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Chemical screening in zebrafish for novel biological and therapeutic discovery

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Abstract

Zebrafish chemical screening allows for an in vivo assessment of small molecule modulation of biological processes. Compound toxicities, chemical alterations by metabolism, pharmacokinetic and pharmacodynamic properties, and modulation of cell niches can be studied with this method. Furthermore, zebrafish screening is straightforward and cost effective. Zebrafish provide an invaluable platform for novel therapeutic discovery through chemical screening.

INTRODUCTION

In the past 15 years, many successful therapeutics have been efficiently discovered by cell-based and biochemical drug screening. However, these screening methods do not consider in vivo small molecule activity. Potential therapeutics from such screens often do not pass in vivo testing in live organisms such as mice, since they have inherent toxicity and poor pharmacoproperties undetectable by the screening process. Also, small molecules may act differently in whole organisms due to their complex biology, as compared to more straightforward biology in cell cultures and purified proteins. Such screens are encountering problems with proteins that are difficult to target, such as transcription factors. These proteins are termed “undruggable”, since they are inept in binding small molecules and often carry out their functions through protein–protein or protein–DNA/RNA interactions.

Zebrafish chemical screening can address the problems inherent in cell-based and biochemical screens. Screening in a whole organism context means drug toxicity and in vivo drug effects are addressed concurrently. Whole organism screening has the advantage of being less targeted than cell-based and biochemical screens, allowing the drug to interact with any biological pathway. The readout is an alteration of a whole organism phenotype that relates well to disease. In contrast, protein–compound binding or cell-based reporters give little indication of disease phenotype modulation. Furthermore, technological advances have made zebrafish screens straightforward and cost effective. It has been 15 years since the first zebrafish screen was attempted, and already, a number of potential therapeutics have been discovered that target processes ranging from hematopoiesis to cancer (Table 1). Zebrafish screening might also provide the ability to discover therapeutic modulators of

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“undruggable” processes, as it explores biology to a complexity unseen in cell-based or biochemical screens. Overall, zebrafish screening is a convenient and ideal technology for novel therapeutic discovery.

1. RATIONALE

Zebrafish screening allows for high-throughput chemical genetics in vivo. This is its greatest advantage over cell-based and biochemical screening. Screening chemicals in the context of the whole organism allows for unique phenotypes to be screened for, other than the traditional alteration of cell state in cell-based assays or target identification (target ID) in protein-binding biochemical assays. Furthermore, small molecules are screened in the context of the complex biology of the whole organism. This allows for assessment of (1) compound toxicity, (2) chemical alteration by metabolism, (3) drug pharmacokinetics and pharmacodynamics, and (4) drug modulation of cell niches (MacRae & Peterson, 2015; Rennekamp & Peterson, 2015; Wheeler & Brändli, 2009; Zon & Peterson, 2005).

In addition, zebrafish embryonic screening is reasonably cost effective, straightforward and biologically relevant (MacRae & Peterson, 2015; Rennekamp & Peterson, 2015; Wheeler & Brändli, 2009; Zon & Peterson, 2005). Firstly, fish husbandry requirements are straightforward and embryos are easily obtained in large numbers of 200–300 per mating pair. Secondly, embryos develop *ex utero* so their development can be monitored easily. Thirdly, embryos are more easily manipulated under a microscope. Fourthly, embryos can be screened at stages with no pigment, so phenotypes are easily observed. Fifthly, drug targets between humans and zebrafish are conserved. Sixthly, in vivo toxicity is observed, eliminating hits that are poor drug candidates.

With the feasibility of high-throughput screening and the advantages associated with in vivo drug assessment, the zebrafish are an ideal organism for whole organism-based therapeutic drug discovery.

2. MATERIALS AND METHODS

2.1 ZEBRAFISH SCREEN SCORING PHENOTYPES

2.1.1 Specific versus nonspecific phenotypes—Most often screens are conducted to generate hypotheses on a specific biological question. These screens are scored based on a chosen morphology change of interest and the aim is to discover specific chemical modifiers of disease or biological pathways. Less frequently conducted are nonspecific screens that score any morphological change observed. These have been carried out to determine compound bioactivity in broad terms; the readouts being any observable perturbation of development (Das, McCartin, Liu, Peterson, & Evans, 2010; Jung et al., 2005; Khersonsky et al., 2003; Moon et al., 2002; Peterson, Link, Dowling, & Schreiber, 2000; Sachidanandan, Yeh, Peterson, & Peterson, 2008; Spring, Krishnan, Blackwell, & Schreiber, 2002; Sternson, Louca, Wong, & Schreiber, 2001; Torregroza, Evans, & Das, 2009; Wong, Sternson, Louca, Hong, & Schreiber, 2004). Often, in-house synthetic libraries containing one specific pharmacophore are screened on wild-type zebrafish embryos. Phenotypes are scored and characterized by eye. For example, Das et al. conducted a screen on wild-type embryos

