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High-Throughput in Vivo Screening for Cardiovascular Drug Discovery

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Expert Opinion on Drug Discovery, 14 (4), 343-353, 2019.

To refer to or to cite this work, please use the citation to the published version:

Zhao Y, Zhang K, Sips P, and MacRae CA (2019). High-Throughput in Vivo Screening for Cardiovascular Drug Discovery. *Expert Opin Drug Discov 14(4)* 343-353. doi: 10.1080/17460441.2019.1577815

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19 Abstract

20 **Introduction:** Our understanding of the complexity of cardiovascular disease 21 pathophysiology remains very incomplete and has hampered cardiovascular drug 22 development over recent decades. The prevalence of cardiovascular diseases and their 23 increasing global burden call for novel strategies to address disease biology and drug 24 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.

2

39 Article Highlights

40 • Modern cardiovascular drug discovery has lagged recently due to the lack of
41 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.

52 **1. Introduction**

53 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 54 55 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 56 some 31% of all global deaths [4-6]. The global burden of CVD was estimated over US\$800 57 billion in direct healthcare costs and productivity losses worldwide in 2010 alone and these 58 costs are projected to reach US\$20 trillion by 2030 [7]. Thus, the burden of cardiovascular 59 disease is both a major public health concern and a growing global challenge [8]. 60

61 Notwithstanding the increasing global cardiovascular disease burden, investment in cardiovascular drug development has stagnated and the approvals for new cardiovascular drug 62 therapies have declined substantially [9–10]. The application of genomic technologies and 63 64 systems biology approaches has identified multiple potential new cardiovascular drug targets, as well as novel molecules with potential cardiovascular applications. However, these 65 scientific advances have not stimulated an increase in drug development for CVD. Fewer drug 66 candidates were found in the cardiovascular research pipeline compared with other 67 therapeutic areas [9]. For instance, as shown in Figure 1, the antineoplastic agents undergoing 68 early-phase development grew +6.9% between two time intervals (1990-1999 vs. 2000-2007), 69 70 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 71 2000-2012) and first-cycle FDA approvals (72%) of oncology drugs, cardiovascular drugs 72 had significantly fewer applications (n=21) and a much lower rate of first-cycle approvals 73

74 (32%) [10].

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

The advancement of systems biology approaches, such as genome editing, functional genomics and computational modeling, is currently accelerating the understanding of disease biology and the exploration of druggable targets. In the meantime, a resurgence of interest in phenotype-based screening for drug discovery such as zebrafish-based *in vivo* screening has also occurred, taking advantage of the tractability of the zebrafish model and of human induced pluripotent stem cell modeling. To date, few drugs have made it to the clinic from zebrafish *in vivo* screening, but several examples are imminent. The combination of these 96 strategies would be expected to bring new enthusiasm and investment, while paving the way
97 for more efficient cardiovascular drug discovery in the near future.

98

99 **2.** Target-based screening in the face of complexity

Target-based approaches are designed to identify biologically active small molecules 100 101 based on systematic, repetitive, and quantitative investigation of their effects on a therapeutic 102 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 103 robust link is identified between a specific human gene and a disease signature, molecules 104 105 that target this gene product are strong candidates to succeed as potential therapeutic drugs. 106 Target-based drug discovery usually relies on a tightly controlled in vitro screening approach, 107 which drives less expensive and less time-consuming experiments compared to whole 108 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 109 molecules, which is often not feasible with the whole organism-based models [12,13]. 110

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 123 parameters quantitating compound absorption, distribution, metabolism and excretion 124 (ADME), is a determining factor for the in vivo efficacy of drug candidates. In whole 125 vertebrate models, such as the zebrafish, the effects of individual human drugs are typically 126 representative of human complexity, including most known drug-drug interactions. In 127 addition, there is increasing evidence that drug distribution across active physiological 128 boundaries such as the blood-brain barrier can also be faithfully observed in animal models 129 130 including the zebrafish [17], while these pharmacokinetic characteristics by definition cannot be established in target-based in vitro drug discovery efforts. 131

132

133 **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 145 examples above, cost and lack of scalability led to the dominance of target-based methods 146 during the last few decades. Currently, there is a resurgence of interest in phenotype-based 147 screening because of the growing number of early discovery successes from such efforts, such 148 149 as the usage of caenorhabditis elegans, drosophila melanogaster and zebrafish disease models. 150 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 151 represented only a small subset of drug discovery efforts [22]. 152

Several benefits may contribute to the success of phenotype-based approaches. First, 153 phenotype-based drug discovery can identify chemical modifiers of virtually any biological 154 process while target-based approaches typically can only discover modifiers of a specific 155 target and then usually in a cell autonomous context. Therefore, phenotype-based approaches 156 provide an opportunity to reveal novel targets and their functions and to obtain fundamental 157 insights into poorly understood biological processes, in contrast to target-based approaches 158 where a prior biological understanding is a prerequisite. Without robust criteria for causation, 159 relevance of any target to a specific disease may be ephemeral. Second, phenotype-based 160 discovery can identify chemical modifiers that produce a therapeutic effect through 161

162 simultaneous activity at multiple targets. For instance, the antiarrhythmic agent, amiodarone, discovered through serendipitous observation, exhibits activities with multiple molecular 163 mechanisms, like actions on ion channels, adrenergic receptors, and possibly via binding to 164 165 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 166 unsuccessful. One possible reason for this discrepancy might relate to the incompletely 167 understood roles of the various types of ion channels [15]. Third, phenotype-based approaches 168 can identify chemical modifiers in the context of a whole organism, which can discover 169 modifiers with acceptable pharmacokinetic/pharmacodynamic profile and parse out chemicals 170 171 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 172 models for effectiveness, side effects, toxicity, and pharmacokinetic profile compared to 173 174 compounds identified in target-based screens.

