STATE-OF-THE-ART REVIEW

Using Zebrafish for High-Throughput Screening of Novel Cardiovascular Drugs

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SUMMARY

Cardiovascular diseases remain a major challenge for modern drug discovery. The diseases are chronic, complex, and the result of sophisticated interactions between genetics and environment involving multiple cell types and a host of systemic factors. The clinical events are often abrupt, and the diseases may be asymptomatic until a highly morbid event. Target selection is often based on limited information, and though highly specific agents are often identified in screening, their final efficacy is often compromised by unanticipated systemic responses, a narrow therapeutic index, or substantial toxicities. Our understanding of complexity of cardiovascular disease has grown dramatically over the past 2 decades, and the range of potential disease mechanisms now includes pathways previously thought only tangentially involved in cardiac or vascular disease. Despite these insights, the majority of active cardiovascular agents derive from a remarkably small number of classes of agents and target a very limited number of pathways. These agents have often been used initially for particular indications and then discovered serendipitously to have efficacy in other cardiac disorders or in a manner unrelated to their original mechanism of action. In this review, the rationale for in vivo screening is described, and the utility of the zebrafish for this approach and for complementary work in functional genomics is discussed. Current limitations of the model in this setting and the need for careful validation in new disease areas are also described. An overview is provided of the complex mechanisms underlying most clinical cardiovascular diseases, and insight is offered into the limits of single downstream pathways as drug targets. The zebrafish is introduced as a model organism, in particular for cardiovascular biology. Potential approaches to overcoming the hurdles to drug discovery in the face of complex biology are discussed, including in vivo screening of zebrafish genetic disease models. (J Am Coll Cardiol Basic Trans Science 2017;2:1-12)

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The identification of novel drugs for cardiovascular disease is a major challenge. Many of today’s cardiovascular drugs are designed to modulate well-known “legacy” targets, in essence pathways far downstream, such as blood pressure, membrane stability, and lipid levels, which may have limited specificity for the underlying disease mechanism. Many such drugs are only modestly effective or are limited in their utility by “on-target” and “off-target” toxicity (1-5). For example, the focus of the current antiarrhythmic armamentarium is the modulation of myocardial automaticity, refractoriness, or conduction. Importantly, these are also the fundamental components of myocardial biology required for the maintenance of a regular rhythm. The clinical strategies applied in therapy for
Arrhythmias ranging from simple atrial premature beats to malignant ventricular tachycardia are remarkably similar, as a consequence of the lack of definitive mechanistic insight into many clinical arrhythmias. The resultant targeting of “final common pathways” or even normal physiology with blunt pharmacological tools rather than the precise manipulation of disease-specific mechanisms leads to predictable problems (6–8). Not surprisingly, many effective antiarrhythmic agents are also highly proarrhythmic in particular contexts, and these “on-target” adverse effects have become all too apparent, with several costly failures in large randomized clinical trials (3,9,10). In this paper, we outline evidence that most existing cardiovascular drug targets are poorly validated, review emerging data on the role of mechanistic insight in drug discovery for specific cardiovascular diseases in humans, and discuss recent advances using direct in vivo chemical screens in zebrafish for the discovery of novel cardiovascular tool compounds and drug leads.

**CHOOSING CARDIOVASCULAR TARGETS**

Chronic cardiovascular diseases pose several fundamental problems for drug development (1,11). Clinical events may present abruptly and often with severe consequences (arterial occlusion, paroxysmal arrhythmia, venous thrombosis, or complex vasomotor syncopal events), but the underlying myocardial, vascular, or systemic substrate may be totally undetectable by conventional technologies (12–16). Indeed, the investigation of many chronic cardiovascular disorders is characterized by difficulty in making a negative diagnosis. This dilemma has driven cardiovascular medicine to exploit the concept of risk factors, treating higher risk cohorts identified by specific downstream biomarkers but without overt manifestations of disease, to enable the prevention of disorders (17).