using a synthetic retinoid analogue library. Their aim was to discover novel retinoids that showed bioactivity in vivo. Hence, they nonspecifically scored any developmental defect they observed. This led to the discovery of BT10, which caused cardiovascular defects in fish and which bound specifically to retinoic acid receptors. This example highlights that undirected-phenotype screens are typically conducted with pharmacophore analogues, to discover better tools or drugs in pathways already known to be modulated by small molecules.

2.2 TYPES OF SCORING PHENOTYPES

Zebrafish are easily manipulated to produce a number of different readouts in chemical screens (Table 1). The types of readout have been subdivided here into three categories: (1) phenotypes that are scored by bright-field (gross morphology scoring), (2) phenotypes that are observable by cellular, genetic, or biochemical manipulation of the zebrafish (cell, genetic, and biochemical scoring), and (3) behavioral phenotypes (behavioral scoring).

2.2.1 Gross morphology scoring—A variety of observable developmental phenotypes have been characterized in zebrafish due to genetic or chemical perturbation. Here we define gross morphology scoring as phenotypic readout scored under a bright-field microscope without the assistance of any fluorescent markers or biochemical assays. Chemicals that effect early developmental patterning processes often produce gross morphological defects that are amenable to this screening process. Dorsomorphin, a bone morphogenic protein (BMP) antagonist, was identified in a screen for factors that disrupt dorsoventral patterning during early embryogenesis (Yu et al., 2008). Often, morphological changes are scored manually but certain morphologies can be scored by automation. For example, in a chemical suppressor screen for inhibitors of polycystic kidney disease (PKD) zebrafish models, Cao et al. designed a computer algorithm that could identify modulation of laterality and curvature in embryos. The error rate was low at 2.2%, suggesting that automating morphology scoring is highly possible (Cao et al., 2009). The screen identified histone deacetylase inhibitors as suppressors of the PKD phenotype, eliciting viable drug candidates for treating PKD. As illustrated by this example, investigating specific morphology changes focuses in on one or a few disease pathways and allows for a more directed and automated screening approach.

2.2.2 Cell state scoring—Cell state is defined here as a molecular phenotype not evident to the naked eye. Examples include mRNA expression levels, protein phosphorylation and cell mitotic state. When scoring a change in cell state, one requires a consequent secondary assay after chemical screening. The three most common secondary assays that have been applied to zebrafish cell state screens are (1) in situ hybridization (North et al., 2007; Paik, de Jong, Pugach, Opara, & Zon, 2010; Yeh et al., 2009), (2) immunohistochemistry (Murphey, Stern, Straub, & Zon, 2006; Stern et al., 2005), and (3) fluorescent protein reporter expression (Molina et al., 2009).

In situ hybridization involves hybridizing an mRNA-specific probe to expressed mRNA transcripts in fixed embryos. A color reaction with the probe localizes expressed transcripts to specific tissues. In addition, color intensity provides a semi-quantitative assessment of transcript levels in the tissue(s) of interest. North et al. utilized in situ hybridization to assess

the expression levels of *cmyb* and *runx1*, two genes required for hematopoietic stem cell (HSC) development. They sought to discover modulators of HSC formation in their chemical screen. Thirty-five and forty-seven compounds increased or decreased *cmyb* or *runx1* expression respectively in the screen. This resulted in the discovery that compounds which modulate prostaglandin E2 levels modulate overall HSC homeostasis (North et al., 2007).

