Zebrafish as tools for drug discovery

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Abstract | The zebrafish has become a prominent vertebrate model for disease and has already contributed to several examples of successful phenotype-based drug discovery. For the zebrafish to become useful in drug development more broadly, key hurdles must be overcome, including a more comprehensive elucidation of the similarities and differences between human and zebrafish biology. Recent studies have begun to establish the capabilities and limitations of zebrafish for disease modelling, drug screening, target identification, pharmacology, and toxicology. As our understanding increases and as the technologies for manipulating zebrafish improve, it is hoped that the zebrafish will have a key role in accelerating the emergence of precision medicine.

Phenotype-based screening
A screen in which the assay output is a complex cellular or organismal phenotype that integrates multiple biochemical pathways and often captures much of the native biological context.

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We are currently witnessing a resurgence of interest in phenotype-based screening in drug discovery. Phenotypic effects of small molecules were historically the basis of all drug discovery, but over the past 30 years this strategy has been largely replaced by target-based approaches. There is now a growing appreciation that 'first-in-class' drug discovery successes are emerging disproportionately from the remaining phenotype-based efforts. For example, a recent analysis of first-in-class drugs approved between 1999 and 2008 revealed that 62% were discovered by phenotype-based screens, despite the fact that such screens represented only a small subset of the overall total[1].

Several factors may explain the apparent superiority of phenotype-based approaches over target-based screening efforts. First, phenotypic screens can discover efficacious drugs in the absence of a validated target. For example, ezetimibe was discovered based on its cholesterol-lowering activity years before Niemann–Pick C1-like protein 1 (NPC1L1) was validated as a therapeutic target[2,3]. Second, phenotypic screens can identify compounds that produce a therapeutic effect through simultaneous activity at multiple targets. Thus, amiodarone, which remains the definitive antiarrhythmic agent decades after its serendipitous discovery, exhibits activity on multiple ion channels, adrenergic receptors, and possibly even nuclear hormone pathways[4]. In addition, there is evidence that the acute effects of the intravenous form may be attributable in part to the solvent Tween 80 (REF. 5). Third, phenotypic screens often combine screening and counter-screening (for example, against relevant organ-specific toxicity reporter lines) in the same assay, discovering compounds that produce a desired effect while parsing out compounds with undesirable qualities.

As a direct result, compounds advancing from phenotypic screens are often of higher quality than hits from *in vitro* target-based screens.

Advantages of screening in zebrafish

Small-molecule screens in zebrafish represent a small but growing fraction of phenotype-based screens (TABLE 1). Zebrafish screens provide the advantages of phenotype-based screens outlined above, but they also offer unique advantages that come from screening in an intact animal.

Broad range of accessible biology. Most phenotypic screens are carried out in cultured cells, which limits the screens to cell-autonomous phenotypes or those end points that can be observed in relatively simple culture systems. Zebrafish screens are typically carried out in living zebrafish embryos or larvae, which exhibit a diverse repertoire of biological processes and possess fully integrated vertebrate organ systems. As such, a much broader range of phenotypes can be assayed in zebrafish than in cultured cells. Pain, sedation, tumour metastasis, vascular tone, and gut motility are examples of disease-relevant phenotypes that are observable in zebrafish yet simply inaccessible to modelling in cultured cells. The advantages of the intact animal as a focus for screening are particularly evident for neuroscience drug discovery, in which the complexities of cell–cell interactions and endocrine signalling defy even modelling with patient-derived induced pluripotent stem cells (iPSCs). By contrast, with emerging automated technologies and zebrafish screening, it is possible to imagine relatively unbiased capture of a substantial proportion of the complete phenotypic repertoire.

Table 1 | **Selected examples of published chemical screens in zebrafish**

Screen type	Phenotypic readout	Refs
Cardiotoxicity screen	Heart rate	19
Suppressors of aortic coarctation	Blood circulation in the aorta	68
Suppressors of mitotic defects	Phospho-histone H3 staining	69
Haematopoiesis	Stem cell marker expression	40
Tissue regeneration	Extent of fin regeneration	77
Angiogenesis	Vascular morphology in transgenic animals	78
Embryogenesis	Embryonic morphology	44
Suppressors of ototoxicity	Number of surviving hair cells	50
Suppressors of leukaemia	Expression of myeloid and erythroid markers	52
Fgf pathway reporter screen	Fgf reporter expression	79
Suppressors of polycystic kidney disease	Presence of laterality defects/body curvature	70
Behavioural sleep screen	Bouts of rest and wakefulness	72
Behavioural screen	Response to photic stimuli	73
Dietary lipid absorption	Processing of fluorescent lipid analogues	80
Modifiers of copper homeostasis	Pigmentation and notochord defects	81
Angiogenesis	Vascular morphology in transgenic animals	82
Suppressors of long QT syndrome	Atrioventricular heart rhythm	55
Suppressors of melanoma	Expression of neural crest markers	83
Learning assay	Habituation to acoustic startle	74
Fluorescent reporter	Fgf signalling, *dusp6* expression	84
Inducers of β-cell differentiation	Number of fluorescent β-cells in pancreas	85
Embryogenesis	Pigment cell patterning and number	86
Hair cell regeneration	GFP expression in hair cells after ablation	87
Modifiers of hypertrophic cardiomyopathy	Natriuretic peptide reporter line	88
Toxicology screen	Death and overt structural defects	89
Suppressors of leukaemia	Fluorescent T cells in thymus	90
Luciferase reporter	Glucocorticoid signalling reporter	91
Suppressors of organophosphate toxicity	Survival	92
Modulators of gluconeogenesis	Luciferase reporter of *pck1* expression	12
Behavioural screen	Motor activity	28
Suppressors of cyanide toxicity	Survival	93
Leukocyte migration	Migration of leukocytes to wound	94
Modifiers of RAS activity	*dusp6* expression by *in situ* hybridization	95
Embryogenesis	Morphological defects in the embryo	58
Embryogenesis	Embryo dorsalization	96
Suppressors of Dravet syndrome	Inhibition of convulsive behaviours	71
Embryogenesis	Inducers of ectopic tail formation	97
Suppressors of leukaemia	Death of MYC-expressing thymocytes	57
Craniofacial development	Craniofacial morphology	98
Suppressors of Wnt-activated cancers	Presence of eyes in BIO-treated zebrafish	99
Suppressors of cardiomyopathy	Normalization of natriuretic peptide levels	30
Stimulators of β-cell proliferation	*In vivo* cell cycle indicator technology	100
Suppressors of cardiomyopathy	Rescue of cardiac function	29
Modifiers of glucose homeostasis	Biochemical measurement of glucose	101

