

Fishing for Drugs from Nature: Zebrafish as a Technology Platform for Natural Product Discovery

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Abstract

Emerging challenges within the current drug discovery paradigm are prompting renewed interest in natural products as a source of novel, bioactive small molecules. With the recent validation of zebrafish as a biomedically relevant model for functional genomics and *in vivo* drug discovery, the zebrafish bioassay-guided identification of

natural products may be an attractive strategy to generate new lead compounds in a number of indication areas. Here, we review recent natural product research using zebrafish and evaluate the potential of this vertebrate model as a discovery platform for the systematic identification of bioactive natural products.

Current Trends and Challenges in Natural Product Discovery

Natural products – referring here to small molecules produced by living organisms, particularly secondary metabolites – are an excellent yet currently underutilized source of chemical diversity for drug discovery. Bioactive compounds isolated from plants, fungi and bacteria have given rise to a wide range of human therapeutics, and numerous others are effective tools in cell biology, biochemistry and pharmacology. Despite this record of success, the field of natural product research has nonetheless witnessed a significant decline in the past two decades, due in large measure to the emergence of target validation, combinatorial chemistry and high-throughput screening as a new paradigm for drug discovery [1]. Several challenges unique to natural products have contributed to this trend as well, including the difficulties associated with isolating pure compounds from crude extracts, identifying their mechanism of action, and synthesizing these oftentimes highly complex structures [2], [3].

Conversely, the overall effectiveness of target-driven drug discovery has recently been called into question [4], especially with respect to the limitations of combinatorial chemistry in terms of providing sufficient structural diversity for the identification of bioactive, drug-like compounds. Furthermore, there is a growing appreci-

ation that natural products are privileged structures that have been evolutionarily selected on the basis of their ability to functionally interact with biological macromolecules [3]. Interest in natural products as a complementary, promising source of novel leads for drug discovery will therefore likely continue to expand.

Another drawback to target-driven drug discovery is the reduced complexity of screening assays – usually involving the ultra-high-throughput analysis of compound interactions with single targets, but too often giving rise to lead compounds that do not perform well *in vivo* [4]. The increasing awareness of this limitation is fueling interest in novel, high-content bioassays which enable target-independent screening and function-based identification of small molecules. Although cell-based screening assays using pathway-specific reporters are already a step in the right direction, bioassays based on intact, living organisms such as zebrafish provide an additional degree of physiological relevance that is difficult to match *in vitro*.

Advantages of Zebrafish as a Model Organism

Zebrafish have recently emerged as a powerful model for the rapid analysis of gene function and small-molecule bioactivity in a variety of in-

dications [5]. Because of their high genetic, physiological and pharmacological similarity to humans, zebrafish are well-suited for the identification of bioactive natural products with therapeutic potential. The primary advantages of zebrafish as a model organism include the small size of embryos and larvae with which most tests can be performed (1–5 mm, depending on developmental stage), the high fecundity of adult zebrafish (hundreds of offspring per breeding pair per week), the optical transparency of embryos and larvae (allowing easy visualization of internal organs and tissues), and the speed at which these develop *ex utero* (● Fig. 1). These features combine to create an ideal *in vivo* model suitable for medium-throughput phenotypic screening in microtiter plates. With regard to testing the bioactivity of small molecules, a key advantage of using zebrafish embryos and larvae is that compounds can simply be added to the (non-sterile) water surrounding them, further increasing screening throughput (for many phenotypic assays, hundreds of samples can be tested per day per investigator).

The zebrafish (*Danio rerio*) – a diploid minnow of the teleost family Cyprinidae, indigenous to the Ganges River Basin – was first established as a genetic model in the 1970s by Streisinger and colleagues [6]. This species was subsequently exploited for large-scale forward genetic screens by the laboratories of Nüsslein-Volhard and Driever using ENU mutagenesis [7], [8], and by Hopkins and colleagues using retroviral insertional mutagenesis [9]. While the primary purpose of these screens was to elucidate the genetic mechanisms of vertebrate development, several were designed to identify biomedically relevant genes, such as those regulating the growth, differentiation and function of tissues and organs. Examples include ENU mutagenesis screens for the isolation of zebrafish mutants with defects in cartilage development [10], hematopoiesis [11], and cardiovascular development [12]. More recent forward genetic screens include assays for gastrointestinal function [13], vascular development [14], and epilepsy [15].

