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HOW TO

How to Apply Translational Models to Probe Mechanisms of Cardiotoxicity

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The number of new anticancer drugs approved annually has more than tripled in the past 3 decades, and there has been a parallel increase in the incidence and diversity of cardiovascular sequelae observed due to these agents.[1] Concurrently, there has been an expansion of knowledge about cardiotoxicity mechanisms and manifestations. This influx of new drugs and information has inspired efforts to model cardiotoxicity for the study of mechanisms, predisposing factors, and mitigation strategies.

Accordingly, this primer focuses on in vitro methods of modeling the mechanisms by which cancer therapies cause cardiotoxicity. Organized according to the primary cardiotoxicity mechanisms and clinical manifestations, models used to study toxicity are introduced in hierarchical order, from simple to complex (**Figure 1**). For each type of toxicity and model, the relevant in vitro readouts are highlighted (**Table 1**).

DIRECT CARDIOMYOPATHY (2-DIMENSIONAL FORMATS)

Direct cardiomyocyte damage and death remains a fairly straightforward form of cardiotoxicity to model. This type of cardiotoxicity predisposes to systolic and diastolic cardiac dysfunction. Anthracyclines, prototypical drugs in this regard, have several mechanisms of direct cellular injury, including free radical generation, disruption of DNA and RNA synthesis, and up-regulation of apoptosis. Her2 pathway antagonists, tyrosine kinase inhibitors (TKIs), and other targeted therapies can also predispose to direct cardiomyocyte toxicity, with mechanisms and severity varying based for each class of agent.[2]

Direct cardiomyocyte toxicity can be modeled in simple 2-dimensional (2-D) cell culture models.[3] Cells commonly employed include 1 of several immortalized cardiomyocyte cell lines, neonatal rodent cardiomyocytes, or cardiomyocytes derived from differentiation of human induced pluripotent stem cells (Hu-iPSCs); each is well-suited for culture.[4] The most common cell culture readouts are biochemical assays designed to detect plasma membrane damage via either substances released into media (lactate dehydrogenase, troponin, creatinine kinase) or entry of extracellular markers into cells (trypan blue and others). Assays based on detection of programmed cell death, or apoptosis, including probes identifying caspase activation and proteins upregulated in apoptosis (p53, Bcl-2, BAX, and others) are also employed as readouts for direct cardiomyocyte toxicity. Assays linked to mitochondrial respiration and cellular energy reserves, including NAD(P)H-dependent cellular oxidoreductase enzymes and assays of mitochondrial membrane potential are also used to identify cardiotoxicity. Finally, to directly measure the contractility of myocytes in 2-D, commercially available software platforms that image sarcomere striations can be used to capture real-time changes in sarcomere length as cultured myocytes contract. Several of these

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HIGHLIGHTS

- Treatment-related cardiovascular disease is a leading cause of morbidity and mortality in cancer survivors.
- For investigators, it is crucial that in vitro models are well-matched to the toxicity studied.
- From simple to complex, we describe how in vitro models are used to model cardiotoxicity.
- Integrating these models will provide opportunities to address growing challenges in cardio-oncology.

assays are compatible with methods of automated high-throughput screening of compounds. Nevertheless, there are concerns that assays employing immature cells and accompanying readouts may tend to distort the functional significance of toxicity. For example, calcium handling is defective in immature cardiomyocytes, and these cells also usually lack the rod-shaped adult phenotype seen in mature cells, making them poorly suited for in vitro assessment of contractility.

DIRECT CARDIOMYOPATHY (3-DIMENSIONAL FORMATS)

To overcome these shortcomings, 3-dimensional (3-D), engineered heart tissue (EHT) models are used to assess the impact of potentially cardiotoxic agents on in vitro force generation. In this context, EHTs are designed to create an in vitro model that mimics the heterocellular organization of adult myocardium and its biomechanical milieu while providing contractile readouts that inform insights into drug toxicity and other applications.[3] Immature cardiomyocytes, frequently derived from Hu-iPSCs,[4] are used to fabricate 3-D microtissues using various scaffolds that permit electrical stimulation and mechanical loading that promote cell and tissue maturation. The contraction and relaxation of EHTs is measured in real time, allowing for cardiac function to be examined via force generation. Contractility is measured by force transducers activated by microtissues, or calculated based on the displacement of a cantilever or beam on which the EHT is mounted. In addition to reductions in force generation, readouts suggesting cardiotoxicity in EHTs include histopathological changes (nuclear and cytoplasmic degeneration), as well as the markers of cell membrane damage and apoptosis, that are employed in 2-D culture formats.[5] Moreover, responses of both 2-D models and EHTs can be interrogated with broader molecular screens, including metabolomics, proteomics, transcriptomics, and epigenomics, that are powerful unbiased tools for identifying cardiotoxicity mechanisms.[6] Though 3-D preparations have many advantages compared with 2-D culture, these models require more cells, are significantly more complex, allow lower throughput, and are less suitable for automation than 2-D cardiomyocyte cultures.