Arrhythmias are an excellent paradigm for much of common complex cardiovascular disease because they are often paroxysmal, limiting the utility of direct approaches to detection and confounding rigorous evaluation of pharmacological or other therapeutic interventions. For many clinically significant arrhythmic syndromes, lifetime risk that an episode will occur may be quite low, but the risk from each individual paroxysm for a morbid or mortal outcome may be quite high (18). Similarly, for arterial occlusive events, even the presence of existing partially obstructive lesions is not a particularly effective predictor of subsequent acute events (19). In addition, the lack of accessibility of cardiac and vascular tissue has left the field to focus on cross-sectional assessment of the final anatomy or physiology, while upstream causal molecular or cellular biology is largely uninterrogated. The balance of risk and benefit in the face of long-term exposure to agents with limited efficacy, even with only a small possibility of severe toxicity, is biased against net benefit. Many clinical trials include aggregates of multiple constituent disorders into single syndromes and so also have likely diluted the effects of new medications, impeding the progress of new drugs that in very heterogeneous conditions must meet very high thresholds of proof. Heterogeneity of etiology results directly in heterogeneity of individual effect sizes, with consequent implications for the magnitude of clinical trials and their costs. These same constraints have also limited enthusiasm for discovery programs for cardiovascular drugs in the pharmaceutical industry (1,20). However, the clinical significance of cardiovascular diseases, and their associated mortality and morbidity, continues to dominate other disorders, including even cancer.

At the core of these concepts is an implied need to carefully balance the risks of a specific condition and the risks of any new therapeutics throughout the development process. These concepts have been framed under the rubric of precision or individualized medicine, which recently has become the focus of major federal initiatives (21,22). Real individualization of medicine dictates remarkable changes in almost everything that we do. It will require a new wave of studies to define the etiologic basis of each disease subset, mechanism-specific diagnostics, transformative approaches to disease modeling, and drug discovery on a previously unimaginable scale (23). Although this is well under way for clonal neoplasia, it will not be feasible in the management of chronic cardiovascular diseases until we have robust approaches to the identification of fundamental mechanisms; detection of subclinical disease; cost-effective, efficient, and predictive disease models; and truly scalable approaches to drug discovery in mechanistically faithful models (22–24).

**BEYOND TRADITIONAL TARGETS**

To date, cardiovascular discovery has focused on a limited repertoire of molecular targets. In myocardial disease, almost every successful agent has transferred from the antihypertensive field, even in situations in which there are intrinsic cellular myocardial abnormalities such as hypertrophic cardiomyopathy (20,25,26). In arrhythmias, almost all of the activities in drug discovery have been focused on transmembrane ion fluxes and the associated channels or
ion exchangers required to generate these (27). In vascular disease, the focus has been on modulating lipids and directly influencing the mechanisms of platelet aggregation or the coagulation cascade. Although in each area there have been successes, the net effects on clinical care have been relatively modest, and there have been numerous relatively late stage failures (28). The precise performance of the pharmaceutical industry in the area of cardiovascular disease is difficult to assess, but by any measure, the average costs have been substantial and have dissuaded many companies from ongoing research or development in this area. Indeed, the prospect of individualized drug discovery seems remote for most cardiovascular disorders or for other chronic medical problems (29,30).

The advent of human genetics led to hope in cardiology, as in oncology, that by defining the causes of specific disorders, highly effective therapies might be developed to reverse the pathobiology of individual diseases. This idea has been sustained through several decades but has yet to be fully realized (23,31). The identification of low-density lipoprotein receptor gene mutations as the major molecular cause of familial hypercholesterolemia and of the associated forms of premature atherosclerosis led to hope that this common scourge might be eliminated. Drugs that were developed to reduce low-density lipoprotein from these insights, specifically the statins, were successful even in the general population and may have created a false sense of translational ease in the cardiovascular field. Subsequent efforts to raise high-density lipoprotein cholesterol have proved uniformly unsuccessful, and as a consequence investigators now are reassessing the strategies for target identification (28,30). Similarly, ion channel gene mutations are known to be the major causes of rare inherited arrhythmias, including the long-QT and Brugada syndromes (14), and drove extensive programs designed to identify specific inhibitors of individual ion channels or currents, on the premise that through modulating basic channel ion physiology it would be possible to adjust arrhythmic risk in a beneficial direction (32). Despite this investment, such ion channel-focused discovery has, to date, largely failed to diminish mortality or morbidity from arrhythmias.

These challenges across multiple fields appear to be the result of several conceptual hurdles, which the drug discovery process itself has uncovered (29,30). For example, the initial assumption that arrhythmias are a direct consequence of abnormalities of passive conductance alone has not borne deeper scrutiny. Definitive genetic manipulations of channel density or net ionic flux have rarely lead to spontaneous arrhythmias, though in truth these experiments are complicated to interpret given the idiosyncrasies of murine cardiac electrophysiology (33). Powerful homeostatic effects counter major effects from null alleles in major ion channel genes. However, targeted knock-in in these same genes of alleles that cause human disease does result in spontaneous arrhythmias (34). These data suggest that arrhythmias result from very specific “gain of function” effects in ion channels rather than simple modulation of ion conductance. Similarly, the knowledge that heart failure is caused by mutations in cardiomyocyte structural proteins has not led to a single new agent’s entering the market, though there have been several notable serendipitous successes during this period, including mineralocorticoid receptor antagonists and neprilysin inhibitors (26). Interestingly, the data supporting reduced high-density lipoprotein as a causal factor in atherosclerosis were remarkably scant, and elegant retrospective analyses have shown that the failure of high-density lipoprotein raising cholesteryl ester transfer protein inhibitors might have been predictable (35). The vagaries of current drug discovery are widely recognized and have stimulated the exploration of new approaches to the entire process (30).