Within the past decade, zebrafish have also become a versatile vertebrate model for reverse genetics. Antisense morpholino phosphorodiamidate oligonucleotides [16] – commonly referred to as “morpholinos” – are effective tools in zebrafish for the specific inhibition of gene expression [17]. Dose-dependent “knock-downs” of gene expression can be achieved by the microinjection of morpholinos into single-cell-stage embryos (● Fig. 1b), wherein the antisense oligonucleotides can be designed to inhibit either the translational initiation of mRNAs or the splicing of hnRNAs. Numerous genes have been functionally analyzed in zebrafish in this manner [18], including several identified within the context of large-scale reverse genetic screens [19], [20], [21]. In addition, it is now possible to use morpholinos for inducible gene knockdowns, via the light-activated cleavage of photolabile complementary oligonucleotides that block their ability to bind their target RNA [22]. Conversely, the efficacy of siRNA in zebrafish embryos remains a topic of debate [23], [24], although it was recently shown that shRNA expressed from an integrated transgene effectively inhibits gene expression in developing embryos [25].

Transgenesis methods in zebrafish are well-established, allowing the straightforward generation of transgenic lines expressing fluorescent reporter proteins under the control of tissue-specific promoters. Insertional mutagenesis screens using enhancer-trap and gene-trap transposons and retroviral vectors are also providing many novel reporter lines [26]. Collectively, this growing collection of transgenic zebrafish lines represents a powerful resource for the systematic elucidation of organogenesis, and for many aspects of cell biology to be performed *in vivo* [27]. In the area of angiogenesis, for example, transgenic lines have been used for the detailed anatomic analysis of embryonic blood vessel formation [28], as well as to carry out both genetic [14] and compound screens [29] for the identification of novel genes and small molecules modulating these processes.

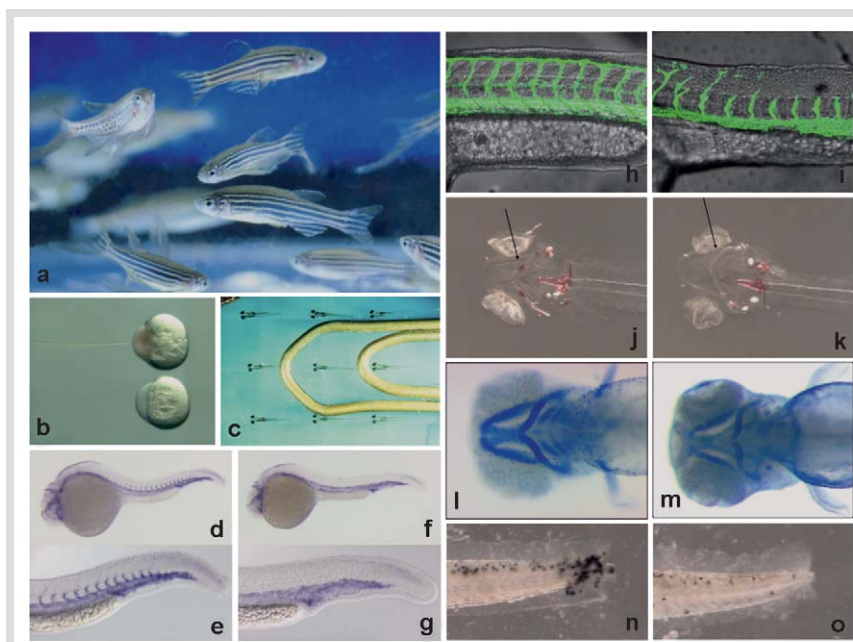


Fig. 1 Zebrafish as a model organism for drug discovery. a Adult zebrafish; b microinjection of one-cell stage embryos; c agarose-embedded larvae at 5 days post-fertilization (dpf), revealing size in relation to paperclip; d, e visualization of developing vasculature in embryo at 30 hours post-fertilization (hpf) by whole-mount *in situ* hybridization with *flk-1* RNA probe, revealing outgrowing intersegmental vessels (ISVs); f, g embryo microinjected with antisense morpholino oligomer (MO) targeting vascular endothelial growth factor (VEGF), exhibiting lack of ISV outgrowth; h visualization of developing vasculature in transgenic *fli-1:eGFP* embryo at 40 hpf by laser multiphoton confocal microscopy; i embryo treated with PI3K inhibitor wortmannin, exhibiting reduced ISV outgrowth; j visualization of bone formation in larva at 10 dpf by whole-mount Alizarin red staining; k larva treated with glucocorticoid prednisolone, exhibiting reduced bone formation; l visualization of cartilage formation in untreated embryo at 3 dpf by whole-mount Alcian blue staining; m embryo microinjected with antisense MO targeting gene involved in craniofacial development, exhibiting reduced staining of, and/or missing, cartilage elements;