Immunohistochemistry can be used to identify levels of modified proteins via specific antibodies. Two screens have been carried out with immunohistochemical readouts to serine-10-phosphorylated histone H3 protein (Murphey et al., 2006; Stern et al., 2005). Histone H3 serine-10 phosphorylation occurs in late G2 to early M phase and is dephosphorylated in anaphase (Hendzel et al., 1997). Both screens were conducted on a *bmyb* zebrafish mutant to identify chemical suppressors of the *bmyb* phenotype. The *bmyb* mutant phenotype entails decreased cyclin B1, mitotic arrest, and genomic instability (Shepard et al., 2005). Mitotic arrest in *bmyb* mutants results in an accumulation of antibody-detectable histone H3 phosphorylation (Murphey et al., 2006; Stern et al., 2005). The screen by Stern et al. identified a small molecule, persynthamide, which reduced histone H3 phosphorylation to wild-type levels, suppressing the *bmyb* phenotype.

It is also possible to screen transgenic zebrafish with a fluorescent reporter for compounds that modulate a pathway of interest. Molina et al. (2009) designed a screen based on Dual specificity phosphatase 6 (Dusp6) expression using Tg(*dusp6:EGFP*)^{pt6} embryos. This transgenic line reports on the fibroblast growth factor (FGF) signaling pathway, since Dusp6 is involved in feedback attenuation of this pathway (Thisse & Thisse, 2005; Tsang & Dawid, 2004). Molina et al. discovered a small molecule, 2-benzylidene-3-(cyclohexylamino)-2,3-dihydro-1H-inden-1-one (BCI), which increased Enhanced Green Fluorescent Protein (EGFP) fluorescence in the embryos and further characterized BCI as a Dusp6 inhibitor.

Zebrafish cell state screening is very versatile and not limited to scoring assays mentioned above. For example, fluorescent lipid analogues were used to in a chemical screen for modulators of dietary lipid absorption (Clifton et al., 2010), and a luciferase reporter of *pck1* was used to identify chemical regulators of whole body energy control (Gut et al., 2013). As new cellular, genetic, and biochemical tools are applied to zebrafish, the assays for screening chemical screen hits will expand.

2.2.3 Behavioral scoring—Zebrafish movement, in response to stimuli, can be characterized. Changes in such movements can be scored for alteration by chemical perturbation. Zebrafish have been screened for psychotropic and neuroactive drugs by characterizing changes in their photomotor response (PMR) (Kokel et al., 2010, 2013), rest/wake behavior (Rihel et al., 2010), habituation to acoustic startle (Wolman, Jain, Liss, & Granato, 2011), and convulsive behaviors and electrographic seizures (Baraban, Dinday, & Hortopan, 2013). This type of screen showcases the robustness of the zebrafish in identifying drugs that target complex pathways in vivo. Such drug discovery is impossible in in vitro screens that cannot recapitulate the biology of an entire organism. Phenotypes are scored by camera recordings and computer analyses. A behavioral screen conducted by Kokel et al. (2010) overcame the inability of cell-based and biochemical chemical screening

to identify compounds that modulate the central nervous system (CNS). CNS biology manifests itself in organism behavior, so a convincing study involves the intact whole organism. Kokel et al. discovered that light-stimulating zebrafish embryos resulted in a PMR that could be easily bar-coded. The PMR was recorded by a camera and bar coding was performed by custom computer scripts. A diverse collection of libraries, including neurotransmitters and ion channel binders were screened and scored for perturbation of the PMR. This study discovered novel neuroactive compounds at a highly efficient rate, illustrating the usefulness of the zebrafish in neuroactive and psychotropic drug discovery.

2.3 ADVANCES SCORING PHENOTYPES

2.3.1 Adult chemical screens—Nearly all chemical screens have been performed on embryos at various stages of development. Screening in adults requires more time and resources while the fish develop to adulthood. The workflow for adult screens often involves anesthetizing individual fish for experimental procedures. Finally, adults are also mobile and pigmented making it difficult to automate these screens and limiting the size of these chemical screens.

Despite these limitations successful adult zebrafish screens have been implemented. Chemical screens for fin regeneration have been performed in adult zebrafish (Mathew et al., 2007; Oppedal & Goldsmith, 2010). In another screen, competitive hematopoietic stem and progenitor cell transplants were performed in adult zebrafish and identified epoxyeicosatrienoic acids as a potent inducer of bone marrow engraftment (Li et al., 2015). Recent advances in administering drugs to adult fish have become more feasible with the use of oral gavage and an anesthetic combination of MS-222 and isoflurane (Dang, Henderson, Garraway, & Zon, 2016). In addition, the transparent fish line *casper*, where pigmentation is ablated, makes the adult zebrafish more amenable to screening (White et al., 2011). As the tool box for manipulating adult zebrafish expands the prevalence of screening adult phenotypes will likely follow.