The complexity of the underlying biology is a major contributor to the current failure rate in drug discovery. For example, drugs that target the conductance of ion channels as antiarrhythmic agents have not proved successful, for several reasons. Unrevealed signaling roles of the various ion channels likely underlie the lack of efficacy seen with simple conductance modulation, while also explaining some of the proarrhythmia observed with such agents (2,27). Toxic effects may also result from “on-target” activity against extracardiac isoforms (e.g., neuronal or smooth muscle) (36).

Careful study of cardiovascular disease in all of its forms also implies that manipulation of single molecules is far from certain to prove a useful strategy for the long-term normalization of chronic disease pathobiology. There is little correlation between the baseline physiologic effects of the causal mutant proteins and clinical events possibly decades later (37). A wide range of homeostatic mechanisms, including autonomic innervation, changes in loading conditions, metabolism, inflammation, and neuro-muscular stimuli, can all act as proximate triggers for downstream clinical events (38-40).

**COMPLEX SYSTEMS**

As genetic and genomic studies flesh out the picture of disease pathophysiology, so the remarkable
interdependence of each aspect of human biology becomes more apparent. Again, these interactions are perhaps best understood in membrane biology, in which the function even of individual molecular domains is accessible through patch clamping, super-resolution microscopy, and other emerging techniques (41). A growing body of data now implicate perturbation of ion channel protein interactions (physical or functional) with partner proteins and distal signaling networks (44,42,43). Human genetics have implicated channel accessory proteins and membrane scaffolding molecules in familial arrhythmias (3,34,44–46). The turnover of ion channel subunits and that of many other membrane molecules (proteins, lipids, carbohydrates, and a host of small molecules) are inextricably linked. Messenger ribonucleic acid editing, splicing, a panoply of post-translational modifications, functional quality control, chaperoning, trafficking, assembly into macromolecular complexes, rates of membrane insertion and dwell time are all under close control across multiple time scales (27,43,47,48). Emerging technologies are uncovering similarly tight regulation of lipid domains and their constituents as well as remarkable physicochemical effects in the membrane that govern much of the membrane’s function (49). As we explore cell biology using unbiased approaches, a host of other proteins from extracellular matrix, cytoskeleton, and even nuclear pore are found to play central roles in cellular electric events (50). Next-generation drug discovery must tackle such complexity directly.

Functional genomics, including transcriptomics, proteomics, and metabolomics, identify metabolic abnormalities in the heart and other tissues in the context of different disorders, including atrial fibrillation and vascular disease (51). Gastrointestinal commensal microbes may contribute to metabolite abnormalities, and whether primary or secondary, these observations suggest dysregulated metabolism may contribute to the initiation or maintenance of different cardiovascular disorders (52). Together these insights suggest that for many cardiac and vascular conditions, the minimal “target” may be an organelle or a large macromolecular complex and difficult to represent in a reductionist in vitro system for traditional high-throughput drug screening.

**CELLULAR HETEROGENEITY IN THE HEART**

Not only are the molecular networks complex, so too are the cellular networks within the heart. Heterogeneous cardiomyocyte populations are coupled in discrete functional networks within the heart (53), including traditional units such as pacemakers, nodal and conduction systems, and unrecognized clusters that are only now being defined (54–56). Acquired heterogeneity may arise through injury (57), but physiological heterogeneity between endocardium, midmyocardium, and epicardium is also well documented (53). The normal and pathological roles for these and other cardiomyocyte subpopulations have yet to be fully understood.

Noncardiomyocytes such as endothelial cells, smooth muscle cells fibroblasts, histiocytes, and infiltrating leukocytes are also beginning to be explored as agents of contractile failure, vascular disruption, and arrhythmogenesis. For example, perturbed interactions between coronary endothelial cells and cardiomyocytes have been implicated in the microvascular ischemia observed in some cardiomyopathies (58). Inflammatory infiltration and associated local or systemic cytokine effects on arrhythmias are observed in myocardial injury or as primary phenomena (59). These effects underpin mechanistic links between myocarditis, Chagas disease, device infection or overt sepsis, and arrhythmias (60,61). Finally, fibroblasts may contribute collagen-based electric barriers but also many other paracrine signals that promote or attenuate discrete functions of cardiomyocytes (62–64).