n visualization of leukocyte migration by whole-mount myeloperoxidase staining in larva at 4 dpf with tail fin injury; o larva with tail fin injury treated with the crude methanolic extract of an anti-inflammatory East African medicinal plant (Fabaceae family), exhibiting reduced leukocyte migration to site of injury.

Recent innovations in zebrafish transgenesis include an elegant approach for targeted ablations – e.g., for the generation of zebrafish larvae lacking pancreatic beta cells, which can then be observed to regenerate – through the tissue-specific expression of bacterial nitroreductase followed by incubation of the transgenic zebrafish embryos in metronidazole [30]. In addition, a novel technique has been reported for the induction of transgene expression in zebrafish using small-molecule agonists of the insect-specific ecdysone receptor (EcR) [31], [32]. This approach allows for the dose-dependent regulation of transgene activity simply through the addition of EcR agonists to the embryo medium – an elegant tool which enables the precise temporal control of gene expression and which will therefore facilitate the molecular dissection of signaling pathways during embryonic development.

As with any experimental system, there are clearly also limitations in working with zebrafish as a model organism. First, given that most screens are carried out at early life stages, it is important to consider the physiological differences between embryonic or larval fish and adult mammals. In any case, bioactive small molecules identified in zebrafish should ideally be validated in an appropriate rodent assay before being prioritized as lead compounds. Second, depending on their molecular weight and hydrophobicity, not all small molecules are readily absorbed by embryos and larvae, possibly leading to false negatives during screening. Finally, given that zebrafish are vertebrates, country-specific animal rights legislation and institutional bioethics regulations need to be taken into account when planning experiments. It is worth noting that there are several jurisdictions which do not consider unhatched fish embryos to be vertebrates in the full legal sense, enabling certain large-scale screens to be carried out with minimal administrative requirements.

Zebrafish-Based Analysis of Natural Products and other Small Molecules

Zebrafish were first proposed as an *in vivo* model for small-molecule drug discovery over fifty years ago [33]. This initial study investigated the utility of zebrafish embryos and larvae for screening both natural products and synthetic compounds, especially with respect to their effects on cell division and differentiation. In addition to numerous studies over the following decades focusing on the detection or characterization of environmental contaminants [34], [35], developing zebrafish embryos have been used to determine the embryotoxicity or teratogenicity of several natural products consumed by humans, including various flavonoids [36]; delta-9-tetrahydrocannabinol, the major psychoactive constituent of marijuana [37]; and arecoline, the major alkaloid in betel nuts [38]. In a related approach, adult zebrafish were recently utilized to confirm the piscicidal properties of aryl-naphthalide lignans isolated from *Phyllanthus piscatorum*, an ethnomedicinal plant used by the Amazonian Yanomami as a fish poison and antifungal remedy [39], [40]. Despite an appreciable history of pharmacological investigation dating back over half a century, it is only within the past decade that large-scale screens of small molecules have been performed using zebrafish-based assays. Initial compound screens have focused on the analysis of combinatorial chemistry libraries – encompassing up to 20,000 molecules – for their ability to induce a variety of developmental phenotypes [41], [42], [43]. More recently, small-molecule screening in zebrafish has been em-

ployed to identify synthetic compounds acting as suppressors of aortic coarctation, a congenital heart defect [44], as modulators of pigmentation [45], [46], [47], as cell cycle inhibitors [48], as regulators of heart rate [49], and as modulators of tissue regeneration [50].

Not all large-scale screens performed in zebrafish have been designed to find potential therapeutics, however. For example, bioactive small molecules can also be used to induce index phenotypes – i.e., disease-like states – which can subsequently be exploited in modifier screens to look for genetic or chemical suppressors of this phenotype. Using this approach, compounds have been identified that induce reversible hemolytic anemia [51]. In a related approach, compounds were screened for their ability to act as radiation sensitizers [52]. Taken together, these studies have clearly established zebrafish as a facile model for *in vivo* drug discovery [5], [53].