175

176 **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.

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

In the past two decades, rapid development of knowledge and technologies have greatly 207 increased the utility of zebrafish as a screenable vertebrate model. The zebrafish genome has 208 209 been sequenced and annotated [35]. The application of DNA microarrays, whole-embryo in situ hybridization (WISH) and RNA sequencing has accelerated transcriptional studies which 210 are often important in validating disease models. More importantly, gene functions can be 211 212 rapidly and robustly studied in zebrafish by use of specific genetic manipulations, such as morpholinos, RNA interference, transcription activator-like effector nucleases (TALENs), 213 zinc finger nucleases, and in recent years also the CRISPR (Clustered regularly interspaced 214 215 short palindromic repeats)-Cas (CRISPR -associated protein) system [36,37]. Morpholinos 216 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 217 218 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 219 the functions of genes without an available mutant allele. Despite concerns regarding the 220 specificity of morpholino effects, larger scale assessments have confirmed biological 221 relevance, albeit requiring careful validation. In addition, the dose-dependent effect of 222 morpholinos on the level of gene knockdown allows for the investigation of intermediate 223 224 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 225 complementary to the genomic target sequence of interest (either produced by the cell after 226 DNA/mRNA transfection or injected directly as a Cas9 protein / guide RNA complex), the 227

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].

235 4.3 Conservation of Cardiovascular Development.

As a vertebrate model, zebrafish share well-conserved genetic pathways that govern 236 cardiovascular development similarly as in humans, which is not as straightforward for 237 invertebrates, such as fruit flies, worms and yeast. The zebrafish heart is the first organ to 238 function, developing rapidly starting around 5 hpf, and fully formed by 48 hpf, compared with 239 12dpf in the mouse and 35dpf in the human embryo [44]. The zebrafish heart is 240 two-chambered, resembling that of a human embryo at 3 weeks gestation [45]. Despite the 241 apparent morphological differences, owing mostly to the lack of a pulmonary circulation, the 242 highly conserved nature of zebrafish and human hearts at anatomical, cellular and 243 membrane-biology levels make it a powerful model for studying cardiac development and 244 related diseases. Interestingly, many human cardiovascular drugs have shown identical effects 245 246 in zebrafish, and several human cardiovascular disorders have been recapitulated in zebrafish models [46]. Compared to rodents, the electrophysiological properties of human 247 cardiomyocytes are more similar to the zebrafish, suggesting higher relevance to human 248 cardiovascular physiology [47-49]. The hematopoietic system and processes in zebrafish are 249

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 254 zebrafish and human. For example, during a screen for the potential toxic effects of small 255 molecules on zebrafish heart rate, 22 of the 23 drugs tested exhibited bradycardia and 256 atrioventricular block effects in zebrafish embryos, which were consistent with the 257 repolarization abnormalities, such as QT prolongation, observed in humans. Classical 258 drug-drug interactions between cimetidine and terfenadine, as well as cisapride and 259 erythromycin, were also reproduced [55]. Similarly, some anti-neoplastic drugs such as 260 doxorubicin with specific effects on human cardiac function, ranging from asymptomatic 261 electrocardiographic changes to pericarditis and decompensated cardiomyopathy, consistently 262 recapitulate these effects in zebrafish [56]. Compounds discovered via zebrafish screening 263 have conserved responses in corresponding rodent disease models. Eight drug candidates 264 identified in separate screens produced the expected effects/toxicities in rodents in follow-up 265 studies providing evidence [57-63], strong that the conservation of 266 pharmacological/toxicological effects between zebrafish and mammals is high for the 267 majority of drugs. 268

269

270 5. Recent Screens in Zebrafish and Examples of Success.

271

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

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

A number of zebrafish phenotypic screens have targeted cardiovascular diseases, 279 including cardiomyopathy, heart failure, long QT syndrome, aortic coarctation, angiogenesis 280 and cerebral cavernous malformations (Table 1) [34, 61, 62, 66-76]. The key concepts of such 281 phenotypic screens are illustrated in a flowchart in Figure 2. Using genetic modification or 282 drug challenge, primary disease characteristics, such as reduced cardiac contraction or other 283 molecular and cellular homeostatic responses, can be recapitulated in zebrafish. Taking 284 advantage of the feasibility of maintaining and manipulating zebrafish embryos or larvae in 285 96 or 384 multi-well plates, a phenotypic screen based on automated video/image capture and 286 287 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 288 morphology can readily be quantified (Figure 2, right). Multiple zebrafish screens have 289 290 identified repurposing opportunities for existing drugs, whereas others have discovered novel therapeutic compound classes (Figure 3). 291

292 5.1 Cardiomyopathy.