Recent work in myocardial regeneration has highlighted potential roles of heterocellular coupling or even cell fusion in the biology of post-natal terminally differentiated cells (65). These insights also bring into sharp relief our limited understanding of the physiological context for cardiomyocyte cell division (even to the limited extent that this occurs) during development or in later life. Adult myocardial responses to stress or injury appear to reflect frustrated cell division, and the physiological consequences of myocardial remodeling may thus represent an intrinsic tension between cellular coupling and autonomous cell behaviors (66).

**DRUG DISCOVERY IN THE FACE OF IRREDUCIBLE COMPLEXITY**

Drug discovery has traditionally involved the isolation of a specific target molecule, the design of a robust and scalable assay for this single target’s activity, and the completion of an empirical screen of large chemical libraries for entities with the desired effect in this refined assay (29,30). Clearly, the choice of target is a major decision node in this process and is often the source of subsequent problems. Target choice is often based on a host of factors that may have little to do with the disease biology in humans, such as prior work in the area, perceived
“drugability” of the specific target, previous successful drugs in the same field, or data from animal models with tenuous mechanistic links with the cognate human disease (29,67). Rarely is the target chosen because it is known to be a specific cause of the underlying disease. Often the target will have been studied intensively in a particular arena, but little may be known of target function in other cell types or tissues or target behavior in the context of commonly encountered stressors or acquired contributors to disease. Many preclinical animal models are expensive or are highly inbred, and it is common for drugs to reach the market after testing in fewer than 1,000 animals. It is perhaps not surprising therefore that many drugs suffer from unanticipated “on-target” effects as well as apparently idiosyncratic reactions in the face of rare genetic variants (2,23,68).

Ironically, amiodarone, the most effective antiarhythmogenic agent identified to date, was discovered serendipitously during its development as an anti-anginal drug (69). Complex effects against multiple “targets” are now known to be important contributors to the final profile of any drug, both to beneficial and adverse outcomes (70,71). As the impetus to more precisely tailor therapy to disease increases, it is increasingly important to closely match any risk associated with the drug with the potential benefit: the acute termination of ventricular tachycardia has a very different risk tolerance than the chronic suppression of a relatively benign atrial arrhythmia. A major impetus to the concept of in vivo drug discovery is the ability to screen a priori for such “dirty” drugs using therapeutically relevant endpoints and counterscreens for toxicities (72,73). In many ways this approach is a systematized search for serendipity in the context of rigorous disease models.

An ideal drug discovery model would recapitulate not just a single target but all the relevant targets in totally native context. It would enable parallel screening for toxic effects; facilitate studies of absorption, distribution, metabolism, and excretion; and encompass drug-drug interactions. Because comorbidities are very common, it would also be useful to be able to model other disease entities as well as environmental stimuli. If the likelihood of success is heightened by the testing of potential therapeutics in the setting of each and every step in the causal chain, then perhaps the only rigorous approach at present is direct in vivo discovery in genetic disease models (30,73,74). Indeed, a recent review of discrete approaches to target identification has suggested that genetically validated targets are twice as likely to result in a drug reaching the market as those without such empirical experimental support.

**IN VIVO DISCOVERY**

In essence, an in vivo discovery strategy, particularly one downstream of a known causal mutant, directly interrogates all of the etiologic components integrated precisely in their native context. The limited feasibility and cost of screening in mammals has allowed this approach only in the later phases of drug discovery to discriminate among small numbers of compounds. More tractable models such as yeast, *Caenorhabditis elegans*, and *Drosophila* are not representative enough for use in phenotypes such as heart failure or arrhythmias, but these models have been successfully used in other disease areas (75).

In the past decade, the emergence of the zebrafish as a screenable vertebrate model has transformed the scale of genetic study that is feasible for complex diseases (74,76–79). Within 48 hours of fertilization, the larval fish has established complex physiology yet can be sustained in large numbers for days in multiwell plates (80,81) and is amenable to both genetic and chemical screening (82). The zebrafish genome has been sequenced and is readily manipulated using zinc finger nucleases, transcription activator-like effector nucleases, and now clustered regularly interspaced short palindromic repeats/Cas9 genomic editing technologies (84). Saturation screens for morphological phenotypes have been successfully performed (85), and more complex screens for physiological and pharmacological traits have begun to emerge (50,80). Embryos can be readily arrayed in multiwell plates, and the development of increasingly sophisticated automated assays has allowed in vivo screens for integrated physiological endpoints at a scale that previously has been feasible only for cell-based assays (50,86).