Zebrafish-Based Analysis of Plant Extracts

With respect to natural product discovery, no large-scale screens of compounds of natural origin have yet been reported in zebrafish. In addition, beyond the natural products mentioned above as having been analyzed in zebrafish, very few zebrafish-based studies of plant extracts have been reported to date. Furthermore, the potential of *in vivo* zebrafish assays for the bioactivity-guided fractionation of complex natural extracts is only beginning to be explored.

One of first reports in this direction describes the use of transgenic zebrafish to characterize the pro-angiogenic properties of the East Asian medicinal plant *Angelica sinensis* (dong quai), but does not mention the isolation of any bioactive constituents. In this study, Lee and colleagues first demonstrate that an *Angelica sinensis* extract stimulates proliferation, migration, and tube formation in human umbilical vein endothelial cells (HUVECs). They then substantiate these findings through the observation of increased subintestinal vessel (SIV) growth in extract-treated *fli-1:EGFP* transgenic zebrafish, which have endothelial cell-specific expression of green fluorescent protein [54].

Another report, presented at the 2006 American Society of Pharmacognosy Meeting, goes one step further with respect to isolating bioactive constituents. Here, Xu and colleagues report the bioactivity-guided purification of three anti-angiogenic compounds from the anti-inflammatory Chinese medicinal plant *Tripterygium wilfordii* (lei gong teng), in this case using whole-mount histochemistry (alkaline phosphatase staining) to visualize blood vessel development in zebrafish embryos [55]. These three molecules include the known angiogenesis inhibitors triptolide (a diterpene triepoxide with immunosuppressive properties and inhibitory activity on NF- κ B) and celastrol (a triterpenoid with anti-inflammatory and antioxidant activity), as well as cangoronine (a triterpenoid with no previously reported bioactivity, but with only marginal anti-angiogenesis activity in this zebrafish assay).

At the 2007 European Zebrafish Genetics and Development Meeting, Patton and colleagues describe the zebrafish bioassay-guided fractionation, using the induction of an early embryonic phenotype as an assay, to partially isolate a cycloamine-like activity from a crude plant extract [56]. It is worth noting that cycloamine, a steroidal jerveratrum alkaloid, was originally isolated from *Veratrum californicum* (corn lilly) by virtue of its ability to induce cyclopia in newborn sheep, and that more recently the

mechanism of action of this teratogen was found to be the potent inhibition of hedgehog signaling [57]. Because of this specific activity, cyclopamine is now an important reagent for the control of embryonic stem cell differentiation *in vitro* [58]. Given the important role of aberrant hedgehog signaling in several human cancers (including basal cell carcinoma, medulloblastoma and rhabdomyosarcoma), these findings qualify cyclopamine and similarly acting natural products as attractive lead compounds for the development of novel anti-cancer therapeutics [59]. Indeed, the zebrafish-based screening of dietary alkaloids recently revealed several of these – most of them similar in structure to cyclopamine – to have similar activities as inhibitors of hedgehog signaling [60]. As such, using zebrafish embryos as an *in vivo* bioassay may be a promising strategy for the identification of novel natural products that inhibit hedgehog signaling or other developmental pathways relevant for human disease.

In order to further examine the potential of zebrafish as a platform for natural product discovery, our laboratory recently initiated an effort based on the zebrafish-mediated screening and bioactivity-guided fractionation of crude plant extracts [61]. Using high-performance thin-layer chromatography (HP-TLC) in combination with high-resolution electrospray ionization mass spectrometry (HR-ESI-MS) and chemical database analysis, we were able to rapidly isolate emodin and an abietane diterpenoid, coleon A lactone, from the crude methanolic extracts of two East African medicinal plants (*Oxygonum sinuatum* and *Plectranthus barbatus*, respectively). The zebrafish assay used for the activity-guided isolation of these two compounds examined their ability to potentiate a sub-effective dose of the anti-angiogenic compound SU5416, an indoline inhibitor of the vascular endothelial growth factor (VEGF) receptor [62], in *fli-1:EGFP* transgenic embryos. In this context it is worth noting that emodin, an inhibitor of the protein kinase CK2 [63], is known to restrict angiogenesis both *in vitro* [64] and *in vivo* [65], and that coleon A lactone was subsequently also found by our investigation to inhibit endothelial cell proliferation and tube formation *in vitro*. This study illustrates the utility of zebrafish, in combination with HP-TLC and HR-ESI-MS, for the rapid and cost-effective bioactivity-guided isolation of natural products from crude plant extracts. The primary advantages of this approach are (1) the requirement for only sub-milligram quantities of crude extracts, fractions, and pure compounds for an *in vivo* analysis of bioactivity, thereby avoiding the use of preparative column chromatography during initial screening stages; and (2) the rapid identification of known compounds, thereby achieving cost-effective dereplication early in the discovery process and facilitating the prioritization of laboratory resources for the pursuit of novel molecules. Furthermore, it highlights the potential of performing natural product discovery in zebrafish using screening assays designed to identify novel modulators of specific signaling pathways and/or novel potentiators of existing drugs.