293

Despite our improved understanding of the pathophysiology of cardiac disorders like

294 hypertrophic cardiomyopathy and arrhythmogenic cardiomyopathy, it is still a great challenge to find novel modifiers of these disease phenotypes. The cardiac natriuretic peptide genes 295 (nppa and nppb), which have been shown to be induced in the heart of embryonic zebrafish 296 297 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 298 recapitulated the expression profile of the *nppb* gene, allowing for a quantifiable read-out of 299 300 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 301 A (TSA, a histone deacetylase (HDAC) inhibitor) and U0126 (a mitogen-activated protein 302 303 kinase kinase (MEK) inhibitor), which could normalize *nppb* induction [34].

304 In another study, a zebrafish model for arrhythmogenic cardiomyopathy (ACM) was generated by transgenic cardiac myocyte-specific expression of the human plakoglobin gene 305 306 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 307 identified three hits that suppressed the disease phenotype. One suppressor, SB216763, 308 previously annotated as an activator of canonical Wnt signaling, with the largest body of 309 extant data, was selected for priority follow-up validation. Early SB216763 therapy could 310 reduce nppb levels, prevent bradycardia and contractility defects and reduce mortality in the 311 312 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 313 phenomenon was also observed in an in vitro neonatal rat ventricular myocyte model 314 overexpressing the mutant 2057del2 plakoglobin [62]. Follow-up studies in mice showed that 315

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

323 *5.2 Arrhythmic heart disorders.*

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

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 342 functional ortholog of the human KCNH2/hERG gene, one of the most frequently affected 343 genes in patients diagnosed with long or short QT syndrome [85]. The channel encoded by 344 this gene is also of great importance from a drug development perspective, since it is sensitive 345 to inhibition by different classes of small molecules. Drug-induced QT prolongation has been 346 the major reason for the withdrawal or restriction of drugs that had already been marketed 347 348 [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 349 350 human electrophysiology, the zebrafish represents an attractive model organism for early preclinical high-throughput in vivo testing of the electrophysiological profile of small 351 molecules [81,87,88]. 352

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].

363 Another screen was designed to further dissect the regulatory mechanisms involved in cardiac Ca²⁺ handling and its effects on cardiac rhythmicity. A library of synthetic compounds 364 was tested for their ability to rescue the irregular, fibrillation-like cardiac rhythm phenotype in 365 zebrafish tremblor (tre) mutant embryos, which carry a mutation in the slc8a1a gene coding 366 for the cardiac-specific Na⁺-Ca²⁺-exchanger 1 (NCX1h) [90]. The compound efsevin, which 367 binds to VDAC2 and potentiates mitochondrial Ca²⁺ uptake from intracellular stores, was able 368 to restore coordinated contraction in tre embryos [74]. This finding suggests that 369 mitochondrial Ca²⁺ uptake can limit Ca²⁺ overload and might therefore represent a new 370 therapeutic target to treat cardiac arrhythmia associated with Ca²⁺ handling disorders. 371

372 *5.3 Angiogenesis.*

Pathologic angiogenesis has emerged as an important therapeutic target in several major 373 diseases, including atherosclerosis, autoimmune diseases, age-related macular degeneration, 374 and cancer [68,91]. A quantitative, automated assay using transgenic zebrafish with 375 fluorescent blood vessels was developed to identify antiangiogenic activities. This assay was 376 designed to automatically administer drugs and collect images of zebrafish in 384-well plates, 377 378 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 379 successfully identified three hit compounds, which included two well-known antiangiogenic 380 compounds, SU4312 (a vascular endothelial growth factor (VEGF) receptor and platelet 381

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

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

397 5.4 Aortic coarctation.

The zebrafish gridlock (*grl*) mutation prevents caudal aortic blood flow in a region and physiological manner akin to aortic coarctation in humans [66]. In a previous study, we arrayed mutant embryos in 96-well plates and exposed them to small molecules from a structurally diverse chemical library to look for hits capable of restoring circulation to the tail. Of the 5000 molecules tested, a novel class of compounds that were not previously known to 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 411 been valuable tools for studying artery/vein specification. They confirmed the importance of 412 413 VEGF signaling in the disease process and revealed that the two downstream components of 414 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 415 416 opposing effect by blocking ERK activation [67]. Thus, phenotype-based screens allowed the discovery of small molecules that ameliorate complex vascular phenotypes in zebrafish 417 embryos without targeting the causal gene directly. 418

419

420 **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

426 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 427 cardiac arrhythmia disorders, drug discovery has focused almost exclusively on modulating 428 429 specific transmembrane conductance identified through human genetics, while the potential of targeting emerging regulators of cellular excitability has largely been ignored [88]. To fully 430 understand disease manifestation and progression, and improve novel drug discovery efforts, 431 432 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. 433

Zebrafish is emerging as an excellent model to explore the genetic and molecular 434 etiology of diseases, perform highly efficient drug discovery and discover novel disease 435 436 mechanisms and therapeutic targets. By use of forward and reverse genetics approaches, numerous cardiovascular disease models have already been established [80]. As outlined in 437 this review, an increasing number of studies have taken advantage of the tractability of these 438 zebrafish models to expedite in vivo drug discovery efforts. In many cases, these studies 439 succeeded in identifying novel therapeutic targets or shedding light on previously 440 441 incompletely understood disease mechanisms.