ENDOTHELIAL DYSFUNCTION AND HYPERTENSION

Endothelial dysfunction is a central mechanism of vascular toxicity associated with some antineoplastic agents, including doxorubicin, platinum-based agents, 5-flurouracil, trastuzumab, and vascular endothelial growth factor (VEGF) inhibitors. To study these toxicities, 2-D endothelial cell culture formats employ primary endothelial cells isolated following in vivo exposure to an anticancer therapy, normal primary cells subjected to in vitro exposures, or endothelial cell lines with in vitro exposures. Readouts include the various viability assays based on development of membrane permeability or markers of apoptosis, as described in the preceding text. Readouts also include endothelium-relevant functional assays including endothelial cell tube formation, assessment of agonist-induced nitric oxide release, or assays examining the ability to limit in vitro platelet aggregation. However, endothelial cell cultures are less suitable for studying vasospasm, a known toxicity of fluoropyrimidines, a phenomenon that requires intact tissue architecture as would be present in vascular segments harvested from animal models or human tissue donors. Alternatively, advances in derivation of vascular smooth muscle cells from Hu-iPSCs permit the use of engineered blood vessels to explore vascular toxicities of cancer therapeutics.

Hypertension, thromboembolism, and myocardial infarction are additional vascular toxicities of antineoplastic agents. In particular, VEGF inhibitors, vinka alkaloids, anthracyclines, and platinum-based agents can cause or exacerbate hypertension and predispose to pathologic myocardial remodeling. In this context, EHTs may be employed to examine how increased biomechanical load, as occurs with hypertension, affects the sensitivity of cardiomyocytes to cancer therapies. In larger scale EHTs, inserting metal braces into the scaffold effectively increases the

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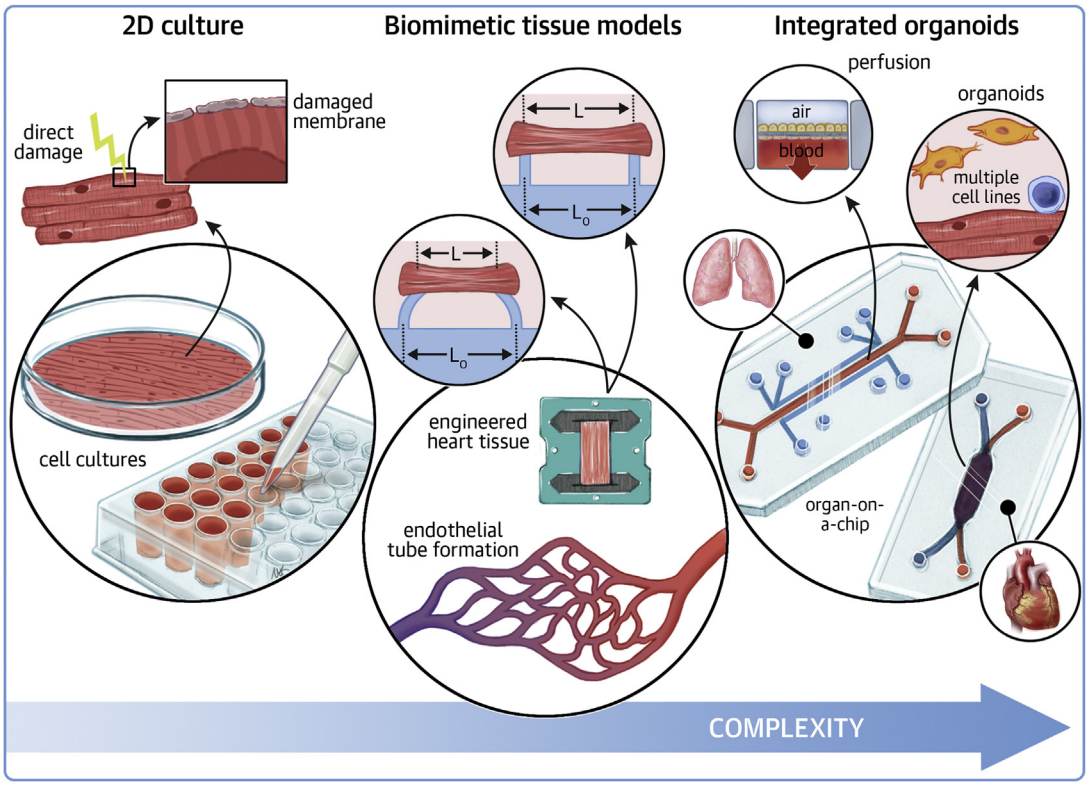
FIGURE 1 Models From Simple to Complex