The permeability of the larval zebrafish to small molecules has popularized chemical screens for modifiers of specific pathways or for the suppression of disease traits that have been modeled in the organism (87,88). The zebrafish is also being developed as a tool in toxicology, and these endpoints can be counterscreened in parallel with ongoing discovery efforts, leveraging even further the representativeness of the intact organism beyond the target organ system (89). Similarly, secondary screens of analog series are immediately feasible after “hits” are identified and facilitate the discovery of lead compounds optimized for a balance between efficacy and toxicity (81,90). The use of existing drugs in combination with new agents enables screens for drug-drug interactions, while outbred lines allow screens for rare gene-drug interactions (80,86). The fish can also be
used for more typical mechanistic studies at lower throughput but still on a scale and at a cost that are competitive with other vertebrate models.

**ZEBRAFISH AND CARDIOVASCULAR DISEASE MODELING**

Techniques have been developed to measure heart rate, contractility, and blood flow at high throughput in the zebrafish, as well as a repertoire of secondary assays, including optical voltage mapping, Ca\(^{2+}\) imaging, and specific transgenic reporters for subcellular Ca\(^{2+}\) compartments, discrete signaling reporters, and even organelle function (50,80,81,91). In addition, it is possible to build reporters for transcriptional events that allow quantitative in vivo assessment of the activity of pathways that may be inaccessible through other means (92). These tools enable efficient large-scale screens for genetic or chemical modifiers of known disease endpoints ranging from specific electrophysiological phenomena through to pathway reporters, all in a completely native context (Central Illustration) (50,92).

In early work it was possible to show that more than 90% of drugs that cause repolarization toxicity in humans result in cognate electrophysiological effects in the zebrafish even as early as 48 hours post-fertilization (81). Initial assays for heart rate using image analysis to explore cardiotoxicity were based on the dominant frequency component of the heart rate but traded specificity for both sensitivity and throughput (81). The complexity of arrhythmogenesis suggested that more sophisticated modeling was necessary to fully understand the underlying biology. To enable mechanistic evaluation of genetic or chemical modifiers, methods were developed to directly measure cardiac action potentials in zebrafish embryos using optical mapping with voltage-sensitive dyes at a stage when the fish are amenable to scalable screening. Normal embryos display subtle differences in atrial and ventricular action potential profiles, as anticipated (50), and these are remarkably representative of adult human cardiac electrophysiology within approximately 48 h of fertilization. Similar studies of contractility and vascular responses were also notable for their recapitulation of much of human physiology (93). Nevertheless, it is always vital to ensure that the endpoints chosen for a specific study are valid for the overall goals of the screen.

Each assay that is developed merits rigorous validation, ideally in genetic models of the diseases of interest. For example, the electrophysiological endpoints in the zebrafish were validated in a comprehensive study of 1 form of human arrhythmia, inherited repolarization perturbation or the long-QT syndrome, using a zebrafish mutant breakdance that carries a missense mutation in the cardiac \(KCNH2\) gene, the major subunit of the potassium channel responsible for inwardly rectifying potassium current (\(I_{Kr}\)) (50). In breakdance homozygote action potentials, there was evidence of significant “triangulation,” or prolongation of the action potential duration 25% to 75%, a phenomenon observed in human repolarization disorders with high arrhythmic risk (Figures 1A and 1B) (94). The mechanism of 2:1 atrioventricular block was evident from recordings that demonstrated alternate atrial impulses encountering refractory ventricular myocardium (Figure 1C, top) (95). Importantly, breakdance homoygotes also exhibited spontaneous early afterdepolarizations, the postulated triggers of fatal arrhythmias in both inherited and acquired repolarization disorders (Figure 1C, bottom). Treatment with doses of dofetilide paralleling effective extracellular concentrations in clinical use (10 nmol/l) caused subtle prolongation of wild-type action potentials (64 ± 45 ms, 28% increase), while the same concentration of dofetilide resulted in marked prolongation of heterozygote action potentials (194 ± 92 ms, 75% increase) (Figure 1D). A final confirmation of the fidelity of the model was the extension of these observations to novel repolarization genes such as \(NOS1AP\), first identified in large human genetic studies (50). Notably, despite some contradictory findings in other experimental systems, these early zebrafish findings for \(NOS1AP\) were subsequently confirmed in elegant studies in higher organisms (96).

