TUULI KARHU

PHARMACOLOGICAL AND TOXICOLOGICAL CHARACTERISATION OF GATA4- AND PROTEIN KINASE C-TARGETED COMPOUNDS IN CARDIAC CELL MODELS

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DOCTORAL PROGRAMME IN DRUG RESEARCH
UNIVERSITY OF HELSINKI
Pharmacological and toxicological characterisation of GATA4- and protein kinase C-targeted compounds in cardiac cell models

Tuuli Karhu

DOCTORAL DISSERTATION

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ABSTRACT

Heart failure is a common costly disease that remains one of the leading causes of mortality in the world. Current treatments benefit heart failure patients by relieving the symptoms and improving the quality of life; however, they do not provide a cure for the disease. The adult human heart has a very limited capacity to regenerate cardiomyocytes. Therefore, following cardiac injury, the cell loss results in the formation of a fibrotic scar and consequent remodelling process, which includes thickening and stiffening of the ventricular wall. Eventually, this leads to impaired cardiac function. The fundamental goal of heart failure therapy is to restore the cardiac function and to prevent the pathological remodelling process. The transcription factor GATA4 plays an important role in cell survival, myocardial regeneration, and cardiomyocyte hypertrophy, and is therefore considered as an attractive drug target for cardiac repair. On the other hand, several studies have suggested that protein kinase C (PKC) plays a role in cardiac fibrosis. However, the results are conflicting and the role of PKC specifically in cardiac fibroblasts is unclear. Early drug discovery projects have traditionally used immortalized cell lines and primary cells from experimental animals to assess the effects of novel compounds. The translatability of the results from these models to human, however, is limited. Today, the emergence of the human induced pluripotent stem cell (hiPSC) technology allows for the production of human-based cell models, which more closely resemble the physiological cell types.

The aim of this thesis project was to characterise pharmacological and toxicological effects of small molecules targeted to GATA4 or PKC, to compare different cell models in in vitro compound testing, and to establish a chronic cardiotoxicity model using human-based cardiomyocytes. In the study, nine different cell models were utilised, including hiPSCs, cardiomyocytes derived from hiPSCs, cardiomyocytes isolated from neonatal rats, and cardiac fibroblasts isolated from adult mice. The effects of the small molecules on cell viability, proliferation, and morphology were investigated using colorimetric cytotoxicity assays, immunocytochemistry, and high-content analysis (HCA). The effects on gene expression, protein levels, and cellular kinase activity were studied using the quantitative polymerase chain reaction (qPCR), western blotting, and AlphaLISA® technology, respectively.

The toxicity screening of GATA4-targeted compounds revealed a characteristic molecular structure that is predominantly responsible for the toxic outcome in hiPSCs, which proved to be the most sensitive screening tool to identify toxicity. The results also demonstrate that long-term low-dose exposure of hiPSC-derived cardiomyocytes can be used as an in vitro model of delayed doxorubicin-induced cardiotoxicity and provide evidence that targeting GATA4 by small molecules may counteract doxorubicin-induced cardiotoxicity. Finally, the results reveal distinct
PKC-dependent regulation of cardiac fibroblast transdifferentiation and proliferation and suggest that fibroblast-targeted PKC activation may be a promising strategy to inhibit cardiac fibrosis. Overall, the results highlight the importance of selecting an appropriate cell type and experimental model for compound testing and support further development of both GATA4-targeted compounds and PKC activators.

Tämän väitöskirjatyön tavoitteena oli karakterisoida GATA4:ään ja PKC:hen kohdentettujen pienmolekyylien farmakologisia ja toksikologisia vaikutuksia, vertailla erilaisia solumalleja yhdisteiden in vitro -testauksessa, sekä kehittää kroonista sydäntoksisuutta kuvaava kokeellinen malli hyödyntämällä ihmisperäisiä sydänlihasvauruja. Työssä hyödynnettiin yhdeksää eri solumallia, mukaan lukien ihmisen, ihmisen indusoidut kantasolut ja niistä erilaistettuja solulinjoja sekä sydänlihasvaurioituneita soluja, rotan poikasten sydämiä ja aikuisten hiirenlähtöisiä solua. Tutkimuksessa osoitettiin, että GATA4:ään kohdentettuja soluja on herkimmäin sydänlihasvaurioituneissa solumalleissa. Lisäksi yhdisteet tunnistettiin rakennettaessa, joka pääasiallisesti aiheuttaa kantasolutoksisuutta. Työ osoittaa, että


LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following publications:


* Equal contribution

The publications are referred to in the text by their Roman numerals.

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<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>α-SMA</td>
<td>α-smooth muscle actin</td>
</tr>
<tr>
<td>ACE</td>
<td>angiotensin-converting enzyme</td>
</tr>
<tr>
<td>Ang II</td>
<td>angiotensin II</td>
</tr>
<tr>
<td>ANP</td>
<td>atrial natriuretic peptide</td>
</tr>
<tr>
<td>aPKC</td>
<td>atypical protein kinase C isoform</td>
</tr>
<tr>
<td>ATP</td>
<td>adenosine triphosphate</td>
</tr>
<tr>
<td>AT1R</td>
<td>angiotensin II type 1 receptor</td>
</tr>
<tr>
<td>Bak</td>
<td>B-cell lymphoma 2 antagonist/killer</td>
</tr>
<tr>
<td>Bax</td>
<td>B-cell lymphoma 2 associated X protein</td>
</tr>
<tr>
<td>Bcl</td>
<td>B-cell lymphoma</td>
</tr>
<tr>
<td>Bcl-xL</td>
<td>B-cell lymphoma extra large</td>
</tr>
<tr>
<td>BH3-only</td>
<td>B-cell lymphoma 2 homology domain 3 only</td>
</tr>
<tr>
<td>BMP</td>
<td>bone morphogenetic protein</td>
</tr>
<tr>
<td>BNP</td>
<td>B-type natriuretic peptide</td>
</tr>
<tr>
<td>BrdU</td>
<td>5-bromo-2’-deoxyuridine</td>
</tr>
<tr>
<td>BSA</td>
<td>bovine serum albumin</td>
</tr>
<tr>
<td>CCN</td>
<td>cysteine-rich protein 61, connective tissue growth factor, and nephroblastoma overexpressed gene</td>
</tr>
<tr>
<td>cPKC</td>
<td>classical protein kinase C isoform</td>
</tr>
<tr>
<td>CTGF</td>
<td>connective tissue growth factor</td>
</tr>
<tr>
<td>cTn</td>
<td>cardiac troponin</td>
</tr>
<tr>
<td>D5EB</td>
<td>mouse embryonic stem cell derivative from day 5 embryoid bodies</td>
</tr>
<tr>
<td>DAG</td>
<td>diacylglycerol</td>
</tr>
<tr>
<td>DAPI</td>
<td>4’,6-diamidino-2-phenylindole</td>
</tr>
<tr>
<td>DB</td>
<td>Dulbecco’s phosphate-buffered saline containing 0.2% bovine serum albumin</td>
</tr>
<tr>
<td>DDR2</td>
<td>discoidin domain receptor 2</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco’s modified Eagle medium</td>
</tr>
<tr>
<td>DMSO</td>
<td>dimethyl sulfoxide</td>
</tr>
<tr>
<td>E8</td>
<td>Essential 8™ medium</td>
</tr>
<tr>
<td>EB</td>
<td>embryoid body</td>
</tr>
<tr>
<td>ECM</td>
<td>extracellular matrix</td>
</tr>
<tr>
<td>EDA-FN</td>
<td>extra domain A containing splice variant of fibronectin</td>
</tr>
<tr>
<td>EDTA</td>
<td>ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>ERK</td>
<td>extracellular signal-regulated kinase</td>
</tr>
<tr>
<td>ESC</td>
<td>embryonic stem cell</td>
</tr>
<tr>
<td>ET-1</td>
<td>endothelin-1</td>
</tr>
<tr>
<td>FBS</td>
<td>foetal bovine serum</td>
</tr>
<tr>
<td>FGF</td>
<td>fibroblast growth factor</td>
</tr>
<tr>
<td>FOG</td>
<td>friend of GATA family member</td>
</tr>
<tr>
<td>Acronym</td>
<td>Definition</td>
</tr>
<tr>
<td>---------</td>
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</tr>
<tr>
<td>GATA</td>
<td>GATA binding protein</td>
</tr>
<tr>
<td>GSK-3</td>
<td>glycogen synthase kinase 3</td>
</tr>
<tr>
<td>HAND</td>
<td>heart and neural crest derivatives expressed</td>
</tr>
<tr>
<td>HCA</td>
<td>high-content analysis</td>
</tr>
<tr>
<td>HEPES</td>
<td>4-(2-hydroxyethyl)-1-piperazinethanesulfonic acid</td>
</tr>
<tr>
<td>HFmEF</td>
<td>heart failure with mid-range ejection fraction</td>
</tr>
<tr>
<td>HFrEF</td>
<td>heart failure with reduced ejection fraction</td>
</tr>
<tr>
<td>HFpEF</td>
<td>heart failure with preserved ejection fraction</td>
</tr>
<tr>
<td>hiPSC</td>
<td>human induced pluripotent stem cell</td>
</tr>
<tr>
<td>hiPSC-CM</td>
<td>human induced pluripotent stem cell-derived cardiomyocyte</td>
</tr>
<tr>
<td>IL</td>
<td>interleukin</td>
</tr>
<tr>
<td>i.p.</td>
<td>intraperitoneal</td>
</tr>
<tr>
<td>IP3</td>
<td>inositol-1,4,5-triphosphate</td>
</tr>
<tr>
<td>iPSC</td>
<td>induced pluripotent stem cell</td>
</tr>
<tr>
<td>JNK</td>
<td>c-Jun N-terminal kinase</td>
</tr>
<tr>
<td>KLF</td>
<td>Kruppel-like factor</td>
</tr>
<tr>
<td>LDH</td>
<td>lactate dehydrogenase</td>
</tr>
<tr>
<td>LV</td>
<td>left ventricle</td>
</tr>
<tr>
<td>MAPK</td>
<td>mitogen-activated protein kinase</td>
</tr>
<tr>
<td>Mcl-1</td>
<td>myeloid cell leukemia 1</td>
</tr>
<tr>
<td>MEF</td>
<td>mouse embryonic fibroblast</td>
</tr>
<tr>
<td>MEF2</td>
<td>myocyte enhancer factor 2</td>
</tr>
<tr>
<td>MEK</td>
<td>mitogen activated protein kinase kinase</td>
</tr>
<tr>
<td>mESC</td>
<td>mouse embryonic stem cell</td>
</tr>
<tr>
<td>MHC</td>
<td>myosin heavy chain</td>
</tr>
<tr>
<td>MLC</td>
<td>myosin light chain</td>
</tr>
<tr>
<td>MMP</td>
<td>matrix metalloproteinase</td>
</tr>
<tr>
<td>MTT</td>
<td>3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide</td>
</tr>
<tr>
<td>NKX2-5</td>
<td>NK2 homeobox 5</td>
</tr>
<tr>
<td>nPKC</td>
<td>novel protein kinase C isoform</td>
</tr>
<tr>
<td>NRCF</td>
<td>neonatal rat cardiac fibroblast</td>
</tr>
<tr>
<td>NRVM</td>
<td>neonatal rat ventricular myocyte</td>
</tr>
<tr>
<td>NT-proANP</td>
<td>N-terminal fragment of pro-atrial natriuretic peptide</td>
</tr>
<tr>
<td>NT-proBNP</td>
<td>N-terminal fragment of pro-B-type natriuretic peptide</td>
</tr>
<tr>
<td>OCT</td>
<td>octamer-binding transcription factor</td>
</tr>
<tr>
<td>p300</td>
<td>histone acetyltransferase p300</td>
</tr>
<tr>
<td>p38</td>
<td>mitogen-activated protein kinase p38</td>
</tr>
<tr>
<td>p53</td>
<td>tumour protein p53</td>
</tr>
<tr>
<td>PBS</td>
<td>phosphate-buffered saline</td>
</tr>
<tr>
<td>PCR</td>
<td>polymerase chain reaction</td>
</tr>
<tr>
<td>PDGF</td>
<td>platelet-derived growth factor</td>
</tr>
<tr>
<td>PFA</td>
<td>paraformaldehyde</td>
</tr>
<tr>
<td>Acronym</td>
<td>Description</td>
</tr>
<tr>
<td>---------</td>
<td>-------------</td>
</tr>
<tr>
<td>PIP₂</td>
<td>phosphatidylinositol-4,5-bisphosphate</td>
</tr>
<tr>
<td>PKC</td>
<td>protein kinase C</td>
</tr>
<tr>
<td>PLC</td>
<td>phospholipase C</td>
</tr>
<tr>
<td>PMA</td>
<td>phorbol 12-myristate 13-acetate</td>
</tr>
<tr>
<td>RAAS</td>
<td>renin-angiotensin-aldosterone system</td>
</tr>
<tr>
<td>RACK</td>
<td>receptor for activated C kinase</td>
</tr>
<tr>
<td>RB</td>
<td>RPMI 1640 medium supplemented with B-27</td>
</tr>
<tr>
<td>ROCK</td>
<td>Rho-associated protein kinase</td>
</tr>
<tr>
<td>ROS</td>
<td>reactive oxygen species</td>
</tr>
<tr>
<td>SEM</td>
<td>standard error of the mean</td>
</tr>
<tr>
<td>SERCA</td>
<td>sarco/endoplasmic reticulum Ca²⁺ ATPase</td>
</tr>
<tr>
<td>SOX</td>
<td>sex determining region Y box</td>
</tr>
<tr>
<td>SRF</td>
<td>serum response factor</td>
</tr>
<tr>
<td>SUMO-1</td>
<td>small ubiquitin-like modifier-1</td>
</tr>
<tr>
<td>TBX</td>
<td>T-box transcription factor</td>
</tr>
<tr>
<td>Tcf21</td>
<td>transcription factor 21</td>
</tr>
<tr>
<td>TGF-β</td>
<td>transforming growth factor β</td>
</tr>
<tr>
<td>TIMP</td>
<td>tissue inhibitor of metalloproteinases</td>
</tr>
<tr>
<td>TNF-α</td>
<td>tumour necrosis factor α</td>
</tr>
<tr>
<td>TTBS</td>
<td>Tris-buffered saline with 0.05 % Tween 20</td>
</tr>
<tr>
<td>Wnt</td>
<td>wingless-related integration site</td>
</tr>
</tbody>
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Heart failure is a common, costly, and potentially fatal condition (Ziaeian and Fonarow 2016). Its estimated prevalence is over 64.3 million individuals globally (Groenewegen et al. 2020). In developed countries 1–2% of the adult population lives with heart failure. Among people ≥65 years old, the prevalence is around 11.8%. Although the age-adjusted incidence of heart failure in the developed world has decreased, the absolute number of patients with heart failure is increasing due to shifts in the global age distribution and improved survival after diagnosis. Still today, heart failure causes significant morbidity and carries a 5-year survival rate of 57%.

Heart failure results from injury to the myocardium due to a variety of causes such as myocardial infarction, hypertension, and cardiotoxic drugs (Kemp and Conte 2012; Ponikowski et al. 2016). The failing heart attempts to maintain adequate function by several compensatory mechanisms, including intrinsic autoregulation of contractility (Frank-Starling mechanism), neurohormonal activation, and ventricular remodelling. Although initially beneficial, the compensatory mechanisms eventually lead to a vicious circle of worsening heart failure. Currently, the treatment of heart failure consists of lifestyle modifications and medical therapies that counteract the compensatory mechanisms, including drugs that inhibit activation of the renin-angiotensin-aldosterone system (RAAS) and the sympathetic nervous system. The current treatments relieve the symptoms, improve the quality of life, and delay the progression of heart failure; however, they do not provide cure for the disease. In the most serious cases of post-infarction heart failure, replacing the whole organ function with a left ventricular assist device or heart transplantation may be necessary. This option is, however, associated with several other complex issues, such as limited availability of replacement organs and considerable financial costs associated with such procedures. In conclusion, there is clearly an unmet medical need for the development of novel cost-effective treatments for the management of heart failure. Therefore, discovering novel drug candidates and drug targets is particularly important.

The fundamental goal in heart failure therapy is restoring the cardiac function by inducing cardiac regeneration and repair (Xin et al. 2013) as well as stopping or reversing the remodelling process (Cohn et al. 2000). Regarding cardiac injury induced by cardiotoxic drugs (e.g. cancer therapies), the focus is also on developing novel cardioprotective strategies (Raber and Asnani 2019). Cardiac regeneration requires the formation of new cardiomyocytes, which can be achieved, for instance, by stimulating proliferation of existing cardiomyocytes or reprogramming non-myocytes into cardiomyocytes (Xin et al. 2013). Several signalling pathways and cardiac transcription factors, such as GATA binding protein 4 (GATA4), participate in these processes. GATA4 plays an important role also in cardiomyocyte survival and...
in the development of pathological conditions, e.g. cardiomyocyte hypertrophy (Pikkarainen et al. 2003; Pikkarainen et al. 2004; Rysä et al. 2010), and is therefore considered an attractive drug target for cardiac repair. On the other hand, protein kinase C (PKC) is known to participate in various signalling pathways and thus regulate, e.g. cell proliferation, differentiation, migration, gene transcription and translation, and cell death (Mochly-Rosen et al. 2012). Previous work suggests that certain PKC isoforms play a role also in cardiac fibrosis and remodelling; however, the results are conflicting (Palaniyandi et al. 2009). More studies are required to help in the identification of the specific isoforms that could serve as therapeutic targets for the treatment of pathological remodelling.

Disease models are essential for understanding the pathogenesis of cardiovascular diseases and developing novel therapeutics. Traditionally, researchers have relied on in vivo and in vitro models that utilise either small animals (e.g. mice and rats) or large animals (e.g. dogs and pigs) and cultures of primary cells isolated directly from animal tissues. However, the translatability of results from these models to human is limited as they are unable to reproduce the complex pathophysiology of human disease (Dixon and Spinale 2009; Houser et al. 2012). Moreover, there are some considerable differences in cardiac function between humans and rodents especially, and therefore human-based models are particularly desirable for cardiovascular research. Because induced pluripotent stem cells (iPSC) can be derived from healthy and diseased human patients and differentiated into cardiomyocytes, they offer a valuable and robust tool for use in disease modelling and drug discovery (Karakikes et al. 2015; Paik et al. 2020; Sayed et al. 2016). Particularly, human iPSCs (hiPSCs) and hiPSC-derived cardiomyocytes (hiPSC-CMs) have the potential of becoming powerful tools for high-throughput screening of drug toxicity (Liu et al. 2017; Magdy et al. 2018).

In the present study, both animal-based in vitro models and novel human-based iPSC-models were utilised to characterise compounds targeting either the transcription factor GATA4 or PKC. For toxicity profiling of the GATA4-targeted compounds, eight different cell types were used and compared. The characterisation of selected GATA4-targeted compounds was continued using hiPSC-CMs to investigate if they have regenerative or cardioprotective potential. Additionally, in order to have an appropriate model for the cardioprotective studies, a chronic cardiotoxicity model using hiPSC-CMs was established. Lastly, to characterise the potential of targeting PKC for the inhibition of cardiac fibrosis, the effects of several different PKC-targeted compounds, both agonists and inhibitors, on cardiac fibroblasts were investigated.
2 REVIEW OF THE LITERATURE

2.1 Heart failure

Heart failure is a clinical syndrome characterised by typical symptoms (e.g. breathlessness, fatigue, reduced exercise tolerance, ankle swelling) and signs (e.g. elevated jugular venous pressure, pulmonary crepitations, peripheral oedema), which are caused by structural or functional cardiac abnormalities leading to elevated intracardiac pressure or reduced cardiac output (Ponikowski et al. 2016). It is a progressive pathological state, in which the heart fails to supply a stable flow of blood for the requirements of the metabolizing tissues (Braunwald 2013). Patients are typically classified based on measurement of the left ventricular (LV) ejection fraction: heart failure with reduced (<40%) ejection fraction (HFrEF, also known as systolic heart failure), heart failure with mid-range (40–49%) ejection fraction (HFmEF, representing a “grey area”), and heart failure with preserved (≥50%) ejection fraction (HFpEF, also known as diastolic heart failure) (Ponikowski et al. 2016). The aetiological profile of heart failure can be diverse and cardiac injury may occur with any cardiovascular disease. One of the leading causes of HErEF is the loss of functional myocardium due to myocardial infarction (Kemp and Conte 2012). Uncontrolled hypertension leading to excessive pressure overload and toxic damage induced by e.g. therapeutic drugs are two other important contributors. Possible causes include also immune-mediated damage (e.g. viral myocarditis), metabolic derangements (e.g. diabetes), genetic abnormalities, valve defects, and arrhythmias.

Cell death is a significant component in the pathogenesis of heart failure, and depending on the stressor, it can occur through different mechanisms (Konstantinidis et al. 2012). Loss of cardiomyocytes leads to increased synthesis of extracellular matrix (ECM) which has an important role in determining the architecture and pumping function of the ventricles (Braunwald 2013). ECM accumulation enhances myocardial stiffness and reduces the rate of ventricular relaxation and contraction, in other words, filling and emptying. Left ventricular dysfunction causes a decrease in cardiac output, which leads to global hypoperfusion (Kemp and Conte 2012). Left ventricular dysfunction causes also an increase in the blood volume in the ventricle and thus an increase in both end-systolic and end-diastolic volumes. This in turn causes increases in left atrial pressure and in pressures of the capillaries of the lungs, eventually leading to pulmonary congestion. In the early stages of heart failure, reduced contractile function leads to activation of the adrenergic nervous system along with the RAAS, which acutely increase cardiac output (via increased heart rate and contractility) and blood pressure (via vasoconstriction and increased blood volume) (Kemp and Conte 2012). Prolonged neurohormonal activation, however, causes further myocardial damage through...
cardiomyocyte hypertrophy, increased energy consumption, cell death, fibrosis, and arrhythmia, thus initiating a vicious cycle (El-Armouche and Eschenhagen 2009).