(Left) Standard 2-D cell culture models can be employed to detect plasma membrane damage, markers of cellular injury, and toxic responses that affect fluorescent or colorometric indicators. 2-D arrays are also used to study the potential of a drug to generate arrhythmia using automated patch clamp platforms. **(Center)** Biomimetic 2-D and 3-dimensional tissue models examining endothelial tube formation **(top)** or force generation by engineered heart tissues **(bottom)** identify toxicity based on the impact on physiologic functions. **(Right)** Integrated organoid models combine microfluidics and multiple cell lines, including representation of both the tumor and the potential toxicity targets, to create a dynamic system with multiple levels of interaction. 2-D = 2-dimensional.

stiffness of the scaffold and thereby the afterload. Alternatively, different degrees of in vitro afterload can be achieved with tissue scaffolds of polydimethylsiloxane (PDMS) by modifying the polymer to increase scaffold stiffness. Using this approach, Truitt et al[5] demonstrated that increased in vitro afterload substantially exacerbates the in vitro caspase activation associated with clinically relevant levels of sunitinib.

INFLAMMATORY/IMMUNE-MEDIATED CARDIOTOXICITY

The advent of cancer therapies modulating the immune system has produced new mechanisms of cardiotoxicity. A prime example is myocarditis associated with clinically used immune checkpoint inhibitors (including anti-CTLA-4, and anti-PD-1 therapies). Myocarditis during immune checkpoint inhibitor therapy reflects induced T-cell reactivity to myocardial tissues and an up-regulated inflammatory response. Accordingly, more complex in vitro models are required to study these mechanisms. For instance, Quagliariello et al[7] employed cocultures of peripheral blood mononuclear cells and cardiomyocytes to test the cardiotoxicity of pembrolizumab and/or trastuzumab. Although the combination of pembrolizumab and trastuzumab produced the greatest cardiomyocyte toxicity, there was minimal toxicity in the absence of peripheral blood mononuclear cells, demonstrating that a coculture of different cell types is required to model the immune injury.

Because immune-mediated cardiotoxicity involves interactions of multiple organ systems and the tumor itself, some are turning toward integrated

TABLE 1 Proposed Models of Cardiotoxicity

Mechanisms of Cardiovascular Toxicity and Proposed In Vitro Models				
Clinical Toxicity	**Manifestation**	**Mechanism**	**Proposed In Vitro Models**	**Measurements**
Cardiomyopathy	HFrEF, HFpEF	Direct cellular death/damage	2-D culture, 3-D culture or ex vivo studies of primary tissues	Biomarkers (LDH, troponin, creatine kinase)
				Signs of membrane damage
				Markers of apoptosis (p53, Bcl-2, BAX, and caspase activation)
				Metabolomic, proteomic, transcriptomic or epigenetic screens
				Mitochondrial readouts (membrane potential, NAD(P)H-dependent enzymes)
		Cellular contractile dysfunction		Calcium handling
				Quantitative image analysis, sarcomere length detection
				Force production
Vascular insults	Hypertension	Increased cardiomyocyte workload	2-D culture and 3-D culture with modifiable stiffness/afterload	Cellular damage and dysfunction models per above
	Myocardial infarction	Ischemic insult, vascular dysfunction	2-D endothelial cell cultures, engineered blood vessels, ex vivo studies of primary blood vessels	Endothelial cell tube formation
	Vasospasm			NO release
	Thromboembolism/vascular events			Platelet aggregation
Inflammatory/ immune-mediated	Pericarditis Myocarditis	Largely not understood, thought to be T-cell mediated	Integrated organ-on-a-chip models	Markers of apoptosis and membrane damage as above
Arrhythmia	Long QT, supraventricular arrhythmias, ventricular arrhythmias	Altered repolarization, increased automaticity, conduction changes	2-D culture, biochemical assays, and in silico models	hERG binding assay
				Voltage clamp experiments
				Multielectrode array platforms

2-D = 2-dimensional; 3-D = 3-dimensional; hERG = human Ether-à-go-go-Related Gene; HFpEF = heart failure with preserved ejection fraction; HFrEF = heart failure with reduced ejection fraction; LDH = lactate dehydrogenase; NAD(P)H = nicotinamide adenine dinucleotide phosphate; NO = nitric oxide.

