Cardiotoxicity of kinase inhibitors: the prediction and translation of preclinical models to clinical outcomes

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Abstract | Targeted therapeutics, particularly those that inhibit the activity of protein kinases that are mutated and/or overexpressed in cancer, have revolutionized the treatment of some cancers and improved survival rates in many others. Although these agents dominate drug development in cancer, significant toxicities, including cardiotoxicity, have emerged. In this Review, we examine the underlying mechanisms that result in on-target or off-target cardiotoxicities of small molecule kinase inhibitors. We also discuss how well the various preclinical safety models and strategies might predict clinical cardiotoxicity. It is hoped that a thorough understanding of the mechanisms underlying cardiotoxicity will lead to the development of safe, effective drugs and consequently, fewer costly surprises as agents progress through clinical trials.

Protein kinases are intriguing molecular targets for therapeutic intervention. Through reversible phosphorylation, kinases regulate a wide array of signalling pathways that control metabolism, cell cycle progression, cell proliferation and cell death, differentiation and survival. There are over 500 kinases in the human kinome, and over 150 of these have been shown or are proposed to be involved in the onset and/or progression of various human diseases including inflammatory diseases, cardiovascular diseases, metabolic diseases, neurodegenerative diseases and cancer[1].

These kinases are therefore putative targets for drug development. Indeed, development of kinase inhibitors — predominantly targeting kinases that are dysregulated in various cancers — is a rapidly growing area of drug discovery, partly as a result of some remarkable early successes (see below), as well as the relative ease with which these agents can be designed. This ease in design is attri-butable to the fact that the structure of the key region of the kinase targeted by most inhibitors (the ATP binding pocket) has been well-studied and is highly conserved across the kinome[2]. Thus the vast majority of approved kinase inhibitors and drugs in development target this ATP binding pocket, preventing ATP from binding to the kinase. If ATP cannot bind, the kinase cannot phosphorylate its downstream targets and signalling is inhibited.

However, as discussed below, the conservation of the ATP binding pocket among kinases means that inhibitors can also inhibit unintended kinases, and if any of these kinases serve important functions in the heart, 'off-target' cardiotoxicity can result. In addition, as we discuss some of the problems inherent to kinase inhibitors, it will become apparent that many of the pathways that regulate cancer cell survival also regulate essential processes in cardiomyocytes, including survival. So, although inhibiting those kinases in cancer is beneficial[3], inhibiting them in the cardiomyocyte may not be.

On the market in the United States, there are currently nine small molecule ATP-competitive inhibitors that target a range of kinases and three inhibitors of mTOR (mammalian target of rapamycin) that work through a novel inhibitory protein–protein interaction (TABLE 1). Numerous additional agents are also in development[4]. Although cases of cardiotoxicity associated with kinase inhibitors (herein defined as drug-related deterioration in cardiac function and/or development of congestive heart failure (CHF)) have been reported for several of these molecules, in general the majority of the approved agents appear to be well-tolerated from a cardiac safety perspective[5]. That said, albeit with the few exceptions that we note below, the true risk of cardiotoxicity is not known because thorough clinical assessments of left ventricular function have not been done. Furthermore, patients in early phase clinical trials typically lack significant cardiovascular co-morbidities. This makes it difficult to predict rates

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Table 1 | **Kinase inhibitors on the US market**

Drug	Year of approval	Intended kinase target(s)	Disease
Sirolimus (Rapamune; Pfizer)	2000	mTOR	Prevention of organ rejection in patients receiving transplants
Imatinib (Gleevec; Novartis)	2001	ABL, ARG, PDGFR-α/β, KIT	CML, GIST, B-ALL, CMML, CEL
Gefitinib (Iressa; AstraZeneca)	2003	EGFR	NSCLC
Erlotinib (Tarceva; Roche/Genentech)	2004	EGFR	NSCL and pancreatic carcinoma
Sorafenib (Nexavar; Bayer)	2005	B-RAF, VEGFRs, PDGFRα/β, FLT3, KIT	RCC, liver carcinoma
Sunitinib (Sutent; Pfizer)	2006	VEGFR, PDGFR, CSF1R, FLT3, KIT	RCC, GIST
Dasatinib (Sprycel; Bristol-Myers Squibb)	2006	ABL, ARG, KIT, PDGFRα/β, SRC	CML with imatinib resistance and/or intolerance
Temsirolimus (Torisel; Pfizer)	2007	mTOR	RCC
Nilotinib (Tasigna; Novartis)	2007	ABL, ARG, KIT, PDGFRα/β	CML with imatinib resistance and/or intolerance
Lapatinib (Tykerb; GlaxoSmithKline)	2007	EFGR (ERBB1 and 2)	HER2 positive breast cancer
Everolimus (Afinitor; Novartis)	2009	mTOR	RCC
Pazopanib (Votrient; GlaxoSmithKline)	2009	VEGFR, PDGFRα/β, and KIT	RCC

ARG, ABL-related gene protein; B-ALL, B-cell acute lymphoblastic leukaemia; CEL, chronic eosinophilic leukaemia; CML, chronic myeloid leukaemia; CMML, chronic myelomonocytic leukaemia; CSF1R, colony-stimulating factor 1 receptor; EGFR, epidermal growth factor receptor; ERBB2, human epidermal growth factor receptor 2 (also known as HER2); ERBB4, human epidermal growth factor receptor 4; FLT3, FMS-related tyrosine kinase 3; GIST, gastrointestinal stromal tumour; mTOR, mammalian target of rapamycin; NSCLC, non-small-cell lung cancer; PDGFR, platelet-derived growth factor receptor; RCC, renal cell carcinoma; VEGFR, vascular endothelial growth factor receptor.

of cardiotoxicity when agents become approved and are subsequently used in a broader population.

To date, the only approved kinase inhibitor that is clearly associated with clinical cardiotoxicity is sunitinib (Sutent; Pfizer). In a retrospective review of a series of patients with gastrointestinal stromal tumours who underwent regular type radionuclide ventriculography, 18% of patients developed either CHF or a decline in left ventricular ejection fraction of ≥15 points — this was a clinically significant decline[6]. The only other large study of cardiotoxicity with a small molecule kinase inhibitor was with lapatinib (Tykerb; GlaxoSmithKline) (which inhibits the epidermal growth factor receptor and HER2 (also known as ERBB2). Although very low rates of cardiotoxicity were reported in the lapatinib trial, the majority of patients had been previously treated with trastuzumab (Herceptin; Roche/Genentech) (a monoclonal antibody targeting HER2 that is associated with cardiac dysfunction) without obvious cardiotoxicity, and thus were presumably less likely to develop left ventricular dysfunction from an anti-HER2 small molecule inhibitor[7]. Other than these two comprehensive cardiovascular safety studies on sunitinib and lapatinib, only data from series of case reports and limited cardiac safety information from Phase III registration trials are available.

Although clinically important cardiotoxicity appears to be limited to only a few currently marketed agents, a major concern is that a large number of drugs in development — many of which are multi-targeted — either intentionally or unintentionally inhibit pathways that maintain cardiomyocyte homeostasis, especially under cardiac stress. Thus, it is imperative to develop strategies that accurately identify problematic agents early in the drug development process.

In this Review, we will discuss the growing connection between preclinical models of kinase inhibitor-induced cardiotoxicity and clinical safety. As protein kinases regulate numerous aspects of cell signalling, developing inhibitors to prevent a tumour from having a selective growth advantage can lead to overlapping deleterious effects on cardiac biology and physiology. We discuss the challenges of making safe and selective kinase inhibitors by examining the cardiac effects of sunitinib. We also explore the vast field of genetically modified mouse models and discuss their merits in predicting more accurately which kinase inhibitors may have the potential to cause cardiotoxicity. Additional *in vitro* models used to predict cardiotoxicity are reviewed, with emphasis on human stem cell-derived cardiomyocytes, as they have the potential to mimic cardiac biology and cardiac energy homeostasis, and be a means of introducing phenotypic and genotypic diversity of the human race into preclinical safety assessment. Lastly, we conclude with future perspectives in clinical studies, including biomarkers and imaging.

Small molecule kinase inhibitors

As noted above, the vast majority of small molecule kinase inhibitors are designed to competitively target the ATP binding domain of the kinase of interest. Given the structural conservation of the ATP binding pocket across the 500+ kinases of the human kinome[2], limited

Regular type radionuclide ventriculography
The use of radionuclides to study left ventricular function.

Multi-targeted
Intentionally designed kinase inhibitor of more than one kinase.

Cardiac stress
Either increased demand (for example, hypertension) or reduced supply (for example, ischaemia) of oxygen to the heart.

selectivity is an inherent property of small molecule inhibitors and a challenge in the design of these agents. Strategies to improve selectivity go beyond selectively targeting the ATP binding pocket and include considering regions adjacent to the pocket (this is the mechanism used by Type II inhibitors) or targeting allosteric regions remote from the pocket (this is strategy adopted by Type III inhibitors)[8–10]. Targeting allosteric regions would be much more selective, but because they exploit novel structural aspects of individual kinases, they are more difficult to design. Although Type III inhibitors constitute a small minority of the kinase inhibitors in development, additional allosteric inhibitors may be identified as screening strategies migrate towards cell-based screening assays[11].