Having established the fidelity of the model in physiological and disease states, it was then possible to exploit the throughput of the zebrafish model system to undertake a pharmacogenetic screen of a library of insertional zebrafish mutants (50). This was designed to identify, in an unbiased manner, new genes that modify the cardiac response to \(I_{Kr}\) blockade. Despite intense efforts, to date few biologically relevant repolarization or drug response modifier loci have been identified. The robust parallels between zebrafish and human cardiac repolarization suggested that formal genetic analysis of this clinically important complex trait might be feasible. To optimize sensitivity, specificity, and throughput, an initial high-throughput screen for abnormal heart rate response to dofetilide was combined with a second high-resolution assay in which confirmed mutants are studied using optical mapping. Subsequent testing in the absence of dofetilide allowed discrimination between pure drug response phenotypes and intrinsic heart rate defects. In the initial shelf screen
of 340 insertional mutants, 15 genes with major effects on repolarization were identified, none of which had been implicated previously in this process. Interestingly, the majority of these genes appear to belong to an integrin-associated network of modulating channels and their adaptor proteins. These findings suggest potential links among mechanical loading conditions, inflammation, and repolarization that may shed light on arrhythmogenesis in a number of conditions. Subsequently, some of these genes have been shown to modify human repolarization, confirming the utility of zebrafish screens for the discovery of genetic modifiers in physiological or pharmacological pathways (50).

This cycle of modeling, assay definition, assay validation, and subsequent screening is vital before embarking on a high-throughput discovery screen in which the sensitivity and specificity of any assay are challenged (74). Where these steps are undertaken and the limits of screen are fully understood prior to its
initiation, it is feasible to mitigate any confounders in the overall design. Pre-specified replication steps can be introduced or individual assays can be combined in series to optimize the sensitivity and specificity of the overall screen. In this construct, an investigator can create a staged series of assays with initial high-throughput and high-sensitivity assays paired with subsequent lower throughput but more stringent high-specificity follow-up assays designed to create an integrated final output with the desired characteristics. These principles are beginning to be applied in a variety of different drug discovery screens for discrete cardiovascular endpoints, with the goal of accelerating the pace of translation to the clinic.

**RECENT SCREENS FOR CARDIOVASCULAR COMPOUNDS IN THE ZEBRAFISH**

The utility of unbiased in vivo screens in cardiovascular disease is now beginning to be realized in different disease models including arrhythmias, heart failure, and cardiotoxicity.
Exploiting viable mutants in the zebrafish ortholog of the human ether-a-go-go potassium channel (KCNH2), Milan’s group has undertaken an impressive screen to look for small molecules capable of suppressing the resultant complex phenotype (97). Testing some 1,200 compounds at 48 h of development by scoring for rescue of the typical 2:1 atrioventricular block at 72 h in a 96-well format, they identified 2 novel classes of compounds that fully suppressed the long-QT phenotype in 3 of 3 fish and were considered hits. Screen compounds were obtained from commercially available small-molecule libraries (Prestwick and Chembridge). Initial hits were subsequently tested with larger numbers of animals across a range of doses and different time courses. Interestingly, the screen identified flurandrenolide and 2-methoxy-N-(4-methylphenyl) benzamide as compounds that reproducibly suppressed the long-QT phenotype. Optical mapping confirmed that treatment with each compound caused shortening of ventricular action potential durations. Structure activity studies and steroid receptor knockdown suggested that flurandrenolide functions via the glucocorticoid signaling pathway (97).

In a robust zebrafish model of the arrhythmogenic cardiomyopathy Naxos disease, which results from mutations in the plakoglobin gene, it was possible to identify abnormalities of heart rate, contractility, and reduced sodium channel conductance at the membrane in the mutant fish (98). However, these phenotypes emerged beyond 5 days post-fertilization, too late for highly efficient screens. Expression profiling of mutants and wild-type siblings revealed substantial elevation of nppb transcription in the mutants compared with their wild-type siblings, and as a result, an nppb::luciferase reporter line was crossed into the plakoglobin mutant background. Subsequent screening identified a compound (SB217639) that has nanomolar activity not only in the original mutant line but also in other zebrafish models of arrhythmogenic cardiomyopathy, 2 murine models of arrhythmogenic cardiomyopathy, and human induced pluripotent stem cell lines derived from patients with arrhythmogenic cardiomyopathy (99) (Figure 2). Interestingly,
this compound has no activity in mutant models of cardiomyopathy caused by mutations in cytoskeletal genes, the nuclear lamin, or metabolic parameters, suggesting a discrete pathway or pathways.

When the primary myocardial insult is less variable, it is feasible to use less quantitative endpoints, and some extremely successful screens have been performed very effectively using rapid visual assessment as an initial screen. This is not surprising in many ways, as 1 of the very first chemical screens in the organism was based on subjective assessment of morphological similarity to bone morphogenetic protein loss-of-function mutants. This screen led to the discovery of dorsomorphin, the first small-molecule antagonist and a harbinger of a class of anaplastic lymphoma kinase inhibitors that is now making its way to the clinic (100,101).