"organ-on-a-chip" models as an investigative strategy. These models are microsystems that link key functional units of living organs within an integrated in vitro model. Such systems are currently being adapted to study off-target drug toxicities in liver, heart, and lung tissues. A particularly exciting advance reported by Weng et al[8] uses a microfluidic device with several connected chambers: 1 culturing paced cardiomyocytes, 1 culturing endothelial cells, and 1 culturing tumor cell spheroids. By exposing microfluidic devices to multiple anticancer compounds, these investigators were able to model both the cardiotoxicity and efficacy of these agents at their therapeutic concentrations. Further development and integration of organ-on-a-chip models will also enable elucidation of cross talk between organs and the tumor itself, and the impact of small molecules, drug metabolites, and cytokines on toxicity and antineoplastic efficacy. Despite these potential advantages and ongoing improvements of integrated organoid models, it is important to acknowledge that in vivo clinical observations and intact tumor-bearing animal models will remain essential for identifying and characterizing these more complex and integrated mechanisms of cardiovascular toxicity.

ARRHYTHMIAS

The potential for cardiotoxicity to manifest as a primary rhythm abnormality is well-established for many classes of antineoplastic therapies. Agents most associated with arrhythmia generation include anthracyclines, antimetabolites such as capecitabine and 5-fluorouracil (5-FU), arsenic trioxide, histone deacetylase inhibitors such as panobinostat, cisplatin and several TKIs.[9] These agents have varied mechanisms of generating arrhythmia, including, but not limited to, altered repolarization, increased automaticity, and changes in conduction. The most common, and best studied, is prolongation of repolarization manifested as an increase in action potential duration in isolated myocytes and prolongation of the QT interval on the surface electrocardiogram.

To detect action potential prolongation, voltage clamp methodologies measure action potential duration in individual cardiomyocytes and reflect the cumulative effect of changes in ion channels. Together, these determine the action potential and QTc prolonging capacity of a given compound. However, these experimental approaches are technically cumbersome. Alternatively, arrhythmogenic

prolongation of the action potential may be examined in vitro using voltage-sensitive fluorescent dyes.

An initiative designed to improve arrhythmia risk assessment by integrating multiple components is exemplified by the Comprehensive in vitro Proarrhythmia Assay (CiPA).[10] CiPA utilizes several methods to determine arrhythmogenicity, including automated patch clamp platforms with a focus on sodium, potassium, and calcium channels, as well as a multielectrode array platform that reflects fluctuations in extracellular field potential. CiPA also incorporates an in silico model of human ventricular action potential and confirms these results using human ventricular myocytes.[10] This platform thereby uses a graduated risk scale to determine the degree of proarrhythmic risk that a drug holds, rather than a binary assessment of QTc prolongation.

CONCLUSIONS

With the proliferation of new and effective targeted antineoplastic agents, there is a corresponding increase in on- and off-target side effects, including cardiotoxicity. Disease modeling to probe mechanisms and explore mitigation strategies is best served by using model systems that are well-matched to the type of toxicity, rather than the class of drug. In this context, advances in 2-D culture techniques, in vitro assays, tissue engineering, organ-on-a-chip technologies, and their integration provide increasing opportunities to address growing challenges in the realm of cardio-oncology. Although these models often employ myocardial and/or vascular cells derived from Hu-iPSCs, future advances might include separate lines of iPSC-derived cells reflecting a variety of genetic backgrounds.[4] Alternatively, patient-specific Hu-iPSC-derived organoids and tumor cells in integrated organ-on-a-chip formats would provide exciting opportunities for personalized assessment of risks and benefits.

Though this review has focused largely on in vitro cell and tissue models available to model chemotherapy-induced cardiotoxicity, these increasingly employed approaches have not altogether replaced in vivo models. For some mechanisms of cardiovascular toxicity, the need to better replicate mature cardiac physiology, interrogate intertissue interactions, or define the effects of neoplastic tissue on heart and vascular toxicity currently favors the use of in vivo models. Nevertheless, the rodent models often used for in vivo studies may not faithfully reproduce cardiovascular toxicity observed in humans, and the use of immunosuppression in tumor-bearing in vivo models may distort the toxicities observed. Accordingly, further advances in integrated organ-on-a-chip models and strategic use of multiple model systems may ultimately be best for comprehensive elucidation of cardiotoxic risks and testing of mitigation strategies.

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