2.3.2 Suppressor chemical screens—The emergence of gene editing tools, such as Clustered regularly interspaced short palindromic repeats and Transcription activator-like effector nucleases, has expanded the possibilities for developmental biology and disease modeling in zebrafish. Chemical suppressor screens have been designed to suppress the phenotypes associated with PKD (Cao et al., 2009), Dravet syndrome (Baraban et al., 2013), and long QT (LQT) syndrome (Peal et al., 2011) in zebrafish disease models. In addition to suppressing genetic phenotypes, chemical screens can also be designed to suppress chemical induced phenotypes. Nishiya et al. (2014) performed a chemical screen on embryos treated with a small molecule that activates Wingless-Type MMTV Integration Site Family (WNT) signaling, 6-bromoindirubin-3'-oxime, and found a novel inhibitor of the WNT signaling pathway. Suppressor screens designed to repress chemical or genetic phenotypes are a useful tool for researches that are limited by the phenotypes available in wild-type fish.

2.4 CHOICE OF SMALL MOLECULE LIBRARY

A number of chemical libraries have been tested in zebrafish (Table 2). Three broad categories of small molecule libraries are available: commercial vendor libraries, natural

product libraries, and synthetic libraries. The majority of zebrafish screens have utilized commercial libraries, specifically the subcategory of bioactive, annotated small molecules. A small number of screens have used personalized synthetic libraries to address specific issues. A description of each library is as follows.

2.4.1 Commercial libraries—Libraries consist of small molecules adhering to Lipinski's rules. These rules describe chemical aspects of small molecules that give them good pharmacokinetics and dynamics. Compounds have low molecular weight, partition coefficient values that afford efficient membrane absorption, and a total number of hydrogen bond donors and acceptors within appropriate limits. These properties predict good bioavailability in organisms (Lipinski, Lombardo, Dominy, & Feeney, 2001).

2.4.2 Bioactive libraries—These libraries are a subset of commercial libraries that are annotated with known protein targets, drug-like molecules, and bioactivity. These libraries are extremely useful in identifying small molecule targets after screening, since the target or pathway is already known. Also, screening such libraries can yield valuable information on multiple pathways in a disease phenotype. Some of the bioactive libraries used in zebrafish screening include the DIVERSet E (ChemBridge), the ICCB Known Bioactives (Biomol), the LOPAC1280 (Sigma–Aldrich), the NINDS Custom Collection (NIH/National Institute of Neurological Disease and Stroke), and the Spectrum Collection (MicroSource) (Table 2). The number of libraries is constantly expanding and companies, such as Selleckchem, ChemDiv, and Chem-Bridge, all have diverse libraries.

2.4.3 In-house synthetic libraries—Some labs synthesize their own compounds depending on their specific goals. In-house libraries are advantageous in (1) discovering novel bioactive small molecules and (2) straightforward target ID. Such libraries are based on known pharmacophores, so one can shortlist candidates of possible targets. The outcome of such a library screen is usually the discovery of novel bioactivity of an analogue of a known pharmacophore. An example of such a study is a zebrafish screen carried out with novel retinoid analogues that discovered a novel retinoid with retinoid receptor specificity. This lead compound is useful for probing the biology of the retinoic acid signaling pathways (Das et al., 2010). A second aspect of a synthetic library is that compounds can be designed with tags, allowing for target ID through protein pull-down. Tagged compounds are screened in the zebrafish to confirm that the tag does not interfere with the bioactivity of the molecule. One such screen with a tagged-triazine library identified a novel inhibitor of mitochondrial ATPase, which induces pigmentation in early zebrafish embryos (Jung et al., 2005). The pull-down protocol was straightforward since the tagged compounds were chemically ideal for binding resin. Also, the tag was already confirmed as noninterfering with target binding by the screen.

2.4.4 Diversity-oriented synthesis libraries—Diversity-oriented synthesis (DOS) libraries expand the boundaries of chemical space by the synthesis of novel pharmacophores (Schreiber, 2000). Such libraries encompass chemical space that is not covered by commercial libraries, hence providing greater potential for novel modulation of “undruggable” pathways and targets if screened.

