High-Throughput in Vivo Screening for Cardiovascular Drug Discovery

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Abstract

Introduction: Our understanding of the complexity of cardiovascular disease pathophysiology remains very incomplete and has hampered cardiovascular drug development over recent decades. The prevalence of cardiovascular diseases and their increasing global burden call for novel strategies to address disease biology and drug discovery.

Areas covered: This review describes the recent history of cardiovascular drug discovery using *in vivo* phenotype-based screening in zebrafish. The rationale for the use of this model is highlighted and the initial efforts in the fields of disease modeling and high-throughput screening are illustrated. Finally, the advantages and limitations of *in vivo* zebrafish screening are discussed, highlighting newer approaches, such as genome editing technologies, to accelerate our understanding of disease biology and the development of precise disease models.

Expert opinion: Full understanding and faithful modeling of specific cardiovascular disease is a rate limiting step for cardiovascular drug discovery. The resurgence of *in vivo* phenotype screening together with the advancement of systems biology approaches allows for the identification of lead compounds which show efficacy on integrative disease biology in the absence of validated targets. This strategy bypasses current gaps in knowledge of disease biology and paves the way for successful drug discovery and downstream molecular target identification.

Article Highlights

- Modern cardiovascular drug discovery has lagged recently due to the lack of understanding of complex disease pathophysiology;
- Target-based *in vitro* screening cannot model the complexity of biological and pathological processes in a whole organism or mimic the pharmacokinetic behaviors of bioactive molecules;
- The resurgence of phenotype-based screening, as represented by zebrafish embryo models, has been a bright spot;
- Using new approaches, such as genome editing technologies, has accelerated the understanding of disease biology and development of zebrafish disease models;
- New molecular entities initially identified in zebrafish screens are expected to represent an increasing proportion of the drug candidates that will enter clinical testing in the near future.

1. Introduction

Despite the significant decline in cardiovascular disease (CVD) mortality over the last several decades due to improved medications and surgical procedures, CVD remains one of the leading causes of death globally [1–3]. Myocardial infarction, heart failure, stroke and other CVDs resulted in an estimated 17.9 million deaths worldwide in 2016, representing some 31% of all global deaths [4–6]. The global burden of CVD was estimated over US$800 billion in direct healthcare costs and productivity losses worldwide in 2010 alone and these costs are projected to reach US$20 trillion by 2030 [7]. Thus, the burden of cardiovascular disease is both a major public health concern and a growing global challenge [8].

Notwithstanding the increasing global cardiovascular disease burden, investment in cardiovascular drug development has stagnated and the approvals for new cardiovascular drug therapies have declined substantially [9–10]. The application of genomic technologies and systems biology approaches has identified multiple potential new cardiovascular drug targets, as well as novel molecules with potential cardiovascular applications. However, these scientific advances have not stimulated an increase in drug development for CVD. Fewer drug candidates were found in the cardiovascular research pipeline compared with other therapeutic areas [9]. For instance, as shown in Figure 1, the antineoplastic agents undergoing early-phase development grew +6.9% between two time intervals (1990-1999 vs. 2000-2007), whereas for cardiovascular agents, there was a significant contraction in the same time period (-4.6%) [9]. Compared to the high number of new molecular entity applications (n=61, year 2000-2012) and first-cycle FDA approvals (72%) of oncology drugs, cardiovascular drugs had significantly fewer applications (n=21) and a much lower rate of first-cycle approvals

74 (32%) [10].

75 Though there are many reasons for this downward trend in cardiovascular drug discovery,
76 a particularly important one is the limits of our understanding of cardiovascular
77 pathophysiology. Promising novel druggable targets are rare. Target-based strategies have
78 been adopted as the method of choice in cardiovascular drug discovery for the past two
79 decades. Recently, the cardiovascular drug development process has become much longer,
80 riskier and more complex. Many diseases exhibit multifactorial etiologies, resulting from the
81 interactions of multiple genetic factors, signaling pathways and various environmental risks.
82 For instance, coronary artery disease (CAD) has been demonstrated to be, at least in part, an
83 intricate chronic inflammatory disease, whose etiopathogenesis is complex including
84 environmental factors, such as diet, smoking, air pollution or physical activity, and genetic
85 factors that modulate the risk of the disease [11]. To data, genome-wide association studies
86 have identified over 100 genetic loci, yet they are able only to explain a proportion of the
87 heritability of CAD [11]. It remains a daunting challenge to discover a single druggable target
88 that can address such complex disease biology.

89 The advancement of systems biology approaches, such as genome editing, functional
90 genomics and computational modeling, is currently accelerating the understanding of disease
91 biology and the exploration of druggable targets. In the meantime, a resurgence of interest in
92 phenotype-based screening for drug discovery such as zebrafish-based *in vivo* screening has
93 also occurred, taking advantage of the tractability of the zebrafish model and of human
94 induced pluripotent stem cell modeling. To date, few drugs have made it to the clinic from
95 zebrafish *in vivo* screening, but several examples are imminent. The combination of these

strategies would be expected to bring new enthusiasm and investment, while paving the way for more efficient cardiovascular drug discovery in the near future.

2. Target-based screening in the face of complexity

Target-based approaches are designed to identify biologically active small molecules based on systematic, repetitive, and quantitative investigation of their effects on a therapeutic target. The target is usually a single gene product or a specific molecular mechanism that has been identified via human genetic studies or basic biological research. In particular, once a robust link is identified between a specific human gene and a disease signature, molecules that target this gene product are strong candidates to succeed as potential therapeutic drugs. Target-based drug discovery usually relies on a tightly controlled *in vitro* screening approach, which drives less expensive and less time-consuming experiments compared to whole organism-based methods. Besides, this type of screening can also be used to assess a large number of variables, such as different experimental parameters or combinations of small molecules, which is often not feasible with the whole organism-based models [12,13].