In establishing the final diagnosis of heart failure, echocardiographic evaluation of cardiac function is the most useful tool as it provides immediate information on chamber volumes, ventricular systolic and diastolic function, wall thickness, valve function, and pulmonary hypertension (Ponikowski et al. 2016). However, as a rule-out-test to identify patients who require further investigation, measurement of plasma concentrations of cardiac natriuretic peptides is useful. Both echocardiography and natriuretic peptide measurements are also recommended as tools to detect myocardial toxicity (Zamorano et al. 2017).

In patients with heart failure, the cardiac hormonal system is activated, leading to elevated plasma levels of atrial natriuretic peptide (ANP), B-type natriuretic peptide (BNP), and the N-terminal fragments of their prohormones (Daniels and Maisel 2007; Ruskoaho 2003). ANP is stored in atrial granules as a prohormone proANP, which upon secretion is split into the N-terminal fragment NT-proANP and biologically active ANP. The storage of ANP in granules enables the rapid release of the peptide with even minor triggers, whereas BNP has only minimal storage and thus increased BNP secretion is preceded by increased BNP mRNA levels. Cardiac stress initiates the synthesis of preproBNP, which is cleaved first to proBNP and then to NT-proBNP and biologically active BNP. The biological actions of ANP and BNP are mediated through membrane-bound natriuretic peptide receptors (NRPs), principally NRP type A (NRP-A), which is linked to a cyclic guanosine monophosphate–dependent signalling cascade (Daniels and Maisel 2007). ANP and BNP act in various tissues inducing vasodilation, natriuresis, and diuresis. Thus, their actions oppose the physiological abnormalities in heart failure. BNP is more stable than ANP in plasma, therefore BNP and NT-proBNP have become the most widely used biomarkers of cardiomyocyte stress in the care of patients with known or suspected heart failure (Braunwald 2013). The synthesis of both ANP and BNP in cardiomyocytes is induced by cellular stress such as mechanical stretch (Pikkarainen et al. 2003), endothelin-1 (ET-1) (Bruneau et al. 1997) and hypoxia (Toth et al. 1994). Furthermore, elevated BNP levels have been reported in response to cytokines (such as interleukin 1β [IL-1β] and tumour necrosis factor α [TNF-α]) (Ma et al. 2004) as well as metabolic stress (Bistola et al. 2008).

### 2.2 Cell death in heart disease

Cells die primarily by apoptosis and necrosis, which have traditionally been referred to as regulated and unregulated/passage, respectively, modes of cell death (Del Re et al. 2019). However, a substantial proportion of necrotic cell deaths are also actively executed by the cell in a highly regulated manner. Both apoptosis and necrosis have been implicated to contribute to the pathogenesis of heart failure; however, their
relative contributions remain unclear. In myocardial infarction the regulated forms of necrosis are critical. A third cell death programme most frequently linked to heart disease is autophagy-associated cell death (Braunwald 2013; Del Re et al. 2019; Konstantinidis et al. 2012).

Both apoptosis and necrosis are mediated through distinct pathways involving cell surface death receptors (referred to as the extrinsic pathway) or mitochondria (the intrinsic pathway) (Del Re et al. 2019; Konstantinidis et al. 2012). The death receptor pathway is regulated by the binding of specific ligands (e.g. TNF-α or Fas ligand) to their cognate cell surface receptors, whereas the mitochondrial pathway can be activated by diverse stimuli, such as oxidative stress, hypoxia, ischaemia, toxins, loss of nutrient/survival factors, and increase in intracellular calcium. The pathways are overlapping and linked by multiple biochemical and functional connections, yet apoptosis and necrosis have their distinct morphological characteristics. Apoptotic cells are characterised by cell shrinkage, fragmentation into membrane-enclosed apoptotic bodies, and phagocytosis of the apoptotic bodies by macrophages and neighbouring cells. When this operation proceeds efficiently, inflammation is avoided. In contrast, necrosis is characterised by cell and organelle swelling, loss of cell membrane integrity, and a marked inflammatory response in the surrounding tissue. In vitro, the apoptotic cleanup system is, however, absent, thus cells undergoing apoptosis in culture conditions may also acquire some necrotic features (such as plasma membrane leakiness) at later time points.

In the case of apoptosis, both mitochondrial and death receptor pathways lead to activation of caspases (Fig. 1), cysteine-dependent proteases, which are synthesized as inactive zymogens termed procaspases (Del Re et al. 2019; Konstantinidis et al. 2012). The enzymes are grouped into three subclasses: initiator (human caspase-8/9/10), effector (human caspase-3/6/7), and inflammatory (human caspase-1/4/5/12). Apoptosis is mediated by the initiator and effector caspases, while inflammatory procaspases are involved in inflammasome signalling. The activation of initiator procaspases is induced by their recruitment into specific multi-protein complexes characteristic of each pathway. The key regulators of the mitochondrial apoptosis pathway are the B-cell lymphoma (Bcl)-2 family proteins, which are divided into three subfamilies: anti-apoptotic Bcl-2 proteins (including Bcl-2, Bcl extra large [Bcl-xL], and myeloid cell leukemia 1 [Mcl-1]), pro-apoptotic Bcl-2 proteins (including Bcl-2 associated X protein [Bax] and Bcl-2 antagonist/killer [Bak]), and pro-apoptotic BH3-only (B-cell lymphoma 2 homology domain 3 only) proteins.

### 2.3 Cardiac fibrosis and remodelling

Besides cardiomyocyte death, the subsequent remodelling process is an essential component of heart failure. Pathological remodelling includes stiffening and
thickening of the ventricular wall due to fibrosis and cardiomyocyte hypertrophy (Sutton and Sharpe 2000). This complex process is regulated by autocrine and paracrine factors generated within the myocardium, hormones derived from the circulation, and mechanical forces occurring within the ventricular wall (Frieler and Mortensen 2015; Nakamura and Sadoshima 2018; Talman and Ruskoaho 2016). Several cell types, including fibroblasts, cardiomyocytes, and immune cells, have an active part in the process.

Figure 1. Overview of apoptosis pathways (Del Re et al. 2019; Konstantinidis et al. 2012). In death receptor apoptosis, binding of death ligands to their cognate receptors results in the formation of sequential protein complexes (I and II), leading to activation of caspase-8 (or -10, not shown), which in turn activate effector caspases, such as caspase-3/7. In mitochondrial apoptosis, extracellular and intracellular death signals lead to permeabilization of the outer mitochondrial membrane, allowing the release of cytochrome c to the cytosol where it stimulates assembly of a protein complex (not shown) in which caspase-9 is activated. Caspase-9 subsequently activates effector caspases. The mitochondrial outer membrane permeabilization is regulated by B-cell lymphoma (Bcl)-2 family proteins which are divided into three subfamilies: anti-apoptotic Bcl-2 proteins (including Bcl-2, Bcl extra large [Bcl-xL], and myeloid cell leukemia 1 [Mcl-1]), pro-apoptotic Bcl-2 proteins (including Bcl-2 associated X protein [Bax] and Bcl-2 antagonist/killer [Bak]), and pro-apoptotic BH3-only proteins. The general idea is that BH3-only proteins transduce death signals to activate Bax and Bak and to inactivate anti-apoptotic Bcl-2 proteins, while anti-apoptotic Bcl-2 proteins oppose these events through sequestering the BH3-only proteins and inactivating Bax and Bak.
Cardiac fibrosis is characterised by the accumulation of extracellular matrix proteins in the cardiac interstitium contributing to both systolic and diastolic dysfunction of the heart (Kong et al. 2014). Depending on the pathophysiological condition, this may occur via different mechanisms. Acute myocardial infarction results in the sudden loss of a large number of cardiomyocytes which triggers an inflammatory response, eventually leading to formation of a collagen-based scar (Shinde and Frangogiannis 2014; van den Borne et al. 2010). This type of fibrotic response is called replacement fibrosis and it is a crucial part of the healing process after a large ischaemic injury (Kong et al. 2014). Other pathophysiological cardiac conditions, such as pressure overload (Berk et al. 2007), induce more insidious reactive interstitial and perivascular fibrosis in the absence of considerable cardiomyocyte loss (Kong et al. 2014). Reactive fibrosis may also take place in remote non-infarcted myocardium following myocardial infarction and thereby lead to progressive remodelling of the left ventricular wall (Talman and Ruskoaho 2016). The expansion of the ECM is accompanied by hypertrophic growth of cardiomyocytes as the cells attempt to compensate for the increased workload (Heineke and Molkentin 2006). Although the pathophysiological mechanism leading to a fibrotic response differs based on the cardiac condition, the cellular effectors and signalling networks involved in cardiac fibrosis are similar.

The cardiac ECM consists of structural proteins and other macromolecules that provide a structural framework for cells, but also participate in biochemical signalling (Klingberg et al. 2013). Type I and III collagens are the primary structural proteins. Collagen I (composing 85% of the collagen matrix) typically forms thick fibres with high tensile strength whereas collagen III (11% of the collagen matrix) forms thin fibres that possess more resilience (Weber 1989). In healthy heart the homeostasis and turnover of ECM is tightly regulated by proteolytic matrix metalloproteinases (MMPs) and their endogenous inhibitors (tissue inhibitors of metalloproteinases, TIMPs) (Lindsey et al. 2016; Vanhoutte and Heymans 2010). In diseased heart the ECM exhibits dynamic alterations in its composition (e.g. upregulation of the glycoprotein fibronectin and growth factors) that, for instance, facilitate proliferation, migration, and activation of fibroblasts (Kong et al. 2014). This leads to increased synthesis of both type I and type III collagens (Weber 1989). In patients with ischaemic cardiomyopathy the ratio of collagen I to collagen III is decreased (Mukherjee and Sen 1991), while in hypertensive rats the ratio has been shown to increase (Mukherjee and Sen 1993), thus suggesting that contextual factors may lead to alterations in expression patterns of collagen subtypes. Another alteration occurring in the ECM in fibrotic conditions is the increased expression of matricellular proteins in the interstitial space (Kong et al. 2014; Talman and Ruskoaho 2016). They are unrelated non-structural proteins, which associate with cytokines and growth factors and bind to cell surface receptors transducing signalling cascades. Central matricellular proteins involved in cardiac diseases are thrombospondins, tenascins,
osteopontin, periostin, and the members of the CCN (cysteine-rich protein 61, connective tissue growth factor, and nephroblastoma overexpressed gene) family.

The key cellular event that drives the fibrotic response is activation and transdifferentiation of fibroblasts into secretory and contractile myofibroblasts, which are characterised by the expression of α-smooth muscle actin (α-SMA) (Kong et al. 2014). Cardiac fibroblasts originate from embryonic epicardium-derived progenitor cells during development (Tallquist and Molkentin 2017) and transcription factor 21 (Tcf21) seems to be essential for their cell fate determination (Acharya et al. 2012). They help to preserve the structural integrity of the heart through proliferation and ECM synthesis and degradation as well as through dynamic cell-ECM and cell-cell interactions (Souders et al. 2009). They interact with ECM through cell surface receptors called integrins and discoidin domain receptor 2 (DDR2), and with other cells through cell surface proteins called connexins and cadherins. Through these connections they are able to respond to a variety of biochemical, mechanical and electrical stimuli, which in a pathological state leads to their activation and transdifferentiation. Certain other cell types can also transdifferentiate to myofibroblasts, which is one reason the origin of myofibroblasts in the fibrotic heart has been under debate (Kong et al. 2014; Tallquist and Molkentin 2017). However, based on current knowledge, the resident fibroblasts represent the most important source.

A prominent characteristic of myofibroblasts is the presence of a contractile apparatus containing actin filaments and non-muscle myosin, which enables their migratory and contractile phenotype (Talman and Ruskoaho 2016; Tomasek et al. 2002). Transforming growth factor β (TGF-β) induces α-SMA expression, likely via Smad3-dependent signalling (Desmoulière et al. 1993; Dobaczewski et al. 2010), and is one of the key factors mediating myofibroblast transdifferentiation. Upregulation of the extra domain A containing splice variant of fibronectin (EDA-FN) mediates acquisition of the phenotype (Serini et al. 1998). Moreover, mechanical force has been shown to stimulate α-SMA mRNA synthesis via the Rho signalling pathway (Zhao et al. 2007), however, this pathway may also require the presence of TGF-β (Kong et al. 2014; Shinde and Frangogiannis 2014). Besides α-SMA, another marker that has been described for activated fibroblasts is the expression of the periostin encoding Postn gene (Kanisicak et al. 2016). Transdifferentiated myofibroblasts synthesize and secrete large amount collagens but also other of ECM proteins and fibrogenic factors (Talman and Ruskoaho 2016). They are the principal cell type responsible for expansion of ECM matrix while other cell types contribute to fibrosis mainly indirectly by secreting pro-fibrotic factors (Kong et al. 2014).

In addition to TGF-β, angiotensin II (Ang II), ET-1, connective tissue growth factor (CTGF/CCN2, a member of the CCN family of matricellular proteins), and platelet-derived growth factor (PDGF) are the central signalling molecules contributing to
myofibroblast persistence and prolonged fibrotic response (Leask 2010; Leask 2015). All of these profibrotic proteins can originate from several different cell sources acting in a complex network. Ang II, which is produced locally by macrophages, cardiomyocytes, and myofibroblasts, exerts its effects by inducing TGF-β1 expression in cardiomyocytes, fibroblasts, and myofibroblasts through the angiotensin II type 1 receptor (AT₁R) (Leask 2015). ET-1 (predominantly produced by endothelial cells but also by, e.g. fibroblasts, cardiomyocytes, and macrophages) is a potent pro-fibrotic factor, which acts downstream of both TGF-β and Ang II. Both Ang II and ET-1 stimulate proliferation and matrix protein synthesis of cardiac fibroblasts (Kong et al. 2014). PDGF has been suggested to play a role especially in fibroblast proliferation, while CTGF seems to promote the fibrogenic activity of TGF-β (Leask 2015).

Considering the well-established role of Ang II in promoting cardiac fibrosis via AT₁R-mediated induction of TGF-β1 expression, it is not surprising that angiotensin-converting enzyme (ACE) inhibitors and AT₁R blockers have been shown to prevent pathological remodelling in various experimental models (Rosenkranz 2004; Weber et al. 2013). Consequently, they are recommended treatments in patients with heart failure (Ponikowski et al. 2016). β-Adrenergic blockers are also recommended for treatment of patients with a history of myocardial infarction and asymptomatic LV systolic dysfunction; however, depending of the selectivity of the β-adrenergic receptor antagonists the beneficial effects are likely to be due to a combination of effects on cardiomyocytes and fibroblasts or due to effects on cardiomyocytes alone (Porter and Turner 2009). Several reports suggest that cardiac fibroblasts express predominantly the β2-receptor subtype (Carter et al. 2014; Porter and Turner 2009; Turner et al. 2003), while β2-adrenergic stimulation enhances cardiac fibroblast proliferation (Porter and Turner 2009; Turner et al. 2003). However, as cardiomyocytes express high levels of the β1-receptor subtype, the preference for using β1-selective β-adrenergic blockers for treatment of heart failure patients ignores the potential benefits of inhibiting the β2-receptor in cardiac fibroblasts. Altogether, currently used cardiovascular drugs bring benefits; however, they are not efficient enough in inhibiting the progression of pathological fibrosis and remodelling.

2.4 Cardiac regeneration

Following cardiac injury, the cell loss leads to the formation of a fibrotic scar because adult humans and other mammals have only limited capacity to regenerate cardiomyocytes (Kikuchi and Poss 2012; Uygur and Lee 2016). On the contrary, several vertebrate species undergo a complete regenerative response upon injury (Poss et al. 2002; Witman et al. 2011). For instance, zebrafish fully regenerate hearts within 60 days of 20% ventricular resection (Poss et al. 2002), as well as within 130 days of 25% ventricular cryocauterization (González-Rosa et al. 2011). The regeneration seems to be driven primarily through proliferation of pre-existing
cardiomyocytes (Jopling et al. 2010; Kikuchi et al. 2010). To facilitate proliferation, the cardiomyocytes undergo limited dedifferentiation by detaching from one another and reducing organization of their sarcomeric structure. The cryoinjury model demonstrated also that in a pathological context the fish heart progressively eliminates scar tissue (González-Rosa et al. 2011).

This regenerative capacity also exists in mammals during development and early neonatal life (Kikuchi and Poss 2012; Uygur and Lee 2016). The hearts of 1-day-old neonatal mice can fully regenerate following 15% ventricular resection or myocardial infarction; however, this capacity is lost by 7 days of age (Porrello et al. 2011; Porrello et al. 2013). As in zebrafish, the regenerative response in 1-day-old mice is characterised by proliferation of pre-existing cardiomyocytes with minimal fibrosis. At day 21 after myocardial infarction, 95% of the lost myocardium was viable and no signs of systolic dysfunction were apparent up to 9 months after the injury (Porrello et al. 2013). Regarding humans, there is a case report of a newborn child developing cardiac damage after a severe myocardial infarction but having a full functional recovery (Haubner et al. 2016). This suggests that, similar to neonatal rodents, newborn humans might have the intrinsic potential to repair myocardial damage. Moreover, Bergmann and colleagues described evidence for cardiomyocyte renewal in humans throughout life (Bergmann et al. 2009); however, the rate was estimated to be only 1% per year at the age of 25 and 0.45% at the age of 75. Mollova and colleagues further demonstrated that in humans, cardiomyocyte cytokinesis gradually decreases to nondetectable levels in adults, who instead show a significant increase in the formation of polyploid cardiomyocytes (Mollova et al. 2013).

It has been suggested that the increase in environmental oxygen is an upstream event which triggers the cell-cycle exit of mammalian cardiomyocytes shortly after birth with subsequent activation of the mitochondria-mediated DNA damage response (Puente et al. 2014). Hypoxia has been shown to prolong the proliferative window of postnatal mouse cardiomyocytes (Puente et al. 2014) and to induce heart regeneration after myocardial infarction in adult mice (Nakada et al. 2017). Along these lines, prolonged mechanical unloading of the human heart resulted in a reduced DNA damage response and induction of cardiomyocyte proliferation (Canseco et al. 2015). Overall, the data demonstrate that the mammalian heart is not a post-mitotic organ, and thus stimulating the renewal of human cardiomyocytes can be seen as a rational strategy for the induction of cardiac regeneration (Leach and Martin 2018; Sharma et al. 2015).

2.5 Transcription factor GATA4

The transcription factor GATA4 is a member of the GATA family of zinc-finger transcription factors, which consists of six proteins (GATA1–6) (Tremblay et al. 2018). Originally, GATA1/2/3 were classified as haematopoietic and GATA4/5/6 as
cardiac GATA factors; however, their function and expression patterns are more complex. GATA4, for instance, is expressed in heart but also in gonad, adrenal gland, intestine and stomach, pancreas, lung, liver, and bone. GATA factors are characterised by two highly conserved zinc-finger domains which recognize and bind to the consensus DNA binding sequence (A/T)GATA(A/G). In addition, they all contain a nuclear localization signal and less conserved N- and C-terminal regions, which contain transcription activation modules. The human GATA4 protein contains 442 amino acids, and the zinc-finger domains are located at residues 217–241 (N-terminal) and 271–295 (C-terminal) (Välimäki and Ruskoaho 2020).

GATA4 was initially discovered as an important regulator of cardiac development and later as a regulator of cardiac hypertrophy, cardiomyocyte survival, and cardiac regeneration (Kikuchi et al. 2010; Malek Mohammadi et al. 2017; Pikkarainen et al. 2004; Rysä et al. 2010; Suzuki 2011; Tremblay et al. 2018). Several essential cardiac genes contain the binding sequence for GATA4 in their promoter, including ANP, BNP, α-myosin heavy chain (α-MCH), β-MCH, cardiac troponin C (cTnC), cardiac troponin I (cTnI), and Na+/Ca2+ exchanger (Charron et al. 1999; Cheng et al. 1999; Grépin et al. 1994; Hasegawa et al. 1997; Herzig et al. 1997; Ip et al. 1994; Murphy et al. 1997). GATA4 interacts directly with other cardiac transcription factors, such as NK2 homeobox 5 (NKX2-5), myocyte enhancer factor 2C (MEF2C), heart and neural crest derivatives expressed 2 (HAND2), serum response factor (SRF), T-box transcription factor 5 (TBX5), and friend of GATA2 (FOG2) to cooperatively regulate gene expression (Välimäki and Ruskoaho 2020). Most of these protein interactions are mediated by the C-terminal zinc-finger, while the N-terminal zinc-finger interacts with FOG2. GATA4 mutations that affect its transcriptional activity or interactions with its cofactors can lead to cardiomyopathies and congenital heart defects (Ang et al. 2016; Garg et al. 2003; Li et al. 2013).