442

443 **7. Expert Opinion**

444 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 454 previously approved for the treatment of rheumatoid arthritis, was identified as a suppressor 455 of the neural crest lineage and melanoma growth [94]. Although a phase I/II clinical trial 456 457 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 458 suppressors of antibiotic-induced ototoxicity, a new class of small molecules was identified 459 [95]. After lead optimization through a medicinal chemistry approach, the compound 460 ORC-13661 was developed [96], which has recently received Investigational New Drug 461 approval from the FDA and is currently being tested in a phase I clinical trial. 462

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.

466 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

470 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 471 zebrafish models has lagged. More investigation is still required to better appreciate the level 472 473 of evolutionary conservation of different organ systems between zebrafish and mammalian species. Although zebrafish correlates are obviously lacking for several mammal-specific 474 tissue types such as lungs and placenta, precluding direct comparisons to diseases affecting 475 these organs, relevant biology can sometimes still be studied in related organ systems [97]. 476 Similarly, proxy phenotypes can often be used as a screenable readout even if the zebrafish 477 phenotype does not completely mirror the human defect. A successful example is the ACM 478 479 model, which used embryonic *nppb* expression as an automatable readout for the cardiac 480 defects caused by mutant plakoglobin overexpression [62].

Another important consideration is the teleost-specific whole genome duplication event. 481 Although most duplicates have been lost during evolution, about 20-30% of human genes still 482 have two zebrafish co-orthologs. In many cases this has resulted in gene sub-functionalization 483 and neo-functionalization of the duplicated zebrafish isoforms [98,99]. Targeted genetic 484 manipulations have recently generated a number of promising zebrafish disease models, 485 although they have been largely restricted to monogenic disorders [43,62]. Modeling complex, 486 multifactorial, and chronic disease processes, such as diabetes, hypertension and rheumatoid 487 488 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 489 screening. Another area that requires systematic approaches is the penetration of chemical 490 compounds into the fish. Drug pharmacokinetics are difficult to measure in zebrafish, and in 491

492 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 493 tissue exposure levels. An important factor is the ability of the molecule to cross the 494 495 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 496 chlorpromazine are concentrated within the fish larvae (up to 1380% for chlorpromazine for 3 497 hours exposure), while others such as aspirin and amoxicillin fail to reach 0.03% of the 498 external concentrations, or may not be detectable [101]. Thus, in vivo drug penetration 499 predictions taking into account cutoff molecular weight, log P partition coefficient, and/or 500 501 polar surface area, might have to be performed to complement zebrafish screening. It should 502 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 503 504 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 505 discover new cyanide countermeasures, we have tested over 140,000 compounds, leading to 506 the discovery of three distinct classes of potential novel antidotes: metal-based chelators, 507 flavin derivatives, and metabolic modulators [102-104]. The majority of these compounds 508 showed efficacy in rodent models, which was improved after lead optimization via medicinal 509 510 chemistry. These results suggest that candidates identified in zebrafish screens are likely to represent drug classes that have suitable pharmacokinetic properties for successful translation 511 to further preclinical studies. 512



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

514 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 515 require follow-up studies to discover the precise molecular targets responsible for the 516 517 observed phenotypes. Target identification can be achieved by multidisciplinary strategies, such as direct biochemical methods, genetic manipulation or computational inferences. In 518 many cases, combinations of approaches may be needed [105]. This strategy has been proven 519 to be a promising approach to fully characterize on-target and off-target effects of the lead 520 molecules identified by zebrafish screening and to understand their mechanisms of action 521 [63,67-74]. 522

523 *7.3 Future perspectives of zebrafish-based drug discovery.*

It can be envisioned that the zebrafish model will serve as an invaluable first-line 524 screening tool in the pre-clinical phase of the drug pipeline, which will reduce the amount of 525 higher vertebrates, mostly rodents, used in early pre-clinical research. Nevertheless, the use of 526 mammalian models, like rodents and primates, is essential to fully understand the efficacy and 527 pharmacokinetic properties of lead compounds and avoid any possible toxicity, and is 528 required to obtain regulatory approval. Ultimately, identifying the precise role of different 529 models in drug discovery for different diseases should increase the efficiency of the entire 530 process. 531

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.

546

547 **Declaration of Interest**

Yanbin Zhao and Kun Zhang are funded by the Starting Research Fund from the Shanghai Jiao Tong University. Patrick Sips has received funding from the European Union's Horizon 2020 research and innovation programme under the Marie Skłodowska-Curie grant agreement No. 794365. Calum Macrae was supported by the NIH (NHLBI, NINDS and OD), the Burroughs Welcome Fund, the American Heart Association, AstraZeneca and Verily Life Sciences. We thank Aaron Kithcart (Harvard Medical School and Brigham and Women's Hospital, MA) for critically reading the manuscript and making valuable suggestions.