The cardiotoxicological properties of small molecule kinase inhibitors will typically be due to (intended or unintended) kinase inhibition, although off-target effects on non-kinases could have a role. In some cases, on-target (intended) inhibition of a kinase which is appealing from a tumour progression and/or angiogenesis perspective could also be physiologically crucial in the heart and/or the vasculature (BOX 1). Off-target kinase inhibitor-mediated cardiotoxicity is a consequence of the inherent challenge of selectivity of most ATP-competitive kinase inhibitors. This leads to unintended inhibition of multiple kinases beyond the intended target kinase (or kinases). Although these off-target kinases may or may not have a role in tumour progression, they may be essential to the function of the heart. Exacerbating this form of toxicity is the current trend of intentional 'multi-targeting' (inhibiting multiple kinases with a single drug)[12]. Members of this class of agents typically target factors driving both tumour progression and angiogenesis, and targets linked to angiogenesis include vascular endothelial growth factor receptor 2 (VEGFR2), placenta growth factor (PLGF), and platelet-derived growth factor receptor-β (PDGFRβ). It has become increasingly apparent that agents targeting VEGFRs, particularly VEGFR2, induce significant hypertension[13]. Indeed, this appears to be a class effect of these agents, be they small molecules or monoclonal antibodies (mAbs), and can require at least temporary cessation of treatment. This additional stress of hypertension can be particularly problematic in patients with an already compromised cardiac reserve or advanced coronary artery disease.

An additional issue that is likely to exacerbate kinase inhibitor toxicity is pathway targeting; that is, the targeting of multiple kinases within a specific pathway to achieve maximal suppression of activity of that pathway. For example, inhibition of multiple components of the phosphoinositide 3-kinase (PI3K)–AKT pathway appears to be a viable strategy in a number of cancers[14]. However, this pathway also maintains cardiomyocyte homeostasis and protects cardiomyocytes from injury and death[15]. Thus, the potential cardiovascular toxicity of compounds that inhibit the PI3K–AKT pathway should be carefully examined.

A third mechanism by which small molecule kinase inhibitors could mediate toxicity would be through the inhibition of non-kinase targets. Any enzyme that requires ATP to perform its various functions could be inhibited by small molecule kinase inhibitors. Thus, even if one knew the complete selectivity profile of a drug against all kinases, it would be impossible (at least at this point in time) to have a full selectivity profile against the non-kinase targets. Even the relatively selective kinase inhibitor imatinib (Gleevec; Novartis) is known to inhibit at least two non-kinase targets, the quinone oxidoreductase NQO2 and the ABC family member BCRP (also known as ABCG2)[16]. NQO2 is believed to have a protective role in the setting of oxidative stress. Although BCRP is not expressed in adult cardiomyocytes, it is expressed in a subset of stem/progenitor cells, the so-called side population cells (in fact,

Box 1 | **Kinase cardiac biology and the potential mechanistic links to cardiotoxicity**

Cardiovascular energy homeostasis is predominantly regulated by protein kinases, including some that are the target of marketed inhibitors or those kinase inhibitors in late phase clinical trials[88]. Beating cardiomyocytes consume vast amounts of ATP *in vivo*. The ATP is utilized to generate contractile activity of the sarcomeres (allowing the heart to contract) and to maintain the necessary gradients of calcium, sodium and potassium, which are driven by the various ion pumps and channels[89]. The most prominent cardiac ion pump is the sarco(endo)plasmic reticulum calcium ATPase (SERCA2A)[89]. To initiate systole, calcium must be released from the sarcoplasmic reticulum into the intracellular environment, and with every diastole, that calcium must be taken back up into the sarcoplasmic reticulum by SERCA2A, otherwise the heart would be unable to relax. It has been estimated that if all ATP production halted in the heart, ATP stores would be depleted within a matter of seconds[90,91]. Any perturbation in the delicate balance of ongoing energy generation and utilization (for example, myocardial ischaemia (reducing supply) or hypertension (increasing demand)), can lead to profound abnormalities in cardiac function.

Kinase signalling pathways are intimately linked with calcium homeostasis, and any dysregulation of calcium handling can lead to profound alterations, culminating in dysrhythmia, altered cardiac conduction and cardiac pathological changes such as cardiac hypertrophy[92]. This dysregulation of calcium can be detrimental, even when it occurs in microdomains within the cytosol and nucleus, and does not require global calcium dysregulation to lead to adverse left ventricular remodelling (that is, dilation and fibrosis). The changes in the ventricle are in part driven by calcium-mediated activation of calcineurin and subsequently, the transcription factors NFAT and GATA4 (REF. 93). Calcium/calmodulin-dependent protein kinase II is a key player in the pathology of hypertrophy and heart failure, as it regulates both calcium handling (via its effects on the sarcoplasmic reticulum and ryanodine receptor) and hypertrophy (via its effects on signalling pathways)[94–96]. Cardiomyocyte survival is also regulated by protein kinases, the most notable being components of the phosphoinositide 3-kinase–AKT pathway[15]. Taken together, there are multiple, critical kinase-regulated pathways that, if inhibited, could exacerbate cardiotoxicity.

Class effect
A toxicity or pharmacological outcome that occurs with all molecules that inhibit a particular target (for example, all β-adrenergic receptor antagonists lead to heart rate slowing).

Side population
A form of stem cell that is characterized by a specific pattern using fluorescent activated sorting.

BCRP-mediated extrusion of Hoechst dye is what produces the side population phenotype of these cells when they are analysed using fluorescence-activated cell sorting)[17]. The toxicological consequences of inhibiting non-kinase targets, if any, are unclear, but this issue is of concern as it significantly increases the potential for side effects and can complicate the toxicological screening strategy. From a cardiotoxicity-focused perspective, alternatives to the traditional medicinal chemistry-based strategies to design Type I (ATP-competitive) inhibitors should be explored.

Sunitinib and cardiotoxicity

To illustrate the complexities inherent in identifying mechanisms of kinase inhibitor toxicity, we will use the example of sunitinib, which appears to have both on- and off-target effects contributing to cardiotoxicity. Clinical issues with this multi-targeted agent are discussed above. It has been presumed that a major contributor to the deterioration in left ventricular function is sunitinib-induced hypertension. In support of this idea, sunitinib-treated mice did not develop cardiomyocyte apoptosis until an increase in blood pressure was induced[18].

A number of studies in various mouse models have also shown that angiogenesis (which is mediated in the heart by both VEGFR2 and PDGFRβ, targets of sunitinib) is key to maintaining cardiac homeostasis in the setting of a pressure load or ischaemia[19–22]. Although this indicates that sunitinib-induced hypertension has an important role in cardiotoxicity, several patients who developed cardiotoxicity on sunitinib did not have hypertension, suggesting that additional mechanisms may be responsible [6]. Although no head-to-head cardiotoxicity study has been conducted with VEGFR antagonists, the cardiotoxicity of sunitinib does appear to be greater than the other agents[23]. Even though this could be due to the greater potency of sunitinib in inhibiting VEGFR2, this difference suggests the possibility of additional off-target effects.

Identifying the mechanisms of sunitinib toxicity is more difficult than for other more selective kinase inhibitors, as sunitinib inhibits at least 50 kinase targets at therapeutically relevant concentrations in *in vitro* assays[24]. Hints to the mechanisms were found in transmission electron micrographs of endomyocardial biopsies from a patient who presented with profound sunitinib-associated heart failure. The biopsies showed marked mitochondrial swelling indicative of the opening of the mitochondrial permeability transition pore, a response that typically leads to a necrotic form of cell death. This swelling appeared to be so widespread that it seemed likely that energy (ATP) homeostasis may have been compromised.

Studies in neonatal rat ventricular myocytes (NRVMs) also confirmed that energy compromise does have a key role in sunitinib-mediated toxicity, but this decrease in available energy did not lead to activation of AMP-activated protein kinase, the central regulator of the response of the cell to energy perturbations. Surprisingly, the lack of a response to the energy compromise appeared to be due to direct inhibition of 5′-AMP activated protein kinase (AMPK) by sunitinib[18]. Gene transfer of an activated mutant of AMPK partially rescued the toxicity in NRVMs, suggesting that inhibition of this off-target kinase contributes to the toxicity. Although this suggests that redesigning sunitinib to prevent it from inhibiting AMPK could solve the problem of off-target toxicity, pivotal work on the protective role of AMPK in maintaining viability of hypoxic cancer cells suggests that this 'off-target' effect may be important in the anticancer efficacy of sunitinib[25]. Although the role of AMPK in cancer cell survival remains unclear[26], AMPK is central to maintaining energy balance in the cardiomyocyte, and thus a compound that alters AMPK activity should be closely scrutinized for cardiotoxicity.