However, despite the thousands of potential therapeutic targets identified since the human genome has been decoded, it remains quite difficult to predict which proteins, when modulated *in vivo*, will reverse a disease phenotype or alter a poorly understood pathological process, especially for complex chronic diseases like cardiovascular disease, diabetes and cancer [14-16]. In the case of cardiovascular arrhythmias for example, despite constantly improving systems biology technologies, the fundamental disease mechanisms are still difficult to elucidate. It remains a particularly challenging problem to uncover the precise

pathways that cause a sudden cardiovascular event after years of quiescence in patients. Another major issue is that many biological processes cannot be faithfully reproduced in target-focused biochemical assays or even in cultured cells. The three-dimensional context and complex interactions with other cells, tissues, or circulating factors are often important factors in disease biology and/or in drug responses.

Furthermore, the pharmacokinetic behavior of the bioactive molecules, as described by parameters quantitating compound absorption, distribution, metabolism and excretion (ADME), is a determining factor for the *in vivo* efficacy of drug candidates. In whole vertebrate models, such as the zebrafish, the effects of individual human drugs are typically representative of human complexity, including most known drug–drug interactions. In addition, there is increasing evidence that drug distribution across active physiological boundaries such as the blood–brain barrier can also be faithfully observed in animal models including the zebrafish [17], while these pharmacokinetic characteristics by definition cannot be established in target-based *in vitro* drug discovery efforts.

3. Resurgence of the *In Vivo* Screens

A phenotype-based approach is designed to study biologically active small molecules based on their interactions with whole organisms. Before *in vitro* approaches made possible by advances in molecular biology, many biologically active molecules were empirically discovered based on their unexpected phenotypes resulting from their effects on whole organisms. For instance, the anticoagulant dicoumarol and its derivatives were first discovered when cattle fed on rotting sweet clover died of internal bleeding, while the

Hedgehog signaling antagonist cyclopamine was discovered because of a fetal deformity, cyclopia, which was observed in offspring from sheep grazing on veratrum californicum [18,19]. Some attempts were also made to accelerate discovery of bioactive small molecules by systematic chemical screening, albeit in costly screens in modest numbers of rodents [20,21].

Despite the power of phenotype-based approaches demonstrated by the successful examples above, cost and lack of scalability led to the dominance of target-based methods during the last few decades. Currently, there is a resurgence of interest in phenotype-based screening because of the growing number of early discovery successes from such efforts, such as the usage of caenorhabditis elegans, drosophila melanogaster and zebrafish disease models. A recent analysis of first-in-class drugs that were approved by the FDA at 1999-2008 revealed that 62% were discovered by phenotype-based screens, despite the fact that such screens represented only a small subset of drug discovery efforts [22].

Several benefits may contribute to the success of phenotype-based approaches. First, phenotype-based drug discovery can identify chemical modifiers of virtually any biological process while target-based approaches typically can only discover modifiers of a specific target and then usually in a cell autonomous context. Therefore, phenotype-based approaches provide an opportunity to reveal novel targets and their functions and to obtain fundamental insights into poorly understood biological processes, in contrast to target-based approaches where a prior biological understanding is a prerequisite. Without robust criteria for causation, relevance of any target to a specific disease may be ephemeral. Second, phenotype-based discovery can identify chemical modifiers that produce a therapeutic effect through

simultaneous activity at multiple targets. For instance, the antiarrhythmic agent, amiodarone, discovered through serendipitous observation, exhibits activities with multiple molecular mechanisms, like actions on ion channels, adrenergic receptors, and possibly via binding to the nuclear thyroid receptor [23]. On the other hand, many molecules that exclusively target the conductance of individual ion channels as antiarrhythmic agents have proved to be unsuccessful. One possible reason for this discrepancy might relate to the incompletely understood roles of the various types of ion channels [15]. Third, phenotype-based approaches can identify chemical modifiers in the context of a whole organism, which can discover modifiers with acceptable pharmacokinetic/pharmacodynamic profile and parse out chemicals with undesirable qualities including obvious toxicities. Thus, hit compounds advancing from phenotype-based drug discovery have a higher probability of passing further tests in other models for effectiveness, side effects, toxicity, and pharmacokinetic profile compared to compounds identified in target-based screens.

4. Zebrafish as a Valuable *in Vivo* Tool for Cardiovascular Drug Discovery

Zebrafish (*Danio rerio*) were first used as a model for the study of developmental biology, and later were increasingly applied to the study of human disease, including cardiovascular disease and cancer [24,25]. In recent years, small-molecule screening for drug discovery in zebrafish has been a fast-growing fraction of phenotype-based screens. The zebrafish not only provides the common advantages of phenotype-based screens described above, but also offers some distinct advantages that are beyond other *in vivo* models, such as flies, worms and yeast.

184 *4.1 Morphology and physiology.*

185 The scale that can be achieved in zebrafish experiments is distinctive for a vertebrate
186 species. First, adult zebrafish are extremely fertile, laying up to 300 eggs per week. Thus, it is
187 feasible to generate thousands of embryos per day even for a small zebrafish facility. Early
188 embryos are approximately 1 mm in diameter, allowing several embryos to fit easily in a
189 single well of a 96 or even 384 well plate [25,26]. Second, the embryogenesis of zebrafish
190 proceeds rapidly. The entire body plan is established by 24 hours post fertilization (hpf) and
191 most of the internal organs are well developed by 96 hpf [27]. For instance, the heart is one of
192 the first organs to form and function during embryogenesis with rapid maturation within the
193 first 48 hpf [28]. Third, zebrafish embryos are transparent, which means organs, cells and
194 tissues can be visualized *in vivo* and functional changes can be investigated in real-time
195 [29,30]. These observations can be further highlighted by the use of transgenic lines and other
196 reporter molecules. For example, the *Tg*(*myl7:GFP*) transgenic line, with myocardial cells
197 expressing green fluorescent protein (GFP), was employed in a range of studies to trace the
198 developmental fates of heart cells, finding new heart specific genes, establishing biological
199 indices of environmental pollutants, and studying the efficacy of therapeutic drugs [31-33]. In
200 another example, a luciferase-based transgenic zebrafish line, *Tg*(*nppb:F-Luc*), enabled *in*
201 *vivo* identification of genetic and chemical modifiers of the expression of cardiac natriuretic
202 peptides. The advantages of zebrafish transparency and the luminescence produced by the
203 transgenic marker were combined to facilitate rapid, large-scale screening for small molecules
204 that could be potentially useful in modifying the pathological response to sarcomeric gene
205 mutations that cause hypertrophic cardiomyopathy [34].