2.5.1 Post-translational modifications of GATA4

The function of the GATA4 protein is regulated through post-translational modifications, which affect its nuclear localization, DNA binding activity, coprotein association, and degradation (Pikkarainen et al. 2004; Välimäki and Ruskoaho 2020). The modifications of GATA4 include phosphorylation, acetylation, SUMOylation, and ubiquitination. Kinases that have been shown to catalyse GATA4 phosphorylation in cardiomyocytes are mitogen-activated protein kinases (MAPK) p38 and extracellular signal-regulated kinase 1/2 (ERK1/2), as well as glycogen synthase kinase 3β (GSK-3β) (Charron et al. 2001; Liang et al. 2001b; Morisco et al. 2001; Tenhunen et al. 2004). For instance, for stretch-induced activation of GATA4-binding both p38 and ERK1/2 are required (Tenhunen et al. 2004). Moreover, both p38 and ERK1/2 mediate the phosphorylation of GATA4 at serine 105 (Charron et al. 2001; Liang et al. 2001b), which has been shown to be critical for the stress-induced cardiac hypertrophic response in vivo (van Berlo et al. 2011). Homozygous Gata4-
S105A mutant mice (whereby serine 105 is mutated to alanine) showed a blunted hypertrophic response to phenylephrine infusion and pressure overload as well as downregulation of several hypertrophy related genes compared to the wild type (van Berlo et al. 2011). There is also evidence that ERK-mediated phosphorylation of serine 105 makes GATA4 more resistant to cellular degradation (Suzuki 2003), while GSK-3β-mediated phosphorylation of GATA4 negatively regulates nuclear expression of GATA4 by stimulating its export from the nucleus (Morisco et al. 2001).

Besides phosphorylation, GATA4 acetylation has been recognized as a mode of post-translational modification regulating cardiac hypertrophy. Histone acetyltransferase p300 has been shown to be involved in phenylephrine-induced acetylation of GATA4 in cardiomyocytes (Yanazume et al. 2003). In vivo overexpression of p300 resulted in increased expression of ANP and MHC-β encoding genes, cardiomyocyte growth, and left-ventricular dilatation, which were associated with increased GATA4 acetylation and DNA binding activity. A mutational analysis later identified the four most critical lysine residues (located between amino acids 311 and 322 in the C-motif) in GATA4 that act as p300-mediated acetylation targets (Takaya et al. 2008). Mutation of all four residues repressed phenylephrine-induced increases in both cell surface area and expression of hypertrophy-responsive genes in cardiomyocytes. On the other hand, ischaemic preconditioning increases GATA4 DNA binding activity, which is associated with GATA4 acetylation (Suzuki et al. 2004). Thus, the possibility was considered that the cardioprotective effect of preconditioning against myocardial infarction is due to modification of GATA4 making the protein more resistant to degradation.

GATA4 is also a target for small ubiquitin-like modifier-1 (SUMO-1), whose major SUMOylation site on the GATA4 protein seems to be lysine 366 (Wang et al. 2004). SUMO modification was shown to increase GATA4 transcriptional activity. On the other hand, mutation at the site reduced GATA4 nuclear occupation without altering its DNA binding activity, thus suggesting SUMOylation may play a role in GATA4 nuclear-cytoplasmic translocalization. Finally, GATA4 ubiquitination by the ubiquitin-proteasome pathway appears to have a role in regulation of GATA4 protein degradation (Li et al. 2018). It has been linked to pathological conditions, such as oxidative stress and hyperglycaemia (Kobayashi et al. 2007; Li et al. 2018).

### 2.5.2 GATA4 in cardiac development

Heart development begins with fusion of the cardiac crescent followed by generation of the heart tube, which eventually forms the chambers and arterial trunk through a looping process (Tremblay et al. 2018). GATA4/5/6 are central regulators of this development process and have specific and redundant roles during heart formation. Among them, GATA4 is one of the earliest transcription factors expressed in the developing heart and abundant GATA4 mRNA continues to be expressed in the adult
heart throughout life (Heikinheimo et al. 1994; Kelley et al. 1993). In the developing mouse embryo, GATA4 mRNA is first apparent at 7.0–7.5 days post coitum (dpc) while GATA4 protein is expressed at 8.0 dpc during formation of the heart tube (Heikinheimo et al. 1994). Homozygous GATA4-deficient mice die between 8.5 and 10.5 dpc due to failure of heart tube formation and ventral morphogenesis (Kuo et al. 1997; Molkentin et al. 1997). Cardiac markers, such as α-MHC, myosin light chain 2A and 2V (MLC2A, MLC2V), and NKX2-5, are normally expressed in mutant embryos, indicating that the differentiation of primitive cardiomyocytes is not impaired due to GATA4-deficiency. Similarly, GATA4-deficient embryonic stem cells have the ability to differentiate into cardiomyocytes in vitro (Narita et al. 1997). It is noteworthy that GATA4-deficiency leads to upregulation of GATA6 in mouse embryos, suggesting that GATA6 may compensate for the lack of GATA4. At later time points of cardiac development, GATA4 regulates the morphogenesis of the right ventricle (Zeisberg et al. 2005). Late deletion of Gata4 results in marked myocardial thinning and decreased cardiomyocyte proliferation. Taken together, GATA4 appears to have an indispensable stage-dependent role in regulation of cardiac morphogenesis but is not essential for cardiomyocyte lineage commitment, in which GATA6 may be capable of replacing it.

2.5.3 GATA4 in cardiac hypertrophy

The involvement of GATA4 in the development of cardiac hypertrophy has been demonstrated by several lines of evidence. First, various hypertrophic stimuli including mechanical forces (aortic constriction and pressure overload in vivo, stretch in vitro) and G protein-coupled receptor agonists (e.g. Ang II, ET-1, phenylephrine, and isoprenaline) activate GATA4 DNA-binding (Hasegawa et al. 1997; Hautala et al. 2001; Kerkelä et al. 2002; Majalahti et al. 2007; Morimoto et al. 2000; Morisco et al. 2001; Pikkarainen et al. 2003). Second, a number of genes, whose expression is altered during cardiac hypertrophy, are mediated by GATA4. Hemodynamic overload induces transcription of the angiotensin II type 1a receptor and β-MHC encoding genes via GATA4 binding elements (Hasegawa et al. 1997; Herzig et al. 1997). Similarly, Ang II infusion and nephrectomy-induced haemodynamic stress activate BNP gene expression via GATA4 binding sites (Majalahti et al. 2007; Marttila et al. 2001). Isoprenaline increases nuclear accumulation of GATA4, while isoprenaline-induced transcription of the ANP gene is attenuated by dominant negative GATA4, demonstrating the role of GATA4 in β-adrenergic-induced ANP transcription (Morisco et al. 2001). Third, it has been shown that GATA4 alone is a sufficient inducer of the hypertrophic response both in vitro and in vivo (Liang et al. 2001a). GATA4 overexpression induces hypertrophic cell growth and gene expression in GATA4 transgenic mice. Similarly, overexpression of GATA4 by adenoviral gene transfer induces cardiomyocyte hypertrophy (Liang et al. 2001a) as well as sarcomere reorganization (Charron et al. 2001) in culture.
### 2.5.4 GATA4 in cell survival

Evidence suggests that the role of GATA4 also extends to the regulation of cardiomyocyte survival. For instance, anthracyclines (Aries et al. 2004; Bien et al. 2007; Esaki et al. 2008; Kim et al. 2003; Kobayashi et al. 2006; Koka et al. 2010; Riad et al. 2008), ischaemia (Suzuki et al. 2004), and mercury (Suzuki 2003) all act as apoptotic stimuli and mediate downregulation of GATA4 in cardiac muscle. The anthracycline-induced downregulation of GATA4 may occur via both transcriptional and post-translational mechanisms. Daunorubicin has been shown to inhibit GATA4 gene transcription via a p53-dependent mechanism (Park et al. 2011), whereas doxorubicin induces caspase-1-mediated degradation of GATA4 protein levels (Aries et al. 2014).

Apoptosis has been shown to increase in mice with conditional GATA4 knockout as well as in neonatal cardiomyocytes whose GATA4 levels have been reduced (Aries et al. 2004; Oka et al. 2006). On the other hand, overexpression of GATA4 prevents anthracycline-induced apoptosis in cardiomyocytes and HL-1 cardiac muscle cells in vitro (Aries et al. 2004; Kim et al. 2003). GATA4 overexpression also prevents myocardial infarction-induced apoptosis along with adverse remodelling in vivo in rats (Rysä et al. 2010). Moreover, the cardioprotective action of the α-adrenergic agonist phenylephrine against doxorubicin toxicity seems to depend on GATA4 (Aries et al. 2004). Phenylephrine protects cardiomyocytes from doxorubicin-induced apoptosis and depletion of GATA4, however, the effect is abrogated in cells lacking GATA. Overall, these findings demonstrate the significance of GATA4 as a regulator of cell death in cardiac muscle.

GATA4 serves as a cell survival factor by regulating the expression of the anti-apoptotic factors Bcl-xL and Bcl-2 (Aries et al. 2004; Kitta et al. 2003; Kobayashi et al. 2006). Adenoviral overexpression of GATA4 alone is enough to increase both mRNA and protein levels of Bcl-xL in cardiomyocytes in vitro (Aries et al. 2004). Moreover, the enhancement of Bcl-xL expression seems to be mediated by phosphorylation of GATA4 at serine 105 (Kitta et al. 2003). Similarly, GATA4 overexpression increases mRNA and protein levels of Bcl-2 both in vitro and in vivo in neonatal rat cardiomyocytes and transgenic mouse hearts (Kobayashi et al. 2006). In vitro GATA4 overexpression also abolishes doxorubicin-induced expression of autophagy-related genes, such as Beclin-1 encoding BCN1 (Kobayashi et al. 2010), which suggests that GATA4 may act as a regulator of both apoptosis and autophagy.

### 2.5.5 GATA4 in cardiac regeneration

The association of GATA4 to heart regeneration and repair in zebrafish was demonstrated by Kikuchi and colleagues (Kikuchi et al. 2010) in a study that identified a subpopulation of cardiomyocytes activating gata4 regulatory sequences
after injury. The expression of\( gata4 \) was triggered in cardiomyocytes throughout the epicardial ventricular layer within a week of trauma but was later localized to proliferating cardiomyocytes within the injury site. Inhibition of GATA4, on the other hand, blocked adult zebrafish heart regeneration and caused severe scarring at the injury site (Gupta et al. 2013). Along these lines, Witman and colleagues noticed increased GATA4 expression in distinct cell populations of newt hearts post-injury (Witman et al. 2011). The impact of GATA4 for mammalian neonatal heart regeneration, on the other hand, was demonstrated by Malek Mohammadi and colleagues, who showed that in neonatal mice cardiac GATA4 becomes strongly downregulated at P7 in parallel with loss of regenerative capacity (Malek Mohammadi et al. 2017). Moreover, loss of \( Gata4 \) entailed reduced cardiomyocyte proliferation and myocardial angiogenesis after cryoinjury at P0 in cardiomyocyte-specific \( Gata4 \) knockout mice. In contrast, overexpression of GATA4 promoted neonatal cardiomyocyte proliferation \textit{in vitro} and improved myocardial regeneration at P7 \textit{in vivo}, suggesting that postnatal GATA4 downregulation is indeed of crucial importance for the impaired regenerative capacity of the heart.

As adult mammalian heart possesses only limited regenerative potential, considerable effort has been done to develop novel approaches for induction of cardiac repair, one of which is reprogramming other cell types into cardiomyocytes (Xin et al. 2013). GATA4 is one of the critical transcription factors, along with MEF2C, TBX5, and HAND2, that are capable of cooperatively reprogramming fibroblasts into cardiomyocyte-like cells \textit{in vitro} and \textit{in vivo} (Ieda et al. 2010; Qian et al. 2012; Song et al. 2012).

### 2.5.6 GATA4 association with NKX2-5

NKX2-5 is a homeodomain-containing transcription factor, which is considered to be cardiac specific (Komuro and Izumo 1993). It is abundantly expressed in embryonic mouse heart and continues to be expressed at a high level in the heart through adulthood. It has an essential role in the regulation of normal cardiac development and mutations of the gene can cause congenital heart diseases (Akazawa and Komuro 2005). In mouse embryos lacking the \( Nkx2-5 \) gene, the heart tube formed normally but the process of looping morphogenesis was not initiated, which led to embryonic lethality (Lyons et al. 1995). Noticeably, commitment to the cardiac muscle lineage and the expression of most myofilament genes were not compromised. On the other hand, NKX2-5 is up-regulated in response to hypertrophic stimulation by adrenoreceptor agonists isoprenaline and phenylephrine (Saadane et al. 1999). Moreover, in transgenic mice overexpressing NKX2-5, transcription of \( ANP \) and \( BNP \) has been shown to increase while the size of the heart stays normal (Takimoto et al. 2000). Overall, the results indicate that NKX2-5 alone is not sufficient to induce cardiac hypertrophy but it regulates the cardiac gene programme in both embryonic and adult hearts.
GATA4 and NKX2-5 cooperate via the physical protein-protein interaction that takes place between the homeodomain of NKX2-5 and the C-terminal zinc finger of GATA4 (Durocher et al. 1997; Lee et al. 1998). First, they activate the ANP promoter synergistically in vitro (Durocher et al. 1997; Lee et al. 1998). Second, mutational studies utilising the BNP promoter have shown that the tandem GATA4 sites of the proximal promoter in combination with the NKX2-5 binding element are both required for stretch-induced BNP transcription (Pikkarainen et al. 2003). Mutation on the NKX2-5 binding site alone had no effect on the stretch response, whereas mutation on the GATA site decreased the stretch-response 40%, and in combination the mutations almost completely abolished the response. It seems that both transcription factors are required to fully activate the mechanical stretch-induced signalling in cardiomyocytes.

2.6 Protein kinase C

PKC is a group of ten related protein kinases, which phosphorylate serine and threonine residues on a large number of proteins and thus participate in various signalling pathways regulating, e.g. cell proliferation, differentiation, migration, gene transcription and translation, as well as cell death (Mochly-Rosen et al. 2012). The expression of different PKC isozymes varies between tissues and cells: some are ubiquitously expressed, whereas some are restricted to specific tissues (Webb et al. 2000). Moreover, the expression of a particular isozyme may vary between species (Rybin and Steinberg 1994; Schreiber et al. 2001; Shin et al. 2000), between developmental stages (Rybin and Steinberg 1994; Schreiber et al. 2001), as well as between normal and diseased states (Bowling et al. 1999; Simonis et al. 2007).

2.6.1 PKC isoform structure

All PKC isozymes share certain basic structural features: a C-terminal catalytic domain and an N-terminal regulatory domain (Steinberg 2008) (Fig. 2). Throughout the polypeptide chains there are a series of conserved (C1–C4) regions and variable (V) regions. The catalytic domains contain binding sites for adenosine triphosphate (ATP) and the phosphoacceptor sequence of the substrate proteins. The regulatory domains contain an autoinhibitory pseudosubstrate sequence whose occupation of the catalytic cleft maintains the enzyme in an inactive state and membrane targeting modules that control the subcellular localization of the enzyme. PKC isoforms are subclassified based on their regulatory region structure and activator requirements. The classical isoforms (cPKCs; α, alternatively spliced βI and βII, and γ) require both diacylglycerol (DAG) and calcium for their activation. Their regulatory domain contains a tandem C1A/C1B domain that is responsible for DAG binding and a C2 domain that binds anionic phospholipids in a calcium-dependent manner. The novel isoforms (nPKCs; δ, ε, η, and θ) are activated by DAG alone. They also have C1A/C1B and C2 domains; however, the C2 domain is insensitive to calcium. A more distant
A subgroup of atypical isoforms (αPKCs; ζ, η, and λ [the mouse homologue of human PKCλ]) does not respond to the same second messengers and shares less homology with the eight other isoforms. They lack a calcium-sensitive C2 domain and contain an atypical C1 domain that does not bind DAG, as well as a Phox and Bem 1 domain that functions as a protein binding module.

**Figure 2.** The domain structures of protein kinase C (PKC) family members (Steinberg 2008). PKC isoforms have a conserved catalytic domain containing motifs for adenosine triphosphate and substrate-binding (C3 and C4). The regulatory domains of classical and novel PKCs (cPKCs and nPKCs, respectively) contain tandem C1A/C1B domains that bind diacylglycerol (DAG), while atypical PKCs (aPKCs) have an atypical C1 domain (aC1) that is unresponsive to DAG. The C2 domain of cPKCs binds calcium, whereas in nPKCs the C2 domain (nC2) is insensitive to calcium. aPKCs contain a Phox and Bem 1 (PB1) domain, while all isoforms contain a pseudosubstrate motif (PS).

### 2.6.2 Activation of PKC

In the traditional model, inactive PKC isoforms localize to the cytosol and interact only weakly with membranes, however, elevation of second messenger levels (DAG and Ca²⁺ for cPKCs, DAG for nPKCs) leads to translocation of the enzyme from the cytosolic fraction to cellular membranes; a process generally considered as the hallmark of PKC activation (Mochly-Rosen et al. 2012; Steinberg 2008). The second messengers are generated following the binding of a variety of stimulators (hormones, growth factors, neurotransmitters) to their respective receptors and subsequent activation of phospholipase C (PLC) resulting in hydrolysis of phosphatidylinositol-4,5-bisphosphate (PIP₂) to DAG and inositol-1,4,5-triphosphate (IP₃). IP₃ further triggers the release of calcium from internal stores to the cytosol. Calcium binds to the C2 domain of cPKCs and increases its affinity for membranes. Binding of membrane-restricted DAG to the C1 domain of both cPKCs and nPKCs induces a conformational change that removes the autoinhibitory pseudosubstrate domain from the substrate-binding pocket. Additionally, in some cases the active conformation of PKC may further interact with receptors for activated C kinase (RACKs), which are membrane-associated anchoring proteins that localize the individual PKC isoforms to specific membrane compartments near their target.
substrates. In addition to the traditional receptor-dependent mechanism, different receptor-independent activation mechanisms involving proteolytic cleavage in response to exogenous stimuli have been identified for PKCα and PKCδ (Steinberg 2012). For instance, PKCδ undergoes caspase-3-dependent cleavage to generate a constitutively active catalytic fragment during apoptosis (Brodie and Blumberg 2003; Emoto et al. 1995; Ghayur et al. 1996).

2.6.3 PKC as drug target

Efforts to target PKC with pharmacological modulators have taken various approaches, including ATP-competitive small molecule inhibitors that bind to the ATP-binding site of the catalytic domain of the kinase; compounds that bind to the C1 domain, thus mimicking the binding of DAG; and peptide drugs that interfere with the protein–protein interactions between PKC and its cognate anchoring proteins (Mochly-Rosen et al. 2012). Because of the high degree of homology of the catalytic region among PKC isoforms and the similarity of the region in other protein kinases as well, generating selective inhibitors for the ATP-binding is challenging (Anastassiadis et al. 2011). A classic example of this is staurosporine, which inhibits all PKC isoforms (Wilkinson et al. 1993) but binds also other kinases (Anastassiadis et al. 2011). Similarly, the C1 domain and DAG binding site are highly homologous among PKC isoforms (Steinberg 2008), thus developing drugs that selectively bind to the C1 domain of only one isozyme is a challenge. Phorbol ester tumour promoters, such as phorbol 12-myristate 13-acetate (PMA), are classic examples of C1 domain ligands (Blumberg et al. 1984). Long-term PKC activation by PMA induces downregulation of PKC protein levels (Young et al. 1987), which is considered to cause the propensity for tumour-promotion (Antal et al. 2015; Newton and Brognard 2017). The third group of PKC modulators are the peptides derived from RACKs (Churchill et al. 2009; Mochly-Rosen and Gordon 1998). These peptides mimic either the PKC binding site on RACKs or the RACK binding site on PKC and act as isoenzyme-specific PKC modulators.

2.6.4 PKC in cardiac remodelling and heart failure

In the adult human heart, all PKC isoforms except for γ and θ have been detected (Shin et al. 2000; Simonis et al. 2007). The expression of all isoforms decreases during ontogenesis; PKCβ being downregulated the most (Simonis et al. 2007). In normal rat heart, the four main PKC isoforms that have been detected are α, δ, ε, and ζ, while the existence of PKCβ has been more debatable, possible due to low abundance and/or methodical differences between studies (Braun et al. 2002; Clerk et al. 1995; Inagaki et al. 2002; Kohout and Rogers 1993; Pucéat et al. 1994; Rybin and Steinberg 1994; Wang et al. 2003). Similarly, in mouse heart PKCα, PKCγ, PKCδ, PKCe, and PKCη have been detected by western blotting (Schreiber et al. 2001).
Based on RNA sequencing data, on the other hand, the main isoforms in mouse heart are \( \alpha, \delta, \epsilon, \eta, \) and \( \lambda \) (Talman et al. 2018a; Talman et al. 2018b).

In a rat model of pressure-overload-induced hypertrophy, aortic banding led to increased total PKC activity along with up-regulation of PKC\( \alpha \) (cytosolic fraction) and PKC\( \delta \) (cytosolic and particulate fraction) (Braun et al. 2002). On the other hand, when hypertensive Dahl salt-sensitive rats developed concentric hypertrophy, the expression and translocation of PKC\( \epsilon \) increased significantly (Inagaki et al. 2002). When the hypertrophy stage progressed to the ventricular dilatation and heart failure stage, the expression of PKC\( \epsilon \) returned to the control level while the expression of PKC\( \beta \text{I} \) and \( \beta \text{II} \) increased. Similarly, in another study conducted in Dahl salt-sensitive rats, the expression of PKC\( \epsilon \) decreased between hypertrophy and heart failure stages. However, in this study, hypertrophy was associated not only with increased expression of PKC\( \epsilon \) but also other isoforms, e.g. PKC\( \alpha \) and PKC\( \delta \) (Koide et al. 2003). Increased PKC activity and PKC\( \beta \text{II} \) levels have also been reported in the aorta and hearts of diabetic rats (Inoguchi et al. 1992). In human patients, aortic stenosis leads to up-regulation of several PKC isoforms except for PKC\( \beta \) (Simonis et al. 2007), while end-stage dilated cardiomyopathy leads to upregulation of PKC\( \beta \) (Bowling et al. 1999; Simonis et al. 2007). Overall, the literature suggests a differential recruitment of PKC isoforms in various forms of myocardial hypertrophy and heart failure.