Given what we know about the role of VEGFRs and PDGFRβ in the heart's response to stressors, inhibition of these kinases most probably contributes to the on-target toxicity associated with sunitinib and the other multi-targeted agents. This toxicity is mediated via adverse effects on the vasculature that, at least in animal models, lead to loss of capillaries, impaired blood flow and regional ischaemia, especially in the setting of cardiac stress[19–22]. These processes are undoubtedly mechanistic contributors to the hypertension that is characteristic of kinase inhibitors. Remarkably, it appears that hypertension is a 'biomarker' of the anticancer efficacy of the multi-targeted agents as several studies have shown that cancer patients who develop hypertension have better outcomes than those who do not[27–30]. That said, it is important to underline that hypertension is an off-target consequence and probably plays no part in the killing of tumour cells; it should therefore be aggressively treated.

This vignette on sunitinib illustrates how it can be difficult to identify the specific mechanisms of cardiotoxicity and manage the toxicity without hampering treatment of the malignancy. However, it also underscores the crucial importance of knowing the kinase selectivity profile of an inhibitor to help develop informed hypothesis-driven research[31,32]. The kinase inhibition profile can be mathematically modelled to associate particular kinases with cardiotoxicity; this is an approach that has been used to correlate kinases with other types of toxicities[33]. One of the limitations of this approach is our limited understanding of the role that the vast majority of kinases expressed in the human kinome have in cardiac biology.

Cardiac biology and stem/progenitor cells

A potential mechanism of kinase inhibitor-mediated toxicity could be to ablate or suppress the proliferation of the stem/progenitor cell compartment of the heart. Given the limited ability of adult cardiomyocytes to re-enter the cell cycle and proliferate[34], and the acute nature of cardiac injury, intrinsic mechanisms to repair the heart are not robust. Although these cardiac stem/progenitor cell populations are relatively quiescent, stress can induce these cells to generate significant numbers of new cardiomyocytes[35–37]. The physiological significance of the replication of small subsets of progenitor cells is still under debate, but it is possible that these cell populations are collateral targets of some of the small molecule kinase inhibitor anticancer agents.

Hoechst dye
A family of fluorescent stains used for labelling DNA and detecting and/or separating cell populations of interest.

Pressure load
A pressure load on the heart can be induced by anything that raises blood pressure. Experimentally, this typically means constricting the transverse aorta or infusing a drug (for example, angiotensin II).

Mitochondrial permeability transition pore
A regulatory opening within the mitochondria induced by certain types of cellular stress (for example, oxidant stress or ischemia). This results in loss of mitochondrial membrane integrity and swelling, ultimately inducing cell death.

Stem/progenitor cell compartment
The small population of undifferentiated cells within a tissue that has the potential to regenerate and replenish the dying cells of an organ.

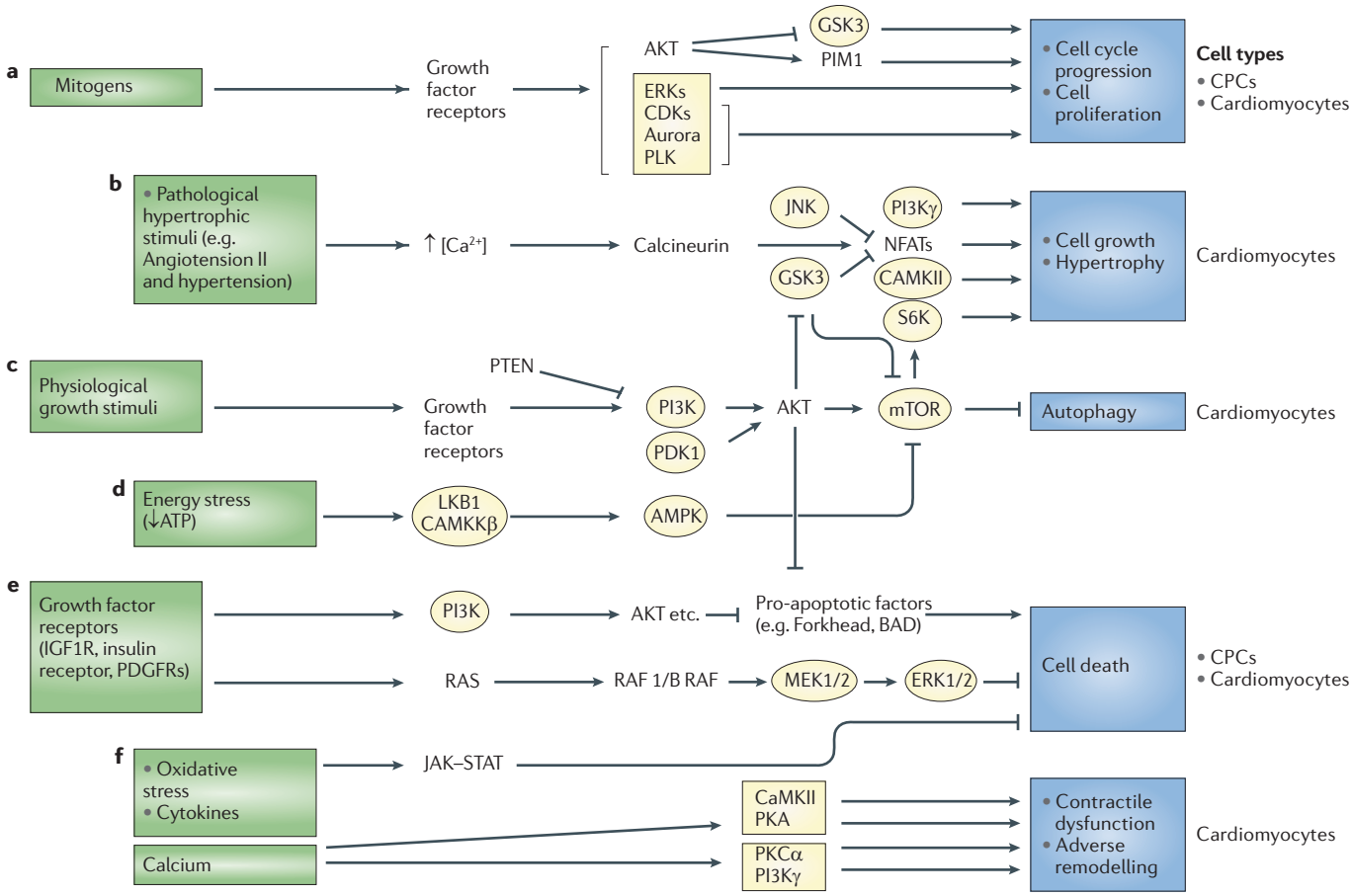

Figure 1 | **Crucial signalling pathways in the heart and consequences of their activation or inhibition.** Various stimuli (green boxes) activate downstream signalling pathways in the heart, leading to physiological or pathological responses (blue boxes). The cell types proposed, or known to be affected, are indicated. **a** | Mitogens activate growth factor receptors that act through various kinases to promote cell cycle progression and cell proliferation. In addition to the core cell cycle regulators (cyclin-dependent kinases (CDKs) and serine/threonine protein kinases (Auroras and PLKs)), extracellular signal-regulated kinases (ERKs) and AKT appear to be key to promoting proliferation of cardiac progenitor cells (CPCs) and, possibly, cardiomyocytes. AKT acts through both activation of a serine/threonine protein kinase (Pim1) and inhibition of glycogen synthase kinase-3 (GSK3). **b** | Pathological hypertrophy is driven by increases in Ca^{2+} concentration that are triggered by various stimuli (angiotensin II, hypertension and so on). This activates calcineurin, a phosphatase. Calcineurin dephosphorylates members of the NFAT family of transcription factors, allowing them to enter the nucleus and activate gene expression programs promoting hypertrophy. Counteracting this are Jun N-terminal kinases (JNKs) and GSK3 family members that phosphorylate NFATs, leading to their exclusion from the nucleus. Other key factors promoting hypertrophy are calcium/calmodulin-dependent protein kinase 2 (CaMKII) and phosphoinositide 3-kinase γ (PI3Kγ). **c** | Physiological growth is driven by various growth factor receptors that activate the PI3K–AKT pathway, leading to inhibition of GSK3. Multiple inputs lead to the activation of mammalian target of rapamycin (mTOR) and downstream targets (for example, ribosomal protein S6 kinase (S6K)) promoting physiological and pathological hypertrophic growth. **d** | Another key regulator of mTOR is 5′-AMP activated protein kinase (AMPK), the master sensor of energy status in the cardiomyocyte. When ATP is depleted, AMP levels rise and this, together with two other kinases (serine/threonine protein kinase 11 (also known as LKB1) and calcium/calmodulin-dependent protein kinase kinase (CAMKKβ), activates AMPK. Along with GSK3, AMPK inhibits mTOR-mediated protein synthesis, thereby conserving energy stores. Autophagy is a complicated response to perturbations in energy status that aims to preserve energy when needed. When the energy status is good, mTOR is active and autophagy (resulting in recycling of damaged organelles including mitochondria) is repressed. During energy stress, mTOR is inhibited and autophagy is activated, restoring homeostasis. **e** | Cardiomyocyte death is regulated by various mechanisms, but focusing on the role of kinases in the heart, the PI3K–AKT pathway (which is downstream of growth factor receptors), is central to prevention of apoptosis, by acting on a number of targets. These include the pro-apoptotic factors BCL2 antagonist of cell death (BAD) and members of the Forkhead family of transcription factors, which are inhibited by AKT. The RAS–RAF–MAPK/ERK kinase (MEK)–extracellular signal-regulated kinase (ERK) pathway is also anti-apoptotic in the heart. **f** | Cytokines and oxidative stress activate members of the Janus kinase (JAK) family and subsequently, their immediate targets, the signal transducer and activator of transcription (STAT) family of transcription factors. In the heart, this pathway protects against cell death via various mechanisms. Contractile function can also be directly regulated by various kinases such as PKA (which is downstream of β-adrenergic receptors in the 'fight or flight' response to stress) and CAMKII. Both kinases enhance contractile function but long-term activation leads to adverse remodelling in the heart. Protein kinase Cα (PKCα) and PI3Kγ (the latter acting via a scaffolding function, not its kinase activity) exert negative inotropic effects on cardiomyocytes. In the setting of heart failure, PKCα inhibition maintains contractile function, whereas inhibition of PI3Kγ appears to be harmful, worsening remodelling. IGF1R, insulin-like growth factor 1 receptor; PDGFR, platelet-derived growth factor. PKA; cAMP-dependent protein kinase.