4.2 Genetic Manipulation.

In the past two decades, rapid development of knowledge and technologies have greatly increased the utility of zebrafish as a screenable vertebrate model. The zebrafish genome has been sequenced and annotated [35]. The application of DNA microarrays, whole-embryo *in situ* hybridization (WISH) and RNA sequencing has accelerated transcriptional studies which are often important in validating disease models. More importantly, gene functions can be rapidly and robustly studied in zebrafish by use of specific genetic manipulations, such as morpholinos, RNA interference, transcription activator-like effector nucleases (TALENs), zinc finger nucleases, and in recent years also the CRISPR (Clustered regularly interspaced short palindromic repeats)-Cas (CRISPR -associated protein) system [36,37]. Morpholinos are among the most commonly used genetic tools in the zebrafish community. They act by "steric blocking", binding to a specific target sequence within an RNA molecule and thereby inhibiting the interaction of ribosomes or spliceosomes with the RNA [38,39]. By producing a reduction or even loss of expression of the gene product, morpholinos can be used to discover the functions of genes without an available mutant allele. Despite concerns regarding the specificity of morpholino effects, larger scale assessments have confirmed biological relevance, albeit requiring careful validation. In addition, the dose-dependent effect of morpholinos on the level of gene knockdown allows for the investigation of intermediate phenotypes [39]. The latest tool for targeted genome editing, the CRISPR-Cas system, is precise and efficient. By delivering the Cas9 nuclease and a synthetic guide RNA complementary to the genomic target sequence of interest (either produced by the cell after DNA/mRNA transfection or injected directly as a Cas9 protein / guide RNA complex), the

genome can be cut at a desired location, which allows existing genes to be edited or removed and/or new ones to be inserted [36,40,41]. The CRISPR-Cas system has been widely adopted in recent years and newer gene editing enzyme discoveries promise only to broaden the repertoire of what is possible. These tools enable targeted mutagenesis by inducing small mutations and even in-frame knock-in to any chromosomal locus of choice [36]. During the past few years, a number of zebrafish models of cardiovascular disease has been established using this method, such as inherited cardiomyopathy and congenital heart defects [42,43].

4.3 Conservation of Cardiovascular Development.

As a vertebrate model, zebrafish share well-conserved genetic pathways that govern cardiovascular development similarly as in humans, which is not as straightforward for invertebrates, such as fruit flies, worms and yeast. The zebrafish heart is the first organ to function, developing rapidly starting around 5 hpf, and fully formed by 48 hpf, compared with 12dpf in the mouse and 35dpf in the human embryo [44]. The zebrafish heart is two-chambered, resembling that of a human embryo at 3 weeks gestation [45]. Despite the apparent morphological differences, owing mostly to the lack of a pulmonary circulation, the highly conserved nature of zebrafish and human hearts at anatomical, cellular and membrane-biology levels make it a powerful model for studying cardiac development and related diseases. Interestingly, many human cardiovascular drugs have shown identical effects in zebrafish, and several human cardiovascular disorders have been recapitulated in zebrafish models [46]. Compared to rodents, the electrophysiological properties of human cardiomyocytes are more similar to the zebrafish, suggesting higher relevance to human cardiovascular physiology [47-49]. The hematopoietic system and processes in zebrafish are

also highly conserved from humans, and drugs affecting hematopoiesis and anemia in humans have similar effects in zebrafish [50,51]. Finally, vascular development is also conserved between zebrafish and higher vertebrates, which has enabled the discovery of new modulators of angiogenesis using zebrafish as a model [52-54].

Multiple drug toxicities, including repolarization cardiotoxicity, are conserved between zebrafish and human. For example, during a screen for the potential toxic effects of small molecules on zebrafish heart rate, 22 of the 23 drugs tested exhibited bradycardia and atrioventricular block effects in zebrafish embryos, which were consistent with the repolarization abnormalities, such as QT prolongation, observed in humans. Classical drug-drug interactions between cimetidine and terfenadine, as well as cisapride and erythromycin, were also reproduced [55]. Similarly, some anti-neoplastic drugs such as doxorubicin with specific effects on human cardiac function, ranging from asymptomatic electrocardiographic changes to pericarditis and decompensated cardiomyopathy, consistently recapitulate these effects in zebrafish [56]. Compounds discovered via zebrafish screening have conserved responses in corresponding rodent disease models. Eight drug candidates identified in separate screens produced the expected effects/toxicities in rodents in follow-up studies [57-63], providing strong evidence that the conservation of pharmacological/toxicological effects between zebrafish and mammals is high for the majority of drugs.

5. Recent Screens in Zebrafish and Examples of Success.

The development of phenotype-based screening highlighted the benefits of using the

zebrafish model for many complex phenotypes inaccessible in other screenable models. Over the past few years, more than 40 small-molecule screens in zebrafish have been published. The phenotypes probed varied widely, including embryogenesis, cardiac function, cardiotoxicity, cell migration, cell proliferation, lipid absorption, regeneration, angiogenesis, cancer and behavior [64,65]. Several of these screens have been related to cardiovascular disease and these are discussed to illustrate some of the successful strategies for drug discovery in this organism.