2.4.5 Natural product libraries—These libraries consist of compounds extracted from nature (Clardy & Walsh, 2004). Famous examples of therapeutics derived from natural products are cancer drugs such as Taxol from the Pacific yew tree or antibiotics such as penicillin from *Penicillium* fungi. Like DOS compounds, natural product libraries increase the potential of novel discovery in screening.

2.5 CHEMICAL SCREENING PLATFORM

The actual screen is performed in a specific order: first, a small-scale optimization screen is conducted to determine the appropriate screening parameters. Second, sufficient zebrafish embryos are generated. Third, the screen is carried out either by hand, for a small screen, or by automation, for high-throughput. Fourth, hits are rescreened for validation.

The optimization screen is performed with a small number of embryos and compounds to determine optimal parameters such as desired plate format, compound concentrations, number of embryos per well, and embryonic stage. It is helpful if a compound that causes a positive phenotype in the assay is available. This would provide an ideal positive control and allow for compound concentrations to be fine tuned, generating an obvious scoring phenotype without causing embryonic lethality. Determining the developmental stage at which embryos are screened is also important since this can affect the phenotypic readout.

Large numbers of zebrafish embryos are needed for high-throughput screens. Traditionally, these are generated by setting up large numbers of mating pairs in multiple tanks. This method takes up a lot of space, makes embryo collection tedious, and may not yield synchronized embryos. Recently, this bottleneck in zebrafish screening has been solved by the introduction of the zebrafish spawning vessel technology (Fig. 1). The zebrafish spawning vessel allows for over 200 fish of any given strain to be spawned simultaneously. This allows for collection of a maximum of 10,500 highly synchronized embryos with a typical spawning time of 10 min. In addition, the apparatus has a small footprint, saving lab space (Adatto, Lawrence, Thompson, & Zon, 2011). Obtaining large numbers of synchronized embryos is now efficient and no longer limits the scale of chemical screening.

In addition to improving embryo collection, advances in technology have also made the handling of large compound libraries easy. Liquid handling robots, such as the TECAN robot (Tecan, Durham, NC), are used to distribute media and chemicals into plate wells rapidly and accurately. These robots are easily calibrated to operate for a range of compound volumes and plate formats. Distributing embryos into plate wells is usually preformed by hand and is a tedious and rate limiting step in the chemical screening process, however some recent chemical screens have automated this process (Truong et al., 2014; Wang et al., 2015).

Depending on the aim of the study, the mechanics of the screen can be conducted in a number of ways. There exist variations in the plate format, the number of embryos per well, and the compound concentration in each well. Embryos are distributed into a variety of plate sizes, ranging from 6-well up to 384-well transparent plates, with the common practice being ~3 embryos per 96-well plate (Fig. 2). Such plates are amenable to liquid handling robotics for compound library addition as well as automated image recording for high-

throughput phenotypic readouts. Compound libraries are added to plates with well concentrations ranging from 1 to 100 μM with the average being $\sim 20 \mu\text{M}$ the most frequent concentration being 10 μM (Fig. 3). Present library sizes range from 10 to 160,000 (Table 2). The size and compound composition frequently depend on the respective study goals.

2.6 TARGET IDENTIFICATION

To develop effective therapeutics, a near complete assessment of the drug candidate is required. This involves identifying the compound's toxicity, pharmacokinetic dynamics, and very importantly, its molecular target(s). A range of target ID methods have been applied to zebrafish chemical screens. These include the traditional techniques of protein pull-down, cell-based assays, in vitro biochemical assays and computer docking simulations. However, the most commonly used target ID method in zebrafish screens is candidate-based ID, which utilizes (1) annotated bioactive libraries, (2) chemoinformatics, and/or (3) genetics to elicit the target.

2.6.1 Candidate-based identification—Most screens address a specific biological question, such as perturbations to the hematopoietic system, pigmentation, or cardiovascular system. This allows one to narrow down the list of possible pathways modulated by the small molecules screened. However, pinpointing the exact protein being targeted is difficult in the context of a whole organism, since traditional biochemical and cell-based target ID methods are not feasible due to the complexity of the organism. Candidate-based ID is achieved by using drug libraries with known targets, chemoinformatic analysis to infer targets and/or genetic experimentation to infer modulated pathways. One or a combination of these methods is used to achieve a more complete understanding of the detailed biological effect(s) exerted by the drug candidate in question.