555 The authors state no conflict of interest and have received no payment in preparation of this 556 manuscript.

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558 **References**

- (1) Roth GA, Johnson C, Abajobir A, et al. Global, regional, and national burden of
 cardiovascular diseases for 10 causes, 1990 to 2015. J Am Coll Cardiol 2017;70(1):1-25.
- (2) Benjamin EJ, Virani SS, Callaway CW, et al. Heart disease and stroke statistics-2018
 update: a report from the American Heart Association. Circulation 2018;137(12):e67–492.
- (3) Townsend N, Bhatnagar P, Wilkins E, et al. Cardiovascular disease statistics 2015.
 London: British Heart Foundation, 2015.
- 565 (4) Fuster V. Global burden of cardiovascular disease: time to implement feasible strategies566 and to monitor results. J Am Coll Cardiol 2014;64:520–2.
- 567 (5) Fuster V, Kelly BB, Vedanthan R. Promoting global cardiovascular health: moving
 568 forward. Circulation 2011;123:1671–8.
- (6) Mathers CD, Loncar D. Projections of global mortality and burden of disease from 2002
 to 2030. PLoS Med 2006;3:e442.
- (7) Bloom DE, Cafiero ET, Jane-Llopis E, et al. The global economic burden of
 noncommunicable diseases. Geneva, Switzerland: World Economic Forum, 2013.
- (8) Fordyce CB, Roe MT, Ahmad T, et al. Cardiovascular drug development: is it dead or just
 hibernating? J Am Coll Cardiol. 2015;65(15):1567-82.
- (9) Pammolli F, Magazzini L, Riccaboni M. The productivity crisis in pharmaceutical R&D.
 Nat Rev Drug Discov 2011;10:428[°]C38.
- (10)Sacks LV, Shamsuddin HH, Yasinskaya YI, et al. Scientific and regulatory reasons for
 delay and denial of FDA approval of initial applications for new drugs, 2000–2012.
 JAMA 2014;311:378–84.
- (11)Sayols-Baixeras S, Lluís-Ganella C, Lucas G, et al. Pathogenesis of coronary artery
 disease: focus on genetic risk factors and identification of genetic variants. Appl Clin
 Genet. 2014;7:15-32.
- (12)Swinney DC. Phenotypic vs. target-based drug discovery for first-in-class medicines.
 Clin Pharmacol Ther 2013;93(4):299-301.
- 585 * This paper demonstrates the superior efficiency of phenotypic screening for the
 586 discovery of new drug classes.
- (13)Zheng W, Thorne N, McKew JC. Phenotypic screens as a renewed approach for drug
 discovery. Drug Discov Today 2013;18(21-22):1067-73.
- 589 (14)MacRae CA, Peterson RT. Zebrafish-based small molecule discovery. Chem Biol
 590 2003;10(10):901-8.
- (15)Macrae CA. Cardiac Arrhythmia: In vivo screening in the zebrafish to overcome
 complexity in drug discovery. Expert Opin Drug Discov. 2010;5(7):619-632.
- (16)Kithcart A, MacRae CA. Using Zebrafish for High-Throughput Screening of Novel
 Cardiovascular Drugs. JACC Basic Transl Sci 2017;2(1):1-12.
- 595 (17)Li Y, Chen T, Miao X, et al. Zebrafish: A promising in vivo model for assessing the

- delivery of natural products, fluorescence dyes and drugs across the blood-brain barrier.
 Pharmacol Res 2017;125(Pt B):246-257.
- (18) Wellens HJ. Cardiac arrhythmias: the quest for a cure: a historical perspective. J Am Coll
 Cardiol 2004;44:1155–63.
- (19) Vaduganathan M, Butler J, Pitt B, et al. Contemporary drug development in heart failure:
 call for hemodynamically neutral therapies. Circ Heart Fail 2015;8:826–31.
- (20)Mueller RL, Scheidt S. History of drugs for thrombotic disease. Discovery, development,
 and directions for the future. Circulation. 1994;89(1):432-49.
- (21)Keeler RF. Teratogenic compounds of Veratrum californicum (Durand) X. Cyclopia in
 rabbits produced by cyclopamine. Teratology. 1970;3(2):175-80.
- 606 (22)Kasten FH. Paul Ehrlich: pathfinder in cell biology. 1. Chronicle of his life and
 607 accomplishments in immunology, cancer research, and chemotherapy. Biotech Histochem
 608 1996;71(1):2-37.
- (23)Tweedy BL, Lesney MS. 1950s: Prescriptions and Polio. The Pharmaceutical Century,
 American Chemical Society, Washington, DC, 2000.
- 611 (24) Ward AC, Lieschke GJ. The zebrafish as a model system for human disease. Front Biosci.
 612 2002;7:d827-33.
- 613 (25)Kari G, Rodeck U, Dicker AP. Zebrafish: an emerging model system for human disease
 614 and drug discovery. Clin Pharmacol Ther 2007;82(1):70-80.
- 615 (26)Zon LI, Peterson RT. In vivo drug discovery in the zebrafish. Nat Rev Drug Discov
 616 2005;4(1):35-44.
- 617 (27)Parng C, Seng WL, Semino C, et al. Zebrafish: a preclinical model for drug screening.
 618 Assay Drug Dev Technol 2002;1(1 Pt 1):41-8.
- 619 (28)Bakkers J. Zebrafish as a model to study cardiac development and human cardiac disease.
 620 Cardiovasc Res 2011;91(2):279-88.
- (29)Barros TP, Alderton WK, Reynolds HM, et al. Zebrafish: an emerging technology for in
 vivo pharmacological assessment to identify potential safety liabilities in early drug
 discovery. Br J Pharmacol 2008;154(7):1400-13.
- (30)Chakraborty C, Hsu CH, Wen ZH, et al. Zebrafish: a complete animal model for in vivo
 drug discovery and development. Curr Drug Metab 2009;10(2):116-24.
- (31)Huang CJ, Tu CT, Hsiao CD, et al. Germ-line transmission of a myocardium-specific
 GFP transgene reveals critical regulatory elements in the cardiac myosin light chain 2
 promoter of zebrafish. Dev Dyn 2003;228(1):30-40.
- (32)Smith KA, Chocron S, von der Hardt S, et al. Rotation and asymmetric development of
 the zebrafish heart requires directed migration of cardiac progenitor cells. Dev Cell
 2008;14(2):287-97.
- (33) Wilson KS, Baily J, Tucker CS, et al. Early-life perturbations in glucocorticoid activity
 impacts on the structure, function and molecular composition of the adult zebrafish