In a recent screen designed to identify compounds that would mitigate the cardiotoxicity of anthracyclines while not attenuating their antineoplastic activity, Liu et al. (102) used the suppression of early pericardial edema to identify potential “hit” compounds that were then studied in more detail using metrics of heart rate, contractility, and survival. In addition, in a reversal of a typical toxicological counterscreen approach, the investigators used tumor cell lines to assay each of the hits to ensure that their antineoplastic activity was conserved. This strategy led to the efficient discovery of visnagin, an agent that completely reverses the acute cardiac toxicity of anthracyclines in the fish and has subsequently been shown to prevent cardiotoxicity in an established murine model of the same syndrome (102).

SCALABLE IN VIVO MODELS COMPLEMENT OTHER EMERGING TECHNOLOGIES

Validated in vivo models of physiology or disease that can be efficiently scaled also complement the broad range of “omics” technologies that are emerging from academia and industry (Figure 2). Functional genomics have identified a host of new ion channels, many of which are now being studied as potential drug targets. In many instances, relatively little is known of the integrated physiology of these channels, and their roles at different stages or in different disease settings may be difficult to define on the scale necessary for prioritization in drug discovery. Gene editing and transgenesis technologies have brought the zebrafish to the forefront in the initial modeling of data from genomewide association studies, expression profiling, metabolomic, and other omics experiments. Although the precise germline manipulations feasible in the mouse are not yet feasible in the zebrafish, the speed and cost of the zebrafish have favored its use in situations in which the phenotypic parallels have been validated carefully (103,104). The ease of modeling extends to many aspects of the pathophysiology of disease, and using specific zebrafish strains, it is possible to test interactions with heart failure, hypertrophy, and ischemia to name but a few (77,105).

SUMMARY

In vivo screening in faithful disease models allows the effects of drugs on integrative physiology and disease biology to be captured during the screening process, in a manner agnostic to potential drug target or targets. This systematic strategy bypasses current gaps in our understanding of disease biology but emphasizes the importance of the rigor of the underlying disease model. Modeling genetic or environmental causes of cardiovascular disease in the zebrafish enables downstream biological investigation at scale and in combination with complementary model systems has the power to transform several aspects of precision medicine.