Chemical Genetics in Zebrafish

▼
A key challenge in natural product discovery is the identification of the mechanism of action, and ultimately the biological target, of bioactive compounds. Chemical genetics – the elucidation of biological function through the use of small molecules – is a particularly powerful strategy in this regard, and is finding increasing application in a number of model systems. Zebrafish represent a powerful system with which to explore the mechanism

of action of natural products and other small molecules, as demonstrated in several recent reports described below. It is worth noting that such studies usually rely on a combination of experiments in zebrafish (for phenotypic data to ascertain pathway specificity) and mammalian cells (for molecular and biochemical data to confirm these findings).

One of these studies identified the biological target of apratoxin A, a cyclodepsipeptide cytotoxin isolated from the marine cyanobacterium *Lyngbya majuscula* [66]. Apratoxin A is one of several cyanobacterial metabolites with potent anti-tumor activity, and although two of these (cryptophycin and dolastatin 10) have been shown to bind microtubules, the other cyanobacterial cytotoxins remain elusive in terms of their mode of action. In an elegant approach, Luesch and colleagues first combined gene expression profiling and genome-wide, arrayed cDNA overexpression to reveal that apratoxin A induces apoptosis in mammalian cells via the modulation of fibroblast growth factor (FGF) signaling. After being shown to inhibit the phosphorylation-dependent activation of STAT3, a downstream effector of FGF signaling, apratoxin A was then found to affect FGF-dependent developmental processes in zebrafish embryos. These phenotypic defects were similar to those induced by the FGF receptor inhibitor SU5402, and included the downregulation of the FGF target gene *mkp3* [67].

Another recent study using zebrafish focused on the mechanism of action of fumagillin, a sesquiterpene antibiotic isolated from the airborne saprophytic fungus *Aspergillus fumigatus* [68] and currently used to treat microsporidiosis in humans and honeybees. Fumagillin and its analogue *O*-(chloroacetyl-carbamoyl)fumagillol (TNP-470) have been characterized as potent angiogenesis inhibitors [69], and an *N*-(2-hydroxypropyl)methacrylamide (HPMA) copolymer conjugate of TNP-470 is currently in late-stage preclinical development as an anti-angiogenesis drug for the treatment of solid tumors [70]. Despite its known inhibition of the methionine aminopeptidase MetAP-2 [71], this interaction alone did not fully explain fumagillin's biological properties. To further explore how the restriction of MetAP-2 activity modulates angiogenesis, Crews and colleagues treated developing zebrafish embryos with TNP-470, inducing a truncated tail dysmorphology that through phenotype database analysis was found to be highly reminiscent of the Wnt5a mutant *pipetail*. A similar phenotype was induced through antisense morpholino oligonucleotides directed against MetAP-2, suggesting a functional link between MetAP-2 and Wnt5a signaling. Further experiments in mammalian cells indicated that this interaction occurs in the context of non-canonical Wnt signaling (i.e., via the receptor Frizzled2), and that the point of action of TNP-470 is downstream of Frizzled2 and upstream of the non-canonical Wnt effectors CamKII, c-Jun, and RhoA [72], [73].

Behavioral assays in zebrafish have also recently been used to examine the mechanism of action of salvinorin A, a physchoactive *trans*-neoclerodane diterpenoid isolated from *Salvia divinorum* [74]. This Lamiaceae species has a long history of traditional use as an entheogen by Mazatec shamans in Central America – an ethnomedicinal application supported by the fact that salvinorin A is a potent kappa-opioid receptor agonist and currently the most powerful known hallucinogen of natural origin. By examining the swimming behavior and conditioned place preference of salvinorin A-treated adult zebrafish, it was possible to ascertain reinforcing properties that were fully reversible by both nor-binaltorphimine (a kappa-opioid receptor antagonist) and rimonabant (a CB1 cannabinoid receptor antago-

nist), implicating both pathways in mediating the rewarding effects of salvinorin A and further establishing zebrafish as a useful model for addiction research (see also [75]).