Target-based screening
A screen in which the assay output is the activity of a specific molecular target in a well-defined but often heterologous context.

Counter-screening
To screen in parallel for secondary end points that might condition the final output of the primary screen. Together, such screens enable the incorporation of simple logic; for example, a counter-screen might identify compounds with a specific form of toxicity, allowing the hits from the primary screen to be weighted appropriately for subsequent evaluation.

Toxicity reporter lines
Genetically modified zebrafish lines expressing specific tissue damage reporters under an organ-specific promoter. These lines will release heterologous reporter peptides that can then be detected using a range of high-throughput detection methods.

Table 1 (cont.) | **Selected examples of published chemical screens in zebrafish**

Screen type	Phenotypic readout	Refs
Embryogenesis	Embryonic morphology	102
Cell migration	Migration of labelled lateral line primordium	103
Tumorigenesis	Liver size	104
Adult transplantation	Imaging of fluorescent stem cell grafts	105
Fluorescent reporter	Number of fluorescent β-cells in pancreas	106

dusp6, dual specificity phosphatase 6; Fgf, fibroblast growth factor; GFP, green fluorescent protein; pck1, phosphoenolpyruvate carboxykinase 1.

Early insight into toxicity. Whereas cell-based assays provide limited information about the absorption, distribution, metabolism, excretion and toxicity (ADME-Tox) of screening compounds, zebrafish screens often reveal insights about these pharmacological characteristics. Zebrafish larvae possess functional livers, kidneys and blood–brain barriers[6–8]. To produce *in vivo* phenotypes in zebrafish assays, compounds must exhibit the ability to be absorbed, reach the target tissue, and avoid rapid metabolism and excretion. This fact may explain the observation that several compounds that were discovered in zebrafish screens have been rapidly translated to *in vivo* mammalian models with minimal optimization of pharmacological properties.

Are zebrafish relevant for human drug discovery?

Being able to perform high-throughput phenotypic screens in an *in vivo* context is theoretically very attractive, but how relevant is the output from zebrafish screens for human biology? Recent studies have cast doubt on the validity of some well-established rodent disease models, reminding us again of the potential to be misled by models of any form[9]. If it is important to understand the capabilities and limitations of mammalian models, the need is even greater for zebrafish, which are phylogenetically further removed from humans. Over the past few years, we have begun to understand how zebrafish compare to humans in terms of targets, physiology, drug metabolism and pharmacology, in particular during the first few days of life when it is possible to house them in multi-well plates suited for screening (FIG. 1). Nevertheless, questions about relevance to humans remain of central importance to the use of zebrafish in drug discovery.

Targets. With a high-quality zebrafish genome now available, it appears that 71% of human proteins (and 82% of disease-causing human proteins) have an obvious orthologue in zebrafish[10]. Orthologous zebrafish proteins are reasonably similar to their human counterparts, particularly within functional domains. For example, the protein targets of the ten most-prescribed drugs have zebrafish orthologues with sequence identity ranging from 54% (glucocorticoid receptor) to 91% (thyroid receptor). Given the sequence divergence between zebrafish and human proteins, one might expect a modest rate of conservation of pharmacological effect. In reality, however, the rates of conservation are relatively high (see below). One potential explanation for this is that the target similarity is greater at active sites in the enzymes, channels and receptors that are frequently the targets of drugs because these sites have retained the ability, through evolution, to bind to the same metabolites, ions and other biomolecules. For example, the zebrafish glucocorticoid receptor, which is only about 50% identical to the human receptor overall, is 74% identical in the carboxy-terminal half of the protein, which contains the ligand-binding domain.