A number of zebrafish phenotypic screens have targeted cardiovascular diseases, including cardiomyopathy, heart failure, long QT syndrome, aortic coarctation, angiogenesis and cerebral cavernous malformations (Table 1) [34, 61, 62, 66-76]. The key concepts of such phenotypic screens are illustrated in a flowchart in Figure 2. Using genetic modification or drug challenge, primary disease characteristics, such as reduced cardiac contraction or other molecular and cellular homeostatic responses, can be recapitulated in zebrafish. Taking advantage of the feasibility of maintaining and manipulating zebrafish embryos or larvae in 96 or 384 multi-well plates, a phenotypic screen based on automated video/image capture and analysis or visual assessment can be undertaken to identify new lead compounds. The typical cardiovascular parameters such as cardiac output, heart rate, blood flow or vascular morphology can readily be quantified (Figure 2, right). Multiple zebrafish screens have identified repurposing opportunities for existing drugs, whereas others have discovered novel therapeutic compound classes (Figure 3).

5.1 Cardiomyopathy.

Despite our improved understanding of the pathophysiology of cardiac disorders like

hypertrophic cardiomyopathy and arrhythmogenic cardiomyopathy, it is still a great challenge to find novel modifiers of these disease phenotypes. The cardiac natriuretic peptide genes (*nppa* and *nppb*), which have been shown to be induced in the heart of embryonic zebrafish by pathological cardiac stimuli, are promising markers of cardiomyocyte hypertrophy and heart failure [34,77]. The transgenic zebrafish reporter line *Tg*(*nppb:F-luc)* faithfully recapitulated the expression profile of the *nppb* gene, allowing for a quantifiable read-out of pathological induction of this marker. The application of this line in a focused screen of a model for hypertrophic cardiomyopathy successfully identified two compounds, Trichostatin A (TSA, a histone deacetylase (HDAC) inhibitor) and U0126 (a mitogen-activated protein kinase kinase (MEK) inhibitor), which could normalize *nppb* induction [34].

In another study, a zebrafish model for arrhythmogenic cardiomyopathy (ACM) was generated by transgenic cardiac myocyte–specific expression of the human plakoglobin gene carrying the pathogenic 2057del2 mutation [62]. Crossing the *Tg(nppb:F-luc)* reporter line into this model enabled high throughput screening of a library of bioactive compounds, which identified three hits that suppressed the disease phenotype. One suppressor, SB216763, previously annotated as an activator of canonical Wnt signaling, with the largest body of extant data, was selected for priority follow-up validation. Early SB216763 therapy could reduce *nppb* levels, prevent bradycardia and contractility defects and reduce mortality in the fish model. The mutant plakoglobin-induced reductions in I_{Na} and I_{K1} current densities were also normalized in zebrafish ventricular myocytes treated with SB216763. In addition, this phenomenon was also observed in an *in vitro* neonatal rat ventricular myocyte model overexpressing the mutant 2057del2 plakoglobin [62]. Follow-up studies in mice showed that

316 the SB216763 compound rescued myocyte injury and cardiac function in two different
317 models of ACM, validating the *in vivo* efficacy of this lead compound in a mammalian system
318 [78]. Importantly, the discovery of SB216763 and subsequent experiments with this
319 compound in different animal models has revealed novel mechanistic pathways responsible
320 for the clinical phenotype of ACM [62,78]. Taken together, these results highlight the utility
321 of zebrafish models for efficient screening of chemical and genetic modifiers of different
322 types of cardiomyopathy.

323 *5.2 Arrhythmic heart disorders.*

324 Zebrafish have been very useful as an excellent animal model to study human disorders
325 related to cardiac arrhythmia [79]. Our initial studies confirmed that drugs causing
326 electrocardiographic QT interval prolongation in humans, a common and serious
327 toxicological issue in drug development, have similar effects in zebrafish [55]. This study
328 hinted to a strong concordance between zebrafish and human cardiac electrophysiology,
329 which was further supported by the discovery of several zebrafish mutations affecting cardiac
330 rhythmicity. The zebrafish mutant strains *breakdance* (*bre*) and *reggae* (*reg*) [80], which
331 demonstrate 2:1 atrioventricular block and cardiac fibrillation respectively, were both found
332 to affect the zebrafish ortholog of the *ether-à-go-go*-related gene (*zERG*). The *bre* missense
333 mutation decreases the activity of the channel responsible for the rapid delayed rectifier K^+
334 current (I_{Kr}), leading to slower cardiac repolarization [81], while the *reg* mutation has a
335 gain-of-function effect, resulting in premature I_{Kr} channel activation and faster repolarization
336 [82]. As such, the *bre* and *reg* zebrafish mutants represented the first *in vivo* models for long
337 and short QT syndrome, respectively. Studies on additional *zERG* mutants [83] confirmed the

relevance of this gene, while positional cloning of the bradycardic zebrafish *hiphop* mutant revealed a mutation in the *atp1a1a.1* gene [84], the ortholog of the human Na^+-K^+-ATPase for which a SNP has been associated with long QT syndrome in several genome-wide association studies.

Of note, the *zERG* gene mutated in *bre* and *reg* is *kcnh6a*, which is considered to be the functional ortholog of the human *KCNH2*/*hERG* gene, one of the most frequently affected genes in patients diagnosed with long or short QT syndrome [85]. The channel encoded by this gene is also of great importance from a drug development perspective, since it is sensitive to inhibition by different classes of small molecules. Drug-induced QT prolongation has been the major reason for the withdrawal or restriction of drugs that had already been marketed [86], leading to the FDA recommendation to test all new chemical entities developed for human use for their potential to affect QT duration. Considering the functional orthology with human electrophysiology, the zebrafish represents an attractive model organism for early preclinical high-throughput *in vivo* testing of the electrophysiological profile of small molecules [81,87,88].