A further example of the power of chemical genetics in zebrafish is the recent description of dorsomorphin, a novel small-molecule inhibitor of bone morphogenic protein (BMP) signaling [76]. In this study, Peterson and colleagues screened 7,500 synthetic compounds, identifying one that perturbed dorsoventral axis formation in early embryos – a phenotype highly reminiscent of transgenic zebrafish overexpressing *noggin* (an extracellular inhibitor of BMP) and *lost-a-fin* mutants (with a loss-of-function allele of the BMP receptor ALK8). Rescue experiments in zebrafish embryos showed that dorsomorphin suppressed the ventralized phenotype induced by antisense-mediated inhibition of *chordin* (another BMP inhibitor) – an elegant confirmation of dorsomorphin's own ability to block BMP signaling. Subsequent biochemical analysis of BMP pathway components in dorsomorphin-treated mammalian cells confirmed that this compound specifically affects SMAD 1/5/8-dependent signaling and not MAPK activity, both of which are stimulated by BMP type I receptor activation. As expected from an inhibitor of BMP signaling, dorsomorphin-treated zebrafish larvae exhibited reduced bone mineralization. Finally, dorsomorphin proved its utility as a new pharmacological tool in mice, helping to elucidate the role of BMP signaling in iron homeostasis.

Zebrafish Assays Useful for Natural Product Discovery

Beyond the plethora of bioassays mentioned above and in **Table 1**, we would like to highlight three areas of particular applicability for zebrafish-mediated natural product discovery: immunity and inflammation, epilepsy, and cardiotoxicity. Despite their clear *in vivo* relevance, rodent-based assays in these areas have inherent disadvantages in terms of cost and throughput that render them less suitable as a primary screening platform for the discovery of novel, bioactive small molecules. Zebrafish larvae, on the other hand, are an ideal *in vivo* system for such applications, and are clearly appropriate for medium-throughput screening of plant extracts and natural products.

Immunity and inflammation

With potential utility for the discovery of novel antimicrobial agents, several zebrafish models for bacterial pathogenesis have been reported, most of which rely on the use of *Mycobacterium marinum* [77], [78]. Other studies have examined the pathogenesis and inflammatory response of zebrafish embryos to bacterial infection, for example, by *Edwardsiella tarda*, which results in the significant upregulation of interleukin-1 β and TNF α as determined by qRT-PCR [79]. Moving towards an easier and lower-risk model for the innate immune response to bacterial infection *in vivo*, Watzke and colleagues have recently shown that bacterial lipopolysaccharides (LPS) are able to induce a very similar upregulation of these two pro-inflammatory cytokines in zebrafish embryos [80].

Zebrafish larvae are now also well-established as an *in vivo* model for inflammation. In 2001, Lieschke and colleagues first showed that leukocytes, including both circulating granulocytes and macrophages, could be readily visualized in zebrafish larvae though whole-mount histochemical staining for myeloperoxidase activity within the first 48 hours after fertilization, and

that transection of part of the larval tail stimulated the migration and accumulation of leukocytes within hours after injury [81]. More recently, two other groups have generated transgenic lines expressing EGFP under the control of the myeloperoxidase promoter, enabling the direct visualization of live leukocytes and therefore the observation of the inflammatory response in real time [82], [83]. In combination with other possibilities to analyze the inflammatory response, for example, using transgenic embryos with EGFP-expressing early macrophages [84], the zebrafish clearly has the potential to be an effective system for the identification of anti-inflammatory natural products and other small molecules.

Epilepsy

Zebrafish have recently also emerged as a novel experimental model of epilepsy [85]. Baraban and colleagues first described the response of zebrafish larvae to pentylentetrazol (PTZ), a convulsant widely used to induce seizures in rodents [86]. This investigation confirmed the ability of PTZ to induce a dose-dependent series of stereotypical behaviors, including clonus-type convulsions characteristic of epilepsy in mammals. Electrophysiological recordings from the brains of PTZ-treated zebrafish larvae indicated epileptiform discharges that were reduced through the application of anti-epileptic drugs such as valproate and diazepam. Baraban and colleagues next performed a forward genetic screen to identify zebrafish mutants insensitive to the effects of PTZ, with the goal of elucidating the genetic basis of seizure resistance [15].