Physiology. Among model systems that are amenable to screening, zebrafish stand out for their highly conserved integrative physiology. Unlike yeast, worms or flies, zebrafish possess recognizable organ systems — livers, hearts, kidneys, pancreases and so on. Although there are some major differences resulting from adaptation to aquatic life, most zebrafish organs perform the same functions as their human counterparts and exhibit well-conserved physiology. For example, the zebrafish pancreas contains islets comprising α, β, δ, and ε cells that regulate glucose homeostasis by secreting glucagon, insulin, somatostatin and ghrelin, just as in humans[11]. Drugs that modulate glucose homeostasis in humans have been shown to have the same effects in zebrafish[12]. The zebrafish haematopoietic system is highly similar to the human system and consists of the same cell types — erythrocytes, neutrophils, eosinophils, lymphocytes, macrophages and so on[13]. Haematopoietic processes are conserved, as are globin switching and iron homeostasis through the hepcidin–ferroportin pathway[14,15]. In fact, key components of these processes were first discovered in zebrafish[16]. Drugs affecting haematopoiesis and anaemia in humans have similar effects in zebrafish[17]. Cardiovascular physiology is also highly conserved between humans and zebrafish at anatomical, cellular and membrane-biology levels. Many human cardiovascular drugs have been shown to have identical effects on zebrafish physiology, and numerous human cardiovascular disorders have been recapitulated in zebrafish genetic models[18]. Interestingly, the cardiac electrophysiology of humans is more similar to that of zebrafish than it is to that of rodents, adding to a list of cases in which zebrafish physiology may be more relevant than the rodent counterpart[19–21]. Many other examples of orthology between humans and zebrafish exist; however, perhaps the ultimate consequence of the throughput feasible in this organism will be a much more global understanding of how representative zebrafish are of human biology compared to most other preclinical models.
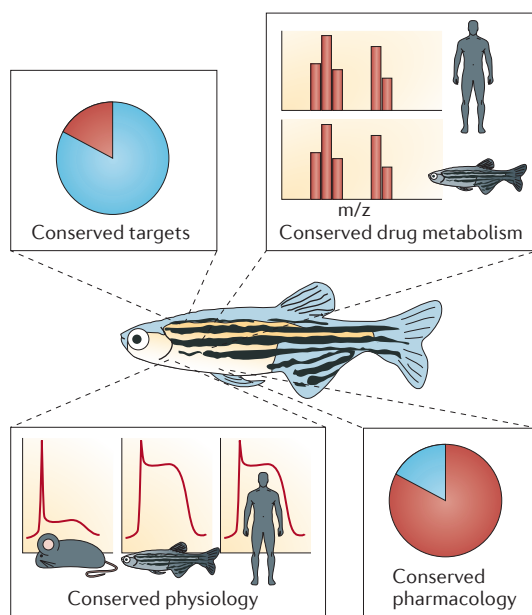
Figure 1 | **How suitable are zebrafish for discovering human drugs?** Although zebrafish enable *in vivo* studies on a large scale, questions remain about how relevant the findings are for drug discovery. Recent studies have begun to elucidate the degree of conservation between humans and zebrafish, which share 82% of disease-associated targets and a large number of drug metabolism pathways (as illustrated by the mass spectrometry of metabolic by-products). Zebrafish physiology is often well conserved (sometimes more so than rodent physiology); for example, cardiac electrophysiology (illustrative electrocardiogram (ECG) tracings from each organism are shown). Several compounds discovered in zebrafish screens have been shown to exhibit similar effects in rodent models and humans, including eight out of ten compounds tested by our groups. m/z, mass to charge ratio.

Some effort has been made to characterize the parallels between different stages of zebrafish development and those of humans. In these cases, we have observed that the key molecular transitions in each of the organs relevant for disease modelling or drug metabolism appear to take place in sequence, but often much more rapidly in zebrafish than in humans. For example, the sequential electrophysiological maturation of the zebrafish heart, which has been characterized at the resolution of individual ionic currents, takes place within 96 hours post-fertilization, whereas some of the parallel events are not completed until adolescence in humans[19,22,23].

Pharmacology. How often will a compound discovered in zebrafish retain its efficacy in humans? This critical question has not yet been answered because only a few compounds discovered in zebrafish have been tested in humans. However, it has been possible to test a reasonable number of human drugs for conserved effects in zebrafish. For example, 23 drugs known to exhibit repolarization cardiotoxicity were tested for their effects on zebrafish, and 22 of the 23 compounds produced repolarization-related toxicity[19]. Similarly, drugs with specific effects on cardiac contractility and vasomotion in humans consistently recapitulate these effects in the zebrafish[24]. Although there may be reporting bias favouring the publication of cases in which pharmacology is conserved, these examples suggest a high probability of direct correlation between effects in humans and zebrafish. In our laboratories, we have tested ten compounds discovered via diverse zebrafish screens in corresponding rodent disease models. Eight of the ten compounds produced the desired effect in rodents, with little or no optimization via medicinal chemistry or formulation[25–31]. Although the sample size is small, these experiences have led us to conclude that conservation of pharmacological effect is high for the majority of drugs where the phenotypic correlation is rigorous.

Drug distribution, metabolism and excretion. As small molecules were systematically tested in the zebrafish, it became apparent that not only were the effects of individual human drugs replicated but so too were the majority of drug–drug interactions. These findings suggested that the distribution, metabolism and excretion of drugs might also be accessible in zebrafish modelling. There is now strong evidence not only of conserved partitioning of drugs into different passive compartments based on physicochemical characteristics but also of the existence of the regulation of drug distribution across active physiological boundaries such as the blood–brain barrier and by conserved tissue-specific transporters[32–35].

Although comparative genomics have established that the zebrafish possesses a full complement of cytochrome P450 (CYP) genes, genome duplication events and functional redundancy have hampered comprehensive study of the conservation of the metabolic processing of small molecules[8,36]. The conservation of other drug metabolism pathways is only beginning to be explored, but to date there is evidence of substantial functional parallels across diverse mechanisms, including multiple non-CYP enzymes[34,37]. Not unexpectedly, there is also evidence that great care must be taken in simple extrapolation, as drug metabolism may vary widely across different developmental stages and, as with other models, the presence of one part of any pathway does not infer the conservation of the remaining components[8,38]. Finally, drug excretion is clearly regulated in the zebrafish, but few studies of drug filtration, reabsorption or excretion have been undertaken to date. As mass spectroscopy techniques advance, more generalizable approaches to ADME in the zebrafish are emerging[36].

Examples of success

To date, more than 65 small-molecule screens in zebrafish have been reported in the literature (TABLE 1). These screens have targeted diverse phenotypes ranging from embryo morphology to cardiac physiology and sleep. Some have identified repurposing opportunities for existing drugs, whereas others have discovered novel compound classes. These screens have been surveyed in

detail elsewhere[39]. Here, we focus on select examples of disease-relevant compounds that have been discovered by zebrafish screens in the hope that they will illustrate the diverse methodologies accessible for drug discovery in this organism.