In fact, adverse effects of cancer therapeutics on stem and progenitor cells may be exacerbated by kinase inhibitor-mediated effects on energy metabolism in the adult cardiomyocyte, as noted above for sunitinib. For example, treatment of juvenile mice with the anthracycline doxorubicin impairs cardiac progenitor cell function and vascularization, leading to cardiotoxicity as the mice age[38]. This is similar to the effect observed in children treated with doxorubicin who developed heart failure later in life[39]. Experimentally, infusion of cardiac stem cells can rescue rats from the cardiotoxicity of doxorubicin, leading the authors of the study to hypothesize that cardiac stem cells are a key target of doxorubicin-induced cardiotoxicity[36].

In other studies, inhibition in mice of KIT, the receptor for stem cell factor, resulted in enhanced cardiomyocyte proliferation and better-preserved left ventricular function when the mice were subjected to pressure overload[25,40]. Although beneficial in the short term, chronic inhibition of KIT in this model raised concerns about long-term maintenance of the progenitor pool in the heart. Although the biological significance of these findings in humans is not known, concerns regarding the effects of kinase inhibitors on stem and progenitor cells or on immature cardiomyocytes that are capable of entering the cell cycle should continue to be an active area of research.

Identification of kinases of concern

In this section we will take a 'virtual cardiotoxicity' approach by briefly examining what is known from the literature concerning the role of kinase inhibitor targets in the heart. The approach towards understanding and predicting cardiotoxicity resulting from kinase inhibition involves a combination of knowing what cell type (or types) of the heart a kinase is expressed in, and the detailed biology of that kinase in the heart. Drug targets that are potentially problematic (or as we refer to them, 'kinases of concern') can often be surmised based on gene targeting studies in mice. Thus, we speculate on possible kinases involved in cardiotoxicity, albeit in some cases with little data beyond genetically modified mice to support our conclusions.

FIG. 1 and TABLE 2 contain several examples of kinases of concern that were identified by studying gene-targeted and/or transgenic mice. A classic example is the HER2 conditional knockout mouse, which developed a dilated cardiomyopathy with ageing that was worsened by thoracic aortic constriction (TAC), mimicking severe acute hypertension[41]. More often, gene deletion may lead to no marked abnormalities unless an additional stressor is added. This pattern also suggests that stress-related recruitment of other normally quiescent pathways may be critical in the mechanism of cardiotoxicity. Other examples are the sorafenib (Nexavar; Bayer) targets RAF1 and BRAF, and the PI3K pathway. In both RAF1 and p110αPI3K dominant negative transgenic mice, there were no obvious phenotypes until the mice were subjected to TAC, at which point they developed left ventricular dysfunction[42,43]. Additional examples in which TAC revealed significant cardiotoxicity are seen in mice lacking the angiogenesis regulators VEGFRs or PDGFRβ or in mice treated with mAbs that sequester VEGF[19,20,22,44]. These mouse models offer insight into the role of stress in promoting cardiotoxicity with sunitinib (and possibly with other VEGF and VEGFR antagonists such as sorafenib, pazopanib (Votrient; GlaxoSmithKline) and bevacizumab (Avastin; Roche/Genentech)).

However, caution must be taken in predicting kinase inhibitor toxicity based solely on findings in genetically modified mice. Many studies involving gene targeting in mice used germline knockouts, meaning that the mouse phenotype could be confounded by unique developmental roles of a given gene in the embryo and/or the placenta. For example, p38α knockout mice die in mid-gestation due to malformed myocardium and vasculature; however, this was attributed to defects in placental development and not cardiac development *per se*[45]. This issue can be addressed by using conditional knockouts in which the gene of interest is deleted very late in gestation or, preferably, in adulthood and selectively within the tissue of interest. A second key confounder is that inhibition of a kinase with a kinase inhibitor is never complete in magnitude or duration; by contrast, kinase inhibition as a result of gene deletion is almost always complete, both in magnitude and duration. Furthermore, protein–protein interactions will be disrupted in the knockout, but will generally not be disrupted with drug treatment. This could cause phenotypes in the knockouts that would not be seen with kinase inhibitor treatment. Finally, the stress of severe TAC is an extreme state that patients rarely — if ever — experience. Thus, the phenotype may be significantly exaggerated in the knockouts exposed to TAC compared to a patient receiving a drug. As discussed extensively by Molkentin and Robbins[46], genetically modified mice are powerful tools that can be used to shed light on the importance of specific genes of interest, but they do not eliminate the need to thoroughly understand the molecular underpinnings of any phenotype.

Although the focus of this article is on the cardiotoxicity mediated by kinase inhibitors, there are several examples of cardiovascular diseases in which kinase inhibitors are predicted to produce beneficial effects (BOX 2).

Can preclinical models predict cardiotoxicity?

Cardiotoxicity is routinely assessed throughout the drug development process, starting during the early stages of drug discovery. Depending on the target and/or the toxicity profile of the compounds being developed, assays that can predict cardiotoxicity can be introduced during lead identification and lead optimization stages of drug development. Initial models often evaluate overt cellular toxicity in basic cell lines and cardiac electrophysiological effects are initially examined using cell lines that overexpress *HERG*. The assessment of potential cardiotoxicity of kinase inhibitors typically involves specialized *in vivo* models in which left ventricular function, electrocardiogram readings and biomarkers of cardiac injury can be quantified.

Attempts to predict clinical cardiotoxicity of kinase inhibitors have had limited success to date. There is a plethora of empirical models available, which include

Anthracycline
A class of drugs (of which doxorubicin is a member) used extensively in treatment of various cancers.

Pressure overload
A consequence of thoracic aortic constriction, resulting in increased blood pressure and subsequent cardiac hypertrophy and LV dysfunction.

Thoracic aortic constriction
A surgical procedure in which the aorta is banded, creating an acute and usually severe increase in blood pressure.

HERG
The human ether-a-go-go gene *HERG* (also known as *KCNH2*) codes for a specific potassium ion channel. Mutations in the gene cause one form of hereditary long QT syndrome.