Cardiac actions of individual PKC isoforms have been investigated using genetic knockdown and overexpression models, as well as pharmacological inhibitors. Studies in genetic models of PKC\( \alpha \) overexpression and knockout in mice identified the role of PKC\( \alpha \) in the regulation of cardiac contraction (Braz et al. 2004). Overexpression of PKC\( \alpha \) reduced cardiomyocyte contractility, which preceded the gradual manifestation of cardiac hypertrophy, whereas \( Prkca^{+/−} \) mice showed increased cardiac function. PKC\( \alpha \)-dependent changes in contraction were attributed to indirect regulation of phospholamban phosphorylation and sarco/endoplasmic reticulum Ca\(^{2+} \) ATPase 2 (SERCA2) activity, leading to alterations in Ca\(^{2+} \) homeostasis. Specifically, in ischaemic hearts, proteolytic processing of PKC\( \alpha \) by calpain may activate pathological signalling through generation of an unregulated catalytic fragment that exhibits promiscuous kinase activity (Kang et al. 2010). Calpain-1 cleaves PKC\( \alpha \) within the V3 hinge region generating a C-terminal fragment, which was shown to induce abnormal myocardial protein phosphorylation compared to the parent PKC\( \alpha \). While expression of PKC\( \alpha \) induced only mild cardiomyopathy, expression of the C-terminal PKC\( \alpha \) fragment induced severe ventricular dilatation and contractile dysfunction.

Transgenic mice overexpressing PKC\( \beta \text{II} \) developed cardiomyopathy that was characterised by histological evidence of cardiac fibrosis as well as functional evidence of decreased left ventricular performance (Wakasaki et al. 1997). Cardiomyocytes isolated from PKC\( \beta \text{II} \) overexpressing mice showed mechanical
dysfunction, which was improved after exposure to the PKCβ inhibitor ruboxistaurin (LY333531) (Takeishi et al. 1998). The degree of phosphorylation of cTnI was significantly increased in transgenic mice compared to wild type, indicating that PKCβII-mediated phosphorylation of cTnI may decrease myofilament Ca\(^{2+}\) responsiveness and contractility. However, although PKCβ activation may drive pathological remodelling, it seems that, at least in mice, PKCβ is not required for the development of cardiac hypertrophy, as PKCβ knockout mice demonstrated a hypertrophic response to both aortic banding and phenylephrine infusion comparable to that of control animals (Roman et al. 2001). Overall, overexpression of PKCβII has rather been linked to the induction of profibrotic cytokines, such as CTGF, and development of cardiac fibrosis (Way et al. 2002). Ruboxistaurin was shown to decrease TGF-β mRNA expression and to reduce myocardial fibrosis and dysfunction after myocardial infarction in rats (Boyle et al. 2005). Ruboxistaurin also showed antifibrotic effects in a rat diabetic cardiomyopathy model (Connelly et al. 2009), as well as in mice subjected to pressure overload (Liu et al. 2009). Finally, selective inhibition of PKCβII with a translocation peptide inhibitor (βIIIV5-3) suppressed myocardial fibrosis, inflammation and cardiomyocyte hypertrophy, as well as improved cardiac function and prolonged survival in a rat model of hypertension-induced cardiac dysfunction (Ferreira et al. 2011). Treatment with the PKCβII inhibitor also corrected the changes detected in Ca\(^{2+}\)-handling proteins (SERCA2 and Na\(^+\)/Ca\(^{2+}\) exchanger) and cTnI phosphorylation in these hypertensive rats. Simultaneously, selective inhibition of PKCβI with the peptide inhibitor βIV5-3 had no effect on these parameters.

PKCS and PKCe are related PKC family members, which in some situations exhibit cross-regulation while in other situations have opposing actions. Studies utilising selective activator and inhibitor peptides showed that these two novel isoforms have opposing cardiac actions in ischaemia-reperfusion injury (Chen et al. 2001; Inagaki et al. 2003; Inagaki et al. 2005). Selective inhibition of PKCS with δVI-1 or selective activation of PKCe with ψeRACK both protected cardiomyocytes from ischaemic damage in vitro as well as reduced infarct size and improved cardiac function in vivo in a porcine model of myocardial infarction. PKCS activation mediates cardiac damage via activation of mitochondrial pyruvate dehydrogenase kinase (PDK), which inhibits pyruvate dehydrogenase, thereby causing reduction in ATP production (Mochly-Rosen et al. 2012). Mitochondrial dysfunction leads to enhanced reactive oxygen species (ROS) production and accumulation of toxic aldehydes, further leading to induction of both apoptosis and necrosis. PKCe activation, on the other hand, protects mitochondrial functions via activation of mitochondrial aldehyde dehydrogenase 2, which removes toxic aldehydes, thus reducing cellular damage.

The reports regarding the role of PKCS and PKCe in cardiac hypertrophy and heart growth have been even more conflicting. In vitro, pharmacological activation of nPKCs increases hypertrophic gene expression in neonatal rat ventricular myocytes
(NRVMs) and hiPSC-CMs (Pohjolainen et al. 2021). In hypertensive rats, expression of PKCε increases after development of left ventricular hypertrophy (Inagaki et al. 2002). However, PKCε is not required for the development of pressure overload-induced hypertrophy as thoracic aortic constriction induced similar hypertrophic responses in PKCε knockout and wild-type mice (Klein et al. 2005). Moreover, PKCε knockout was associated with increased fibrosis and elevated expression of collagen I and III along with compensatory upregulation of PKCδ and increased activation of p38 MAPK and c-Jun N-terminal kinase (JNK) signalling pathways, which suggests that myocardial fibrosis in knockout mice is likely mediated via activation of PKCδ as well as through cross-regulation by PKCε and PKCδ isoforms. On the other hand, pharmacological inhibition of PKCε with a selective translocation peptide inhibitor εV1-2 reduced fibrosis and improved cardiac function in hypertensive Dahl rats as well as inhibited collagen secretion from cultured primary cardiac fibroblasts stimulated with TGF-β (Inagaki et al. 2008), while pharmacological inhibition of PKCδ with δV1-1 promoted neonatal rat cardiac fibroblast proliferation in vitro (Braun and Mochly-Rosen 2003). Lastly, Song and colleagues showed that PKCε and PKCδ have a central role in limiting both developmental and stress-reactive heart growth (Song et al. 2015). Combined cardiomyocyte specific deficiency of these isoforms provoked exaggerated hypertrophy, adverse remodelling, and cardiac dysfunction in adult mouse hearts subjected to haemodynamic overloading as well as evoked cardiomyocyte proliferation in embryonic mouse hearts, while deficiency of either isoform alone had only a slight effect.

2.7 Small animal models of heart failure

In vivo models cannot be totally replaced when studying cardiac function and the cardiovascular effects of drugs. Numerous animal models have been used to mimic different pathological mechanisms that contribute to heart failure. However, there is no ideal model available for cardiac research, as each model is accompanied with its own advantages and disadvantages (Milani-Nejad and Janssen 2014; Riehle and Bauersachs 2019). Small animal (mouse and rat) models have some unique properties which make them especially valuable to cardiovascular research. For instance, mice and rats are easier to handle and house, have shorter gestation times and lifespans, and have lower maintenance costs compared to larger animals. Moreover, genetic modifications using viral vectors are usually easier to introduce into the genome of small rodents compared to larger animals. On the other hand, a major advantage of using mice compared to rats is the availability of numerous transgenic strains as well as readily available tools to generate novel ones. However, despite these several advantages there are also limitations that should be considered. For instance, in vivo studies are typically conducted in animals with a defined genetic background, which does not reflect the genetic heterogeneity of the human patient population. Moreover, in several experimental models young healthy animals are exposed to the stressor rapidly (especially in surgical models), which is in contrast to
the typically slow disease progression in humans with comorbidity. There are also some prominent differences in rodent and human hearts that should be kept in mind when utilising mouse and rat models. Many of the differences result from difference in heart rate, which is inversely correlated to body weight. While humans have a heart rate of approximately 60–80 beats per minute (bpm), the rate in rats is approximately 300–400 bpm, and in mice 500–600 bpm. In order to maintain cardiac output at high heart rates, hearts of smaller animals contract and relax more rapidly compared to larger animals. This further associates with differences in myofilament protein isoforms and calcium handling. While mouse ventricular cardiomyocytes express the fast α-MHC, human ventricular cardiomyocytes predominantly express the slow β-MHC (Reiser and Kline 1998; Reiser et al. 2001). Similarly, while in mouse the contribution of SERCA to calcium removal from the cytosol during relaxation is >90%, in human SERCA contributes 77% and the Na+/Ca2+ exchanger 23% (Li et al. 1998; Piacentino et al. 2003).

Some of the commonly used small animal models of heart failure are listed and summarized in Table 1. These models utilise surgical and pharmacological approaches, and genetic modifications to study HFrEF or HFpEF. Additional models with, for instance, genetic gain-of-function and loss-of-function modifications have been developed. Surgical models (Table 1) of ischaemic injury, left ventricular pressure overload, and volume overload, as well as toxic cardiomyopathy models (e.g. doxorubicin-induced cardiotoxicity) typically provoke HFrEF. The models of hypertension, obesity, and diabetes mellitus, on the other hand, are commonly used to study HFpEF. It should be noted, however, that the pathogenesis of HFrEF and HFpEF are multifactorial and often overlapping, thus drawing a strict line between the models is not possible. Some of the commonly used models of HFrEF induce also HFpEF, which precedes the later onset of HFrEF. Similarly, systolic contractile dysfunction may also be present in the commonly used models of HFpEF.
Table 1. Generally used small animal models of heart failure (Houser et al. 2012; Katz et al. 2019; Milani-Nejad and Janssen 2014; Riehle and Bauersachs 2019).

<table>
<thead>
<tr>
<th>Model</th>
<th>Induction of heart failure</th>
<th>Causes and associated features</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ischaemic injury</td>
<td>Permanent LAD ligation</td>
<td>MI, DCM</td>
</tr>
<tr>
<td></td>
<td>Temporary LAD ligation (I/R)</td>
<td>MI, DCM</td>
</tr>
<tr>
<td>Pressure overload</td>
<td>Ascending aortic constriction (acute increase in LV overload)</td>
<td>Aortic stenosis, hypertrophy</td>
</tr>
<tr>
<td></td>
<td>Transverse aortic constriction (gradual increase in LV overload)</td>
<td>Aortic stenosis, hypertrophy</td>
</tr>
<tr>
<td></td>
<td>Abdominal aortic constriction (reduced renal blood flow)</td>
<td>Aortic stenosis, gradual progression of heart failure</td>
</tr>
<tr>
<td>Volume overload</td>
<td>Aorta-venous shunt</td>
<td>Progressive ventricular dilatation</td>
</tr>
<tr>
<td>Toxic cardiomyopathy</td>
<td>Doxorubicin (oxidant stress, mitochondrial dysfunction)</td>
<td>DCM</td>
</tr>
<tr>
<td></td>
<td>Isoprenaline (chronic stimulation of β-adrenergic receptor signalling)</td>
<td>Hypertrophy, DCM</td>
</tr>
<tr>
<td>Hypertension</td>
<td>Angiotensin II infusion (chronic stimulation of AT1R signalling)</td>
<td>Slow progression of hypertension and heart failure</td>
</tr>
<tr>
<td></td>
<td>Dahl salt-sensitive rats fed with high-salt diet</td>
<td>Slow progression of hypertension and heart failure</td>
</tr>
<tr>
<td>Metabolic syndrome</td>
<td>ob/ob mice with leptin deficiency</td>
<td>Obesity, T2D, diastolic dysfunction</td>
</tr>
<tr>
<td></td>
<td>db/db mice with leptin resistance</td>
<td>Obesity, T2D, diastolic dysfunction</td>
</tr>
<tr>
<td></td>
<td>High-dose STZ (pancreatic β-cell failure)</td>
<td>T1D</td>
</tr>
<tr>
<td></td>
<td>High-calorie diet + low-dose STZ (pancreatic β-cell failure)</td>
<td>Late stage T2D</td>
</tr>
</tbody>
</table>

LAD, left anterior descending artery; I/R, ischaemia/reperfusion; MI, myocardial infarction; DCM, dilated cardiomyopathy; LV, left ventricle; AT1R, angiotensin II type 1 receptor; ob/ob, mutation in the obese (ob) gene encoding leptin; db/db, mutation in the diabetes (db) gene encoding leptin receptor; T2D, type 2 diabetes; T1D, type 1 diabetes; STZ, streptozotocin
In the present study, an experimental model of doxorubicin-induced cardiomyopathy was used. Doxorubicin belongs to the group of anthracyclines which have a prominent role in treating various forms of cancer (solid tumours and leukaemia), albeit its clinical applications are limited by dose-dependent cardiotoxicity (Corremans et al. 2019). Anthracyclines can cause acute, subacute, or late toxicity (Senkus and Jassem 2011). Acute morbidity (typically supraventricular arrhythmia and transient LV dysfunction) develops immediately after infusion and tends to be reversible. The subacute form of morbidity develops within a few weeks, clinically resembling myocarditis. These two forms are, however, rare (<5%). Clinically the most significant form is the chronic cardiotoxicity, which leads to congestive heart failure. The symptoms may appear during the first year post-treatment or only after 10–20 years. In clinical use, doxorubicin is usually administered at the doses of 40–90 mg/m² (4–8 cycles) as continuous (48–72 h) or bolus (15 min) infusions every third week (Vejpongsa and Yeh 2014). For doxorubicin, the incidence rate of heart failure rises over 5% when the cumulative lifetime dose exceeds 400 mg/m² and increases up to 48% at 700 mg/m² (Zamorano et al. 2017). The exact mechanism of anthracycline-induced cardiotoxicity remains unclear. However, topoisomerase IIα-mediated DNA double-strand breaks, leading to mitochondrial dysfunction, generation of reactive oxygen species, and apoptosis, seem to play important roles (Renu et al. 2018; Zhang et al. 2012).

In order to be sufficiently clinically relevant, preclinical studies of late doxorubicin cardiotoxicity should be long-term studies in which the effects require weeks to appear (Corremans et al. 2019; Gianni et al. 2008). However, this is often not the case. Many of the studies investigating the mechanisms of doxorubicin cardiotoxicity have evaluated in vitro and in vivo effects, which appear within hours or days. Usually, these studies also have used relatively high drug concentrations. For instance, the role of GATA4 in doxorubicin cardiotoxicity has been studied using models where mice were given a single 15 mg/kg or 20 mg/kg dose of doxorubicin i.p. and neonatal cardiomyocytes were exposed to 300 nM or 1 μM doxorubicin for ≤24 h (Aries et al. 2004; Kobayashi et al. 2006). The clinical value of these studies has been questioned, since they simulate acute cardiotoxicity (Corremans et al. 2019; Gianni et al. 2008).

### 2.8 In vitro cardiomyocyte models

In contrast to in vivo models, cell culture models have the advantage of allowing more precise control of environmental conditions and manipulations (Parameswaran et al. 2013; Peter et al. 2016). Study of a single cell type instead of the whole heart makes it possible to attribute findings to a specific cell type rather than interactions with other cardiac cell types. In cardiac research, the most commonly used in vitro cardiomyocyte models include primary cardiomyocytes, immortalized cardiac cell lines, and cardiomyocytes derived from embryonic stem cells (ESCs) or hiPSCs.
2.8.1 Primary cardiomyocytes

Isolated cardiomyocytes from small animals provide a valuable tool for studying cellular level functions and regulation of electrophysiology, intracellular Ca\textsuperscript{2+}-handling, contractile mechanics, and protein expression (Parameswaran et al. 2013). They also enable mechanistic studies of compounds that are toxic to cardiomyocytes. NRVMs and neonatal mouse ventricular myocytes have been popular in in vitro cardiac research as they are cost-effective, relatively easy to isolate, and have a great ratio of cell number per animal (Peter et al. 2016). Neonatal cardiomyocytes beat spontaneously in culture (Peter et al. 2016) and respond to hypertrophic stimuli, including epinephrine, norepinephrine, isoprenaline, phenylephrine, ET-1, and mechanical stretch (Huang et al. 2015; Menaour et al. 2014; Sadoshima et al. 1993; Simpson et al. 1982; Simpson 1983; Simpson 1985). Their disadvantage, however, is the immature phenotype. Moreover, the cultures are typically overgrown by proliferating non-myocytes after a few days following isolation, thus limiting their usage (Parameswaran et al. 2013). A major advantage adult primary cardiomyocytes have over neonatal cardiomyocytes is their mature phenotype (Peter et al. 2016). However, they do not beat spontaneously in culture and the isolation is technically more challenging compared with neonatal cells. Moreover, their morphology and functional characteristics change over the course of few days, which limits their utility for investigation of phenotypes (Louch et al. 2011; Zhou et al. 2000).

2.8.2 Immortalized cardiac cell lines

As primary cardiomyocytes cannot be passaged and their phenotype changes over time, attempts have been made to develop immortalized cardiac cell lines (Parameswaran et al. 2013; Peter et al. 2016). Although they enable homogenous and rapidly expanded cultures, these cell lines do not precisely recapitulate the structural and functional physiology of primary cardiomyocytes. The most commonly used immortalized cardiac muscle cell lines are AT-1, HL-1, and H9c2 cells. The AT-1 cells, derived from mouse atrial cardiomyocyte tumours, do contract spontaneously in culture; however, their atrial phenotype and immature sarcomeres limit their utility (Delcarpio et al. 1991; Lanson et al. 1992). Moreover, they cannot undergo freeze-thaw cycles and exhibit very limited capacity for passaging in culture (Lanson et al. 1992; Peter et al. 2016). The HL-1 cell line, derived from the AT-1 cell lineage, can be recovered from frozen stocks and passaged indefinitely (Claycomb et al. 1998). They maintain their phenotype and contractile activity in culture media supplemented with retinoic acid and norepinephrine. The gene expression pattern of HL-1 cells resembles that of adult mouse atrial myocytes while morphologically they resemble immature mitotic cardiomyocytes. The H9c2 cell line, derived from embryonic rat ventricular tissue, has characteristics of both cardiac and skeletal muscle cells (Ménard et al. 1999). The cardiac phenotype can be enhanced and maintained with chronic retinoic acid treatment. H9c2 cells respond to hypertrophic stimuli (Watkins
et al. 2011); treatment with Ang II or ET-1 induced similar increases in cell surface area and hypertrophic gene expression, including upregulation of BNP, in both H9c2 cells and NRVMs. However, H9c2 cells do not beat spontaneously and they display immature sarcomere organization; therefore their utility in contraction-based studies is limited. A comparative study between HL-1 and H9c2 cells suggested that in regard to energy metabolism H9c2 cells are more similar to primary cardiomyocytes than are HL-1 cells (Kuznetsov et al. 2015).

### 2.8.3 Human pluripotent stem cell-derived cardiomyocytes

**Induced pluripotent stem cells.** iPSCs are generated by reprogramming somatic cells into a state where they hold the developmental capacity to generate all cell types in the body (Karagiannis et al. 2019). The iPSC technology was pioneered by Dr. Shinya Yamanaka’s research group who showed that by retrovirally introducing four transcription factors OCT3/4 (octamer-binding transcription factor 3/4), SOX2 (sex determining region Y box 2), KLF4 (Kruppel-like factor 4), and c-MYC into mouse fibroblasts they could generate ESC-like cells (Takahashi and Yamanaka 2006). The following year, they showed that the same four factors are also sufficient to generate hiPSCs (Takahashi et al. 2007). Approximately at the same time, Dr. James Thomson’s research group demonstrated their own hiPSCs by lentivirally introducing OCT3/4, SOX2, NANOG, and LIN28 into human fibroblasts (Yu et al. 2007). Since these landmark studies, various other approaches have been established for producing iPSCs more efficiently, including virus, DNA, RNA, and protein-based reprogramming methods and varying cocktails of reprogramming factors (Karagiannis et al. 2019). Moreover, the number of cell sources and species that have been used to produce iPSCs is increasing. Human iPSCs provide a unique tool for disease modelling, drug discovery, and cell therapy development (Shi et al. 2017). The advantages hiPSCs have over traditional cell-based methods include their human origin, easy accessibility, expandability, avoidance of ethical concerns associated with human ESCs, potential to give rise to almost any cell type, and the potential of developing patient specific iPSCs. Moreover, the combination of hiPSCs with recent developments in gene-editing technologies and 3D organoids makes hiPSC-based platforms even more powerful. Finally, the potential applicability of hiPSCs for regenerative medicine has attracted considerable interest. The overwhelming enthusiasm for the use of hiPSCs in advancing basic and clinical research has, however, raised concerns regarding how different these cells are from ESCs. Based on current knowledge, hiPSCs and ESCs have similar molecular and functional characteristics, with possible differences rising from different genetic backgrounds (Sayed et al. 2016). They express similar markers, have self-renewal capacity, and differentiate into the three embryonic germ layers (Avior et al. 2016). However, the epigenetic memory of hiPSCs, different DNA methylation profiles and a different extent of genetic aberrations, may lead to some differences between hiPSCs and ESCs.
Cardiomyocyte differentiation from pluripotent stem cells. Human PSCs are capable of differentiating into beating cardiomyocytes through exposure to various stimuli. In vitro the differentiation process mimics the sequential stages of embryonic cardiac development, which are controlled by three families of growth factors, bone morphogenetic proteins (BMPs), wingless-related integration site proteins (Wnts), and fibroblast growth factors (FGFs) (Mummery et al. 2012). Differentiation via embryoid body (EB) formation in a serum-containing culture medium was the first method described for producing beating cardiomyocytes from human stem cell sources (Kehat et al. 2001). The same EB method, originally devised using ESCs, was later used for producing hiPSC-derived cardiomyocytes as well (Zhang et al. 2009). These cells show downregulation of pluripotency genes (OCT4, NANOG), upregulation of several cardiac genes (e.g. NKKX2-5), and stain positive for sarcomeric myosin light and heavy chains, cardiac troponin, and α-actinin following 60 days in culture. Electrophysiological recordings show three types of action potentials characteristic of ventricular, atrial, and nodal cells (predominantly ventricular). This method is, however, rather inefficient as spontaneously beating areas were found in <10% of the EBs. As a means to offer more controllable and reproducible environments in which defined growth factors can be applied, monolayer differentiation techniques for cardiomyocyte production have been developed (Mummery et al. 2012). In these 2-dimensional systems, application of activin A and BMP4 growth factors in RPMI-B27 medium supplemented with a B27 component mix was proven efficacious, yielding >30% contracting cardiomyocytes (Laflamme et al. 2007; Yang et al. 2008; Zhu et al. 2011).