- 634 (*Danio rerio*) heart. Mol Cell Endocrinol 2015;414:120-31.
- (34)Becker JR, Robinson TY, Sachidanandan C, et al. In vivo natriuretic peptide reporter
 assay identifies chemical modifiers of hypertrophic cardiomyopathy signalling.
 Cardiovasc Res 2012;93(3):463-70.
- (35)Howe K, Clark MD, Torroja CF, et al. The zebrafish reference genome sequence and its
 relationship to the human genome. Nature 2013;496(7446):498-503.
- ⁶⁴⁰ * This paper reports the completion of the first zebrafish genome sequence.
- (36)Koester R, Sassen WA. A molecular toolbox for genetic manipulation of zebrafish. Adv
 Genom Genet 2015;5:151–163.
- 643 (37)Gao Y, Liu J, Wang X, et al. Genetic manipulation in zebrafish. Sheng Wu Gong Cheng
 644 Xue Bao 2017;33(10):1674-1692.
- (38)Summerton J. Morpholino antisense oligomers: the case for an RNase H-independent
 structural type. Biochim Biophys Acta 1999;1489(1):141-58.
- 647 (39)Stainier DYR, Raz E, Lawson ND, et al. Guidelines for morpholino use in zebrafish.
 648 PLoS Genet 2017;13(10):e1007000.
- 649 (40)Ledford H. CRISPR, the disruptor. Nature 2015;522(7554):20-4.
- (41)Hendel A, Bak RO, Clark JT, et al. Chemically modified guide RNAs enhance
 CRISPR-Cas genome editing in human primary cells. Nat Biotechnol
 2015;33(9):985-989.
- (42)Zou J, Tran D, Baalbaki M, et al. An internal promoter underlies the difference in disease
 severity between N- and C-terminal truncation mutations of Titin in zebrafish. Elife
 2015;4:e09406.
- (43)Farr III GH, Imani K, Pouv D, et al. Functional testing of a human PBX3 variant in
 zebrafish reveals a potential modifier role in congenital heart defects. bioRxiv 2018; doi:
 https://doi.org/10.1101/337832.
- (44) Stainier DYR. Zebrafish genetics and vertebrate heart formation. Nat Rev Genet 2001; 2:39-48.
- (45)Epstein FH, Epstein JA. A perspective on the value of aquatic models in biomedical
 research. Exp Biol Med 2005; 230(1): 1-7.
- (46)Asnani A, Peterson RT. The zebrafish as a tool to identify novel therapies for human
 cardiovascular disease. Dis Model Mech 2014;7(7):763-7.
- (47) Vornanen M, Hassinen M. Zebrafish heart as a model for human cardiac
 electrophysiology. Channels (Austin) 2016;10(2):101-10.
- (48)Burns CG, Milan DJ, Grande EJ, et al. High-throughput assay for small molecules that
 modulate zebrafish embryonic heart rate. Nat Chem Biol. 2005;1(5):263-4.
- (49) Chi NC, Shaw RM, Jungblut B, et al. Genetic and physiologic dissection of the vertebrate
 cardiac conduction system. PLoS Biol 2008;6(5):e109.
- 671 (50) Jagannathan-Bogdan M, Zon LI. Hematopoiesis. Development 2013;140(12):2463–7.

- (51)Paffett-Lugassy N, Hsia N, Fraenkel PG, et al. Functional conservation of erythropoietin
 signaling in zebrafish. Blood 2007;110(7):2718-26.
- (52) Childs S, Chen JN, Garrity DM, et al. Patterning of angiogenesis in the zebrafish embryo.
 Development. 2002;129(4):973-82.
- (53) Wang M, Sips P, Khin E, et al. Wars2 is a determinant of angiogenesis. Nat Commun
 2016;7:12061.
- (54) Hasan SS, Tsaryk R, Lange M, et al. Endothelial Notch signalling limits angiogenesis via
 control of artery formation. Nat Cell Biol 2017;19(8):928-940.
- (55)Milan DJ, Peterson TA, Ruskin JN, et al. Drugs that induce repolarization abnormalities
 cause bradycardia in zebrafish. Circulation. 2003;107(10):1355-8.
- ** This is one of the first papers highlighting the functional orthology between
 zebrafish and human cardiovascular electrophysiology.
- (56)Ma X, Ding Y, Wang Y, et al. A doxorubicin-induced cardiomyopathy model in adult
 zebrafish. J Vis Exp 2018;(136).
- 686 (57)Yu PB, Deng DY, Lai CS, et al. BMP type I receptor inhibition reduces heterotopic
 687 ossification. Nat Med 2008;14(12):1363-9.
- (58) Ren B, Deng Y, Mukhopadhyay A, et al. ERK1/2-Akt1 crosstalk regulates arteriogenesis
 in mice and zebrafish. J Clin Invest 2010;120(4):1217-28.
- (59)Zhang Y, Wang J, Wheat J, et al. AML1-ETO mediates hematopoietic self-renewal and
 leukemogenesis through a COX/β-catenin signaling pathway. Blood 2013;12:4906-16.
- (60)Kokel D, Cheung CY, Mills R, et al. Photochemical activation of TRPA1 channels in
 neurons and animals. Nat Chem Biol 2013;9(4):257-63.
- (61)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(266):266ra170.
- 697 (62)Asimaki A, Kapoor S, Plovie E, et al. Identification of a new modulator of the
 698 intercalated disc in a zebrafish model of arrhythmogenic cardiomyopathy. Sci Transl Med
 699 2014;6(240):240ra74.
- ** This paper describes successful screening of an arrhythmogenic cardiomyopathy
 model using a genetically-encoded reporter.
- (63)Shin JT, Pomerantsev EV, Mably JD, et al. High-resolution cardiovascular function
 confirms functional orthology of myocardial contractility pathways in zebrafish. Physiol
 Genomics 2010;42(2):300-9.
- (64) Rennekamp AJ, Peterson RT. 15 years of zebrafish chemical screening. Curr Opin Chem
 Biol 2015;24:58-70.
- (65)MacRae CA, Peterson RT. Zebrafish as tools for drug discovery. Nat Rev Drug Discov
 2015;14(10):721-31.
- (66) Peterson RT, Shaw SY, Peterson TA, et al. Chemical suppression of a genetic mutation in