Prohema. Prohema is a stabilized derivative of prostaglandin E2 (PGE2) that is currently in Phase II trials in patients undergoing umbilical cord blood (UCB) transplantation for leukaemia or lymphoma. Prohema was discovered in a zebrafish screen for compounds that increased or decreased the numbers of haematopoietic stem cells (HSCs)[40]. Automated *in situ* hybridization was used to stain HSCs in the aorta–gonad–mesonephros region of the embryo, and compounds that enhanced PGE2 synthesis were found to increase HSC numbers. The ability of PGE2 to enhance production of HSCs was later tied to its interaction with WNT signalling[41].

The therapeutic potential of Prohema was first demonstrated in adult zebrafish, in which it enhanced the rate of marrow recovery after sub-lethal irradiation. In mice, whole bone marrow exposed to Prohema *ex vivo* formed more spleen colony-forming units when transplanted into irradiated mice than did untreated marrow[40]. Similarly, Prohema-treated whole bone marrow more effectively reconstituted the haematopoietic system and contributed more HSCs to short-term and long-term repopulation than did untreated whole bone marrow.

For humans undergoing HSC transplantation as therapy for leukaemia or lymphoma, UCB has become an important source of transplantable HSCs. However, the number of HSCs present in a typical UCB sample is often suboptimal for treating adults. Therefore, the apparent ability of Prohema to boost HSC numbers and its repopulating ability may enhance the effectiveness of UCB transplantation. On the basis of this idea, Prohema is currently in a Phase II clinical trial for the *ex vivo* conditioning of UCB before transplantation into patients with leukaemia or lymphoma[42,43].

Dorsomorphin. Dorsomorphin and its derivatives are inhibitors of the bone morphogenetic protein (BMP) receptor: in humans this protein is activin receptor-like kinase 2 (ALK2, also known as ACVR1) and in zebrafish it is Alk8 (also known as Acvr1l). These compounds are currently in development as therapies for fibrodysplasia ossificans progressiva (FOP) and for anaemia of inflammation (FIG. 2). Dorsomorphin was discovered in a zebrafish screen that sought to identify compounds that perturb the establishment of the basic body organization during early embryogenesis[44]. In screening a library of a few thousand compounds, a pyrazolopyrimidine was identified that causes dorsalization — the expansion of dorsal tissues at the expense of ventral tissues. Treated embryos developed with reduced or missing tails, and the pyrazolopyrimidine was named dorsomorphin to reflect its dorsalizing activity.

The dorsalizing pyrazolopyrimidine had previously been described as an inhibitor of AMP-activated protein kinase (AMPK), but other AMPK inhibitors failed to replicate the dorsalized phenotype in zebrafish embryos, suggesting that AMPK was not the dorsalizing target. Instead, dorsomorphin-treated animals were indistinguishable from a previously identified genetic mutant called *lost-a-fin*, which harboured a mutation in *Alk8* (REF. 44). This phenotypic similarity suggested that dorsomorphin may inhibit Alk8 in zebrafish and ALK2 in humans — a hypothesis that has since been confirmed. As the first small-molecule inhibitor of the BMP pathway, dorsomorphin and derivatives such as LDN-193189 have become widely used probes for manipulating BMP signalling[45].

Hyperactive BMP signalling causes a variety of disease states, including FOP and anaemia of inflammation. The dorsomorphin derivative LDN-193189 has proven to be effective in treating rodent models of FOP and anaemia of inflammation[15,25,46]. In addition, it has been shown to be effective in treating mammalian models of other BMP-linked conditions, including arterial calcification and inflammatory bowel disorder[47–49]. Because of the apparent promise of BMP inhibitors for treating these conditions, the US National Institutes of Health has selected dorsomorphin and its derivatives for preclinical development through its TRND (Therapeutics for Rare and Neglected Diseases) and BrIDGs (Bridging Interventional Development Gaps) programmes, which will support preclinical development through early clinical trials in FOP and anaemia of inflammation, respectively.

PROTO-1. PROTO-1 and its derivatives are benzothiophene carboxamides that are currently in development for preventing antibiotic-induced hearing loss. Because aminoglycoside antibiotics can cause hearing loss by killing hair cells in the human ear, a zebrafish screen of 10,960 compounds was conducted to identify those compounds that could protect zebrafish hair cells from aminoglycoside-induced death[50]. PROTO-1 and a structurally related benzothiophene carboxamide were found to be potent otoprotectants in zebrafish, and to protect hair cells from aminoglycoside death in cultured murine utricles.

PROTO-1 has been licensed to Oricula Therapeutics, a company dedicated to developing the benzothiophene carboxamides as otoprotectants. Oricula reports that lead optimization has yielded analogues with 100 times higher potency than PROTO-1, a wide safety margin and efficacy in rat models of aminoglycoside-induced hearing loss. Investigational new drug (IND)-enabling studies are ongoing (Oricula Therapeutics and M. Gleser, personal communication).

Repurposing existing drugs

In addition to discovering novel compounds with therapeutic potential, zebrafish screens have proven useful for identifying novel uses for existing drugs. As the following examples illustrate, repurposing screens can provide an abbreviated path to clinical investigation because the compounds they identify have already been approved for human use or at least have been subjected to prior pharmacokinetic analysis and safety testing.

Fibrodysplasia ossificans progressiva
(FOP). A rare autosomal dominant condition caused by gain-of-function mutations in the gene encoding activin receptor-like kinase 2 (ALK2). These mutations result in chronic activation of the bone morphogenetic protein (BMP) pathway with resultant formation of ectopic bone in muscle tissue. Restriction of the thoracic skeleton then leads to respiratory failure, usually in childhood.