Next, it was demonstrated that temporal modulation of Wnt signalling with a GSK-3 inhibitor and Wnt signalling inhibitors is sufficient to produce a high yield of almost pure functional cardiomyocytes in 14 days (Lian et al. 2013). This approach was further optimized by Burrige and colleagues (Burrige et al. 2014), who developed a differentiation strategy using a chemically defined medium consisting of three components. Using this medium in conjunction with small molecule-based induction of differentiation and lactate-based enrichment (Tohyama et al. 2013) of hiPSC-derived cardiomyocytes led to the generation of up to 95% TNNT2 positive (cTnT encoding gene) cells (Burrige et al. 2014). They also demonstrated that the essential signalling pathways for early mesoderm induction include FGF, activin-Nodal, BMP, and Wnt, as inhibition of these pathways impeded efficient differentiation. After mesoderm induction, however, inhibition of these pathways had no effect on the differentiation efficiency. Characterisation of the cells revealed that the expression of early cardiomyocyte markers (e.g. GATA4) increased from days 5–6, the expression of later markers (NKKX2-5, TBX5, and MEF2C) peaked at days 8–9, and finally the expression of myofilament encoding genes TNNT2 and MYH6 (α-MHC encoding gene) peaked at days 8–10. At day 20, many cells coexpressed markers of atrial, ventricular, and nodal cardiomyocytes, suggesting that at this point the cell population still consisted of unspecified cardiomyocytes. Further analysis of MLC2
isoform expression (atrial MLC2A and ventricular MLC2V) and electrophysiological characteristics demonstrated that after day 30, the cardiomyocytes begin to have a more heterogeneous phenotype, with ventricular-like cells becoming the predominant type. Atria-like specification can be promoted, for instance, by modulating retinoic acid signalling during the differentiation process (Lee et al. 2017).

**Cardiomyocyte maturation.** Today, hiPSC-CMs can be produced routinely with high yield and purity, solving the problems related to inter-species comparisons when using murine cardiomyocytes. However, the differentiation status of hiPSC-CMs is still underdeveloped, as they lack some of the structural and functional characteristics of primary human cardiomyocytes, suggesting they resemble more foetal than adult cardiomyocytes (Karbassi et al. 2020). Cardiomyocytes undergo major structural and functional changes during heart development, particularly during the neonatal period. Foetal cardiomyocytes proliferate actively and are adapted to the hypoxic intrauterine environment. At birth they undergo changes that are linked to greater availability of oxygen and need for more efficient pump function. Moreover, they gradually lose their proliferative capacity after which the further growth of the heart occurs primarily by increasing the size of the cardiomyocytes. A summary of differences between mature human cardiomyocytes and immature hPSC-CMs is presented in Table 2. In order to enhance hPSC-CM maturation, several different approaches have been presented, such as long-term culture (Snir et al. 2003), *in vivo* transplantation in rat hearts (Kadota et al. 2017), biophysical cues (e.g. substrate stiffness and shape) (Ribeiro et al. 2015), culturing as 3D engineered heart tissue (Ulmer et al. 2018), and the addition of factors, e.g. tri-iodo-L-thyronine (Yang et al. 2014). However, despite considerable progress and advances, producing hiPSC-CMs with an adult-like phenotype is still not possible *in vitro*. Different approaches induce different features of maturation, suggesting that a combinatorial use of approaches might be beneficial. The immaturity of hiPSC-CMs should be kept in mind also when utilising these cells for drug screening as their phenotype might have an effect on drug responses. On the other hand, the degree of hiPSC-CM maturity that is needed for testing drug responses might vary depending on the question of interest. For instance, it has been shown that drug-induced arrhythmias (Navarrete et al. 2013), as well as the predilection of breast cancer patients to doxorubicin-induced cardiotoxicity (Burridge et al. 2016) can be recapitulated in hiPSC-CMs. Overall, hiPSC-CM-based drug screening and toxicity assays have shown good accuracy in evaluation of drug toxicity (Doherty et al. 2015; Sirenko et al. 2013). These studies for their part demonstrate the opportunities hiPSC-CMs have as a drug screening technology.
Table 2. Comparison of mature cardiomyocytes and human pluripotent stem cell-derived cardiomyocytes (hPSC-CMs) (Denning et al. 2016; Karbassi et al. 2020; Paik et al. 2020; Sayed et al. 2016; Yang et al. 2014).

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Mature cardiomyocytes</th>
<th>hPSC-CMs</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Morphology</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Size</td>
<td>Large</td>
<td>Small</td>
</tr>
<tr>
<td>Shape</td>
<td>Rod-shaped</td>
<td>Circular or polygonal</td>
</tr>
<tr>
<td>Nucleus</td>
<td>Multinucleated</td>
<td>Mononucleated</td>
</tr>
<tr>
<td><strong>Sarcomeres</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sarcomere alignment</td>
<td>Organised</td>
<td>Disorganised</td>
</tr>
<tr>
<td>Sarcomeric protein MHC</td>
<td>β (MYH7) &gt;&gt; α (MYH6)</td>
<td>Mixed</td>
</tr>
<tr>
<td>Sarcomeric protein titin</td>
<td>N2B &gt; N2BA</td>
<td>N2BA &gt; N2B</td>
</tr>
<tr>
<td>Sarcomeric protein troponin I</td>
<td>cTnI (TNNI3)</td>
<td>ssTnI (TNNI1)</td>
</tr>
<tr>
<td><strong>Calcium handling</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>T-tubules</td>
<td>Present</td>
<td>Absent</td>
</tr>
<tr>
<td><strong>Electrophysiology</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Resting membrane potential</td>
<td>–80 to –90 mV</td>
<td>–20 to –60 mV</td>
</tr>
<tr>
<td>Upstroke velocity</td>
<td>150–350 V/s</td>
<td>10–50 V/s</td>
</tr>
<tr>
<td>Spontaneous beating</td>
<td>Absent</td>
<td>Exhibited</td>
</tr>
<tr>
<td>Conduction velocity</td>
<td>Faster (0.3–1.0 m/s)</td>
<td>Slower (=0.1 m/s)</td>
</tr>
<tr>
<td>Gap junction distribution</td>
<td>Polarized</td>
<td>Circumferential</td>
</tr>
<tr>
<td><strong>Mitochondrial bioenergetics</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mitochondrial network</td>
<td>Regular, 20–40% of cell volume</td>
<td>Irregular, small fraction of cell volume</td>
</tr>
<tr>
<td>Metabolic substrate</td>
<td>Fatty acid (β-oxidation)</td>
<td>Glucose (glycolysis)</td>
</tr>
<tr>
<td><strong>Proliferative capacity</strong></td>
<td>Low</td>
<td>Declines within a month</td>
</tr>
</tbody>
</table>

MHC, myosin heavy chain; cTnI, cardiac troponin I; ssTnI, slow skeletal muscle troponin I
3 AIMS OF THE RESEARCH

The main aim of the present study was to characterise the properties of GATA4- and PKC-targeted small molecule compounds and the potential of using them for the induction of cardiac regeneration and inhibition of cardiac remodelling. More specifically, the objectives were:

1. To investigate the \textit{in vitro} toxicity and structure–toxicity relationships of novel GATA4-targeted compounds and to compare different cardiac and stem cell types for toxicity screening.
2. To establish a chronic doxorubicin-induced cardiotoxicity model using hiPSC-CMs.
3. To investigate if a GATA4-targeted compound can protect cardiomyocytes from doxorubicin-induced toxicity \textit{in vitro}.
4. To characterise the potential of targeting PKC for the inhibition of cardiac fibrosis and to elucidate the roles of classical and novel PKC isoforms in fibroblast activation and proliferation.
4 MATERIALS AND METHODS

A summary of the experimental methods used in the original publications is presented in Table 3.

Table 3. Summary of the experimental methods.

<table>
<thead>
<tr>
<th>Experimental method</th>
<th>Study</th>
</tr>
</thead>
<tbody>
<tr>
<td>LDH assay</td>
<td>I, III</td>
</tr>
<tr>
<td>MTT assay</td>
<td>I, I, III</td>
</tr>
<tr>
<td>Automated fluorescence microscopy and high-content analysis</td>
<td>I, I, II, III</td>
</tr>
<tr>
<td>Non-automated fluorescence microscopy and phenotypic analysis</td>
<td>III</td>
</tr>
<tr>
<td>Quantitative real-time PCR</td>
<td>III</td>
</tr>
<tr>
<td>Western blotting</td>
<td>III</td>
</tr>
<tr>
<td>AlphaLISA® immunoassay</td>
<td>III</td>
</tr>
</tbody>
</table>

LDH, lactate dehydrogenase; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; PCR, polymerase chain reaction

4.1 Compounds

The small molecule compound 3i-1000 (Table 4), which inhibits GATA4 and NKX2-5 interaction (Välimäki et al. 2017), was purchased from Pharmatory LTD (Oulu, Finland). The other derivatives targeting GATA4 and its interaction with NKX2-5 were synthesized at the Division of Pharmaceutical Chemistry and Technology, Faculty of Pharmacy, University of Helsinki (Finland). The PKC-targeted isophthalic acid derivatives HMI-1a3 and HMI-1b11 were designed and synthesized at the Division of Pharmaceutical Chemistry and Technology, Faculty of Pharmacy, University of Helsinki (Boije af Gennäs et al. 2009). PMA and bryostatin-1 were purchased from Sigma-Aldrich. The pan-PKC inhibitor Gö6983 and the inhibitor of classical PKC isoforms, Gö6976, were bought from Merck Millipore. The small molecule inhibitors Y-27632, CHIR99021, and Wnt-C59 used in hiPSC-CM differentiation, as well as doxorubicin hydrochloride, were purchased from Tocris Bioscience. All drugs were dissolved in dimethyl sulfoxide (DMSO) and stored at -20 °C, with the exception of doxorubicin hydrochloride stocks, which were stored at -80 °C. The molecular structures of the GATA4-targeted compounds and the isophthalate derivatives are presented in Figure 3.
Table 4. Summary of the pharmacological tools and experimental compounds used in the study.

<table>
<thead>
<tr>
<th>Compound name</th>
<th>Description</th>
<th>PubChem CID</th>
</tr>
</thead>
<tbody>
<tr>
<td>3i-1000*</td>
<td>Inhibits GATA4 and NKX2-5 interaction</td>
<td>753168</td>
</tr>
<tr>
<td>HMI-1a3</td>
<td>Activates protein kinase C</td>
<td>44186531</td>
</tr>
<tr>
<td>HMI-1b11</td>
<td>Activates protein kinase C</td>
<td>44186619</td>
</tr>
<tr>
<td>Phorbol 12-myristate 13-acetate</td>
<td>Activates protein kinase C</td>
<td>27924</td>
</tr>
<tr>
<td>Bryostatin-1</td>
<td>Activates protein kinase C</td>
<td>5280757</td>
</tr>
<tr>
<td>Gö6976</td>
<td>Inhibits classical PKC isoforms</td>
<td>3501</td>
</tr>
<tr>
<td>Gö6983</td>
<td>Inhibits classical and novel PKC isoforms</td>
<td>3499</td>
</tr>
<tr>
<td>Y-27632</td>
<td>Selective inhibitor of Rho-associated protein kinase</td>
<td>448042</td>
</tr>
<tr>
<td>CHIR99021</td>
<td>Selective inhibitor of glycogen synthase kinase 3, acts as Wnt activator</td>
<td>9956119</td>
</tr>
<tr>
<td>Wnt-C59</td>
<td>Inhibits Wnt signalling pathways</td>
<td>57519544</td>
</tr>
<tr>
<td>Doxorubicin hydrochloride</td>
<td>Cardiotoxic antineoplastic drug</td>
<td>443939</td>
</tr>
</tbody>
</table>

*The other derivatives targeting GATA4–NKX2-5 interaction are presented in Figure 3A.

4.2 Cell cultures

All cell cultures were maintained at 37 °C in a humidified atmosphere of 5% carbon dioxide (CO₂). For compound exposures, regular growth medium was used.

4.2.1 Human induced pluripotent stem cells

The induced pluripotent stem cell line iPS(IMR90)-4 (Yu et al. 2007) was purchased from WiCell (Madison, Wisconsin, USA). The cells were cultured in Essential 8™ medium (E8) on 6-well plates coated with Matrigel® (1:50) and passaged every fourth day at a 1:15 split ratio (Fig. 4). For passaging and seeding the cells were dissociated with Versene® and resuspended in E8 containing 10 μM Rho-associated protein kinase (ROCK) inhibitor Y-27632.
Figure 3. Molecular structures of (A) the GATA4-targeted compounds and (B) the PKC-targeted isophthalate derivatives.
4.2.2 Human induced pluripotent stem cell-derived cardiomyocytes

Cardiomyocytes were produced from hiPSCs using small molecule induction, a method developed by Lian and colleagues (Lian et al. 2012). The stem cells were grown until 80–95% confluent after which differentiation was started by adding 6 \( \mu \text{M} \) CHIR99021 in RPMI 1640 medium supplemented with B-27 without insulin (RB-ins) to the cells (day 0; Fig. 4). After 24 h (day 1) the medium was replaced with fresh RB-ins. On day 3 the medium was changed to RB-ins containing 2.5 \( \mu \text{M} \) Wnt-C59 for 48 h. On day 5 the medium was again replaced with RB-ins in which the cells were maintained until day 11. Generally, beating cardiomyocytes were observed from days 7–8 onwards. The cardiomyocyte cultures were purified with metabolic selection by feeding the cells on day 11 and 13 with RPMI 1640 without glucose supplemented with B-27 (RB-glu). From day 15 onwards the cells were maintained in RPMI 1640 supplemented with B-27 (RB). Moreover, from day 15 onwards beating hiPSC-CMs were ready to be dissociated by incubating them in cell dissociation solution containing 40% enzyme-free cell dissociation buffer (Gibco™), 40% RPMI 1640 and 20% trypsin-ethylenediaminetetraacetic acid (trypsin-EDTA) (final trypsin concentration 0.01%) for 7–8 min. Trypsin was inactivated with RB supplemented with 10% foetal bovine serum (FBS) followed by centrifugation. The cardiomyocytes were resuspended in RB with 10% FBS containing 10 \( \mu \text{M} \) ROCK inhibitor Y-27632 and seeded on gelatin-coated well plates. The cells were allowed to attach for 2 days, after which they were maintained in RB (without FBS or ROCK inhibitor).

**Figure 4.** Timeline of human induced pluripotent stem cell (hiPSC) growth and subsequent cardiomyocyte differentiation. The timeline shows the medium and small molecules used in each day (D). hiPSCs were grown four days in Essential 8™ medium (E8) after which differentiation towards the cardiomyocyte lineage was induced using small molecules to modulate Wnt signalling, first with a glycogen synthase kinase 3 inhibitor CHIR99021 (Gi; D0) to potentiate Wnt signalling and then two days later with a Wnt inhibitor Wnt-C59 (Wi; D3) to attenuate Wnt signalling. The culture medium used throughout differentiation was basal RPMI 1641 medium supplemented with B-27 without insulin (RB-ins). Cardiomyocyte cultures were purified with metabolic selection by removing glucose from the culture medium (RB-glu; D11). A Rho-associated protein kinase inhibitor Y-27632 (Ri) was used upon passaging to improve cell survival.
4.2.3 Neonatal rat cardiomyocytes

NRVMs were isolated from 1–3 day old Wistar rats. The animals were sacrificed by decapitation. Ventricles were dissected, cut into small pieces, and enzymatically digested by incubating them for 1–1.5 h at 37 °C under 600 rpm shaking conditions in a solution containing 100 mM NaCl, 10 mM KCl, 1.2 mM KH₂PO₄, 4.0 mM MgSO₄, 50 mM taurine, 20 mM glucose, 10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), 2 mg/ml collagenase type II, 2 mg/ml pancreatin, 100 U/ml penicillin, and 100 μg/ml streptomycin. The cell suspension was collected and centrifuged for 5 min at 160 × g after which the supernatant and the top layer of the pellet were discarded. The isolated cardiac cells were resuspended in Dulbecco’s modified Eagle medium/Nutrient mixture F-12 (DMEM/F-12) supplemented with 10% FBS, 100 U/ml penicillin, and 100 μg/ml streptomycin. Myocytes and fibroblasts were separated based on a difference in rate of attachment (Polinger 1970). The cells were pre-plated onto cell culture flasks and allowed to attach for 45–60 min in cell culture conditions after which unattached cells (enriched cardiomyocytes) were collected with the medium and seeded on gelatin-coated well plates. The following day the medium was changed to complete serum free medium, consisting of DMEM/F-12 supplemented with 2.5 mg/ml bovine serum albumin (BSA), 5 μg/ml insulin, 5 μg/ml transferrin, 5 ng/ml selenium, 2.8 mM sodium pyruvate, 0.1 mM triiodo-L-thyronine, 100 U/ml penicillin, and 100 μg/ml streptomycin, for 24 h prior to compound exposures.

4.2.4 Neonatal rat cardiac fibroblasts

Neonatal rat cardiac fibroblasts (NRCFs) were isolated simultaneously with cardiomyocytes. After collecting the unattached cardiomyocytes with the medium (see above), the attached cell fraction, which were regarded as fibroblasts (Polinger 1970), were grown in DMEM/F-12 supplemented with 10% FBS, 100 U/ml penicillin, and 100 μg/ml streptomycin for 3 days. The cells were dissociated with trypsin-EDTA and seeded on well plates after which they were allowed to grow for another 3 days prior to drug exposures.

4.2.5 Adult mouse cardiac fibroblasts

Adult mouse cardiac fibroblasts were isolated from 10–20 week old C57BL/6JOlaHsd mice weighing 20–22 g (Envigo, Horst, The Netherlands). The protocol utilised MACS® technology and a skeletal muscle dissociation kit by Miltenyi Biotec. Cell isolation was done by combining mechanical dissociation with enzymatic degradation of the extracellular matrix. First, the animals were euthanized by CO₂ narcosis followed by cervical dislocation. Thoracic cavities were opened and hearts were perfused through aortas with 2.5 ml of 500 U/ml collagenase II in phosphate-buffered saline (PBS) using a 27 G 12 mm needle. Ventricles were dissected, cut into
small pieces, and transferred into gentleMACS C tubes (two hearts per tube) containing 5 ml of enzyme solution; DMEM supplemented with 400 U/ml collagenase II, 60 U/ml DNase I, and 10 μl/ml enzyme P from the skeletal muscle dissociation kit. Tissue pieces were incubated for 20 min at 37 °C under 300 rpm shaking conditions followed by mechanical dissociation with a gentleMACS Dissociator using the program m_muscle_01. Enzymatic digestion was continued with a 30-min incubation at 37 °C under 600 rpm shaking conditions followed by another mechanical dissociation with a gentleMACS Dissociator (program m_muscle_01). After a short centrifugation step, tissue debris was separated from the cell suspension using a 250 μm tissue strainer. The cell suspension was centrifuged again at 300 × g, 4 °C for 10 min after which the supernatant was aspirated and the cell pellet was resuspended in a buffer solution containing PBS supplemented with 0.5% BSA and 2 mM EDTA, pH 7.2. Red blood cell lysis solution (Miltenyi Biotec) was used according to the manufacturer’s protocol to remove erythrocytes. After centrifuging the sample for 10 min (300 × g, 4 °C), the supernatant was aspirated and the cells were washed with cell culture medium containing DMEM/F-12 supplemented with 10% FBS, 100 U/ml penicillin, and 100 μg/ml streptomycin. The suspension was centrifuged again for 10 min (300 × g, 4 °C), the supernatant was aspirated and the cells were resuspended in the cell culture medium. The cells were plated on well plates and were allowed to attach for 2 h in cell culture conditions. To remove dead cells, the medium was changed once more before allowing the cells to grow overnight prior to compound exposures.