2.6.1.1 Annotated bioactive libraries: Annotated bioactive libraries are often used in chemical screens. These libraries contain therapeutic compounds with known targets and/or drug-like compounds. With these libraries, compound effects and targets are already known or can be easily predicted. This allows for straightforward target ID upon hit confirmation.

2.6.1.2 Chemoinformatics: When a hit with unknown targets is identified, it can be subjected to chemoinformatic analysis to predict its possible target(s). Often, from a chemical screen with diverse compound libraries, one finds interesting hit compounds that are either not well annotated or unannotated. The simplest way to hypothesize the target pathway or protein of such compounds is to find well-annotated compounds with similar structural features. Classes of compounds with similar chemical structures typically target similar pathways. Chemoinformatics can compile structural similarity information for the hit compound of interest, allowing the researcher to use pre-existing structure–activity information from other similar molecules to hypothesize the possible activity of the compound of interest.

Chemoinformatic analysis can be applied by utilizing the many chemical databases with integrated search options. These databases compile useful information such as structural information, bioactivity, 3D molecular models, literature and patent links, material safety

data sheets, and commercial availability. Liao, Sitzmann, Pugliese, and Nicklaus (2011) reviewed the software and databases available for drug design and discovery. Some examples of the useful database include PubChem (<https://pubchem.ncbi.nlm.nih.gov/>), ChemBank (<http://chembank.broadinstitute.org>), chemical identifier resolver (<https://cactus.nci.nih.gov/chemical/structure>), Fiehn Lab (<http://cts.fiehnlab.ucdavis.edu/conversion/batch>), Mcule (<https://mcule.com/>), CrossFire Beilstein, and DiscoveryGate (<http://www.discoverygate.com>). DiscoveryGate uses a proprietary algorithm to search its compound databases, which encompass many sources such as journal articles, patent information, and commercial and proprietary chemical databases. One can perform structural or text-based searches on DiscoveryGate to obtain vast amounts of pharmacological and biological information on chemicals related to the search compound. This tool requires a flat fee for usage (Trompouki & Zon, 2010). Searches can be performed with the chemical structure of interest to find analogous chemicals with known bioactivity. This might elicit related structures such as known pharmacophores or reactivity groups with biochemical activity. Also, hits obtained from screens performed by others can be compared for similar compound activity. In this way, the bioactivity of the hit compound can be predicted, so that the appropriate validation experiments can be conducted (Brown, 2005; Parker & Schreyer, 2004; Trompouki & Zon, 2010).

Chemical structures of search compounds can be entered into chemoinformatics tools in a variety of ways. The most common molecular format that gives detailed structural information in a highly simplified manner is the Simplified Molecular Input Line Entry Specification (SMILES) format. The SMILES format involves a simple textual representation of chemical features such as bonds, aromaticity, stereochemistry, branching, and isotopes without the use of complicated chemical drawing software. This format allows for rapid data interpretation by computers with little ambiguity in the chemical structure. Other chemical formats used are the Chemical Markup Language, GROMACS, CHARMM, the chemical file format, and the SYBYL Line Notation. To convert between the formats, one can use open source tools such as Open Babel and JOELib. Integrated applets for drawing traditional two-dimensional chemical structures into the search engine are also available (Trompouki & Zon, 2010).

There are different search algorithms one can use to search chemical databases. Examples include the commonly used Tanimoto similarity scoring, the Similarity Ensemble Approach algorithm, or the Tversky similarity algorithm. Some databases also use proprietary algorithms unique to their services. Different algorithms often lead to varying results so one might need to test more than one algorithm should the first one prove unsuccessful. Although not always consistent with each other, these algorithms are all equally important in hypothesis generation through the compilation of structurally similar compounds to the small molecule of interest. This information allows the researcher to test if the shortlisted compounds can phenocopy the compound of interest, thus narrowing down the activity of the compound of interest (Brown, 2005; Parker & Schreyer, 2004; Trompouki & Zon, 2010).

Chemoinformatics was used for target ID by Hong et al. In their chemical screen, Hong et al. obtained a hit compound GS4898 that rescued tail and trunk circulation in zebrafish *gridlock* mutant embryos. GS4898 had not been previously characterized so Hong et al. used

chemoinformatics to predict that GS4898 might be a protein kinase inhibitor, since the molecule was structurally related to flavone kinase inhibitors. They then tested structurally related flavones kinase inhibitors and found that a specific phosphatidylinositol-3 kinase (PI3K) inhibitor phenocopies the rescue of the *gridlock* mutation by GS4898. This allowed Hong et al. to further validate that GS4898 did indeed inhibit PI3K.