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11. Spooner PM, Albert C, Benjamín EJ, et al. Sudden cardiac death, genes, and arrhythmogenesis: consideration of new population and mechanistic approaches from a National Heart, Lung, and Blood Institute workshop, part I. Circulation 2001;103:2361–4.
12. MacRae CA, Ellison PT. Genetic screening and risk assessment in hypertrophic cardiomyopathy. J Am Coll Cardiol 2004;44:2236–8.
13. Vatta M, Dumeaine R, Varghese G, et al. Genetic and biophysical basis of sudden unexplained nocturnal death syndrome (SUNDS), a disease allelic to Brugada syndrome. Hum Mol Genet 2002;11:337–45.
14. Keating MT, Sanguinetti MC. Molecular and cellular mechanisms of cardiac arrhythmias. Cell 2001;104:569–80.
15. Amaral N, Okonko DO. Metabolic abnormalities of the heart in type II diabetes. Diab Vasc Dis Res 2012;15:239–48.
16. Gregory SA, MacRae CA, Aziz K, et al. Myocardial blood flow and oxygen consumption in patients with Friedreich’s ataxia prior to the onset of cardiac hypertrophy. Coron Artery Dis 2003;14:15–22.
17. Kapur NK, Ashen D, Blumenthal RS. High density lipoprotein cholesterol: an evolving target of therapy in the management of cardiovascular disease. Vasc Health Risk Manag 2008;4:39–57.
18. Lloyd-Jones DM, Wang TJ, Leip EP, et al. Assessment of diastolic function with Doppler imaging techniques to predict progression of atherosclerosis in heart disease. Drug Discov Today 2012;17:419–24.
19. Shah M, Akar FG, Tomaselii GF. Molecular basis of arrhythmias. Circulation 2005;112:2517–29.
20. Morgan P, Van Der Graaf PH, Arrowsmith J, et al. Can the flow of medicines be improved? Fundamental pharmacokinetic and pharmacological principles toward improving phase II survival. Drug Discov Today 2012;17:419–24.
21. Collins FS, Varmus H. A new initiative on precision medicine. N Engl J Med 2015;372:793–5.
22. Shah SH, Arnett D, Houser SR, et al. Opposite regulatory roles for FGF21 in preventing and delineation of the chambers and the nodes. Trends Cardiovasc Med 2004;14:301–7.
23. van der Merwe PL, Rose AG, van der Walt JJ, et al. Progressive familial heart block type I. Clinical and pathological observations. S Afr Med J 1991;80:34–8.
24. Brink PA, Ferreira A, Moolman JC, et al. Gene for progressive familial heart block type I maps to chromosome 19q13. Circulation 1995;91:1633–40.
25. de Bakker JM, van Capelle FJ, Janse MJ, et al. Reentry as a cause of ventricular tachycardia in patients with chronic ischemic heart disease: electrophysiologic and anatomic correlation. Circulation 1988;77:589–606.
26. Heydemann A, Huber JM, Kakkar R, Wheeler MT, McNally EM. Functional nitric oxide synthase mislocalization in cardiomyopathy. J Mol Cell Cardiol 2004;36:213–23.
27. Frustaci A, Chimenti C, Bellocchi F, Morgante E, Russo MA, Maseri A. Histological substrate of atrial biopsies in patients with lone atrial fibillation. Circulation 1997;96:1180–4.
60. Issac TT, Dokainish H, Lakiss NM. Role of inflammation in initiation and perpetuation of atrial fibrillation: a systematic review of the published data. J Am Coll Cardiol 2007;50:2017–8.
61. Ramos-Mondragon R, Galindo CA, Avila G. Role of TGF-beta on cardiac structural and electrical remodeling. Vasc Health Risk Manag 2008;4:1289–300.
62. Lin CS, Pan CH. Regulatory mechanisms of atrial fibrillatory remodeling in atrial fibrillation. Cell Mol Life Sci 2008;65:1489–508.
63. Ellinor PT, Sasse-Klaassen S, Probst S, et al. A novel locus for dilated cardiomyopathy, diffuse myocardial fibrosis, and sudden death on chromosome 10q25–26. J Am Coll Cardiol 2006;48:106–11.
64. Weber KT. Fibrosis and hypertensive heart disease. Curr Opin Cardiol 2000;15:264–72.
65. Wu SM, Chien KR, Mummery C. Origins and fates of cardiovascular progenitor cells. Cell 2008;132:337–43.
66. Ahuja P, Sdek P, MacLellan WR. Cardiac myocyte cell cycle control in development, disease, and regeneration. Physiol Rev 2007;87:521–44.
67. Milan DJ, MacRae CA. Animal models for arrhythmias. Cardiovasc Res 2005;67:426–66.
68. Lin CS, Pan CH. Regulatory mechanisms of atrial fibrillatory remodeling in atrial fibrillation. Cell Mol Life Sci 2008;65:1489–508.
69. Langheinrich U. Zebrafish: a new model on the pharmaceutical catwalk. Bioessays 2003;25:904–12.
70. Lieschke GJ, Currie PD. Animal models of human disease: zebrafish swim into view. Nat Rev Genet 2007;8:353–67.
71. Zon LI, Peterson RT. In vivo drug discovery in the zebrafish. Nat Rev Drug Discov 2005;4:35–44.
72. MacRae CA, Peterson RT. Zebrafish-based small molecule drug discovery. Chem Biol 2003;10:901–8.
73. Wu SM, Chien KR, Mummery C. Origins and fates of cardiovascular progenitor cells. Cell 2008;132:337–43.
74. Asimaki A, Kapoor S, Plovie E, et al. Identification of a new modulator of the intercalated disc in a zebrafish model of arrhythmogenic cardiomyopathy. JCI Insight 2016;1(5).
75. Yu PB, Hong CC, Sachidanandan C, et al. Dorzolomorph inactivates BMP signals required for embryogenesis and iron metabolism. Nat Chem Biol 2008;4:33–41.
76. Yu PB, Deng DY, Lai CS, et al. BMP type I receptor inhibition reduces heterotopic [corrected] ossification. Nat Med 2008;14:1363–6.
77. Liu Y, Asnani A, Zou L, et al. Visnagin protects against doxorubicin-induced cardiomyopathy through modulation of mitochondrial malate dehydrogenase. Sci Transl Med 2014;6:240ra74.
78. Sho SY, Thorpe JL, Deng Y, Santana E, DeRose RA, Farber SA. Lipid metabolism in zebrafish. Methods Cell Biol 2004;76:87–108.
79. Weinstein BM. What guides early embryonic blood vessel formation? Dev Dyn 1999;215:2–11.
80. Shin JT, Fishman MC. From zebrafish to human: modular medical models. Annu Rev Genomics Hum Genet 2002;3:311–40.

KEY WORDS drug discovery, high-throughput screening, translational medicine, zebrafish