- a zebrafish model of aortic coarctation. Nat Biotechnol 2004;22(5):595-9.
- (67)Hong CC, Peterson QP, Hong JY, et al. Artery/vein specification is governed by
 opposing phosphatidylinositol-3 kinase and MAP kinase/ERK signaling. Curr Biol
 2006;16(13):1366-72.
- (68)Tran TC, Sneed B, Haider J, et al. Automated, quantitative screening assay for
 antiangiogenic compounds using transgenic zebrafish. Cancer Res 2007;67(23):11386-92.

** In this study a fully automated screen was able to identify a novel antiangiogenic
 compound with potency in human cells.

- (69) Alvarez Y, Astudillo O, Jensen L, et al. Selective inhibition of retinal angiogenesis by
 targeting PI3 kinase. PLoS One 2009;4(11):e7867.
- (70) Wang C, Tao W, Wang Y, et al. Rosuvastatin, identified from a zebrafish chemical
 genetic screen for antiangiogenic compounds, suppresses the growth of prostate cancer.
 Eur Urol 2010;58(3):418-26.
- (71)Peal DS, Mills RW, Lynch SN, et al. Novel chemical suppressors of long QT syndrome
 identified by an in vivo functional screen. Circulation 2011;123(1):23-30.
- (72) Huang CC, Monte A, Cook JM, et al. Zebrafish heart failure models for the evaluation of
 chemical probes and drugs. Assay Drug Dev Technol 2013;11(9-10):561-72.
- (73)Tang C, Xie D, Feng B. Zebrafish as a new model for phenotype-based screening of
 positive inotropic agents. Chem Biol Drug Des 2015;85(3):253-8.
- (74) Shimizu H, Schredelseker J, Huang J, et al. Mitochondrial Ca(2+) uptake by the
 voltage-dependent anion channel 2 regulates cardiac rhythmicity. Elife 2015;4.
- (75)Reynolds AL, Alvarez Y, Sasore T, et al. Phenotype-based discovery of
 2-[(E)-2-(Quinolin-2-yl)vinyl]phenol as a novel regulator of ocular angiogenesis. J Biol
 Chem 2016;291(14):7242-55.
- (76)Otten C, Knox J, Boulday G, et al. Systematic pharmacological screens uncover novel
 pathways involved in cerebral cavernous malformations. EMBO Mol Med 2018;10(10)
 pii: e9155.
- (77) Sergeeva IA, Christoffels VM. Regulation of expression of atrial and brain natriuretic
 peptide, biomarkers for heart development and disease. Biochim Biophys Acta
 2013;1832(12):2403-13.
- (78) Chelko SP, Asimaki A, Andersen P, et al. Central role for GSK3β in the pathogenesis of
 arrhythmogenic cardiomyopathy. JCI Insight 2016;1(5).
- (79)Pott A, Rottbauer W, Just S. Functional genomics in zebrafish as a tool to identify novel
 antiarrhythmic targets. Curr Med Chem. 2014;21(11):1320-9.
- (80)Chen JN, Haffter P, Odenthal J, et al. Mutations affecting the cardiovascular system and
 other internal organs in zebrafish. Development. 1996;123:293-302.
- 746 * The original paper describing the cardiovascular phenotypes of zebrafish mutants
 747 generated by random mutagenesis.