Anaemia of inflammation
A form of anaemia that is characterized by a block in iron availability for haematopoiesis and is observed in many chronic inflammatory diseases.
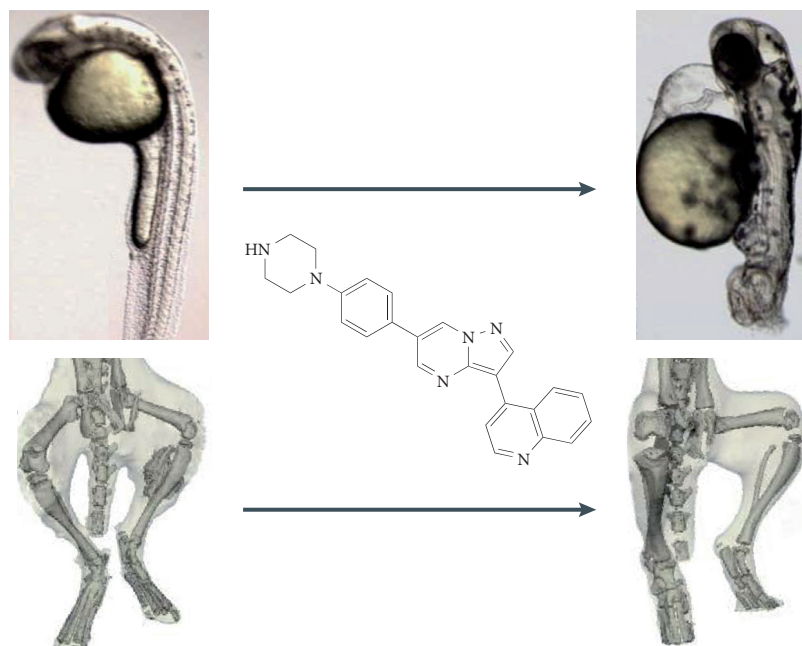
Figure 2 | **BMP pathway inhibitors discovered by *in vivo* screens in zebrafish.** A screen for disruptors of early embryogenesis identified a pyrazolopyrimidine called dorsomorphin that dorsalized zebrafish embryos by inhibiting the zebrafish orthologue of the bone morphogenetic protein (BMP) receptor activin receptor-like kinase 2 (ALK2). Dorsomorphin derivatives such as LDN-193189 (shown) have since been shown to be efficacious in several mammalian models of BMP-associated diseases, including the bone overgrowth disorder fibrodysplasia ossificans progressiva (FOP). Figure from REF. 25 and REF. 44, Nature Publishing Group.

Long QT syndrome
A disorder in which restoration of the membrane potential to equilibrium after a cardiac action potential is delayed as a result of abnormal ion fluxes. This delay can be detected from the simple measurement, on a surface electrocardiogram (ECG), of the time from depolarization onset until the return to baseline membrane potential (the QT interval).

2:1 atrioventricular heart block
A cardiac rhythm disorder characterized by the failure of every second electrical impulse to propagate from the atrium to the ventricle. This can result from extreme forms of the long QT syndrome in which the delay in restoration of the membrane potential in the ventricle is such that it is refractory to the next electrical impulse from the atrium.

COX inhibitors in leukaemia. A zebrafish model of acute myeloid leukaemia (AML) was generated by transgenic expression of the human AML oncogene *AML1–ETO*[51]. Transgenic zebrafish accumulated myeloid blast cells reminiscent of those from patients with AML. A zebrafish screen of existing drugs identified cyclooxygenase (COX) inhibitors as potent suppressors of the leukaemia-like phenotype and implicated WNT–β-catenin signalling in leukemogenesis[52]. Subsequent studies in mice confirmed that WNT–β-catenin signalling is required for self-renewal of leukaemia stem cells[53]; moreover, treatment of murine AML models with COX inhibitors suppressed leukaemia xenograft formation and inhibited *in vivo* progression of transplanted human leukaemia cells[27]. Because COX inhibitors are clinically approved and well tolerated in humans, it was possible to quickly carry out a Phase I trial to test the clinical hypothesis that COX inhibitors would provide a therapeutic benefit in patients with AML[54].

Glucocorticoids in long QT syndrome. A zebrafish model of genetic long QT syndrome (LQT syndrome), generated by a mutation in the *kcnh2* gene, exhibits prolonged action potential duration and 2:1 atrioventricular heart block[55]. A zebrafish screen of existing drugs identified the glucocorticoid flurandrenolide as a potent suppressor of the LQT-like phenotype and implicated glucocorticoid signalling in LQT syndrome. High-resolution physiology, comparable to that in mammalian models,

was used to demonstrate that glucocorticoids shorten action potential duration in LQT hearts[55]. Because glucocorticoids are clinically approved and well tolerated in humans, it was possible to quickly test the clinical hypothesis that glucocorticoids would provide therapeutic benefit in patients with LQT syndrome. A clinical trial of the glucocorticoid dexamethasone in patients with LQT syndrome was initiated at Massachusetts General Hospital, USA; early results are consistent with the hypothesis that dexamethasone may shorten QT intervals in humans (D. Milan, personal communication).

Challenges in determining mechanisms of action

As with other types of phenotype-based small-molecule discovery, determining the mechanism of action (MOA) remains one of the most substantial hurdles for small molecules discovered in zebrafish screens. Although challenging, MOA studies are among the most rewarding aspects of phenotype-based drug discovery because they often reveal unexpected and transformative new biological insights into the disease under investigation. Other reviews have described the rich variety of computational, biochemical and genetic techniques that can be used to discover MOAs[56]. Each of these techniques can be used for small molecules discovered in zebrafish screens, and several excellent examples have been reported. For example, affinity chromatography coupled with mass spectrometry was used to identify the Aα subunit of protein phosphatase 2A (PP2A) as the target of perphenazine, an antipsychotic found to kill MYC-overexpressing thymocytes in a zebrafish model of T cell acute lymphoblastic leukaemia (T-ALL)[57]. Similarly, affinity chromatography was used to determine that mitochondrial malate dehydrogenase is the target of visnagin, a cardioprotective compound discovered in a zebrafish heart-failure screen[30].