In order to discover new genetic determinants modulating cardiac repolarization, we screened a genetic library to identify zebrafish mutant embryos that were sensitized or resistant to the 2:1 atrioventricular block which is uniformly induced in wild-type controls after exposure to the KCNH2/hERG inhibitor dofetilide. Using an automated assay to measure heart rate [48], we discovered a network of 15 genes modulating repolarization, of which one gene (*GINS3*) was also found to be associated with QT variation in humans [89]. A subsequent small molecule screen identified two compounds that reproducibly rescued the 2:1

atrioventricular block in *bre* mutant zebrafish embryos. One of these compounds functions via the glucocorticoid signaling pathway, representing a new potential therapeutic option to treat long QT syndrome [71].

Another screen was designed to further dissect the regulatory mechanisms involved in cardiac Ca^{2+} handling and its effects on cardiac rhythmicity. A library of synthetic compounds was tested for their ability to rescue the irregular, fibrillation-like cardiac rhythm phenotype in zebrafish *tremblor* (*tre*) mutant embryos, which carry a mutation in the *slc8a1a* gene coding for the cardiac-specific Na^{+}-Ca^{2+}-exchanger 1 (NCX1h) [90]. The compound efsevin, which binds to VDAC2 and potentiates mitochondrial Ca^{2+} uptake from intracellular stores, was able to restore coordinated contraction in *tre* embryos [74]. This finding suggests that mitochondrial Ca^{2+} uptake can limit Ca^{2+} overload and might therefore represent a new therapeutic target to treat cardiac arrhythmia associated with Ca^{2+} handling disorders.

5.3 Angiogenesis.

Pathologic angiogenesis has emerged as an important therapeutic target in several major diseases, including atherosclerosis, autoimmune diseases, age-related macular degeneration, and cancer [68,91]. A quantitative, automated assay using transgenic zebrafish with fluorescent blood vessels was developed to identify antiangiogenic activities. This assay was designed to automatically administer drugs and collect images of zebrafish in 384-well plates, followed by custom algorithm-based image analysis to quantify the number of blood vessels as a read-out for angiogenesis [68]. A screen of 1280 small molecules with this assay successfully identified three hit compounds, which included two well-known antiangiogenic compounds, SU4312 (a vascular endothelial growth factor (VEGF) receptor and platelet

382 derived growth factor receptor antagonist) and AG1478 (an epidermal growth factor receptor
383 antagonist), and one previously unknown antiangiogenic compound, indirubin-3'-monoxime
384 (IRO). Each of these compounds had dose-dependent antiangiogenic activity in zebrafish and
385 IRO displayed the highest potency among them [68].

386 Using a similar strategy, the *Tg(kdrl:EGFP)* line, which expresses GFP specifically in
387 endothelial cells, was also employed in a library screen with 2000 small molecules for
388 angiogenic inhibitors [70]. Seven hit compounds were identified that could inhibit the growth
389 of the zebrafish intersegmental vessels, which could be classified into three groups: rotenoids,
390 aristolochic acid, and statins. Among these, rosuvastatin was further demonstrated to decrease
391 the viability, inhibit the migration, and dose-dependently inhibit the capillary-like tube
392 formation *in vitro* in human umbilical endothelial cells (HUVEC). In addition, it also
393 significantly suppressed prostate cancer growth in a mouse xenograft tumor model by
394 decreasing the tumor microvessel density and causing tumor cell apoptosis. These results
395 offered initial evidence of a potential therapeutic use of rosuvastatin in the treatment of
396 human prostate cancer [70].

397 *5.4 Aortic coarctation.*

398 The zebrafish gridlock (*grl*) mutation prevents caudal aortic blood flow in a region and
399 physiological manner akin to aortic coarctation in humans [66]. In a previous study, we
400 arrayed mutant embryos in 96-well plates and exposed them to small molecules from a
401 structurally diverse chemical library to look for hits capable of restoring circulation to the tail.
402 Of the 5000 molecules tested, a novel class of compounds that were not previously known to
403 influence vasculogenesis or angiogenesis, as represented by GS4012, was identified to

suppress the disease phenotype in a dose-dependent manner. It was postulated to function via activation of the VEGF signaling pathway during the specification and migration of angioblasts [66].

In a subsequent study, a larger screen was performed using a similar approach, and identified a distinct compound class that was also capable of suppressing the gridlock phenotype. A representative compound GS4898, (structurally distinct from GS4012) is a flavone that likely acts through AKT inhibition [67].

These two classes of compounds identified by unbiased mutant zebrafish screening have been valuable tools for studying artery/vein specification. They confirmed the importance of VEGF signaling in the disease process and revealed that the two downstream components of VEGF signaling surprisingly have opposite effects on artery/vein specification of endothelial progenitor cells [67]. ERK signaling promotes the arterial cell fate, whereas PI3K has an opposing effect by blocking ERK activation [67]. Thus, phenotype-based screens allowed the discovery of small molecules that ameliorate complex vascular phenotypes in zebrafish embryos without targeting the causal gene directly.

6. Conclusion.

Identification of novel and highly specific therapeutics tailored to individual needs is one of the major challenges in modern cardiovascular medicine. Even the best available cardiovascular models do not perfectly mimic human biology. This knowledge gap is a central issue in all drug discoveries. To decipher the precise disease mechanisms, merely focusing on a list of druggable targets is largely insufficient. Even where a specific molecular cause has

been identified, it is often not feasible to progress directly to a viable therapeutic discovery strategy due to the limited understanding of the downstream pathophysiology. For example, in cardiac arrhythmia disorders, drug discovery has focused almost exclusively on modulating specific transmembrane conductance identified through human genetics, while the potential of targeting emerging regulators of cellular excitability has largely been ignored [88]. To fully understand disease manifestation and progression, and improve novel drug discovery efforts, we need to capture a comprehensive picture of the underlying biology, including the complex interconnections of molecular and cellular contributions in different cell types or tissues.