In order to facilitate the discovery of novel anti-epileptic drugs, Goldsmith and colleagues have recently developed an automated tracking system to monitor the movement of zebrafish larvae in microtiter plates [87]. Using this methodology, 13 of 14 known anti-epileptic drugs were found to suppress PTZ-induced behavioral abnormalities, thereby validating the suitability of zebrafish for the high-throughput screening of potential anti-epileptic compounds. Intriguingly, domoic acid (DA, a phycotoxin produced by *Pseudo-nitzschia* diatoms) was recently found to increase the susceptibility of zebrafish larvae to PTZ, further confirming the role of DA in seizure induction in marine mammals exposed to algal blooms [88]. Taken together, the progress in this area underscores the potential of zebrafish as a promising *in vivo* bioassay for the discovery of anti-epileptic natural products.

Cardiotoxicity

Another noteworthy advantage of using zebrafish larvae for compound screening is that various aspects of cardiotoxicity can be investigated at a very early stage in the drug discovery process. Cardiotoxicity is one of the more common adverse effects seen for many new chemical entities, and the clinical development of numerous drug candidates has been discontinued because of their tendency to induce cardiac arrhythmias such as QT prolongation and torsades de pointes. One of the primary causes of cardiotoxicity are unforeseen interactions of compounds with the HERG potassium channel (the protein product of the *KCNH2* gene, and also referred as the $K_v11.1$ potassium channel), responsible for the repolarizing I_{Kr} current in the cardiac action potential. Despite this appreciable risk, drugs are normally only examined relatively late in pre-clinical testing using, for example, isolated guinea pig hearts, which requires multi-milligram amounts of each compound to be tested.

With their requirement for only microgram quantities of any given compound to be analyzed, combined with the ability to

Table 1 Overview of selected zebrafish assays with potential relevance for natural product discovery

Indication	Zebrafish Assay	Description	Reference	
Cancer	Oncogene-induced tumors	Heat-shock induction of Ras-dependent tumors in Cre/loxP transgenics	[93], [94]	
		Cre RNA induction of Myc-dependent tumors in loxP transgenics	[95]	
		T-cell leukemia induced by Myc overexpression	[108]	
	Tumor suppressor-induced tumors	Target-selected ENU mutagenesis screen for p53 mutations	[96]	
		Mutagen-induced tumors	Transplantation of isogenic tumor cells between clonal zebrafish lines	[97]
	Tumor imaging	Ultrasound imaging of liver tumors in adult zebrafish	[98]	
	Tumor metastasis	Transplantation of human tumor cells into zebrafish embryos	[99], [100], [101], [102]	
	Radiation sensitivity	Small-molecule screens for radiation sensitizers and protectants	[52], [103], [109]	
		Genomic instability	ENU mutagenesis screen for genomic instability mutants	[104], [105]
		Cell migration	Antisense screen for genes affecting primordial germ cell migration	[19]
		Cell cycle	Small-molecule screen for cell cycle inhibitors	[48]
			ENU mutagenesis screen for mitosis mutants	[105]
			Small-molecule screen for suppressors of mitosis mutant (<i>crash and burn</i>)	[106]
	Skeletal disorders	Cartilage formation	ENU mutagenesis screen for cartilage mutants	[10]
Osteoporosis		Glucocorticoid-induced reduction of bone formation; characterization of bone anabolic compounds	[110], [111]	
Blood disorders	Hematopoiesis	ENU mutagenesis screen for hematopoiesis mutants	[11]	
		Small-molecule screen for inducers of reversible hemolytic anemia	[51]	
	Thrombosis	Laser-induced thrombosis in larvae	[112]	
Cardiovascular disorders	Heart formation and function	ENU mutagenesis screen for heart and cardiovascular mutants	[12]	
	Angiogenesis	Small-molecule screen for suppressors of aortic coarctation mutant (<i>gridlock</i>)	[44]	
		ENU mutagenesis screen for angiogenesis mutants using transgenic reporter line	[14]	
		Small-molecule screen for anti-angiogenic compounds using transgenic reporter line	[28], [29]	
	Cardiotoxicity	Characterization of anti-angiogenic natural products in transgenic larvae	[107]	
		ENU mutagenesis screen for QT prolongation mutants	[92]	
		Characterization of QT-prolonging drugs in zebrafish larvae	[89], [90], [91]	
Small-molecule screen for modulators of heart rate		[49]		
Neurological disorders	Epilepsy	Validation of anti-epileptic drugs in larvae with PTZ-induced seizures	[86], [87]	
		ENU mutagenesis screen for seizure-resistant mutants	[15]	
	Addiction	Characterization of psychoactive drugs and drugs of abuse	[74], [75], [113]	
		ENU mutagenesis screen for differences in cocaine sensitivity	[114]	
		ENU mutagenesis screen for embryos/larvae with behavioral defects	[115]	
	Behavior and learning	Characterization of drug effects on non-associative learning in larvae	[116]	
		Automated detection of behavioral defects	[117]	
	Neurodevelopment	ENU mutagenesis screen for enteric nervous system defects	[118]	
		Axon myelination	ENU mutagenesis screen for axon myelination defects	[119], [120]
		Neurodegeneration	Transgenic line with overexpression of mutant human Tau protein	[121]
		ALS	Transient transgenic model with overexpression of mutant human SOD protein	[122]
Immunity and inflammation	Bacterial infection	Characterization of immune response to bacterially infected larvae	[77], [79], [80]	
		Small-molecule screening for antimicrobials in bacterially infected larvae	[78]	
	Inflammation	Histochemical visualization of leukocyte migration after injury	[81]	
		Transgenic zebrafish lines for visualization of leukocyte migration	[82], [83], [84]	
		Metabolic disorders	ENU mutagenesis screen for defects in digestive physiology	[13]
Diabetes	ENU mutagenesis screen for defects in pancreas formation using transgenic reporter lines	[123]		
	Liver disease	ENU mutagenesis screens for liver formation and hepatomegaly	[124], [125]	
Other	Pigmentation	Small-molecule screens for modulators of pigmentation and melanocyte differentiation	[45], [46], [47], [129]	
		Tissue regeneration	Small-molecule screen for modulators of tissue regeneration	[50]
	Hearing loss	Characterization of ototoxic and otoprotective agents on larval hair cells	[126], [127]	
	Circadian rhythms	ENU mutagenesis screen for circadian rhythm mutants	[128]	