Table 2 | **Kinases of importance in the heart and vasculature: findings in genetically modified or KI-treated mice**

Kinase	Mouse model(s)	Role of kinase in heart/vasculature	Other notes	Refs
RAF1/BRAF	KO; DNTG	Anti-apoptotic; preserves LV function under stress. KO: LV dysfunction and HF in the absence of additional stress; DNTG: reduced hypertrophy but LV dysfunction due to cell death	KO: effects mediated by ASK1; RAF inhibits ASK1 via a non-kinase-dependent mechanism. RAF mutations account for some cases of Noonan syndrome (HCM phenocopy)	42,113
PI3K (p110α)	CATG; DNTG	Physiological heart growth; cardiomyocyte survival	Greater LV dysfunction after TAC in DNTG and improved LV function in CATG mouse	43,114,115
PI3K (p110γ)	KO	Regulates contractility and pathological hypertrophy	Mice protected from isoproterenol-induced injury	116–118
PDK1	KO	Cardiomyocyte survival and β-adrenergic responsiveness	Cardiac-specific KO displayed HF and DCM	119
AKT1, 2 or 3	CATG; DNTG; KO	Regulators of cardiomyocyte survival, growth and metabolism	AKT1 promotes physiological hypertrophy and suppresses pathological hypertrophy; AKT2 promotes survival and insulin sensitivity	120,121
mTOR	Kinase inhibitor (KI) administered	mTORC1 regulates protein synthesis, inhibition leads to energy preservation under stress; mTORC2 regulates AKT activation	Rapamycin is well-tolerated and blocks cardiac hypertrophy. It will be used in combination regimens in cancer (long-term administration inhibits mTORC2 and AKT)	122,123
AMPK	Transgenic; KO	Sensor of energy stress; inhibits mTORC1, preserving energy stores. KO of AMPKα2 increased hypertrophy and LV dysfunction after TAC	Activated by tumour suppressor LKB1 or CAMKKβ. Activated mutant leads to glycogen storage hypertrophic cardiomyopathy	124,125
GSK3α/β	Transgenic; knock-in; KO	Together with AMPK, inhibits mTORC1; deletion of GSKβ protective in post-MI remodelling; deletion of GSK3α leads to HF in setting of stress	GSK3α important in β-adrenergic responsiveness. Deletion leads to HF with stress	126–128, 181
CDKs	Conditional KO; KO	CDK2 inhibition reduces ischaemia–reperfusion injury, mediated via effects on retinoblastoma protein	Deletion of CDK2 and CDK4 during development leads to HF, but conditional deletion of CDK2 in adult mice lacking CDK4 does not lead to abnormalities	129,130
Aurora kinases	Kinase inhibitor administered	M phase regulators	KIs expected to disrupt CPC proliferation, karyokinesis and any cytokinesis of cardiomyocytes resulting in cell death; associated with cardiotoxicity	131,132
PLKs	Kinase inhibitor administered	PLK1 involved in activation of CDC2, chromosome segregation, centrosome maturation, bipolar spindle formation and cytokinesis	As above	132,133
PDGFRs	KO	β isoform is crucial in angiogenesis and heart's response to PO	Conditional KO: HF with pressure overload	21
VEGFRs	VEGF trap; (monoclonal antibody); KO; Kinase inhibitor administered	Crucial in angiogenesis and the heart's response to PO; antihypertensive effects	Heart-specific KO of VEGFR1 or VEGFR2 showed impaired response to ischaemia	19, 20,22, 44,53
EGFR (ERBB1)	Kinase inhibitor administered	Helps to maintain LV function in setting of chronic catecholamine stimulation; mediates pro-survival signalling	HF uncommon in clinical use	134
ERBB2 ERBB4	KO; conditional KO; transgenic	Cardiomyocyte survival and homeostasis; maintenance of LV function	KOs of HBEGF (ERBB ligand), neuregulin, ERBB2 and ERBB4 each develop DCM and HF. Neuregulin 1 KOs are more sensitive to doxorubicin-induced cardiotoxicity	135, 136–138
KIT	*W/W*^{v} mouse, kinase inhibitor administered	Promotes CSC and immature cardiomyocyte differentiation; promotes homing to sites of MI, promoting repair.	Imatinib reduced restenosis after arterial injury; *W/W*^{v} mouse protected versus HF induced by PO	40, 104,106, 139
ABL/ARG	Kinase inhibitor administered	Maintains ER homeostasis. LV dysfunction is seen in rodents treated with imatinib	Minimal HF with ABL inhibitors noted in clinical use	56
JAK2	Transgenic; KO	JAK2 and STAT3 protective in many pathological settings	KO: displayed decreased ischaemia–reperfusion injury, PO hypertrophy and peripartum cardiomyopathy	140–142
FAK	KO	Antihypertrophic and antifibrotic in heart	Eccentric hypertrophy and dilated cardiomyopathy with PO or angiotensin II	143

Table 2 | (cont) **Kinases of importance in the heart and vasculature: findings in genetically modified or KI-treated mice**

Kinase	Mouse model(s)	Role of kinase in heart/vasculature	Other notes	Refs
DMPK	Transgenic	Myotonic dystrophy type 1 is caused by excess repeats of the 3′ UTR region of DMPK	Overexpression in mouse heart leads to hypertrophic cardiomyopathy with dysrthymia	144
LTK	Transgenic	Activation of LTK results in cardiac hypertrophy and cardiomyocyte degeneration	Overexpression led to severe cardiac concentric hypertrophy	145
ROCK	Transgenic; KO	Pro-fibrotic and pro-apoptotic in the setting of PO	KO is protected from fibrosis, whereas transgenic shows accelerated hypertrophic cardiac decompensation	146,147
LKB1	KO	Activates AMPK which is pro-angiogenic in heart	Cardiac-specific deletion leads to LV dysfunction, atrial fibrillation and death	148
LDB3, ZASP and/or Cypher	Conditional KO	Mutations in ZASP are associated with skeletal muscle myopathies	Deletion in cardiomyocytes leads to a DCM	149
ERK1/2	KO; transgenic	Generally promotes survival and may modulate physiological (but not pathological) hypertrophy	Activation of this pathway protects aginst cardiac ischaemic injury; inactivation promotes LV dysfunction after PO	150–153
PKCα	KO; kinase inhibitor administered	Adverse effects on heart in setting of PO	PKCα KO (as opposed to PKCβ or PKCγ) protects against TAC-induced CHF. Ruboxistaurin protects against TAC-induced CHF	154
cGMP-dependent PK	KO	One of the four nodal kinases in HF; activated by PDE5 inhibitors; inhibits apoptosis, hypertrophy and □-adrenergic responses	Clinical trial with sildenafil in HF showed improved LV function	155,156
PIM Kinase	KO	Pro-survival; activated by AKT; regulated at level of gene expression	Promotes cardiac resident stem cell proliferation and survival	157
CAMKII	KO	Nodal kinase in HF; pro-hypertrophic; promotes decompensation in setting of PO	Mechanism of cardiotoxicity involves regulation of CAMKII gene expression and Ca^{2+} handling	94,158,159
GRK2 and/or GRK5	KO	Downregulates □-adrenergic signalling through recruitment of □-arrestin	Peptide inhibitor protects against HF	160,161
ASK1	KO	Promotes pathological hypertrophy and remodelling; pro-apoptotic	KO protected from PO and MI-induced remodelling	162,163
PTEN phosphatase	KO	Anti-hypertrophic, impairs cardiomyocyte survival with stress and antagonizes PI3K signalling	PO in PTEN KO mice reduced pathological hypertrophy, interstitial fibrosis and apoptosis with preservation of LV function	163

AMPK, 5′ AMP-activated protein kinase; ARG, abl-related gene; ASK1, apoptosis signal-regulating kinase 1; CAMKII, $Ca2^{+}$/calmodulin-dependent protein kinase II; CATG,constitutively active transgenic; CDK, cyclin dependent kinase; CHF, congestive heart failure; CPC, cardiac progenitor cells; CSC; DCM, dilated cardiomyopathy; DMPK, dystrophia myotonica protein kinase; DNTG, dominant negative transgenic; EGFR, epidermal growth factor receptor (also known as ERBB1); ERBB2, human epidermal growth factor receptor 2; ERBB4, human epidermal growth factor receptor 4; ERK2, extracellular signal-regulated kinase 2 (also known as MAP kinase 1); FAK, focal adhesion kinase; GSK3, glycogen synthase kinase 3; HCM, hypertrophic cardiomyopathy; HF, heart failure; JAK2, Janus kinase 2; LDB3, LIM domain binding 3; LKB1, serine threonine kinase 11 (also known as STK11); LTK, leukocyte receptor tyrosine kinase; LV, left ventricular; MI, myocardial infarction; mTOR C1, mammalian target of rapamycin complex 1; PDE5, phosphodiesterase 5; PDGFR, platelet-derived growth factor receptor; PDK1, phosphoinositide-dependent protein kinase 1; PI3K, phosphoinositide 3-kinase; PKC, protein kinase C; PLK, polo-like kinase; PO, pressure overload; PTEN, phosphatase and tensin homologue; ROCK, RHO-associated kinase (RHO-kinase); TAC, trans-aortic constriction; VEGFR, vascular endothelial growth factor receptor.

smooth and cardiac muscle cell lines, primary cardiomyocytes (neonatal and adult, and rodent and human), isolated three-dimensional organoids, isolated progenitor cardiomyocytes and, more recently, human embryonic stem cell (ESC) and inducible pluripotent stem (iPS) cell-derived cardiomyocytes[47,48]. Additional models include isolated heart preparations from various species and mammalian models used for short-term *in vivo* toxicology studies.

In vitro models of cardiotoxicity

Cell lines. Cell culture, especially with cell lines, is a frequently relied upon approach for predicting clinical side effects, including the potential risk of cardiotoxicity. A number of rodent and human cell lines, including myocardial or skeletal muscle, have been developed to detect cardiotoxicity (TABLE 3). Importantly, most of the cardiomyocyte models listed in the table continue to contract *in vitro*, thereby maintaining the primary physiological and functional role of the cardiomyocyte, including the key *in vivo* demand of constant ATP production. This is a crucial feature for detecting any cardiotoxicity of small molecule kinase inhibitors *in vitro*, as energy stress is likely to contribute to the toxicity.