Zebrafish is emerging as an excellent model to explore the genetic and molecular etiology of diseases, perform highly efficient drug discovery and discover novel disease mechanisms and therapeutic targets. By use of forward and reverse genetics approaches, numerous cardiovascular disease models have already been established [80]. As outlined in this review, an increasing number of studies have taken advantage of the tractability of these zebrafish models to expedite *in vivo* drug discovery efforts. In many cases, these studies succeeded in identifying novel therapeutic targets or shedding light on previously incompletely understood disease mechanisms.

7. Expert Opinion

7.1 Key achievements to date.

Since the use of *in vivo* zebrafish screens is growing, the question arises whether this approach has a real impact on drug discovery and further development leading to approval for clinical use. Although the field is still relatively young, several successful screening programs

have already led to the identification of new compound classes and repurposed drugs that have started to make the transition to the clinic. An example in the cardiovascular field comes from the identification of the glucocorticoid receptor as a pathway involved in the modulation of cardiac repolarization [71], which has led to the testing of the effects of cortisone on QT interval in patients [92]. Interestingly, the glucocorticoid dexamethasone was confirmed to show efficacy for the suppression of drug-induced long QT syndrome in a case report [93].

Zebrafish screens have also been successful in other medical fields. Leflunomide, a drug previously approved for the treatment of rheumatoid arthritis, was identified as a suppressor of the neural crest lineage and melanoma growth [94]. Although a phase I/II clinical trial aimed at repurposing this drug for melanoma treatment was terminated, new clinical trials are being planned to evaluate its effects in breast cancer and myeloma. In a zebrafish screen for suppressors of antibiotic-induced ototoxicity, a new class of small molecules was identified [95]. After lead optimization through a medicinal chemistry approach, the compound ORC-13661 was developed [96], which has recently received Investigational New Drug approval from the FDA and is currently being tested in a phase I clinical trial.

Many more zebrafish drug screens and follow-up validations of initial hits are currently underway, which will undoubtedly lead to more drugs identified through zebrafish research making their way to market in the future.

7.2 Remaining challenges.

Despite the advantages of the zebrafish model highlighted in the review, several key limitations are evident that call for further creative solutions. Perhaps the most important concern is the limit of our knowledge to create faithful zebrafish models for specific human

diseases. While the understanding of human diseases at anatomical, cellular and molecular biology levels has increased dramatically, our ability to map these variables to relevant zebrafish models has lagged. More investigation is still required to better appreciate the level of evolutionary conservation of different organ systems between zebrafish and mammalian species. Although zebrafish correlates are obviously lacking for several mammal-specific tissue types such as lungs and placenta, precluding direct comparisons to diseases affecting these organs, relevant biology can sometimes still be studied in related organ systems [97]. Similarly, proxy phenotypes can often be used as a screenable readout even if the zebrafish phenotype does not completely mirror the human defect. A successful example is the ACM model, which used embryonic *nppb* expression as an automatable readout for the cardiac defects caused by mutant plakoglobin overexpression [62].

Another important consideration is the teleost-specific whole genome duplication event. Although most duplicates have been lost during evolution, about 20-30% of human genes still have two zebrafish co-orthologs. In many cases this has resulted in gene sub-functionalization and neo-functionalization of the duplicated zebrafish isoforms [98,99]. Targeted genetic manipulations have recently generated a number of promising zebrafish disease models, although they have been largely restricted to monogenic disorders [43,62]. Modeling complex, multifactorial, and chronic disease processes, such as diabetes, hypertension and rheumatoid arthritis, is still difficult, particularly when aiming to achieve quantifiable readouts during the early stages of zebrafish development, when the organism is amenable to high-throughput screening. Another area that requires systematic approaches is the penetration of chemical compounds into the fish. Drug pharmacokinetics are difficult to measure in zebrafish, and in

many cases, compounds lacking activity in an *in vivo* zebrafish assay may not have the proper absorption, distribution, metabolism and/or excretion characteristics to achieve sufficient tissue exposure levels. An important factor is the ability of the molecule to cross the biological barrier of larval zebrafish skin, which is known to be a function of the specific physicochemical properties of the compound [14,100]. Drugs like cisapride and chlorpromazine are concentrated within the fish larvae (up to 1380% for chlorpromazine for 3 hours exposure), while others such as aspirin and amoxicillin fail to reach 0.03% of the external concentrations, or may not be detectable [101]. Thus, *in vivo* drug penetration predictions taking into account cutoff molecular weight, log P partition coefficient, and/or polar surface area, might have to be performed to complement zebrafish screening. It should be noted that the penetration properties may also indirectly reflect the utility of the molecule as a drug. As a general rule, compounds compliant with the Lipinski rules have the highest likelihood of showing both a favorable bioavailability profile for zebrafish exposure as well as having reasonable drug-like properties. In a zebrafish high-throughput screen designed to discover new cyanide countermeasures, we have tested over 140,000 compounds, leading to the discovery of three distinct classes of potential novel antidotes: metal-based chelators, flavin derivatives, and metabolic modulators [102-104]. The majority of these compounds showed efficacy in rodent models, which was improved after lead optimization via medicinal chemistry. These results suggest that candidates identified in zebrafish screens are likely to represent drug classes that have suitable pharmacokinetic properties for successful translation to further preclinical studies.

A third issue is that target identification is still required *post-hoc* for most hits identified

by *in vivo* screening. Unlike target-based screening, phenotypic screens in zebrafish allow small-molecule action to be tested in a more disease-relevant setting at the outset, but they require follow-up studies to discover the precise molecular targets responsible for the observed phenotypes. Target identification can be achieved by multidisciplinary strategies, such as direct biochemical methods, genetic manipulation or computational inferences. In many cases, combinations of approaches may be needed [105]. This strategy has been proven to be a promising approach to fully characterize on-target and off-target effects of the lead molecules identified by zebrafish screening and to understand their mechanisms of action [63,67-74].