4.3 Colorimetric cell viability assays

4.3.1 LDH assay

The effect of the compounds on cell membrane integrity was investigated using the lactate dehydrogenase (LDH) assay (Korzeniewski and Callewaert 1983). After drug exposures, 50 μl of culture medium was transferred from each well onto a new 96-well plate followed by the addition of 50 μl substrate solution containing 1.3 mM β-nicotinamide adenine dinucleotide, 660 μM iodonitrotetrazolium, 54 mM L(+)-lactic acid, 280 μM phenazine methosulphate, and 0.2 M Tris-HCl (pH 8.0). After a 30-min incubation, 50 μl of 1 M acetic acid was added to each well to stop the reaction. Absorbance was measured at 490 nm with a Victor2 plate reader (PerkinElmer). Spontaneous LDH release was determined from untreated cells, maximal LDH release from cells lysed with 0.9% Triton X-100, and background absorbance from wells without cells (medium only). After subtracting background, cytotoxicity was calculated as follows: cytotoxicity % = [(sample – spontaneous LDH release) / (maximal LDH release – spontaneous LDH release)] × 100.
4.3.2 MTT assay

The effect of the compounds on mitochondrial metabolism was investigated using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay (Mosmann 1983). After drug exposures, MTT was added to the cells at a final concentration of 0.5 mg/ml followed by a 2-h incubation in cell culture conditions. The medium was aspirated and the formed formazan crystals were solubilized in DMSO. Absorbance was measured at 550 nm while absorbance at 650 nm was subtracted as background.

4.4 Automated fluorescence microscopy and high-content analysis

For high-content analysis (HCA), the cells were plated on 96-well plates and exposed to compounds. The cells were fixed with 4% paraformaldehyde (PFA) for 15 min at room temperature (rt) and permeabilized with 0.1% Triton X-100 for 10 min. Non-specific binding sites were blocked with 4% FBS in PBS for 45 min at rt. The cells were incubated 60 min (rt) with primary antibodies (Table 5) diluted in PBS. After 3 x 5-min washes with PBS, the cells were incubated 45 min (rt) with Alexa Fluor-conjugated secondary antibodies (Table 5) and 4',6-diamidino-2-phenylindole (DAPI; at 1 μg/ml concentration) followed by 3 x 5-min washes with PBS. The cells were imaged and analysed with the CellInsight CX5 High-Content Screening Platform (Thermo Scientific) using a 10× objective (Olympus UPlanFL N 10×/0.3). For quantification, the cells were first identified based on DAPI fluorescence, which defined the nuclear area (Fig. 5A). In cardiomyocyte cultures, non-myocytes were excluded based on the absence of α-actinin or cTnT staining and data were collected only from α-actinin or cTnT positive cells. To adjust for minor variation in staining intensity and to allow optimal exclusion of non-myocytes, the threshold for α-actinin or cTnT fluorescence intensity was set manually in each experiment.

4.4.1 DNA content

DAPI binds to A-T rich sequences of double-stranded DNA, thus the fluorescence depends on the DNA content of the cells (Kapuscinski 1995). Increased intensity of DAPI staining was used as a marker for DNA replication (see also 4.4.2 Cell cycle activity) whereas decreased intensity was an indication of decrease in the amount of double-stranded DNA in the cells (Darzynkiewicz et al. 2010). Intranuclear variability in DNA staining intensity, on the other hand, was measured as an indication of DNA fragmentation (Bacsó et al. 2000; Doan-Xuan et al. 2013).
Table 5. Antibodies used in immunofluorescence stainings (IF) and western blotting (WB).

<table>
<thead>
<tr>
<th>Primary antibodies</th>
<th>Species</th>
<th>Manufacturer</th>
<th>Product #</th>
<th>Application</th>
<th>Dilution</th>
</tr>
</thead>
<tbody>
<tr>
<td>anti-α-Actinin</td>
<td>Mouse</td>
<td>SA</td>
<td>A7811</td>
<td>IF</td>
<td>1:600</td>
</tr>
<tr>
<td>anti-α-Smooth muscle actin</td>
<td>Mouse</td>
<td>SA</td>
<td>A2547</td>
<td>IF</td>
<td>1:200</td>
</tr>
<tr>
<td>anti-β-Actin</td>
<td>Rabbit</td>
<td>CST</td>
<td>4967</td>
<td>WB</td>
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<td>anti-PKC alpha</td>
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<td>anti-PKC epsilon</td>
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<td>ab124806</td>
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<tr>
<td>anti-proBNP</td>
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<td>ab13115</td>
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<table>
<thead>
<tr>
<th>Secondary antibodies</th>
<th>Manufacturer</th>
<th>Product #</th>
<th>Application</th>
<th>Dilution</th>
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</thead>
<tbody>
<tr>
<td>Alexa Fluor 488 goat anti-mouse lgG</td>
<td>LT</td>
<td>A11029</td>
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<tr>
<td>Alexa Fluor 488 goat anti-rabbit lgG</td>
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<tr>
<td>Alexa Fluor 546 donkey anti-rabbit lgG</td>
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<td>IF</td>
<td>1:200</td>
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<tr>
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A, Abcam; BrdU, 5-bromo-2′-deoxyuridine; CST, Cell Signaling Technology; DDR2, discoidin domain receptor 2; HRP, horseradish peroxidase; I, Invitrogen; LT, Life Technologies; PKC, protein kinase C; proBNP, pro-B-type natriuretic peptide; SA, Sigma-Aldrich; SCB, Santa Cruz Biotechnology
Materials and methods

Figure 5. Principles of the image analyses. (A) High-content analysis was performed using a CellInsight CX5 High-Content Screening Platform. The cells were identified based on DNA staining with DAPI, which defined the nuclear area (blue circle in the image). The perinuclear area was defined by a ring around the nucleus (green line in the image). (B) Phenotype analysis was performed using CellProfiler™ cell image analysis software. The cells were first identified based on DAPI fluorescence, which defined the nuclear area. Discoidin domain receptor 2 (DDR2) staining was used to define the cell area (red or turquoise line in the image) from which the intensity of α-smooth muscle actin (α-SMA) staining was measured. Representative images of (A) human induced pluripotent stem cell-derived cardiomyocytes and (B) primary cardiac fibroblasts. proBNP, pro-B-type natriuretic peptide; cTnT, cardiac troponin T.

4.4.2 Cell cycle activity

Cell cycle activity was studied by measuring 5-bromo-2’-deoxyuridine (BrdU), Ki67 and DAPI staining intensities within the nucleus. For BrdU stainings, 10 μM BrdU was added to the culture medium for the last 1 h (hiPSCs) or 24 h (cardiomyocytes and cardiac fibroblasts) before fixation. Moreover, after permeabilization with 0.1% Triton X-100, DNA was hydrolysed with 2 M HCl for 30 min followed by neutralization with 0.1 M sodium borate (pH 8.5) for 30 min. The thresholds for BrdU and Ki67 positive cells and cells with high DNA staining (DAPI) intensity were set manually in each experiment to adjust for slight variation in staining intensity. The cells with high DNA staining intensity represent the cells that have >2N chromosomes (Darzynkiewicz et al. 2010).

4.4.3 Mitochondrial function

Cell viability was assessed using the MitoTracker™ probe to investigate mitochondrial function. The accumulation of the MitoTracker™ Orange CMTMRos probe is dependent upon mitochondrial membrane potential, which allows selective
staining of active mitochondria. The MitoTracker™ reagent at 400 nM concentration was added to the cells for the last 30 min (at 37 °C) before fixation. MitoTracker intensity was analysed in the perinuclear area, which in hiPSCs was defined by a 3-pixel ring and in hiPSC-CMs by a 5-pixel ring around the nucleus. The threshold for MitoTracker positive and MitoTracker negative cells was set manually in each experiment based on visual inspection and estimation that 2% of control hiPSC-CMs and 5% of control hiPSCs were MitoTracker negative.

4.4.4 Caspase activity

To study caspase activation in hiPSCs, the cells were exposed to the test compounds after which they were incubated with 3–5 μM CellEvent™ Caspase-3/7 Green Detection Reagent solution in PBS with 5% FBS for 50 min and stained with Hoechst for 10 min at 37 °C. After this the cells were fixed, or alternatively, the staining solution was replaced with fresh 5% FBS in PBS followed by live cell imaging. Hoechst fluorescence was used to identify the cells and to define the nuclear area. To study caspase activation in cardiomyocytes, the cells were exposed to the compounds after which they were incubated with 7 μM caspase-3/7 detection reagent solution in PBS with 5% FBS for 60 min at 37 °C prior to fixation. Fixed cells were further stained with DAPI to identify the cells and to define the nuclear area. The intensity of green fluorescence within the nucleus was used as a measure of caspase-3/7 activity. The threshold for caspase-3/7 positive and caspase-3/7 negative cells was set manually in each experiment.

4.4.5 GATA4 levels

To evaluate the effect of compounds on GATA4 protein levels in cardiomyocytes, a primary antibody against GATA4 was used. Based on the staining, GATA4 was principally located inside the nucleus, the cytosolic fraction being only minor. The intensity of GATA4 staining was thus measured within the nucleus.

4.4.6 proBNP expression

To investigate the expression of BNP precursor proBNP in response to compound exposures, proBNP staining was used. Besides compound treatments, the cells were additionally treated with Brefeldin A solution for 3 h prior to fixation. The intensity of proBNP staining was measured in the perinuclear area defined by a 4-pixel ring around the nucleus (Fig. 5A). The threshold for proBNP positive cells was set manually in each experiment to adjust for minor variation in staining intensity.
4.5 Non-automated fluorescence microscopy and phenotypic analysis

Immunocytochemistry and non-automated fluorescence microscopy was used to study the phenotype of cardiac fibroblasts. The cells were grown on gelatin-coated microscopy cover glasses on 24-well plates and fixed with 4% PFA for 15 min at rt. After permeabilization with 0.1% Triton X-100 for 10 min followed by 2 × 10-min washes with Dulbecco’s PBS containing 0.2% BSA (DB), the cells were incubated 60 min at rt with anti-α-SMA and anti-DDR2 primary antibodies diluted in DB. After 3 × 5-min washes with DB, the cells were incubated 45 min with Alexa Fluor-conjugated secondary antibodies and DAPI at rt. The microscope cover glasses were mounted on microscope slides with Prolong™ Gold Antifade Mountant (Thermo Fisher Scientific). The cells were imaged with a Leica DM6000B fluorescence wide field microscope (Leica Microsystems) and CMOS camera (Hamamatsu Orca-Flash4.0 V2, Hamamatsu Photonics) using a 20×/0.7 HC PL APO CS objective, and Leica Application Suite X software (Leica Microsystems). The images were analysed using CellProfiler™ cell image analysis software. For quantification, the cells were first identified based on DAPI fluorescence, which defined the nuclear area. DDR2 staining was measured (Fig. 5B).

4.6 Quantitative real-time PCR

The effects of the compounds on gene expression in cardiac fibroblasts was analysed with quantitative polymerase chain reaction (qPCR). The cells were grown on gelatin-coated microscopy cover glasses on 24-well plates. Samples were lysed in 350 μl lysis buffer containing 1% β-mercaptoethanol, and RNA was isolated using a NucleoSpin® RNA kit (Macherey-Nagel) according to the manufacturer’s protocol. Total RNA was transcribed into complementary DNA (cDNA) with a Transcriptor First Strand cDNA Synthesis kit (Roche) according to the manufacturer’s protocol while using random hexamer primers. Quantitative PCR was performed using TaqMan™ assays (Thermo Fisher Scientific; Table 6), LightCycler® 480 Probes Master kit (Roche), and a LightCycler® 480 Real-Time PCR machine (Roche). The results were analysed using the ΔΔCt method and adjusted to the average of two housekeeping genes (18S and Actb) from the same samples.
**Materials and methods**

**Table 6.** TaqMan™ gene expression assays used in qPCR.

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**4.7 Western blotting**

The effect of the compounds on PKC protein levels in cardiac fibroblasts was studied with western blotting. The cells were grown on 6-well plates and were lysed with 1% sodium dodecyl sulphate in 50 mM Tris-Cl (pH 7.5). Genomic DNA was sheared using a 25 G needle. Protein concentrations were determined using a bicinchoninic acid protein assay kit (Thermo Scientific). From each sample 10 μg of total protein was separated on 12% Mini-protean® TGX Stain-Free™ gels (Bio-Rad) and transferred by a Trans-Blot® Turbo™ transfer system (Bio-Rad) to polyvinylidene difluoride membranes. Nonspecific background was blocked with 5% non-fat dry milk in Tris-buffered saline with 0.05% Tween 20 (TTBS) for 1 h at rt, after which the membranes were incubated with primary antibodies (in 5% milk-TTBS) overnight at 4 °C. The membranes were washed with TTBS, followed by 1-h incubation with horseradish peroxidase-conjugated secondary antibody (in 5% milk-TTBS) at rt. Bands were detected with an enhanced chemiluminescent substrate (SuperSignal™ West Femto Maximum Sensitivity Substrate, Thermo Scientific) using a ChemiDoc XRS+ (Bio-Rad) or Luminescent Imager Analyzer LAS-3000 (Fujifilm). Optical densities of the immunoreactive bands were quantified using the Fiji ImageJ 1.52 program (Schindelin et al. 2012). The densities of the PKC bands were adjusted to the corresponding β-actin bands from the same membranes.
4.8 Cellular kinase assay

Activation of the mitogen activated protein kinase kinase–extracellular signal-regulated kinase (MEK–ERK) pathway in cardiac fibroblasts exposed to PKC-targeted compounds, was studied by measuring ERK1/2 phosphorylation using Alpha technology. The cells were exposed to the compounds for 30 min after which they were lysed and the amount of phospho-ERK1/2 (p-ERK1/2) and total ERK1/2 were detected using AlphaLISA® SureFire® Ultra™ p-ERK 1/2 (Thr202/Tyr204) and AlphaLISA® SureFire® Ultra™ Total ERK 1/2 assay kits (PerkinElmer) according to the manufacturer's protocol. The cells were lysed in 80 μl lysis buffer and 30 μl of the lysate was transferred from each well to two 96-well 1/2 AreaPlates™ (PerkinElmer) for assays. The lysates were then incubated with the Acceptor bead mix for 1 h at rt, followed by addition of the Donor bead mix and a further 5-h incubation at rt. The Alpha signal was measured using an EnSpire Alpha plate reader (PerkinElmer) with standard AlphaLISA settings.

4.9 Statistics

The results are expressed as the mean from at least three independent experiments with error bars representing the standard error of the mean (SEM). Statistical analyses were performed using IBM SPSS Statistics 24 or 25 software. In study I, statistical significance was evaluated with one-way ANOVA followed by Tukey’s HSD post hoc test. In studies II and III, statistical significance was evaluated with randomized block ANOVA (experiment and treatment as factors) followed by Tukey’s HSD or Dunnett’s post hoc test. If the data did not meet the assumptions of the Levene’s test, Welch ANOVA and Games-Howell post hoc test were performed. Regarding the unpublished data, statistical significance between two groups was determined using independent samples t test. Differences at the level of P<0.05 were considered statistically significant.
5 RESULTS

5.1 Toxicological characterisation of the compounds

5.1.1 Effects on cell viability (I, II, III, unpublished)

Cardiomyocytes and stem cells. Acute toxicity of a structurally conserved set of eight GATA4-targeted compounds was investigated with the MTT assay after a 24-h treatment. Toxicity profiling was carried out in eight cell types, including both cardiac and non-cardiac cell models: the H9c2 myoblast cell line derived from rat myocardium, NRVMs, NRCFs, mouse embryonic stem cells (mESCs), mouse embryonic fibroblasts (MEFs), mESC derivatives from day 5 embryoid bodies (D5EBs), hiPSCs, and hiPSC-CMs. The effects of individual GATA4-targeted compounds on cell viability differed markedly between the cell types studied. As an example, compound 3i-1000 induced significant reductions in cell viability in mESCs (56% at 30 μM concentration, P=0.006) and hiPSCs (93–94% at ≥3 μM concentrations, P<0.001) as well as a notable 46–56% reduction in metabolic activity of D5EBs at ≥10 μM concentrations (Fig. 6A). However, it had no significant effect on the viability of NRVMs, NRCFs, MEFs, or hiPSC-CMs. Compound 3i-1047, on the other hand, induced only 35–45% reductions in cell viability, even at the highest 30 μM concentration, in mESCs (not statistically significant), hiPSCs (P=0.021), and D5EBs (not statistically significant) (Fig. 6B). It also increased the metabolic activity of hiPSC-CMs 51% compared to control (P=0.033). Overall, the comparison between the eight cell types demonstrated that H9c2 cells, cardiomyocytes (NRVMs and hiPSC-CMs), and fibroblasts (NRCFs and MEFs) are the most resistant cell types against the toxicity of GATA4-targeted compounds. Moreover, the two fibroblast lines exhibited comparable responses to the small molecules. Similarly, the two cardiomyocyte lines acted comparably. The only notable difference was the 3i-1047-induced increase in hiPSC-CM viability, which was not observed in primary cardiomyocytes. Contrary to cardiomyocytes, fibroblasts, and H9c2 cells, several GATA4-targeted compounds induced significant toxicity in D5EBs, mESCs, and hiPSCs. In general, based on the toxicity induced at the highest concentrations tested (10 μM concentration in Fig. 6C), hiPSCs were the most sensitive cell type to identify acute toxicity of GATA4-targeted compounds.

The effects of selected GATA4-targeted compounds on cell viability was further characterised using HCA after a short-term treatment as well as the MTT assay after a long-term treatment. For HCA, compounds 3i-1000 and 3i-1047 were chosen as representatives of the more toxic and non-toxic compounds, respectively, and the experiments were conducted in hiPSCs and hiPSC-CMs representing the sensitive and resistant cell types, respectively. Correspondingly to the MTT assay results, 3i-1000 at 10 μM concentration impaired mitochondrial function in hiPSCs, as reflected
**Figure 6.** The effects of GATA4-targeted compounds on cell viability. The effects of two representative compounds (A) 3i-1000 and (B) 3i-1047 on the viability of eight cell types after a 24-h treatment. (C) Heat map of the effects of all test compounds on the viability of eight cell types after a 24-h treatment at the concentration of 10 μM. (D) The effect of 3i-1000 on the viability of human induced pluripotent stem cell-derived cardiomyocytes (hiPSC-CMs) after long-term treatment. The cells were exposed to 3i-1000 for 1–21 days after which the MTT assay was performed. The results are expressed as mean +/− SEM (n=3–4). ***P<0.001 vs. DMSO-control; **P<0.01 vs. DMSO-control; *P<0.05 vs. DMSO-control (one-way ANOVA followed by Tukey’s HSD post hoc test or randomized block ANOVA followed by Tukey’s HSD).

by a 5.7-fold increase (P=0.001) in the percentage of MitoTracker negative cells compared to control, while 3i-1047 had no effect on MitoTracker staining in this cell type. On the other hand, neither of the compounds had an effect on the proportion of MitoTracker negative hiPSC-CMs, which corresponds with the results from the MTT
Results

Despite not showing signs of acute toxicity in cardiomyocytes after a 24-h treatment either in the MTT assay or HCA, long-term treatments (up to 21 days) revealed the delayed toxicity of 3i-1000 also in hiPSC-CMs (Fig. 6D). At 10 μM concentration 3i-1000 reduced hiPSC-CM viability 34% (P=0.001), 50% (P<0.001), and 65% (P<0.001) after 7, 14, and 21 days of exposure, respectively. At 3 μM concentration the decrease of cell viability was 16% (not statistically significant) after a 21-day exposure.

Adult cardiac fibroblasts. Acute toxicity of the PKC-targeted compounds along with selected GATA4-targeted compounds (including 3i-1000 and 3i-1047) was investigated in adult mouse cardiac fibroblasts. Compound 3i-1000 at ≤30 μM concentrations had no effect on fibroblast viability after a 24-h treatment (Fig. 7A). GATA4-targeted 3i-0595, on the other hand, induced 23% (P=0.001) and 37% (P<0.001) decreases at 10 μM and 30 μM concentrations, respectively, whereas 3i-0777 and 3i-1047 increased the metabolic activity of the cells 45% and 42% (P=0.018) at 30 μM concentration, respectively. All PKC-targeted compounds were relatively non-toxic to mouse cardiac fibroblasts. Among the proprietary PKC agonists, HMI-1b11 at 30 μM concentration decreased metabolic activity 70% (P<0.001), but at 10 μM concentration slightly increased it (28%; P=0.025) compared to control, while HMI-1a3 had no effect on the viability of cardiac fibroblasts (Fig. 7A). PMA at the concentrations of 10 nM and 100 nM caused small 23% (P=0.004) and 14% (P=0.062) decreases, respectively, in viability of cardiac fibroblasts, while bryostatin-1 had no effect (Fig. 7B–C). A 24-h exposure to the pan-PKC inhibitor Gö6983 had no effect on cardiac fibroblast viability, while the inhibitor of cPKCs, Gö6976, decreased fibroblast viability concentration-dependently: 17% at 1 μM concentration (not statistically significant) and 39% (P=0.004) at 10 μM concentration (Fig. 7D).

5.1.2 Cell death mechanisms (I, II, III, unpublished)

GATA4-targeted compounds did not cause significant LDH release from any of the eight cell types after a 24-h treatment. The compound-induced cytotoxicity was always less than 15%, suggesting that none of the compounds exhibited a necrotic response regardless of the cell type. In adult mouse cardiac fibroblasts only HMI-1b11 at the highest 30 μM concentration exhibited a statistically significant cytotoxic response; after a 24-h exposure the LDH release was 35% of the maximal (P<0.001). The other PKC-targeted compounds as well as selected GATA4-targeted compounds (3i-0595, 3i-0777, 3i-1000, 3i-1047) induced less than a 10% increase in cytotoxicity even at the highest concentrations.