A point worthy of consideration with *in vitro* models is the use of media containing high concentrations of glucose. Many cell lines are cultured in a manner that is suitable for immortalized, tumour-derived cell lines, which are metabolically adapted to grow quickly and generate energy with little reliance on mitochondrial fatty acid oxidation. Cells in this environment generate ATP through glycolysis and are less reliant on oxidative phosphorylation and mitochondria in general[49]. In cardiomyocytes, mitochondria are a crucial toxicological

Box 2 | **Potentially beneficial roles of approved kinase inhibitors in the cardiovascular system**

Below we outline potential uses of kinase inhibitors in the treatment of diseases other than cancer.

Inflammation and autoimmune diabetes mellitus

Imatinib (Gleevec; Novartis) has anti-inflammatory effects in mouse models, and early phase studies in patients with rheumatoid arthritis and psoriasis are in process[97]. Furthermore, imatinib therapy improved type I autoimmune diabetes in the non-obese diabetic mouse model[98], probably through inhibition of platelet-derived growth factor receptors (PDGFRs), as neutralizing PDGF also reversed the disease[98]. Sunitinib (Sutent; Pfizer) was also shown to be protective against diabetes in this study. The exact mechanism by which PDGFR inhibition modulates autoimmune diabetes is not clear, but these studies suggest that other kinase inhibitors that inhibit PDGFR would also be beneficial in treating diabetes. However, small clinical studies have reported inconsistent results with imatinib in the control of blood glucose levels [99,100].

Pulmonary artery hypertension

Pulmonary artery hypertension (PAH) can either be primary or secondary to a range of disorders, including chronic obstructive pulmonary disease, congenital heart disease and collagen vascular diseases. PAH is a particularly complex disease with limited therapeutic options. Imatinib reverses monocrotaline-induced PAH, and this has been attributed to inhibition of PDGFR signalling[101]. However, in another study, sorafenib (Nexavar; Bayer) was shown to be more effective than imatinib, possibly owing to the additional inhibition of the RAF–MAPK/ERK kinase (MEK)–extracellular signal-regulated kinase (ERK) pathway by sorafenib[102]. Indeed, sorafenib reduced not only the remodelling of the pulmonary artery but also the right ventricular hypertrophy that follows PAH. Given the role of the ERK pathway in promotion of hypertrophy in various mouse models, this is likely to be at least part of the therapeutic mechanism. Recently a Phase II clinical trial of imatinib was completed in patients with PAH, and results suggest that imatinib could be efficacious in PAH treatment[103] (Clinicaltrials.gov identifier: NCT00477269).

Restenosis following percutaneous coronary intervention

Although the use of clopidogrel (Plavix; Sanofi Aventis / Bristol-Myers Squibb) in patients with drug eluting stents has significantly reduced the incidence of stent thrombosis and in-stent restenosis, these adverse outcomes are still observed. Imatinib reduces post-arterial injury stenosis[104,105]. Importantly, imatinib prevented stenosis without blocking endothelial repair, which is a well-described effect of drug-eluting stents and has led to more prolonged use of clopidogrel (1 year versus 3–6 months for bare metal stents). This was hypothesized to be attributable to PDGFR inhibition.

Post-myocardial infarction (MI) remodelling

Kit–deficient *W/W^v* mice have worse remodelling (left ventricular dilatation and reduced function) post-MI compared to wild-type mice[106], although as noted, these mice have better-preserved left ventricular function in the pressure overload model[40]. It is important, therefore, to identify the specific targets of kinase inhibitors (such as imatinib, for example) that mediate the disparate effects of the drug in the injured artery and stressed heart in order to more effectively target therapy and reduce adverse effects.

Genetic forms of cardiac hypertrophy: the hypertrophic cardiomyopathy phenocopies

Familial hypertrophic cardiomyopathy is largely attributable to mutations in genes encoding sarcomere proteins[107]. However, there are phenocopies that are attributable to mutations in protein kinases. These genes include the *PRKAG2* gene which encodes a regulatory subunit of AMPK[108]. The mutation seems to lead to constitutive activation of AMPK and results in a glycogen storage hypertrophic myopathy that is associated with ventricular pre-excitation and atrial tachyarrythmias (as observed in Wolfe–Parkinson–White syndrome). This disorder is extremely difficult to treat and early mortality is common[109]. Given the potent inhibition of AMPK by sunitinib, we suggest that preclinical studies should be done to examine whether sunitinib can prevent, delay or even reverse the disease phenotype.

Another group of HCM phenocopies is the Noonan, Costello and Cranio-facio-cutaneous syndromes, which are attributable to mutations involving the RAS–RAF pathway and tyrosine protein phosphatase non-receptor type 11 (PTPN11), which regulates this pathway. Mutations lead to constitutive activation of the RAS/RAF pathway[110–112]. These disorders are also very difficult to treat and lead to early mortality. It is conceivable that RAF pathway inhibitors — including sorafenib — could be used in the treatment of these patients, who otherwise have very limited treatment options.

Phenocopies
A phenotype or trait that is similar among different individuals.

Noonan, Costello and Cranio-facio-cutaneous syndromes
A group of syndromes that affect a number of organ systems and cause severe cardiac hypertrophy.

Ischaemia–reperfusion injury
A complex phenomenon that occurs when the blood supply from an organ is restored after a period of ischaemia.

target, and thus the use of galactose-containing media is optimal because it forces the generation of ATP through oxidative phosphorylation.

One of the more frequently used models to study cardiac biology has been the rat embryonic ventricle-derived H9C2 cell line. These cells have been studied as an *in vitro* model of cardiac muscle for biochemical and pathophysiological processes, including ischaemia–reperfusion injury, oxidative stress and tissue differentiation[50,51]. In addition, these cells have the versatility to allow the study of action potentials and cardiac electrophysiology, as the voltage channels that are expressed are similar to embryonic or neonatal cells, including L-type Ca^{2+} channels and ATP-sensitive K^+ channels[52]. Studies using H9C2 cells grown in high or low glucose-containing media showed that sorafenib was significantly more cytotoxic in galactose-containing media versus glucose-containing media, whereas imatinib, dasatinib (Sprycel; Bristol-Myers Squibb) and sunitinib showed no difference across the two media types[53].

Primary cells. NRVM isolation was optimized in the early 1980s[54,55]. This model has been extensively used to study hypertrophy, stress, cardiac failure, cardiac ion channels, contractility and basic cardiac biology, including the role of kinases in a large number of processes (including hypoxia/reoxygenation (which mimics ischaemia), hypertrophy and apoptosis) as well as toxicity. An assessment of seven marketed kinase inhibitors (lapatinib, erlotinib (Tarceva; Roche/Genentech), gefitinib (Iressa; AstraZeneca), imatinib, sorafenib, sunitinib

Table 3 | **Advantages and disadvantages of the most commonly used models of cardiomyocytes**

In vitro model	Derivation or source	Key feature	Advantage	Disadvantage	Refs
H9C2	Embryonic BDIX rat heart	Replicating cell line used to assess cardiac biology and electrophysiology	Expresses cardiac and skeletal muscle ion channels, and similar metabolic enzymes	Morphologically distinct from cardiomyocytes; embryonic phenotype can differentiate into myotube-like structures	50,164,165
HL1	Derived from atrial cell isolated from transgenic mice expressing SV40 large T antigen controlled by an atrial natriuretic factor promoter	Spontaneously contract and proliferate, maintain an adult cardiac phenotype. Maintain organized sarcomeric structures suitable for contraction yet are able to proliferate	Energy metabolism is different from cardiomyocytes, including lack of complex I activity in mitochondria and glycolytic phenotype	Limited toxicity screening conducted; mouse repolarization cascade is different from human cascade, so more relevant CV safety pharmacology screening systems are also used	166–169
Neonatal rat ventricular myocytes	Neonatal rat	Readily available, can maintain contractility	Well characterized, long utility in culture during which the cells maintain the ability to beat	Many variables, including strain used, day postpartum used, isolation procedures, timing of post-isolation studies, media constituents, whether studies done in buffer or media and concentration and duration of drug exposure studied	54,55
Adult rat cardiomyocytes	Rat	Can be maintained in culture for several days and maintain their contractile state	Cell yield can be increased using reversible transfection resulting in transient dedifferentiation	Limited toxicological data available	170,171
Neonatal mouse ventricular myocytes	Mouse	Increasing use due to the ability to generate transgenic, KO and knock-in models from which cells can be isolated	Lack of response to certain stress stimuli, a lack of secretion of atrial natreutic factor, autonomous hypertrophy in serum-free growth factor deprived media	Cardiac repolarization of cardiomyocytes uses the Ito and ISUS instead of IKr (predominates in human cardiomyocyte repolarization); use in toxicological studies limited	172–174
Adult mouse ventricular myocytes	Mouse	As above	Studies are limited to contractile function, calcium handling and electrophysiology	Survival time in culture is very short so experimental study design has to be adjusted accordingly	182
Human cardiomyocytes	Human	Electrophysiological properties can be maintained, albeit briefly	Most experiments of short duration and focus on life span, cells maintain their rod-like shape and striated, sarcomeric structure. Cells can dedifferentiate and proliferate if cultured for long time periods	Long-term duration culture and study of human primary cardiomyocytes has been complicated by dedifferentiation, which is attributed to media containing high concentrations of serum	175–179
AC16 line	Human cells derived from fusion with SV40 transfected, mitochondrial devoid fibroblasts	Cells proliferate readily until placed in growth factor-deficient media, which leads to differentiation and acquisition of adult cardiac markers	Expandable source of maturing cells	Limited data have been published on toxicity screening	180
Stem cell-derived human cardiomyocytes	Derived through directed differentiation of pluripotent cells into cardiomyocytes	Have electrophysiological properties similar to embryonic cardiomyocytes, but are a maturing population of cells	Most methods yield cardiomyocytes with sarcomeric structures and channel activity similar to immature cardiomyocytes; require longer duration culturing to mature	Although early data are encouraging, more validation data are needed	47,48