7.3 Future perspectives of zebrafish-based drug discovery.

It can be envisioned that the zebrafish model will serve as an invaluable first-line screening tool in the pre-clinical phase of the drug pipeline, which will reduce the amount of higher vertebrates, mostly rodents, used in early pre-clinical research. Nevertheless, the use of mammalian models, like rodents and primates, is essential to fully understand the efficacy and pharmacokinetic properties of lead compounds and avoid any possible toxicity, and is required to obtain regulatory approval. Ultimately, identifying the precise role of different models in drug discovery for different diseases should increase the efficiency of the entire process.

The continuing further refinement of the already widely adopted CRISPR/Cas9 system, which allows for highly efficient, specific, and permanent manipulations of the zebrafish genome, provides exciting possibilities for more individualized disease modeling [106,107] beyond conventional gene knockdown or knockout strategies. Particularly the possibility to

generate specific point mutations in the genome using the CRISPR/Cas-based “base editing” technology is very promising [108]. This technology as well as other emerging genome editing tools will enable precise modeling of specific genetic variants identified in patients, paving the way for personalized drug discovery.

Taken together, individual, tailored therapies to treat cardiovascular diseases will become attainable through further technological improvements in the near future. Though a complete understanding and widespread application of zebrafish as an integral component of drug discovery platforms will still need time, the identification of new molecular entities that make it to market will pave the way for a wider incorporation of zebrafish technology into drug discovery.

Declaration of Interest

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*** This paper demonstrates the possibilities of single-base editing in zebrafish.**

818 **Table 1.** Chemical screens in zebrafish for cardiovascular drug discovery.

Screening Type	Readout	Major findings	Year	Refs
Aortic coarctation	Blood circulation in the aorta	Two hits (suppressors) targeted on VEGF expression; activation of VEGF pathway is sufficient to suppress the gridlock phenotype	2004	66
Aortic coarctation	Blood circulation in the aorta	Two compound classes were identified, targeted on VEGF pathway; uncovered opposing roles of PI3K and ERK in artery/vein specification	2006	67
Angiogenesis	Vascular morphology	Three hits with antiangiogenic activity	2007	68
Angiogenesis	Vascular morphology	One hit as PI3 kinase inhibitor	2009	69
Angiogenesis	Vascular morphology	Seven hits (represented by Rosuvastatin) with antiangiogenic activity	2010	70
Long QT syndrome	Atrioventricular heart rhythm	Two suppressors of atrioventricular block, one targeted on glucocorticoid receptor–mediated pathway	2011	71
Cardiomyopathy	Natriuretic peptide reporter	Two alleviators of the disease phenotype	2012	34
Heart failure	Heart morphology	Three compound classes with distinct targets	2013	72
Cardiomyopathy	Natriuretic peptide reporter	One suppressor of the disease phenotype; aberrant trafficking of intercalated disc proteins as a central mechanism	2014	62
Cardiomyopathy	Rescue of cardiac function	Two suppressors of the disease phenotype; MDH2 is a new druggable target	2014	61
Heart failure	Heart morphology	Several hit compounds (represented by AF-001)	2014	73
Cardiac rhythmicity	Cardiac contraction	One suppressor of the cardiac fibrillation; uncovered the critical role of VDAC2-dependent mitochondrial Ca2+ uptake	2015	74
Angiogenesis	Vascular morphology	One hit targeted on cysteinyl leukotriene receptors	2016	75
Cerebral cavernous malformations	Endothelial-specific reporter and heart morphology	One alleviator of CCM; uncovered several novel related pathways	2018	76

Figure 1. Comparison of drug discovery for cardiovascular and other diseases. (A) The percentage of total number of drugs undergoing early-phase development between two separate intervals (2000-2007 vs. 1990-1999). (B) The first-cycle approval rate of new molecular entities for each medical specialty from 2000 to 2012. Raw data is from Pammolli et al. [9] and Sacks et al. [10].