To further elucidate the mechanism of cell death caused by the GATA4-targeted 3i-1000, caspase-3/7 activation was investigated in both stem cells and cardiomyocytes using HCA. In hiPSCs, 3i-1000 at 10 μM concentration induced a 3.2-fold increase (P=0.009) in cells positive for the fluorescent caspase-3/7 activity reporter compared
Results

to DMSO already after a 4-h treatment (Fig. 8A). In hiPSC-CMs and NRVMs, 3i-1000 at 10 μM concentration induced 2.5 (not statistically significant) and 1.4-fold (P=0.037) increases, respectively, in the percentage of caspase+ cells after a 24-h exposure compared to the DMSO-control (Fig. 8B–C). After a 4-day treatment at 10 μM concentration, the 3i-1000-induced increase in hiPSC-CMs positive for the caspase reporter was 5.0-fold (P=0.007). The level of caspase+ hiPSC-CMs stayed high compared to DMSO also after 7 and 14-day treatments. At 3 μM concentration, the 3i-1000-induced increase in caspase+ hiPSC-CMs was only 1.8-fold compared to the control at day 14. The results suggest caspase-3/7 dependent apoptosis as a mechanism contributing to both stem cell and cardiomyocyte death caused by the GATA4-targeted compound.

![Graph showing the effects of selected GATA4- and PKC-targeted compounds on the viability of primary cardiac fibroblasts (CF) isolated from adult mice. The cells were exposed to test compounds 24 h after plating for 24 h. At 48 h after plating the MTT assay was performed. The results are expressed as mean + SEM (n=3). ***P<0.001 vs. DMSO-control; **P<0.01 vs. DMSO-control; *P<0.05 vs. DMSO-control (randomized block ANOVA followed by Dunnett’s post hoc test). Bryo, bryostatin-1.](image)

Figure 7. The effects of selected GATA4- and PKC-targeted compounds on the viability of primary cardiac fibroblasts (CF) isolated from adult mice. The cells were exposed to test compounds 24 h after plating for 24 h. At 48 h after plating the MTT assay was performed. The results are expressed as mean + SEM (n=3). ***P<0.001 vs. DMSO-control; **P<0.01 vs. DMSO-control; *P<0.05 vs. DMSO-control (randomized block ANOVA followed by Dunnett’s post hoc test). Bryo, bryostatin-1.
5.1.3 Structure–toxicity relationships of GATA4-targeted compounds

When comparing the effects of the selected GATA4-targeted compounds within the eight different cell types, the compounds can be grouped into two classes: toxic (3i-1000, 3i-1120, 3i-1148, and 3i-1229) and non-toxic (3i-1047, 3i-1051, 3i-1165, and 3i-1228). The classification was based on their effects on stem cell and progenitor cell viability (Fig. 6C). Based on the toxicity data from hiPSCs (the most sensitive cell type), compound 3i-1148 can be considered as the most toxic compound with an IC50 value of 0.30 μM. The respective IC50 values of 3i-1000, 3i-1120, and 3i-1229 were 1.46 μM, 0.60 μM, and 2.07 μM.

The structures of all eight compounds are characterised by northern and southern parts linked via a linker part (Fig. 3A and 9A). The northern part is typically comprised of a phenylenediamine whereas the southern part is typically comprised of a substituted heterocycle. Analysis of the structures of the toxic compounds reveals that the stem cell toxicity of the hit compound 3i-1000 was preserved despite major structural modifications in the middle part (3i-1120) and northern part (3i-1229). However, a structure that is characteristic to the toxic compounds is the six-membered ring bound to isoxazoles of the southern part (Fig. 3A and 9B). The non-toxic compounds, on the other hand, have a five-membered ring in the same position (Fig. 3A and 9C). A parallel conformational analysis of the two compound classes suggests that the compounds with a five-membered ring in the southern part adopt a flatter ring geometry (torsional angle ranging from 0° to 19°) as compared to the six-membered ring-systems (torsional angles ranging from 28° to 51°) (Fig. 9B–C).
5.2 Establishment of a chronic cardiotoxicity model (II)

An *in vitro* model of chronic doxorubicin-induced cardiotoxicity was developed utilising hiPSC-CMs, which can be cultured for extended periods of time in contrast to primary cardiomyocytes. Long-term doxorubicin treatments were carried out as repeated consecutive 3–4-day exposures, i.e. the culture media were replaced with fresh drug-supplemented media every 3–4 days (Fig. 10). The doxorubicin concentration used in the final model was chosen based on cell viability data evaluation. Exposure to doxorubicin at 1 μM and 3 μM concentrations markedly reduced (approximately 60%, *P*<0.001) hiPSC-CM viability already within 48 h (Fig. 11A). Exposure to 300 nM doxorubicin was less toxic but led to considerable cell death within 21 days. A 14-day treatment with 100 nM doxorubicin, on the other hand, induced only a modest 26% (*P*=0.201) reduction in the viability of hiPSC-CMs. In other words, at 100 nM concentration, doxorubicin reduces cardiomyocyte viability over long-term exposure without causing excessive cell death, thus allowing the evaluation of the efficacy of potential cardioprotective or restorative therapies *in vitro*.

The effects of long-term low-dose doxorubicin treatment on cell density, DNA content, GATA4 protein levels, and caspase-3/7 activity in hiPSC-CMs was further investigated using DAPI and GATA4 stainings, the fluorescent caspase reporter, and HCA. DAPI staining was used to evaluate hiPSC-CM density and DNA content in
hiPSC-CMs after a treatment with 100 nM doxorubicin. After a 4-day treatment, 100 nM doxorubicin induced a 12% (not statistically significant) reduction in cell density compared to control, while decreasing the average total intensity of DNA staining by 18% (P<0.001; Fig. 11B–C). A 14-day exposure to doxorubicin decreased cell density by 49% (not statistically significant) and total DNA staining intensity by 28% (P=0.003) compared to control. Based on the average intensity of GATA4 staining in the nucleus, doxorubicin at 100 nM concentration had no effect on nuclear GATA4 levels in hiPSC-CMs even after a 14-day exposure (Fig. 11D). Doxorubicin at 100 nM concentration had no effect on the percentage of hiPSC-CMs positive for the caspase-3/7 activity reporter after a 4-day exposure compared to DMSO (Fig. 11E). However, a 7-day treatment tended to increase the proportion of caspase+ cells, while a 14-day exposure produced a significant 3.1-fold (P=0.001) increase compared to DMSO.

Figure 10. Experimental design for long-term drug exposures to human induced pluripotent stem cell-derived cardiomyocytes. The cells were treated with doxorubicin and/or an experimental compound for up to 21 days. The treatments were carried out as a single exposure period (≤4 days) or as several consecutive exposure periods, consisting of 2–6 culture media changes every 3–4 days (total exposure time 7–21 days). Red arrows indicate drug-supplemented culture media change, black arrows indicate analysis.

To compare the effects of doxorubicin in different cardiomyocyte models, NRVMs were exposed short-term to doxorubicin. In the MTT assay, a 48-h exposure to 1 μM and 3 μM concentrations induced 79% (P<0.001) and 82% (P<0.001) reductions, respectively, in the viability of NRVMs (Fig. 12A). After a 24-h treatment, doxorubicin at 100 nM concentration had no significant effect on the percentage of NRVMs positive for the caspase reporter; however, it is notable that in NRVM cultures the basal level of caspase+ cells (on day 3 after cell isolation) was higher compared to hiPSC-CM cultures; 8% and 1%, respectively. Moreover, analysis of GATA4 levels with HCA revealed that doxorubicin at 100 nM concentration induced a 14% (P=0.039) decrease in the average intensity of GATA4 staining in NRVM nuclei already after a 24-h treatment (Fig. 12B). When compared to the effects seen in hiPSC-CMs (Fig. 11A and 11D), the data suggest that primary cardiomyocytes are more sensitive to doxorubicin toxicity than hiPSC-CMs.
Figure 11. Doxorubicin (DOX)-induced toxicity in human induced pluripotent stem cell-derived cardiomyocytes (hiPSC-CMs). (A) To study the effect of long-term DOX exposure on hiPSC-CM viability, the cells were exposed to DOX for 2–21 days after which the MTT assay was performed. For high-content analysis of (B) cell density, (C) DNA content, (D) GATA4 levels, and (E) caspase activation in hiPSC-CMs, the cells were exposed to 100 nM DOX for 4, 7 or 14 days after which they were fixed and stained. Results are expressed as mean ± SEM (n=3–5). ***P<0.001 vs. DMSO-control; **P<0.01 vs. DMSO-control; *P<0.05 vs. DMSO-control (randomized block ANOVA followed by Tukey’s HSD post hoc test).
Figure 12. Doxorubicin (DOX)-induced toxicity in neonatal rat ventricular myocytes (NRVMs). (A) To study the effect of short-term DOX exposure on NRVM viability, the cells were exposed to 100 nM–3 μM DOX for 48 h after which the MTT assay was performed. (B) For high-content analysis of GATA4 levels in NRVMs, the cells were exposed to 100 nM DOX for 24 h after which they were fixed and stained. Results are expressed as mean + SEM (n=3–4). ***P<0.001 vs. DMSO-control; **P<0.01 vs. DMSO-control; *P<0.05 vs. DMSO-control (randomized block ANOVA followed by Tukey’s HSD post hoc test).

5.3 Pharmacological characterisation of the GATA4-targeted compounds (I, II)

The potential of GATA4-targeted compounds to induce cardiac repair and/or to protect cardiomyocytes from doxorubicin toxicity was investigated using HCA and the MTT assay. The effects of selected compounds 3i-1000 and 3i-1047 on cell cycle activity was studied in healthy cardiomyocytes as well as stem cells after short-term exposure. The effects of 3i-1000 on doxorubicin-induced decreases in cell number, viability, and DNA content, as well as increases in caspase activity and proBNP expression were studied using the chronic cardiotoxicity model. In order to investigate if the GATA4-targeted compound has cardioprotective potential, the cells were exposed simultaneously to both doxorubicin and 3i-1000.

Both 3i-1000 and 3i-1047 at 30 μM concentration increased the percentage of Ki67+ hiPSC-CMs (1.7- and 1.5-fold increases, respectively), compared to the DMSO-control after a 24-h exposure, suggesting that the compounds may promote cell cycle activity (Fig. 13A). However, this increase was not linked to parallel increases in the proportion of BrdU+ cardiomyocytes (Fig. 13B). Compound 3i-1047 had no effect on the proportion of BrdU+ cells, while 3i-1000 at 30 μM concentration tended to decrease the percentage (43%) compared to DMSO. These results also indicate that the 3i-1047-induced 51% increase in metabolic activity of hiPSC-CMs (Fig. 6B) was not due to an increase in cell number. Furthermore, 3i-1000 at 10 μM concentration tended to increase caspase-3/7 activity in hiPSC-CMs after a 24-h exposure (Fig. 8B), thus suggesting the increase seen in Ki67+ cells after a 24-h exposure to 30 μM concentration is not due to a promoting effect on cardiomyocyte proliferation. In
hiPSCs, 3i-1000 at 10 μM concentration induced a significant decrease in the percentage of Ki67+ and BrdU+ cells after a 24-h exposure (Fig. 13C–D). This decrease was associated with a significant decrease in cell number and thus corresponded with the MTT assay results that showed a significant decrease in cell viability by 3i-1000 (Fig. 6A). Compound 3i-1047, by contrast, had no significant effect on Ki67+ or BrdU+ stem cells even at the concentration of 10 μM (Fig. 13C–D).

Figure 13. The effects of compounds 3i-1000 and 3i-1047 on cell cycle activity of hiPSC-CMs and hiPSCs. For high-content analysis, the cells were exposed to GATA4-targeted compounds for 24 h after which they were fixed and stained. Quantifications for the proportion of hiPSC-CMs positive for (A) Ki67 and (B) BrdU, and hiPSCs positive for (C) Ki67 and (D) BrdU are expressed as mean ± SEM (n=3–4). ***P<0.001 vs. DMSO-control; **P<0.01 vs. DMSO-control; *P<0.05 vs. DMSO-control (one-way ANOVA followed by Tukey’s HSD post hoc test or Welch ANOVA followed by Games-Howell post hoc test).

In the chronic cardiotoxicity model, doxorubicin at the concentration of 100 nM induced a 3.1-fold increase (P<0.001) in the percentage of hiPSC-CMs positive for proBNP after a 4-day exposure compared to control (Fig. 14A and 14C). When the cells were exposed simultaneously to doxorubicin and 10 μM 3i-1000, the percentage of proBNP+ cardiomyocytes decreased 60% (P<0.001). At a 3 μM concentration of 3i-1000, the decrease was 20%. Correspondingly, at day 4, the 3i-1000-induced (at 10 μM concentration) 5.0-fold increase in cardiomyocytes positive for the caspase-3/7 reporter was attenuated when the cells were simultaneously exposed to 10 μM 3i-1000 and 100 nM doxorubicin (Fig. 14B and 14D). The cell density in 3i-1000 and
3i-1000 plus doxorubicin treated groups, however, did not differ significantly; the reduction in cell number was 32–37% compared to DMSO (Fig. 14E).

Figure 14. The effects of doxorubicin (DOX) and 3i-1000 on expression of pro-B-type natriuretic peptide (proBNP) and caspase-3/7 activation in hiPSC-CMs after a 4-day exposure. For high-content analysis, the cells were exposed simultaneously to 100 nM DOX and 3i-1000 for 4 days after which they were fixed and stained. Representative images of (A) proBNP staining and (B) caspase staining. Quantifications for the proportion of (C) proBNP positive hiPSC-CMs and (D) hiPSC-CMs positive for fluorescent caspase-3/7 activity reporter, as well as (E) hiPSC-CM density. Results are expressed as mean ± SEM (n=3–4). ***P<0.001 vs. DMSO-control; **P<0.01 vs. DMSO-control; †††P<0.001 vs. DOX-control (randomized block ANOVA followed by Tukey’s HSD post hoc test). cTnT, cardiac troponin T.
After a 7-day exposure the proportion of proBNP+ cardiomyocytes was similar in both doxorubicin and doxorubicin plus 3i-1000 (3 μM and 10 μM) treated groups. However, after exposing the cells simultaneously to doxorubicin (100 nM) and 3i-1000 at 10 μM concentration for 14 days, the proportion of proBNP+ cardiomyocytes increased 19.3-fold compared to a 7.7-fold increase seen in cardiomyocytes exposed to 100 nM doxorubicin alone. In accordance with the 4-day exposure, after 7 and 14-day exposures the percentage of caspase+ cardiomyocytes was lower when treated simultaneously with 3i-1000 (10 μM) and doxorubicin compared to cells treated with 3i-1000 alone. Similarly, 3i-1000 had no effect on doxorubicin-induced reduction in hiPSC-CM viability measured by the MTT assay. Moreover, compound 3i-1000 had no significant effect either alone or in combination with doxorubicin on the total intensity of DNA staining or the average intensity of nuclear GATA4 staining. Overall, the results show that 3i-1000 protected cardiomyocytes from doxorubicin-induced elevation of proBNP, but also that doxorubicin protected cardiomyocytes from 3i-1000-induced caspase activation. Importantly, the cardioprotective potential of 3i-1000 was also demonstrated in a chronic in vivo rat model, in which treatment with 3i-1000 (30 mg/kg/day, i.p.) for 2 weeks inhibited doxorubicin-induced cardiotoxicity by restoring the left ventricular ejection fraction and fractional shortening. 3i-1000 treatment had no significant effect on doxorubicin-induced elevation of ANP and BNP mRNA levels.

5.4 Pharmacological characterisation of the PKC-targeted compounds (I.1., unpublished)

The possibility of using pharmacological tools to inhibit pathological fibrosis was characterised by investigating the effects of selected PKC- and GATA4-targeted compounds on adult mouse cardiac fibroblast transdifferentiation, proliferation, and gene expression. Based on the toxicological characterisation and previously published data, a set of PKC-targeted and GATA4-targeted compounds was chosen for a preliminary analysis on their effect on fibroblast transdifferentiation using α-SMA as a marker of myofibroblasts. Based on α-SMA protein expression, the effect of GATA4-targeted compounds on cardiac fibroblast transdifferentiation was variable. Compound 3i-0595 at 10 μM concentration induced a 1.4-fold increase in the relative intensity of α-SMA staining compared to DMSO-control after a 48-h treatment, while 10 μM 3i-0777 and 10 μM 3i-1000 decreased the intensity 37% (P=0.036) and 12%, respectively (Fig. 15A). All PKC-agonists, on the other hand, inhibited fibroblast transdifferentiation to myofibroblasts. HMI-1a3 at 10 μM concentration induced a 25% reduction and bryostatin-1 at 10 nM concentration a 34% reduction (P=0.043) in the α-SMA staining intensity after a 48-h treatment (Fig. 15B).

Examples of even more effective proprietary and commercially available PKC-agonists in the preliminary analysis were, however, HMI-1b11 and PMA. These two
compounds were thus selected for a more detailed analysis to further clarify the role of different PKC subfamilies in the phenotypic change. The potent PKC activator PMA at 10 nM concentration reduced the intensity of α-SMA staining by 56% (P=0.011) compared to the control after a 48-h treatment (Fig. 16A and 16C). The effect was prevented with both the pan-PKC inhibitor Gö6983 and the inhibitor of cPKCs, Gö6976, suggesting the decrease was cPKC-dependent. The weaker PKC agonist HMI-1b11 at 10 μM concentration induced a 34% decrease (P=0.018) in the expression of α-SMA, and the effect was partially attenuated with the inhibitors. The inhibitors alone had no significant effect on α-SMA protein expression at 1 μM concentration after a 48-h treatment.

**Figure 15.** The effects of selected GATA4- and PKC-targeted compounds on α-smooth muscle actin (α-SMA) expression in primary cardiac fibroblasts. The cells were isolated from adult mice and 24 h after plating they were exposed to the compounds at 10 μM concentration (with the exception of bryostatin-1 at 10 nM concentration) for 48 h. At 72 h after plating, the cells were fixed and stained. Quantified intensity of α-SMA staining in cells treated with (A) GATA4-targeted compounds and (B) PKC-targeted compounds. The results are expressed as mean + SEM (n=3). *P<0.05 vs. DMSO-control (independent samples t test). Bryo, bryostatin-1.

The effect of the selected PKC-targeted compounds on cell cycle activity was studied using BrdU and Ki67 stainings and HCA. After a 48-h exposure, PMA at 10 nM concentration induced a 33% decrease (P=0.018) in the percentage of BrdU positive cells compared to the DMSO-control (Fig. 16B and 16D). This was paralleled by a 36% decrease (P=0.008) in the percentage of Ki67+ cells. Both decreases were inhibited with Gö6983 but not with Gö6976, thus suggesting the effects were nPKC-dependent. HMI-1b11 (10 μM) alone had no effect on BrdU+ cells but induced a non-significant 15% decrease in Ki67+ cells. Interestingly, co-exposure to HMI-1b11 and Gö6976, however, tended to decrease the proportion of BrdU+ cells (29%) compared to HMI-1b11-exposure alone. The inhibitors alone had no significant effect on the proportion of BrdU+ or Ki67+ cells after a 48-h exposure.
Figure 16. The effects of PKC agonists and inhibitors on the phenotype of primary cardiac fibroblasts. The cells were isolated from adult mice and 24 h after plating they were exposed to PKC agonists (10 nM PMA or 10 μM HMI-1b11) with or without PKC inhibitors (1 μM Gö6976, 1 μM Gö6983) for 48 h. At 72 h after plating the cells were fixed and stained. Representative images of (A) α-smooth muscle actin (α-SMA) and (B) BrdU stainings. Quantifications for (C) the intensity of α-SMA staining and (D) the proportion of BrdU positive cells. Results are expressed as mean + SEM (n=3). *P<0.05 vs. DMSO-control (randomized block ANOVA followed by Dunnett’s post hoc test). DDR2, discoidin domain receptor 2.

To study the effects of the PKC-targeted compounds on the expression of genes related to cardiac fibrosis, mRNA levels were analysed with qPCR after treating fibroblasts with the compounds for 48 h. Both PKC agonists decreased the expression of several genes that encode structural proteins of cardiac ECM. Both PMA (10 nM) and HMI-1b11 (10 μM) caused a significant decrease in the expression of the type III collagen encoding gene Col3a1; 64% (P<0.001) and 53% (P=0.007), respectively (Fig. 17). Moreover, PMA decreased the expression of type I collagen encoding Col1a1 by 47% (P=0.003). Slight but not significant decreases were also detected in
the expression of Col1a2 and fibronectin encoding Fn1. All these decreases were inhibited or attenuated with the pan-PKC inhibitor Gö6983 but not with the cPKC inhibitor Gö6976, suggesting they were nPKC-dependent. Interestingly, co-exposure to Gö6976 tended to further decrease the mRNA levels of these structural protein encoding genes, particularly with the weaker PKC activator HMI-1b11.

Figure 17. The effects of PKC agonists and inhibitors on gene expression in primary cardiac fibroblasts. Heat map of relative gene expression levels on a log10 scale. The cells were isolated from adult mice and 24 h after plating they were exposed to PKC agonists (10 nM PMA or 10 μM HMI-1b11) with or without PKC inhibitors (1 μM Gö6976, 1 μM Gö6983) for 48 h; mRNA was then extracted and measured by qPCR. The transcript levels were adjusted to the average of two housekeeping genes (18S and Actb) from the same samples and normalized to the DMSO-control (n=3). G76, Gö6976; G83, Gö6983; 1b11, HMI-1b11.