Beyond the generic approaches to identify the MOA, there are zebrafish-specific tools that can aid in MOA determination. One of the most effective is the large collection of zebrafish phenotypes that have been associated with specific gene mutations and knockdowns. Identifying similarity between a drug-induced phenotype and a genetic phenotype can often provide clues as to the principal drug target or targets (FIG. 3). In the case of dorsomorphin described above, similarity to the *lost-a-fin* phenotype revealed the BMP pathway as the target of dorsomorphin. Embryos treated with dorsomorphin developed missing the ventral side of their tail fins — an unusual phenotype that almost perfectly replicates the phenotype of *lost-a-fin* mutants[44]. The ability of dorsomorphin to phenocopy *lost-a-fin* revealed that it inhibits ALK2, the protein disrupted by the mutation.

In a more recent example, a zebrafish embryogenesis screen identified kalihinol F as causing several distinctive developmental defects, including undulation of the notochord and defects in pigmentation, haematopoiesis and neural development[58]. This collection of phenotypes was identified as highly similar to those of *calamity,* a mutant with disrupted function of the copper-transporting ATPase *atp7a*. The kalihinol F/*calamity* phenocopy
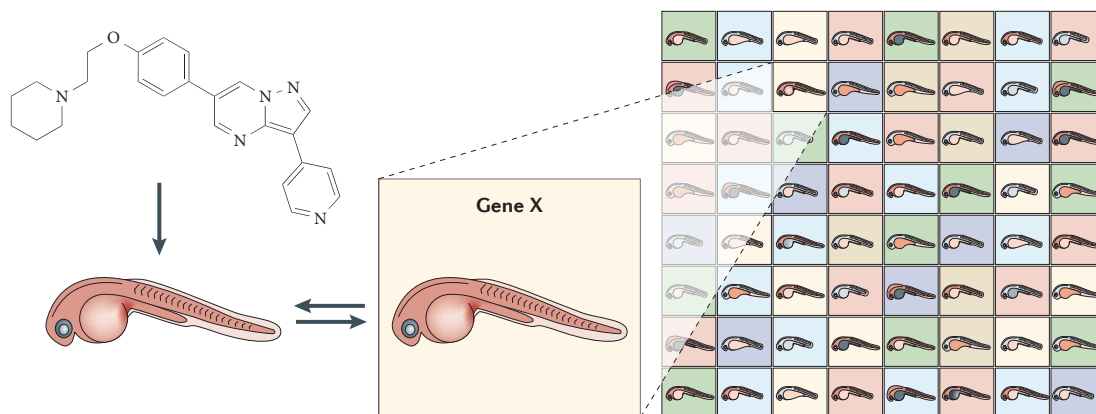
Figure 3 | **Zebrafish phenotypes reveal drug targets.** Drug-induced phenotypes in embryonic or larval zebrafish can be compared with databases containing thousands of mutation-associated zebrafish phenotypes. On several occasions, the discovery of phenotypic similarity between drug- and mutation-induced phenotypes has revealed the mechanism of action of a poorly understood compound.

led to the hypothesis that kalihinol F chelates copper, which was confirmed biochemically. In fact, the addition of exogenous copper was able to rescue the kalihinol F-induced developmental defects in zebrafish, and treatment with kalihinol F was able to rescue copper overload toxicity in zebrafish embryos and mammalian cells.

It should be noted that this type of multidimensional phenotype matching, or phenoclustering, in zebrafish can also be used to determine MOAs for compounds that were not originally discovered using zebrafish screens. Fumagillin, an anti-angiogenic natural product, was discovered more than 60 years ago, and its binding target, methionine aminopeptidase 2 (MetAP-2), was discovered more than 15 years ago. However, phenotype matching in zebrafish revealed a key insight into its MOA. Zebrafish embryos treated with fumagillin exhibited a distinctive gastrulation phenotype previously observed in zebrafish with mutations in the non-canonical Wnt5, revealing that MetAP-2 inhibition by fumagillin ultimately disrupts non-canonical WNT signalling downstream of the Frizzled receptor[59].

Because the number and variety of phenotypes that can be distinguished in a whole organism greatly exceed the number distinguishable in cultured cells, phenotype matching is a powerful way of determining small-molecule MOAs. As the number of described gene–phenotype pairs continues to rapidly increase, the utility of this approach should continue to grow. We anticipate that phenoclustering will be an important tool for MOA determination, not only for compounds discovered in zebrafish screens but also for drugs discovered by any other means. These observations also suggest the potential benefits of more systematic approaches to compound annotation in the zebrafish.

Other uses for zebrafish in drug development
Although much of the academic effort in zebrafish chemical biology has been focused on small-molecule discovery, it is by no means the only way in which zebrafish are contributing to drug discovery and development. In fact,

toxicology may be the most prevalent use of zebrafish in industry, with the majority of large pharmaceutical companies reporting some use of zebrafish for toxicology.

It should be noted that the role of zebrafish in toxicology is often different from that of mammalian species. Owing to the cost and effort associated with mammalian toxicology, it is often performed relatively late in preclinical development, with the goals of determining the safety of advanced preclinical lead compounds and obtaining regulatory approval for a clinical trial. By contrast, zebrafish toxicology is much less expensive and can be performed rapidly on large numbers of compounds in parallel. As such, zebrafish can be deployed much earlier in preclinical development. For example, toxicology testing can be performed on hundreds or thousands of hits from a primary high-throughput screen to eliminate toxic compounds at an early stage and to prioritize hits for further development. Indeed, this implicit counterscreen for toxicity is an integral part of disease suppressor screens in the organism. Therefore, zebrafish can perform an invaluable function in enabling toxic compounds to 'fail fast', before substantial resources have been wasted on their preclinical advancement. As suggested earlier, zebrafish screens may generate lead compounds from primary screens that are already 'pre-evaluated' for several major forms of toxicity.

