate that rofecoxib-induced hidden cardiotoxicity is primarily driven by proteomic and post-translational mechanisms rather than transcriptional and posttranscriptional regulation. The observed

alterations in protein expression and phosphorylation, particularly in pathways related to calcium handling and cardiac conduction, provide a mechanistic basis for the previously observed increased arrhythmia susceptibility under stress conditions and thus hidden cardiotoxicity of rofecoxib (Brenner et al., 2020).

5. Conclusion

This thesis demonstrates that hidden cardiotoxicity represents a critical limitation in current preclinical drug-safety assessments. Standard testing in healthy models often fails to detect adverse cardiac effects because drug-induced cardiac alterations may only manifest when additional comorbidities or stress, such as I/R injury, are present (Ferdinandy et al., 2019). This concept is exemplified by rosiglitazone and rofecoxib, whose cardiotoxic effects became apparent only after widespread clinical use, as observed in post-marketing studies (Bresalier et al., 2005; Nissen & Wolski, 2007).

In this work, rosiglitazone did not exacerbate infarct size, arrhythmias, or mortality during I/R injury; however, it abolished the antiarrhythmic benefits of ischemic preconditioning. This finding reveals a subtle form of hidden cardiotoxicity, where drug treatment interferes with cardioprotective mechanisms.

In parallel, the molecular investigation of hidden cardiotoxicity exemplified by rofecoxib demonstrated that

28-day treatment leads to significant alterations in protein expression and phosphorylation. Importantly, transcriptomic analyses revealed only minor changes, and miRNA–mRNA correlation network analysis indicated that post-transcriptional regulation by miRNAs is unlikely to significantly influence gene expression. This indicates that the observed effects are primarily driven by translational and post-translational mechanisms, rather than transcriptional or post-transcriptional regulation, resulting in a primed myocardial state with increased vulnerability.

A key mechanistic insight from the integrative multi-omics analysis is the dysregulation of calcium-handling pathways and proteins involved in cardiac conduction. These alterations provide a plausible link to the previously observed increased susceptibility to arrhythmias in an I/R injury model (Brenner et al., 2020). Thus, by applying an unbiased multi-omics network analysis, we were able to identify how the previously detected hidden cardiotoxicity of rofecoxib may prime the myocardium at baseline through protein-level and phosphoregulatory

disturbances. Moreover, greater emphasis should be placed on detecting the proarrhythmic effects of drugs on calcium-handling proteins in preclinical safety testing.

Collectively, these findings underscore the need to refine preclinical safety testing strategies by incorporating disease-relevant models, such as I/R injury, and testing calcium-mediated arrhythmias. In addition, the application of unbiased multi-omics and network-based approaches enables the identification of underlying molecular mechanisms that remain undetected by traditional hypothesis-driven methods.

Ultimately, this work emphasises that early identification of hidden cardiotoxicity requires relevant experimental models and mechanistic molecular insight. Integrating these approaches into drug development pipelines has the potential to improve translational predictability, reduce late-stage drug attrition, and enhance patient safety, particularly for those most susceptible to cardiac adverse drug reactions.

6. 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) IF: 4,6

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) IF: 2,0

Hambalkó Szabolcs, Pelyhe Csilla, Kovácsházi Csenger, Kenyeres Bence, Kiss Bernadett, Ágg Bence, Gergely Tamás G, Weber Bennet Y, Makkos András, Brenner Gábor B, Komlódi Tímea, Tretter László, Horváth Csaba, Jarabíková Izabela, Adameová Adriana, Görbe Anikó, Poggi Paola, Chatgililoglu Alexandros, Horváth Ildikó, Máthé Domokos, Szigeti Krisztián, Oláh Attila, Radovits Tamás, Varga Zoltán V, Merkely Béla, Gary F Baxter, Rainer Schulz, Ferdinandy Péter, Giricz Zoltán MAO-B inhibition by selegiline blunts cardiac functions improved by High-Fat Diet: Role of inflammation, apoptosis, and calcium-handling CURRENT RESEARCH IN PHARMACOLOGY AND DRUG DISCOVERY 9 Paper: 100237, 9 p. (2025)

Kovács házi Csenger, Hambalkó Szabolcs, Sayour Nabil V, Gergely Tamás G, Brenner Gábor B, Pelyhe Csilla, Kapui Dóra, Weber Bennet Y, Hültenschmidt Alexander L, Pállinger Éva, Buzás Edit I, Zolcsák Ádám, Kiss Bálint, Bozó Tamás, Csányi Csilla, Kósa Nikolett, Kellermayer Miklós, Farkas Róbert, Karvaly Gellért B, Kieran Wynne, David Matallanas, Ferdinandy Péter, Giricz Zoltán Effect of hypercholesterolemia on circulating and cardiomyocyte-derived extracellular vesicles SCIENTIFIC REPORTS 14: 1 Paper: 12016, 14 p. (2024) IF: 3,9

The cumulative impact factor: 18,2