- (81)Langheinrich U, Vacun G, Wagner T. Zebrafish embryos express an orthologue of HERG
 and are sensitive toward a range of QT-prolonging drugs inducing severe arrhythmia.
 Toxicol Appl Pharmacol. 2003;193(3):370-82.
- (82)Hassel D, Scholz EP, Trano N, et al. Deficient zebrafish ether-à-go-go-related gene
 channel gating causes short-QT syndrome in zebrafish reggae mutants. Circulation.
 2008;117(7):866-75.
- (83)Arnaout R, Ferrer T, Huisken J, et al. Zebrafish model for human long QT syndrome.
 Proc Natl Acad Sci U S A. 2007;104(27):11316-21.
- (84)Pott A, Bock S, Berger IM, et al. Mutation of the Na+/K+-ATPase Atp1a1a.1 causes QT
 interval prolongation and bradycardia in zebrafish. J Mol Cell Cardiol. 2018;120:42-52.
- (85)Leong IU, Skinner JR, Shelling AN, et al. Identification and expression analysis of kcnh2
 genes in the zebrafish. Biochem Biophys Res Commun. 2010;396(4):817-24.
- (86)Roden DM. Drug-induced prolongation of the QT interval. N Engl J Med.
 2004;350(10):1013-22.
- (87)Lee SH, Kim HR, Han RX, et al. Cardiovascular risk assessment of atypical antipsychotic
 drugs in a zebrafish model. J Appl Toxicol. 2013;33(6):466-70.
- (88)Dhillon SS, Dóró E, Magyary I, et al. Optimisation of embryonic and larval ECG
 measurement in zebrafish for quantifying the effect of QT prolonging drugs. PLoS One.
 2013;8(4):e60552.
- (89)Milan DJ, Kim AM, Winterfield JR, et al. Drug-sensitized zebrafish screen identifies
 multiple genes, including GINS3, as regulators of myocardial repolarization. Circulation.
 2009;120(7):553-9.
- (90)Langenbacher AD, Dong Y, Shu X, et al. Mutation in sodium-calcium exchanger 1
 (NCX1) causes cardiac fibrillation in zebrafish. Proc Natl Acad Sci U S A.
 2005;102(49):17699-704.
- (91)Folkman J. Angiogenesis: an organizing principle for drug discovery? Nat Rev Drug
 Discov 2007;6(4):273-86.
- (92)Bandorski D, Hoeltgen R, Becker N, et al. Follow-up (measurement) of corrected qt
 interval in adult patients before and after lung transplantation. Biomed Res Int.
 2017:4519796.
- (93) Winterfield JR, Milan DJ. Dexamethasone suppresses long QT phenotype in patient with
 acute promyelocytic leukemia treated with arsenic. Heart Rhythm Case Rep.
 2015;2(4):280-282.
- (94) White RM, Cech J, Ratanasirintrawoot S, et al. DHODH modulates transcriptional
 elongation in the neural crest and melanoma. Nature. 2011;471(7339):518-22.
- (95)Owens KN, Santos F, Roberts B, et al. Identification of genetic and chemical modulators
 of zebrafish mechanosensory hair cell death. PLoS Genet. 2008;4(2):e1000020.
- * Successful zebrafish screening leading to the identification of a new drug class

786 which has recently received Investigational New Drug approval.

- (96)Chowdhury S, Owens KN, Herr RJ, et al. Phenotypic optimization of urea-thiophene
 carboxamides to yield potent, well tolerated, and orally active protective agents against
 aminoglycoside-induced hearing loss. J Med Chem. 2018;61(1):84-97.
- (97)Santoriello C, Zon LI. Hooked! Modeling human disease in zebrafish. J Clin Invest
 2012;122(7):2337-43.
- (98)Postlethwait J, Amores A, Cresko W, et al. Subfunction partitioning, the teleost radiation
 and the annotation of the human genome. Trends Genet 2004;20(10):481-90.
- (99) Mathias JR, Saxena MT, Mumm JS. Advances in zebrafish chemical screening
 technologies. Future Med Chem 2012;4(14):1811-22.
- (100) Berghmans S, Butler P, Goldsmith P, et al. Zebrafish based assays for the assessment
 of cardiac, visual and gut function--potential safety screens for early drug discovery. J
 Pharmacol Toxicol Methods 2008;58(1):59-68.
- (101) Nath AK, Roberts LD, Liu Y, et al. Chemical and metabolomic screens identify
 novel biomarkers and antidotes for cyanide exposure. FASEB J 2013;27(5):1928-38.
- (102) Nath AK, Shi X, Harrison DL, et al. Cisplatin analogs confer protection against
 cyanide poisoning. Cell Chem Biol 2017;24(5):565-575.
- 803 (103) Sips PY, Shi X, Musso G, et al. Identification of specific metabolic pathways as
 804 druggable targets regulating the sensitivity to cyanide poisoning. PLoS One
 805 2018;13(6):e0193889.
- 806 (104) Schenone M, Dančík V, Wagner BK, et al. Target identification and mechanism of
 807 action in chemical biology and drug discovery. Nat Chem Biol 2013;9(4):232-40.
- 808 (105) MacRae CA. Recent advances in in vivo screening for antiarrhythmic drugs. Expert
 809 Opin Drug Discov 2013;8(2):131-41.
- 810 (106) Dow LE. Modeling disease in vivo with CRISPR/Cas9. Trends Mol Med.
 811 2015;21(10):609-621.
- (107) Liu J, Zhou Y, Qi X, et al. CRISPR/Cas9 in zebrafish: an efficient combination for
 human genetic diseases modeling. Hum Genet 2017;136(1):1-12.
- 814 (108) Zhang Y, Qin W, Lu X, et al. Programmable base editing of zebrafish genome using
 815 a modified CRISPR-Cas9 system. Nat Commun. 2017;8(1):118.
- 816 * This paper demonstrates the possibilities of single-base editing in zebrafish.
- 817

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

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

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.