Zebrafish have been shown to be a good model for predicting several types of drug toxicities (FIG. 4). Cardiotoxicity, for example, appears to be highly conserved between zebrafish and humans. Systematic studies of drugs that cause QT prolongation in humans show a >95% conservation of effect in zebrafish[19]. In the identification of clinically significant repolarization toxicity, this single assay performed as well as the combination of an assay for human ERG (hERG) inhibition and toxicological experiments on rabbit whole heart and canine Purkinje fibres. Similarly close correlations have been observed for hepatotoxicity, nephrotoxicity and reproductive toxicity, in which all of the known toxicants in preclinical mammalian models or humans have similar effects in zebrafish[60,61]. Nevertheless, it will require considerable
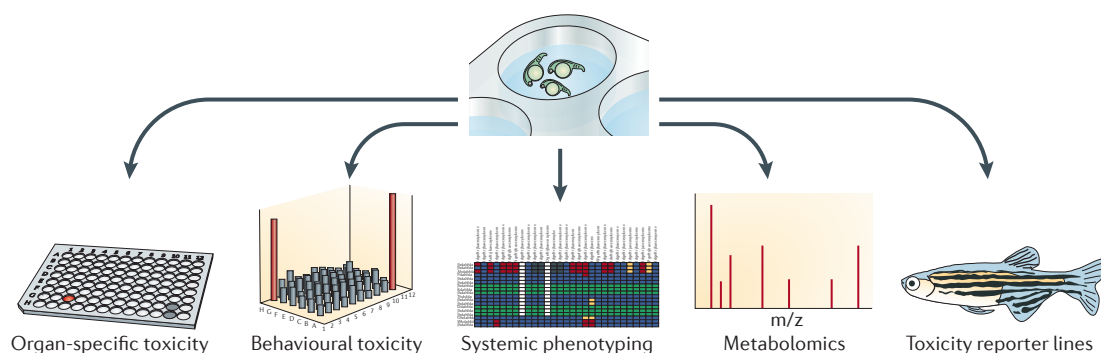
Figure 4 | **Use of zebrafish in toxicology.** Zebrafish have been validated for high-throughput screening focused on specific organ toxicity (liver, kidney and heart) or behavioural toxicity. Alternatively, they have been used to profile compounds systematically for toxicity across multiple systems simultaneously. New technologies, including metabolomic profiling and toxicity reporter lines, promise to extend the utility of zebrafish further. m/z, mass to charge ratio.

additional effort, and much higher-resolution assessment of the mechanisms of toxicity in each system, to establish the full extent of the homology between zebrafish and human toxicology.

Future opportunities for zebrafish

Technologies for working with zebrafish are evolving rapidly and opening new opportunities to contribute to improved drug development. Zebrafish are poised to make a difference in a number of areas in the near future, as outlined below.

Genome engineering to generate sensitized models for screening. The advent of transcription activator-like effector nucleases (TALENs) and CRISPR–Cas (clustered regularly interspaced short palindromic repeat–CRISPR-associated) nucleases has dramatically changed the landscape for zebrafish-based research. Within the past 2 years, CRISPR–Cas technology in particular has made it possible to rapidly generate targeted loss-of-function mutants and knock-in lines[62,63]. For the first time, it is now possible to recreate human disease alleles in zebrafish[64–67]. The potential impact of this advance on drug discovery is immense. With thousands of human disease-associated mutations being discovered by human geneticists, the challenge now is to figure out the functions of these mutations and determine how to translate that knowledge into new therapies. Because targeted mutants can be generated and phenotyped so much more efficiently in zebrafish than in rodents, zebrafish are likely to be a popular choice for testing the relevance of candidate disease genes.

As genome editing technologies continue to improve, it will be possible to reconstruct tens or hundreds of human mutations in zebrafish, followed by rapid assessment of their phenotypic effects. Perhaps more importantly, lines that are found to be disease-relevant can quickly form the basis of large-scale chemical suppressor screens. It has already been demonstrated that zebrafish screens can identify compounds that reverse the effects of genetic mutations[30,52,55,68–71]. Thus, any line that produces a disease-relevant phenotype could be quickly expanded and used to screen for chemical suppressors of the disease-associated phenotype.

Behavioural screens for novel neuroactive drugs. Diseases of the nervous system are arguably the area of greatest challenge for drug discovery. Despite the prevalence of nervous system disorders, there are generally few effective pharmacological therapies. Developing new central nervous system (CNS) drugs is slow, expensive, and fraught with failures. Challenges include the fact that there are few well-validated targets for most nervous system disorders. Furthermore, it is difficult to create and assess faithful models of many CNS disorders. Psychiatric end points such as depression and psychosis are difficult to assess in animals, and neurodegenerative end points are often slow to develop and laborious to quantify.

Zebrafish behavioural screens offer an intriguing alternative approach to CNS drug discovery. Zebrafish possess a rich repertoire of behaviours, including simple stimulus–response behaviours and more complex behaviours such as learning and sleep. Many of these behaviours have been shown to be amenable to assessment in a 96-well format, and high-throughput screens have discovered compounds that alter these behaviours in specific, reproducible ways[72–74].

Although zebrafish and human behaviours differ, the underlying circuits, cells and receptors are frequently conserved. Therefore, it may be possible to identify compounds that modulate disease-relevant processes by their ability to modulate zebrafish behaviours controlled by homologous pathways. For example, it may be difficult to model schizophrenia in a zebrafish or to assess schizophrenia-like symptoms directly. However, most of the proteins and cell types associated with human schizophrenia are conserved in zebrafish[75,76]. Antipsychotic drugs would be expected to modulate such proteins and cell types, causing specific and distinctive behavioural changes in zebrafish. With a sufficiently granular understanding of the correlations between human disease and zebrafish behaviour, it would theoretically be possible to identify neuroactive drugs simply by screening for compounds that produce the desired behavioural profile in zebrafish.