Note that several mutant/transgenic lines are described above in terms of how they were generated (e. g., via ENU mutagenesis screens); the assays used for these screens and/or the mutant/transgenic lines themselves may be useful for characterizing screening natural products or other small molecules.

readily observe heart function in transparent larvae, zebrafish are well-suited as a front-line assay for cardiotoxicity. In 2003, two laboratories validated zebrafish larvae as a reliable model to test compounds for their ability to induce QT prolongation [89], [90], [91]. These studies showed that 22 out of 23 compounds known to cause QT prolongation in humans induced bradycardia or atrioventricular block in zebrafish, and that similar effects were observed by the antisense-mediated knockdown of Zerg (encoded by *kcnh2*, the zebrafish ortholog of human *KCNH2*, both of which share 99% amino acid identity in the pore domain of their protein products). In a related effort, one of the groups went on to establish a transgenic line with myocardium-specific expression of EGFP, and used this model together with automated fluorescence microscopy for the high-throughput screening of small molecules that modulate heart rate in zebrafish embryos [49]. More recently, another group isolated the mutation responsible for an inherited arrhythmia in zebrafish, determining the affected gene to be *kcnh2* by genetic mapping and direct sequencing [92]. Together, these findings underscore the suitability of zebrafish to serve as a useful model for prescreening natural products and other small molecules for their potential risk to cause QT prolongation and other arrhythmias.

Outlook

Zebrafish are now firmly established as a powerful research platform for many areas of biology and drug discovery. Given the relative ease with which natural products and other small molecules can be analyzed in zebrafish, it is conceivable that within the next decade the large majority of currently available plant and fungal extracts (and increasing numbers of microbial extracts) can be screened in a plethora of biomedically relevant assays – an undertaking not currently feasible using other *in vivo* models. Zebrafish-based assays have the capacity to facilitate the bioassay-guided fractionation of large numbers of bioactive extracts identified in such *in vivo* screens, and thereby to enable the isolation of numerous novel, bioactive natural products – several of which are likely to be attractive lead compounds for the development of new, effective drugs.

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