IKr, inward delayed rectifier potassium current; I_{SUS}, sustained current during depolarization; Ito, transient outward k^+ current; mTORC1, (derivative of) mammalian target of rapamycin complex 1; SV40, simian virus 40.

and dasatinib) in NRVMs suggested that sunitinib and dasatinib are more cytotoxic compared to the others at equivalent concentrations. In addition, the rank order of cytotoxicity appeared to inversely correlate with selectivity, with less selective molecules being more prone to cause cytotoxicity *in vitro* and cardiac injury *in vivo*[31].

In another study, imatinib treatment of NRVMs at low micromolar concentrations led to endoplasmic reticulum stress, energy rundown, collapse of mitochondrial membrane potential and activation of apoptosis and cell death[56]. The cytotoxicity of imatinib was largely inhibited when NRVMs were transduced with imatinib-resistant ABL, suggesting that the on-target inhibition of ABL by imatinib contributes to the cytotoxicity[57]. Sunitinib increased lactate dehydrogenase release in NRVMs at submicromolar concentrations[57], and pretreatment with the AMPK-activating molecule metformin did not attenuate sunitinib-mediated cytotoxicity. In another study, sunitinib inhibited AMPK activity at low nanomolar concentrations and caused cell death; this was attenuated by adenoviral transduction with constitutively active AMPK[6,18]. Given the crucial role that ATP and energy homeostasis have in the contraction of cardiomyocytes, the role of AMPK as a general modulator of cardiotoxicity should be explored further (FIG. 1).

Embryonic stem cells. New insights into how stem cells mature into cardiomyocytes have enabled us to generate stem cell-derived cardiomyocytes[58]. Human ESCs will grow into aggregates in suspension and differentiate into embryoid bodies that contain spontaneously beating cardiomyocytes[59] with electrophysiological properties that are similar to embryonic cardiomyocytes[60]. Newer methods are based on directing embryological differentiation towards cardiomyogenic precursors[47]. In addition, depending on the derivation and purification methods, the resulting cells are a heterogeneous population of atrial, ventricular and/or nodal-like cells. Most methods yield cardiomyocytes with immature sarcomeric structures and channel activity similar to immature or embryonic cardiomyocytes[47]. The most prominent difference between ESC-derived cardiomyocytes and adult cardiomyocytes is the lack of a prominent IKr in ESC-derived cardiomyocytes[60,61].

As noted above, no suitable models for adult human cardiac biology exist, but it is now feasible to use stem cell-derived cardiomyocytes for safety, pharmacology and toxicology assessment[62]. Initial publications have focused on the electrophysiological properties of stem cell-derived cardiomyocytes, noting that known cardioactive molecules like terfenadine, cisapride, verapamil, E4031, sotalol and quinidine show detectable, predicted changes using patch clamping and multi-electrode arrays[63,48]. Preliminary data suggest that the gene expression pattern of stem cell-derived cardiomyocytes is similar to the gene expression pattern in the intact adult heart (K.L.K., unpublished observations). Although data are still forthcoming, stem cell-derived cardiomyocytes appear to be a considerable breakthrough in studying cardiac biology, pharmacology and toxicology.

In vivo models of cardiotoxicity

Zebrafish. Zebrafish *(Danio rerio)* have been extensively employed in embryology and vertebrate genetics, establishing a well-conserved linkage to mammalian genetics. They have been used to attempt to predict drugs that will either cause reproductive or teratogenic effects or alter cardiac conduction and prolong the QT interval[64]. In addition, zebrafish are beginning to be explored as a means to predict overt cellular cardiotoxicity, although we are not aware of any published reports using this model.

The molecular aspects of the development of the cardiovascular system are well-conserved throughout vertebrate evolution, including in zebrafish (although the zebrafish heart has only two chambers). Of note, the heart maintains the ability to regenerate throughout adulthood, through dedifferentiation of adult cardiomyocytes and subsequent polo-like kinase-dependent proliferation[65]. Nonetheless, zebrafish cardiomyocytes express voltage-gated sodium channels, L-type and T-type calcium channels and potassium channels, in a similar way to other vertebrate hearts[66,67]. In addition to relevant cardiac biology and ion channels, zebrafish are small, amenable to screening in 384 well plates, and are transparent for up to several days of age, allowing for visual inspection of the contractile function of the heart[68]. It can be difficult to parse out the cardiotoxic effects of drugs on the heart that are a result of a disturbance in development (and therefore not likely to be applicable to adult cancer patients taking that drug). However, the use of the casper zebrafish, which remain transparent throughout adulthood, circumvents this problem[69]. Another problem arises from differences between fish and human kinases at the ATP binding pocket, which could, in some cases, invalidate the use of the zebrafish as a model. Thus, the value of zebrafish in predicting kinase inhibitor toxicity remains to be defined.

Rodent models. Although the cell-based models described above were developed to improve the speed at which one could identify and understand the deleterious effects of a molecule on the heart, some of the earliest studies were conducted in rodents. Rabbit models were initially considered the standard model in which to profile cardiotoxicity — for example, the cardiotoxicity of doxorubicin was first confirmed in rabbits[70,71]. Studies with anthracyclines in rats and mice, including the spontaneously hypertensive rat model, confirmed that smaller rodent models could also be employed to study cardiac injury[72].

Rodents offer an ideal model for examining cardiotoxicity, as extensive pharmacokinetic and pharmcodynamic data are available from studies examining the anticancer efficacy of kinase inhibitors in rodents. This, plus the extensive data from patients treated with kinase inhibitors, allows for matching of plasma levels in rodents with plasma levels in patients. However, there are many caveats to the use of rodents. First, appropriate end points to measure cardiotoxicity are not clearly defined. For example, left ventricular ejection fraction (LVEF) has been examined in rodents treated with imatinib and sunitinib. For imatinib, deteriorations in

Endoplasmic reticulum stress
A conserved response to excessive misfolded proteins resulting in an effort to repair and correct the situation; failing to do so leads to programmed cell death.

ABL
An oncogene associated with chronic myelogenous leukaemia.

Embryoid bodies
Aggregates of differentiating and undifferentiated cells formed from embryonic stem cells.

IKr
The inward delayed rectifier potassium current that is regulated in humans by *HERG* and is responsible for repolarization of cardiac action potential.

LVEF were seen in two studies[56,73], although the magnitude was small. With sunitinib, no abnormalities in LVEF were seen, despite the fairly extensive mitochondrial abnormalities observed using transmission electron microscopy. In fact, not until a pressure load was added was it possible to see increased cardiomyocyte apoptosis in the sunitinib-treated mouse[6,18]. This response was similar to the response observed in many of the genetically modified mice discussed above. This clearly suggests that there is a 'reserve' functional capacity present in the mouse heart, and when this is taken together with the lack of cardiovascular co-morbidities, it seems that there is no perfect predictive model when it comes to detecting the cardiotoxicity of kinase inhibitors *in vivo*. We believe that the addition of a pressure load will increase sensitivity, and this approach should be considered in future studies, even though TAC is a technically demanding procedure. However, more sensitive measures of left ventricular function (see below) should also be tried.

Future preclinical directions. A current trend in safety assessment is to develop human cell-based models early in drug development. This has the potential to improve the predictive accuracy of preclinical models by introducing models that truly resemble the genotypic and phenotypic diversity present in the human population and, ultimately, the propensity to develop disease. This is an important transition, as early safety assessment has historically relied heavily upon animal models[74]. A plausible strategy incorporating this approach would be to create stem cells from adult cells (that is, iPS cells)[75,76]. By using iPS cells to derive cardiomyocytes, for example, one could prospectively explore the differences in electrophysiological and toxicological phenotypes of the various normal and disease states of interest[48].