For such an approach to work, the challenge will be to determine what behavioural phenotypes are indicative of a therapeutic candidate. Two approaches seem
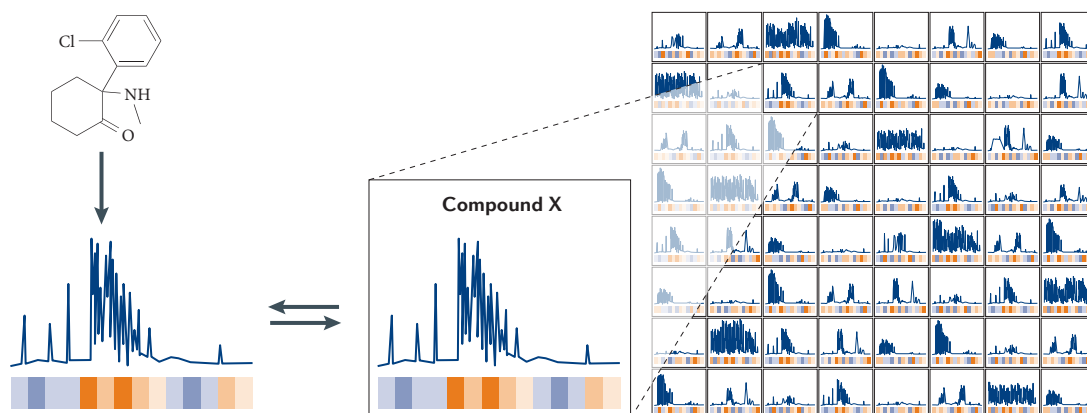
Figure 5 | **Shared behavioural phenotypes reveal shared mechanisms and utilities.** Neuroactive compounds often produce distinctive behavioural changes in zebrafish that can be quantified in high-throughput behavioural assays. As databases of compound-induced behavioural profiles grow, it is becoming possible to identify mechanistic relationships between compounds and search large chemical libraries for desirable profiles that indicate therapeutic potential for nervous system disorders.

promising in this regard. The first is to use existing drugs to establish what the behavioural profile is for a particular therapeutic class. It has been shown that some classes of known neuroactive drugs produce distinctive and reproducible behavioural signatures in zebrafish behavioural assays[72,73]. Establishment of a behavioural signature for a therapeutic class would enable subsequent high-throughput screening for novel compounds that produce a similar behavioural effect in zebrafish (FIG. 5). A second potential approach is to use genome engineering to recreate human disease-associated mutations in zebrafish, then phenotype these animals carefully for any resulting behavioural deficits. Mutant lines could then be subjected to behavioural screening to identify compounds that suppress the abnormal behaviour and return the behavioural profile to normal.

Unresolved questions and remaining challenges

The zebrafish has filled the niche for a screenable vertebrate in biology and drug discovery, but the emergence of the model and its rational appraisal as a tool for drug discovery has also served to highlight several challenges that remain. One of the most substantial issues is the challenge of controlling and quantifying drug exposures. Although it is easy to control the concentration of a drug in the water bathing the zebrafish, it is more difficult to predict how much drug will be absorbed. Some drugs appear to be absorbed poorly, whereas others are taken up readily. Drug levels are often measured directly in serum or tissues of mammalian models, but such measurements are more challenging to make in microscopic zebrafish, especially for high-throughput applications. Although some physicochemical attributes of compounds, especially logP, have been shown to be predictive of drug uptake in zebrafish, predictions are still imperfect. As a consequence, false negatives are possible in zebrafish screens, whereby a compound that could be a good drug candidate fails to produce a biological effect owing to poor uptake. Generally speaking, such

false negatives are not problematic for drug discovery screens, in which the occasional false negative is well tolerated. In toxicology screening, however, false negatives can be more troubling, as they could potentially result in a failure to identify a compound's significant toxicity. Improved methods for predicting or quantifying drug uptake could dramatically improve the utility of zebrafish for drug discovery.

Of course, several other challenges remain. Natural variation exists in many *in vivo* phenotypes, especially complex phenotypes such as animal behaviours. Creating robust, high-throughput assays to measure such phenotypes is challenging, but has been overcome in a variety of creative ways by numerous different research groups (TABLE 1). Another challenge is producing enough zebrafish to enable truly large-scale, high-throughput screens. This can be particularly difficult when working with fragile or inbred transgenic lines. To date, most screens have involved production and screening of a few hundred to tens of thousands of zebrafish per day[39]. Improved methods for mass production and handling of zebrafish embryos would be highly beneficial to the field.

Moving forward, substantial innovation and investment will be required to generate phenotypes that are both relevant to human disease traits and scalable for high throughput. Even as genome editing enables truly mechanistic modelling in different organisms (overcoming some of the limitations inherent to target choice), the development of adequately characterized phenotypes will be a rate-limiting step in zebrafish chemical screens. It will also be important to ensure that the precise details of each screen are well documented, as numerous variables can influence screen output. These include the well volume, timing and mode of exposure, temperature, lighting, pH, assay sensitivity and specificity, and linearity of assay response characteristics. As experience accumulates, assays will hopefully become standardized, and more formal

relationships with other preclinical animal models and with quantitative metrics of human efficacy or toxicity will be established.

A new scale and efficiency of drug discovery is necessary to move towards the individualized preventive therapeutics that medicine will demand in the future. To achieve these ends, diseases must be stratified and specific drugs must be tailored for each new disease 'entity'. As we intervene earlier in disease, we will also require nuanced drug discovery with the goal of pathway normalization rather than target inhibition. Successful integration of the zebrafish with traditional approaches to drug development will help to realize this vision of medicines that empirically balance efficacy and toxicity, facilitating the discovery of new classes of therapeutics.

Note added in proof

While this review was going to press, several important zebrafish chemical screens were published, including screens for modifiers of hedgehog signalling[102], cell migration[103], liver tumorigenesis[104], marrow engraftment[105], and pancreatic β-cell mass[106].

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