The expression of periostin encoding Postn and α-SMA encoding Acta2, both associated with fibroblast transdifferentiation, were decreased significantly after a 48-h exposure to a PKC activator. PMA decreased the expression of Postn by 60% (P=0.005) and HMI-1b11 by 48% (P=0.022) compared to the DMSO-control. The effect was inhibited with both PKC inhibitors, suggesting it was cPKC-dependent. The mRNA level of Acta2 was not affected after a 48-h exposure to PMA but was 36% lower (P=0.004) in HMI-1b11-treated cells compared to control. The expression of
Results

*Mmp2* (matrix metalloproteinase encoding), *Tcf21* (transcription factor 21 encoding), and *Myh10* (myosin heavy chain 10 encoding) genes, on the other hand, were not affected significantly by the PKC activators, while the expression of the TGF-β1 encoding gene *Tgfb1* was up-regulated significantly by HMI-1b11 (54%; *P*=0.019). This effect was attenuated with both PKC inhibitors, especially Gö6983. Lastly, the expression of *Mmp9* increased after a 48-h exposure to the PKC agonists: 2.6-fold (*P*=0.035) in PMA-treated cells and 1.3-fold (not statistically significant) in HMI-1b11-treated cells compared to control. In both cases the effect on the expression of *Mmp9* was attenuated with co-exposure to Gö6976 and Gö6983, suggesting it was cPKC-dependent.

In order to confirm that the effects on transdifferentiation, proliferation, and gene expression are due to PKC activation and not inhibition by downregulation, ERK1/2 phosphorylation and PKC protein levels were studied using Alpha technology and western blotting, respectively. A 30-min exposure to PMA at 10 nM concentration and HMI-1b11 at 10 μM concentration induced 2.9-fold (*P*=0.016) and 2.6-fold (*P*=0.037) increases, respectively, in the amount of p-ERK1/2 compared to the DMSO-control. The increases were inhibited with the pan-PKC inhibitor Gö6983 but not with the cPKC inhibitor Gö6976, suggesting that PKC agonist-induced activation of the MEK–ERK pathway is mediated by nPKCs in cardiac fibroblasts. Neither PMA (at 10 nM) nor HMI-1b11 (at 10 μM) had an effect on PKCa, PKCe or PKCγ protein levels after a 48-h treatment. Both PKC activators induced a minor downregulation of PKCδ, which was not inhibited by co-exposure to the pan-PKC inhibitor Gö6983. The PMA-induced decrease of PKCδ was 25% (*P*=0.040) and HMI-1b11-induced decrease 34% (*P*=0.003) compared to control.

Overall, the results show that PKC agonists inhibit mouse cardiac fibroblast transdifferentiation into myofibroblasts, decrease cardiac fibroblasts proliferation, and decrease expression of collagen encoding genes in cardiac fibroblasts without noticeably downregulating the PKC isoymes. Moreover, the results suggest that cardiac fibroblast transdifferentiation and proliferation are distinctly regulated by classical and novel PKC isoforms, respectively.


6 DISCUSSION

Toxicity and lack of efficacy are two major causes of failure in preclinical and clinical drug development (Kola and Landis 2004; Laverty et al. 2011). This indicates that the existing and routinely used screening methods and in vitro cell models are not appropriate, which leads to misjudgements in lead selection. Typically, early drug discovery projects have relied on tumour-derived and genetically immortalized cell lines and primary cells from experimental animals (Horvath et al. 2016). Toxic compounds are most often identified during the preclinical phase; however, they may only come up in the clinical phase or during post-approval follow-up with detrimental results (Kerbrat et al. 2016; Suntharalingam et al. 2006; Wysowski and Bacsanyi 1996). More precise in vitro toxicity assessment using human cell models would improve the validity of the results as well as reduce the use of experimental animals (Sison-Young et al. 2012). Improved sensitivity and specificity in the early detection of toxicity risks would also reduce the likelihood of mistakenly discarding viable drug candidates (Gintant et al. 2016). Similarly, the use of appropriate preclinical models for efficacy testing could help the progression of beneficial drug candidates. As the monetary cost of failure increases with progression towards clinical trials (Paul et al. 2010), more efficient and precise compound screening in early phases could noticeably improve research and developmental productivity.

The present study demonstrates how the cell model selection can have a major effect on the results, and thus highlights the importance of selecting an appropriate experimental model and cell type for compound screening and testing already in early phases of drug discovery projects. The present study also brings novel information on small molecule compounds targeted to two different potential drug targets and helps to clarify the role and significance of these targets in pathological cardiac remodelling. Overall, the data support further development of both GATA4-targeted 3i-1000 derivatives and PKC activators.

6.1 In vitro model of delayed doxorubicin-induced cardiotoxicity

Cardiotoxicity is a well-recognized adverse outcome related to doxorubicin therapy and can lead to long-term morbidity (Senkus and Jassem 2011). The exact mechanisms of doxorubicin-induced cardiotoxicity are still unclear but involve DNA damage and apoptosis (Arola et al. 2000; Lyu et al. 2007; Rochette et al. 2015; Zhang et al. 2012). Among other mechanisms, cardiomyocyte death has been connected with downregulation of the transcription factor GATA4 (Aries et al. 2004; Bien et al. 2007; Esaki et al. 2008; Kim et al. 2003; Kobayashi et al. 2006; Kobayashi et al. 2010; Koka et al. 2010; Riad et al. 2008). One reason for the lack of full understanding of the mechanisms may be that conventional preclinical models are not appropriate or clinically relevant (Madonna et al. 2015). For instance, most studies have evaluated
In the present study, an in vitro model of long-term low-dose administration of doxorubicin utilising hiPSC-CMs was established to more accurately mimic long-term doxorubicin dosing and delayed cardiotoxic effects in clinical practice. In the model, cardiomyocytes were exposed to doxorubicin at 100 nM concentration for up to 21 days. While micromolar concentrations of doxorubicin caused severe acute toxicity leading to considerable cell death already after two days, the 100 nM concentration reduced cell viability over long-term exposure without causing excessive cell death. Over long-term treatment, 100 nM doxorubicin also induced a significant reduction in DNA content as well as an increase in caspase-3/7 activity in hiPSC-CMs, which further validates long-term low-dose exposure of hiPSC-CMs as a novel model of doxorubicin-induced cardiotoxicity. When compared with the other hiPSC-CM-based models that have recently been used to study doxorubicin toxicity (Burridge et al. 2016; Chaudhari et al. 2016a; Chaudhari et al. 2016b; Louisse et al. 2017; Zhao and Zhang 2017), the concentration was lower and the exposure time longer, which can be expected to more accurately model chronic dosing in human cancer patients and the cardiotoxicity that ensues. The 100 nM concentration of doxorubicin compares to the plasma levels that are reached within a few hours after doxorubicin administration and are maintained by continuous infusion in clinical use (Creasey et al. 1976; Greene et al. 1983; Muller et al. 1993; Speth et al. 1987). Interestingly, 100 nM doxorubicin had no significant effect on GATA4 protein levels in hiPSC-CMs under the present experimental conditions. In previous in vitro studies in which doxorubicin was shown to decrease GATA4 mRNA and protein levels, doxorubicin concentrations were higher and treatment times ≤24 h (Aries et al. 2004; Aries et al. 2014; Kim et al. 2003; Kobayashi et al. 2006; Kobayashi et al. 2010). Similarly, in the prior in vivo studies, mice were treated with a single high-dose injection of doxorubicin (Aries et al. 2004; Kobayashi et al. 2006), thus suggesting that the changes in GATA4 levels are related to short-term high-dose doxorubicin treatments. On the other hand, in the present study, doxorubicin at 100 nM concentration did induce a statistically significant reduction in GATA4 levels in NRVMs already after a 24-h treatment, indicating possible interspecies differences. Moreover, the immaturity of the hiPSC-CMs and the potential limitations that may entail should also be considered. However, the finding that doxorubicin had no effect on GATA4 protein levels either in vitro in hiPSC-CMs after long-term treatments or in vivo in a rat model of chronic doxorubicin cardiotoxicity in the present study suggests that the mechanism of chronic doxorubicin cardiotoxicity is not related to obvious changes in GATA4 protein levels. More investigations are still needed in the future to fully understand the role of GATA4 in doxorubicin-induced toxicity.
6.2 Stem cell-based models in compound testing

Stem cells proved to be the most sensitive screening tool to identify the toxicity of GATA4-targeted compounds. The toxicity of the lead compound 3i-1000 could be detected with HCA using the caspase-3/7 activity reporter already after a 4-h treatment as well as by the MTT assay after a 24-h treatment in hiPSCs. The result is not unexpected, as stem cells are known to be highly sensitive to rapid apoptosis in response to DNA damage (Dumitru et al. 2012; Liu et al. 2013; Liu et al. 2014). Significant toxicity was observed with several of the GATA4-targeted compounds in hiPSCs, mESCs, and D5EBs, of which hiPSCs were the most sensitive cell type as demonstrated by the maximal toxicity induced at the highest concentration tested. In the toxicity screening, none of the selected GATA4-targeted compounds induced significant toxicity in cardiomyocytes, fibroblasts, or H9c2 cells after a 24-h treatment as measured by the MTT assay, indicating these cell types are more resistant compared with stem cells. The two fibroblast types, NRCFs and MEFs, acted comparably, which suggests that the GATA4-targeted compounds do not exhibit species-specific acute toxicity in cardiac fibroblasts. However, interspecies differences cannot be entirely ruled out based on these results, as was demonstrated in cardiomyocytes.

In cardiomyocytes, the 3i-1000-induced toxicity was more delayed. A 24-h exposure to 3i-1000 at 30 μM concentration had no effect on mitochondrial metabolism in cardiomyocytes as measured by the MTT assay; however, the compound-induced increase in caspase-3/7 activity could be detected already at this time-point using HCA, even at the concentration of 10 μM. While active caspases can be involved in other cellular processes, including cell division and differentiation (Hashimoto et al. 2008; Sztiller-Sikorska et al. 2009), effector caspase-3/7 activation is considered a hallmark of apoptosis (Del Re et al. 2019). Thus, the present results demonstrate the utility of image-based high-content analysis to detect compound toxicities earlier compared to the more traditional MTT assay. Overall, the delayed toxicity of the GATA4-targeted 3i-1000 demonstrates the significance of using longer exposure times and multiple parameters in in vitro toxicity screening.

A comparison between the two cardiomyocyte models suggests that some differences exist and neonatal primary cardiomyocytes are more sensitive compared with hiPSC-CMs. This is in line with a previous report by Mioulane and colleagues showing that hESC-CMs are generally more resistant to chelerythrine-stimulated apoptosis than NRVMs (Mioulane et al. 2012). In the present study, doxorubicin-induced reduction in cell viability was more prominent in primary cardiomyocytes. Other notable differences were the doxorubicin-induced decrease in GATA4 levels in NRVMs (not detected in hiPSC-CMs) and the 3i-1047-induced increase in hiPSC-CM viability (not detected in NRVMs). The findings suggest that the molecular mechanisms of doxorubicin-induced toxicity to some extent differ between hiPSC-CMs and NRVMs. The mechanism and significance of the 3i-1047-induced increase remains to be
established. Otherwise, in the toxicity screening of the GATA4-targeted compounds, the two cardiomyocyte models acted comparably. Regarding preclinical drug development, the lack of GATA4 protein depletion in response to long-term low-dose doxorubicin treatment in hiPSC-CMs as well as in vivo in rats demonstrates that selecting a suitable model is a key element also in investigating the mechanisms of actions of drugs. The present study suggests that long-term exposure of hiPSC-CMs can be utilised as an in vitro model of delayed doxorubicin-induced toxicity to study the mechanisms of cardiotoxicity as well as the effects of novel cardioprotective drug candidates.

6.3 Cardioprotective potential of the GATA4-targeted compound

Currently, the main strategies to prevent left ventricular dysfunction and heart failure induced by doxorubicin include reduction in the cumulative dose, use of continuous infusions to decrease peak plasma levels, liposomal formulations and less toxic analogues of anthracyclines, as well as the cardioprotective agent dexrazoxane (Zamorano et al. 2017). Unfortunately, these strategies fail to prevent a subset of cancer patients from developing heart failure. Thus, there is a need for novel therapies for prevention and management of doxorubicin-induced cardiotoxicity.

In this study, the aim was to investigate if the GATA4-targeted compound 3i-1000 has cardioprotective potential against doxorubicin cardiotoxicity. Previously, 3i-1000 has been reported to improve cardiac function in experimental models of myocardial infarction and Ang II-mediated hypertension (Kinnunen et al. 2018; Välimäki et al. 2017). It inhibits GATA4–NKX2-5 interaction by directly binding to GATA4 protein (Jumppanen et al., unpublished) and attenuates stretch-, ET-1-, and phenylephrine-induced gene expression of ANP and BNP, as well as hypertrophic cell growth in cardiomyocytes (Kinnunen et al. 2018; Välimäki et al. 2017). In the present study, 3i-1000 attenuated the doxorubicin-induced increase in proBNP expression in hiPSC-CMs after a 4-day treatment. Importantly, in the in vivo rat model of doxorubicin cardiotoxicity, a 2-week treatment with 3i-1000 inhibited doxorubicin-induced cardiac damage by restoring left ventricular ejection fraction and fractional shortening. Taken together, these results show that compound 3i-1000 exhibits cardioprotective actions against doxorubicin cardiotoxicity both in vitro and in vivo.

However, the present results also reveal that further structural optimization is required. The structure–toxicity relationship analysis revealed a phenyl ring in the southern part of the GATA4-targeted compounds as a major cause for the observed progenitor and stem cell toxicity. On the other hand, a structure–activity relationship analysis has revealed that the inhibition of GATA4–NKX2-5 transcriptional synergy is regulated by the aromatic isoxazole substituent in the southern part (Jumppanen et al. 2019). Together these data support further lead optimization towards more efficacious non-toxic derivatives, i.e. compounds with a five-membered ring in the
southern part of the molecule. Additionally, the mechanism of action of 3i-1000 remains to be investigated in future experiments.

6.4 Antifibrotic potential of PKC agonists

The role of PKC in heart failure has been investigated in numerous studies and the results suggest that PKC isoforms play a role in cardiac fibrosis and remodelling. However, the results are sometimes conflicting and difficult to interpret. In knockdown models, the partially overlapping roles and compensatory changes in the expression of PKC isoforms complicate the analysis of the results (Klein et al. 2005; Song et al. 2015). On the other hand, many of the pharmacological tools that have been used to define the cardiac actions of PKC exert cellular actions via several PKC isoforms as well as other kinases, thus limiting the conclusions from such studies (Steinberg and Sussman 2005; Wu-Zhang and Newton 2013). Lastly, the effect of PKC activation or inhibition is often cell type and stimulus-dependent, which makes the identification of the precise PKC isoforms that could serve as therapeutic targets for the treatment of adverse myocardial remodelling particularly challenging. Interestingly, not much is known about the role of PKC specifically in cardiac fibroblasts. Most reports concerning the role of PKC in cardiac fibrosis are in vivo studies, leading to difficulties in deciphering whether the effects on fibroblasts are direct or indirect. Particularly, the roles of PKC isoforms in cardiac fibroblast transdifferentiation and proliferation, two central processes in the development of fibrosis, and the potential of targeting PKC with pharmacological compounds to inhibit pathological fibrosis have not been fully evaluated.

In the present study, the aim was to investigate the effects of pharmacological PKC activation on phenotype, cell cycle activity, and gene expression of adult mouse cardiac fibroblasts in vitro. To clarify the role of different PKC subfamilies, two PKC inhibitors, which differ in their selectivity towards classical and novel PKC isoforms, were used. Based on the changes observed in both α-SMA protein levels and periostin mRNA levels after a 48-h treatment with the PKC modulators, both PKC agonists used in this study (PMA at 10 nM and HMI-1b11 at 10 μM concentration) inhibited cardiac fibroblast transdifferentiation into myofibroblasts cPKC-dependently. The potent PKC activator PMA also decreased the proportion of BrdU positive cells as well as downregulated the expression of collagen encoding genes nPKC-dependently, indicating that PKC activation decreases cardiac fibroblast proliferation as well as attenuates the production of new matrix proteins. The weaker PKC agonist isophthalate derivative HMI-1b11 induced parallel changes in cell cycle activity and gene expression. Taken together, the results reveal distinct PKC-dependent regulation of cardiac fibroblast transdifferentiation and proliferation and suggest that PKC agonists exhibit potential as an antifibrotic treatment (Fig. 18). In the present study, PMA proved out to be more effective in inhibiting cardiac fibroblast activation compared with HMI-1b11. However, considering the propensity of the
ultrapotent PKC agonists, such as PMA, to promote tumours by downregulating PKC (Antal et al. 2015; Newton and Brognard 2017), weaker agonists that do not downregulate PKC completely, such as the isophthalate HMI-1b11 (Sarajärvi et al. 2018), could serve as better leads for PKC-targeted therapies.

**Figure 18.** The antifibrotic effects of PKC agonists in mouse cardiac fibroblasts. cPKC, classical protein kinase C isoforms; nPKC, novel protein kinase C isoforms; α-SMA, α-smooth muscle actin.

### 6.5 Limitations

When assessing results from cell culture studies involving only one cell type, it should be kept in mind that cardiac remodelling is a multifactorial process. What eventually takes place on the tissue level, depends on the cooperation and interaction between different cell types present in the cardiac tissue. For instance, coculture studies have shown that crosstalk between fibroblasts and cardiomyocytes influence both fibrotic and hypertrophic responses (Fredj et al. 2005; Zhang et al. 2019). Moreover, depending on the cell type used, various factors should be considered in view of the applicability of the present results to human patients, the obvious ones being the immature phenotype of hiPSC-CMs as well as the potential interspecies differences between human, rat, and mouse cells. Expressions of the PKC isoforms and thereby PKC-mediated signalling may change in disease states, likely resulting in changes in the effects of PKC-targeted compounds as well. Similarly, the sex and age of the animals could potentially have an effect on the drug responses demonstrated here, and it should be noted that the adult mouse cardiac fibroblasts used in this study were isolated from healthy female mice. Thus, further studies in a multicellular and
pathophysiological context are required to confirm the antifibrotic effects of PKC activators. Coculture studies could be carried out using, for instance, a hiPSC-based platform (Zhang et al. 2019). Another limitation of the present study is the fact that the pharmacological tools targeting multiple PKC isoenzymes also target other kinases. However, parallel use of Gö6976 and Gö6983 allows analysis of whether an effect is nPKC-dependent, cPKC-dependent, or PKC-independent. Alternatively, isoenzyme-specific silencing could be used to investigate the role of individual PKC isoforms in cardiac fibroblasts. However, as discussed above, conclusions from such experiments are also limited. Furthermore, PKC has also non-catalytic functions (Cameron et al. 2008; Ling et al. 2007; Zeidman et al. 1999) and therefore genetic silencing and pharmacological inhibition of certain PKC isoforms may not always result in similar outcomes.

It is also noteworthy to point out some challenges that would come from attempting to modify either GATA4 or PKC activity in clinical practice. Both targets are expressed and functional also in many other tissues besides heart, and thus strategies to limit off-targeting of other organs should be developed. Regarding PKC activators, drug therapy should be targeted specifically to cardiac fibroblasts, as pharmacological nPKC activation has been shown to induce cardiomyocyte hypertrophy (Pohjolainen et al. 2021). One opportunity for targeted drug delivery could be developing nanotherapies targeted to the injury site of the myocardium (Ferreira et al. 2017). Development of nanoparticles targeted specifically to fibroblasts/myofibroblasts would require a cell type specific cellular marker, which unfortunately has not been identified (Kalluri and Zeisberg 2006; van den Borne et al. 2010).
Summary and conclusions

The aim of the present study was to compare different cell models in in vitro toxicity screening, to establish an in vitro model of delayed doxorubicin-induced cardiotoxicity using hiPSC-CMs, as well as to characterise pharmacological and toxicological effects of small molecules targeted to the transcription factor GATA4 or PKC. The main findings are summarized as follows:

1. The screening of GATA4-targeted small molecules for cytotoxicity in several cardiac and stem cell models revealed profound differences in the sensitivity of different cell types. The results highlight the importance of cell type selection for toxicity screening and indicate that stem cells represent the most sensitive screening model to identify toxicity of small molecules targeted to the transcription factor GATA4. The toxicity screening of the eight novel GATA4-targeted compounds also revealed significant structure-dependent toxicity profiles. The data suggest that a six-membered ring in the southern part of the molecule is predominantly responsible for the toxic outcome in stem cells. Based on these results, further compound development may be directed towards non-toxic derivatives, i.e. compounds with a five-membered ring in the southern part of the molecule.

2. To study chronic doxorubicin cardiotoxicity in vitro, hiPSC-CMs were treated with doxorubicin at 100 nM concentration for up to 21 days. The concentration was selected based on cell viability data evaluation. Over long-term treatment, doxorubicin at 100 nM concentration decreased DNA content and increased caspase-3/7 activity, while inducing a modest reduction in cell viability in hiPSC-CMs. The data demonstrate that long-term low-dose exposure of hiPSC-CMs can be utilised as an in vitro model of delayed doxorubicin-induced cardiotoxicity.

3. The GATA4-targeted lead compound 3i-1000 exhibited cardioprotective potential by attenuating the doxorubicin-induced increase in proBNP expression in hiPSC-CMs. Moreover, it restored cardiac function in an in vivo rat model of chronic doxorubicin cardiotoxicity. Together, the results suggest that targeting GATA4 may counteract doxorubicin-induced cardiotoxicity. However, as the compound 3i-1000 was toxic to cardiomyocytes over long-term treatment, further structural optimization is required to develop non-toxic derivatives.

4. Characterisation of mouse cardiac fibroblasts in response to several different PKC agonists and inhibitors revealed that PKC agonists inhibit cardiac fibroblast transdifferentiation into myofibroblasts, decrease cardiac fibroblast proliferation, and decrease expression of collagen encoding genes in cardiac fibroblasts. Moreover, the data suggest that cardiac fibroblast
transdifferentiation is regulated via cPKCs, whereas cardiac fibroblast proliferation and collagen gene expression are regulated via nPKCs. Overall, the results suggest that fibroblast-targeted PKC activation may be a promising strategy to inhibit cardiac fibrosis.
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