The most meaningful and direct approach, however, would require prospective planning in clinical trial design, which would include skin punch biopsies to create iPS-derived cardiomyocytes from patients with positive or negative cardiovascular outcomes. In this direct post-hoc clinical trial, the ability to glean valuable cardiovascular, functional and toxicological information across an entire clinical trial could lead to the development of improved biomarkers. The eventual goal is to use these biomarkers to determine which patients should be included or excluded in future clinical trials.

Studies in patients

Before exploring strategies to predict or detect patients at risk of cardiotoxicity, there are a few caveats worth mentioning. First, there are no guidelines for oncologists (or cardiologists) that address how to approach patients who will be treated with cancer therapeutics. Strategies for follow-up do not exist, and approaches involving treatment of patients who develop left ventricular dysfunction generally rely on guidelines written for patients with more traditional forms of heart failure. There is, however, a significant amount of interest in this issue, and position papers are beginning to appear[77]. Second, although recovery of left ventricular function has been seen in patients treated with trastuzumab and sunitinib, it is crucial to follow patients who have developed cardiotoxicity for longer periods of time. This lesson was learned from the studies on adult survivors of childhood cancer, in whom early recovery of left ventricular function was followed by significant and persistent declines in function[39]. Although it appears that the cardiotoxicity of kinase inhibitors may be intrinsically different to the cardiotoxicity resulting from anthracyclines or radiation, that conclusion can only be drawn after longer-term follow-up of patients.

The third caveat is that postmarketing surveillance and vigilance are crucial. Patients typically enrolled in trials that are conducted before regulatory approval have limited co-morbidities, and they are often excluded if they have, for example, coronary artery disease (CAD) or any left ventricular dysfunction. Although it makes sense to control variables during drug development, after approval many patients with significant co-morbidities will be treated. Furthermore, experience with sunitinib, albeit limited in patient number, shows that the only predictor of patients who would go on to develop CHF was a history of CAD[6].

Imaging studies. LVEF is a very insensitive marker of cardiotoxicity because of the multiple compensatory mechanisms that can be recruited to maintain contractile function. Thus, LVEF can be maintained even when the intrinsic contractile function is depressed. Therefore, determinations of LVEF may only detect those patients whose cardiovascular compensatory mechanisms are already compromised by co-morbidities (for example, CAD or uncontrolled hypertension). Furthermore, LVEF determinations do not detect heart failure with normal systolic function (previously termed diastolic dysfunction), which is a major cause of admissions to hospital for heart failure. There is some evidence that tissue Doppler imaging/strain rate may be more sensitive than LVEF, but the true utility of that approach remains to be determined[78]. Below we examine other potential markers and biomarkers that are being explored.

Biomarkers. A few cardiovascular safety biomarker studies have been conducted, with troponin I emerging as a potential candidate. Elevations in troponin I levels are the major biomarker for cardiomyocyte necrosis in acute coronary syndromes. New technology now allows us to detect serum levels of troponin I in the picomolar range, but the significance of small, transient drug-related changes in troponin I is not clear[79,80]. That said, elevations appear to be predictive of cardiotoxicity with anthracyclines[81]. In one study involving patients receiving high-dose chemotherapy, an early elevation in troponin I predicted future left ventricular dysfunction, and treatment of troponin I-positive patients with an angiotensin-converting enzyme inhibitor led to better preserved left ventricular function[82].

Although this is encouraging, it is not clear that using troponin I as a biomarker of cardiotoxicity to follow patients receiving cancer therapies that are less cardiotoxic (including kinase inhibitors) will be as predictive. For example, in the study of sunitinib in gastrointestinal

Tissue Doppler imaging/strain rate
A form of cardiac ultrasound used to assess functional parameters of the direction and speed of blood flow.

stromal tumours, troponin I elevations did not predict which patients would go on to develop CHF or significant declines in LVEF[6]. By contrast, Cardinale *et al.* found that a troponin I elevation was the best predictor of trastuzumab-induced cardiotoxicity (the hazard ratio was 22.9) and also predicted those patients in whom ejection fraction (EF) would not improve after stopping drug treatment (the hazard ratio was 2.88)[83]. It is our view that in the long-term monitoring of patients taking kinase inhibitors, using biomarkers with increased sensitivity will increase the false positive rate. Although the above studies are encouraging, the value of using troponin I as a biomarker in the monitoring of patients taking kinase inhibitors chronically is unclear.

Biomarkers of heart failure (for example, the various measurements of B-type natriuretic peptide) are also being explored, but it is currently unclear whether these biomarkers will be prognostic or whether they will be able to identify patients significantly earlier than clinical examination. That said, given the shared symptom complexes of dyspnea, fatigue and peripheral oedema in both cancer and heart failure, it is likely that B-type natriuretic peptide will be of clinical value in sorting out the individuals who have heart failure from those who do not. However, it is unclear whether such biomarkers will be of value in preclinical studies and/or have sufficient credibility for regulators in preapproval trials to identify agents that have the potential to cause cardiotoxiticty.

Genetic evaluations: polymorphisms. Gene polymorphisms can contribute to cardiotoxicity and account for the significant variability in the susceptibility to drug-induced cardiotoxicity to certain drugs such as anthracyclines. For example, doxorubicin can lead to severe or fatal CHF in some patients treated with cumulative doses that are approximately one-fifth of the dose that other patients can tolerate with little to no cardiac toxicity[84]. In doxorubicin-treated patients, a number of suspected genetic polymorphisms have been identified, including in genes regulating oxidative stress (NADPH oxidase) and transport (multidrug resistance protein 1 and 2) and in gene products that generate reactive metabolites of doxorubicin (carbonyl reductase 1 and 3 and aldehyde reductase 1A1)[85]. Research on genetic polymorphisms will grow with increased understanding of cardiotoxicity at the molecular level, aided by tools such as iPS cells derived from afflicted patients. This will allow investigators to directly explore the mechanisms of toxicity associated with the polymorphism. It seems highly likely that polymorphisms will also have a central role in kinase inhibitor-induced cardiotoxicity.

Metabolomics and metabolic imaging

As discussed above, left ventricular dysfunction is an insensitive and late-appearing marker of cardiotoxicity. Biomarkers of injury (for example, troponin I) are only starting to be evaluated, and we have already noted our concerns regarding their predictive accuracy when used to monitor chronic kinase inhibitor therapy.

The field of metabolomics is expanding rapidly. This process can identify metabolic signatures in the blood that are indicative of stress and injury, for example[86,87]. We believe, although this has not yet been verified, that metabolomic signatures of kinase inhibitor-induced stress will eventually be identified, and they will allow the identification of patients with incipient cardiotoxicity, well before left ventricular dysfunction is evident.

Other strategies that could be used to examine alterations in metabolic state include cardiac magnetic resonance imaging (MRI) and FDG positron emission tomography (FDG-PET). These strategies can clearly detect alterations in energetics, but their ability to detect profiles predictive of stress in large-scale screens of patients will be offset by the cost of the procedures. Therefore, we believe that metabolomic biomarkers, if identified, could significantly move the field forward.

Future perspectives and conclusion

We have outlined the practical concerns associated with, and the screening models related to the cardiotoxicity of small molecule kinase inhibitors. The crucial role of kinases in normal cardiovascular development and their involvement during cardiovascular stress responses is highlighted by the work done in genetically modified mice. This delicate balance of energy shuttling, contraction and kinase-regulated stress pathways creates a cause for concern when one attempts to pharmacologically intervene with a molecule that can impair the coordinated function of this network of structurally related kinases. Although the direct translation of findings in genetically modified mice — to predict clinical outcomes in patients treated with small molecule kinase inhibitors — has a multitude of caveats, the clinical data on cardiotoxicity of poorly selective kinase inhibitors demand further study using most, if not all of the strategies we have outlined above.

Nevertheless, the cardiac safety profile of current small molecule kinase inhibitors is relatively good. But the early and significant successes of these agents demand that oncologists, cardiologists and patients must agree to go forward with more aggressive strategies, including targeting of multiple kinases along one or more pathways. To achieve success with this strategy, we believe that the pharmaceutical industry should move towards more selective agents, thereby minimizing off-target (and therefore unnecessary) toxicity. For every time that one serendipitously takes advantage of off-target inhibition to treat a particular cancer, the inherent non-selectivity of the agent may well lead to cardiac (or other) toxicities. Despite all of the preclinical models that are available, current models are not adequate. Thus, an opportunity exists for technological breakthroughs (for example, stem cell-derived cardiomyocyte models) to provide insight into and understanding of the prediction of cardiotoxicity, from preclinical assessment stages through to the postmarketing stage. Ultimately, the predictive value of improved preclinical models will be revealed through diligent patient monitoring, and although we do not yet know the best way to do this, it is clear that we will in the near future.

Hazard ratio
An explanatory factor used to assess the risk of a given event or disease.

B-type natriuretic peptide
A protein secreted from the heart in response to stress, including stretch.

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Competing interests statement
The author declares competing financial interests: see web version for details.

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