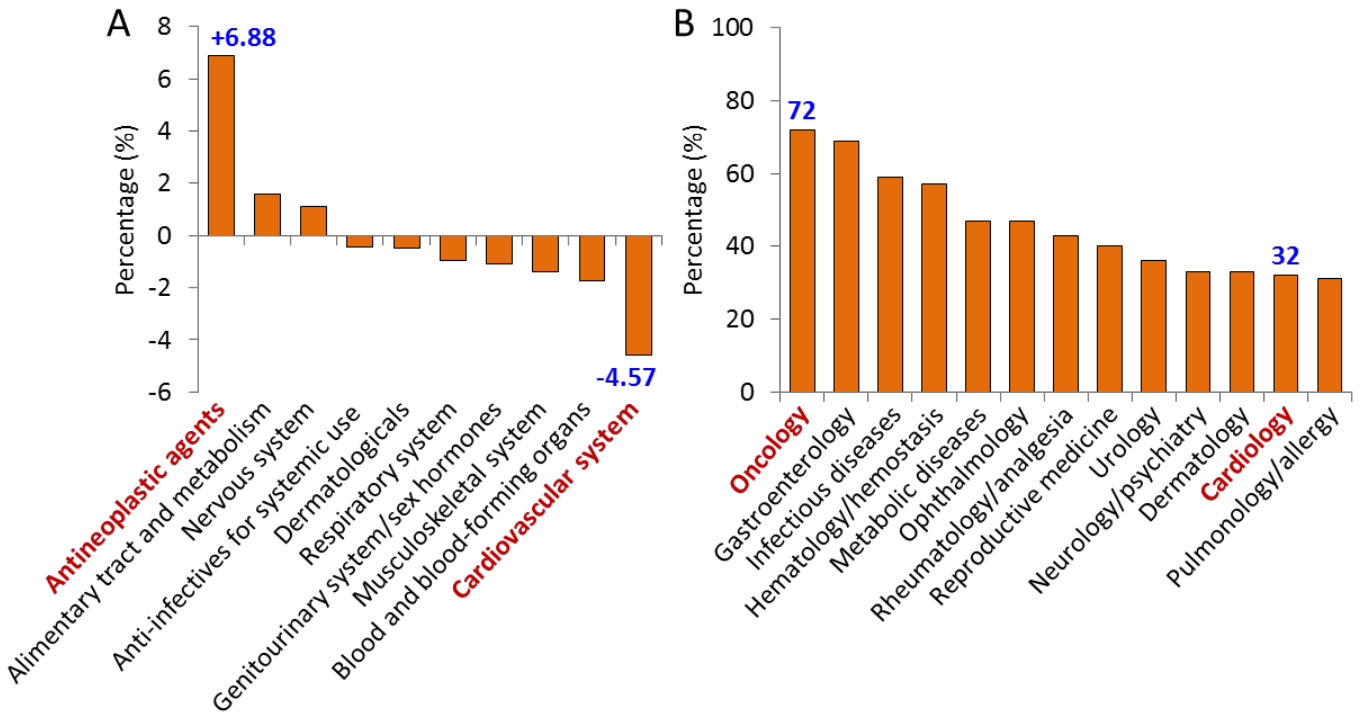

Figure 2. The flow chart of *in vivo* chemical screening in zebrafish cardiovascular disease models. Multiple phenotypes can be quantified via the established auto Video/Image analysis approaches, as highlighted on the right. a.u.: arbitrary units.

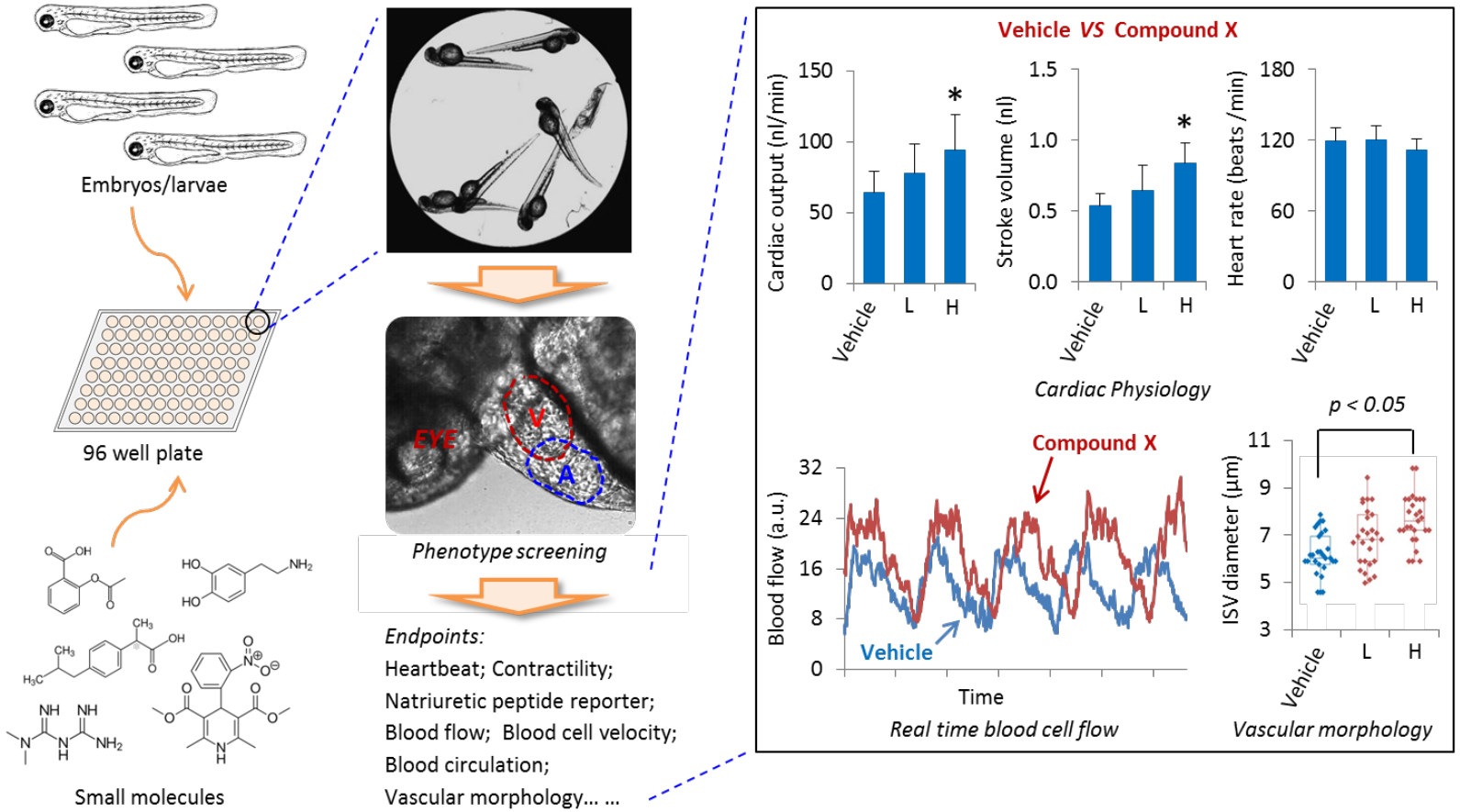

Figure 3. Representative hit compounds discovered by *in vivo* screens in zebrafish. Structure information was obtained from Ref. 34, Ref. 61, Ref. 62, Ref. 66, Ref. 68, Ref. 69, Ref. 70, Ref. 71, Ref. 72, Ref. 73, Ref. 75, and Ref. 76.