2.6.1.3 Genetics: If the small molecule of interest is poorly annotated and chemoinformatics does not provide a viable hypothesis, one can perform genetic studies to predict the mode of action of the small molecule. Target ID via genetics first involves identifying a candidate pathway or pathways most likely modulated by the chemical. Secondly, the expression levels of genes in each pathway are analyzed to see if they are perturbed by the chemical. Thirdly, if chemical inhibitors of various steps of the pathway are available, these can be used to see if known inhibitor treatment phenocopies the effect of the chemical of interest. If inhibitors are not available, gene knockdowns and knockouts can be performed to try and phenocopy the effect of the chemical of interest. Genetic studies give evidence for what pathway the chemical of interest is modulating and serves as the basis for other follow-up biochemical and cell-based experiments directed at a specific pathway.

Gene expression changes can be measured in a number of ways. Microarrays are used to generate a large data set for gene expression changes encompassing a myriad of pathways. This is useful when either (1) a significant number of pathways are responsible for the phenotypic change or (2) the pathways responsible are unclear. In cases where a small number of candidate pathways can be shortlisted, real-time PCR is used to evaluate the expression changes of individual genes in the pathways of interest. In situ hybridization, which allows for visual observation of gene expression changes in an intact zebrafish, allows for assessment of spatial changes in gene expression if present.

Yu et al. (2008) took a multistep genetics approach to identify the target of dorsomorphin, a chemical they obtained from their screen that looked for small molecule effectors of zebrafish embryo dorsalization. The dorsoventral axis is established by BMP signaling gradients, and excess BMP signaling causes ventralization while reduced BMP signaling causes dorsalization (Fürthauer, Thisse, & Thisse, 1999; Mintzer et al., 2001; Mullins et al., 1996; Nguyen et al., 1998). The screen by Yu et al. was based on the hypothesis that BMP signaling antagonists would cause dorsalization in zebrafish. To identify the target of their chosen molecule, dorsomorphin, they first performed in situ hybridization to investigate the effects of dorsomorphin on dorsal and ventral gene markers. They noticed that the level of ventral marker *eve1* was reduced while dorsal markers such as *egr2b* and *pax2a* underwent lateral expansion during dorsomorphin treatment. Since dorsomorphin phenocopies BMP antagonism in fish, the second experiment they performed was to use dorsomorphin treatment to rescue zebrafish that were deficient in the endogenous BMP antagonist, chordin. Dorsomorphin was able to rescue the phenotype of chordin morphants, thus validating that dorsomorphin is indeed a BMP antagonist. From this genetic evidence, Yu et al. (2008) proceeded with biochemical and cell-based assays to show that dorsomorphin inhibits Suppressor of Mothers Against Decapentaplegic-dependent BMP signals and BMP type I receptor function.

2.6.2 General target identification methods applied to zebrafish screens

2.6.2.1 Protein pull-down: This method is relatively straightforward for target ID. It involves immobilizing a compound of interest to resin, by means of a chemical linker group, and incubating the resin with cell lysates. This allows intracellular binders to associate tightly with the immobilized compound, pulling it out from suspension. The resin is then washed to remove nonspecific binders, and the bound proteins are analyzed by mass spectrometry to identify them. Protein pull-down is often used with synthetic libraries, which are screened with a linker group already present on the compounds. This ensures that in vivo activity is unaffected by the linker. Compounds from other libraries are more difficult to modify for resin linkage.

2.6.2.2 Cell-based assays: Cell-based assays are used to confirm that drug candidates can bind their target in vivo. Such an assay would involve addressing a specific target of interest in a cellular environment. For example, if the hit compound is hypothesized to inhibit an enzyme that phosphorylates a certain substrate, then one can design an experiment that indicates that compound addition inhibits substrate phosphorylation. Cell-based assays are used as a confirmation for target ID, rather than for broad spectra target discovery.

2.6.2.3 In vitro biochemical assays: Similar to cell-based assays, in vitro biochemical assays are more suited to confirm targets than for discovering them. Biochemical assays are nearly identical to cell-based assays but do not involve complex cell biology. These assays require purification of the target of interest and if necessary, the target's substrate. The ability of the target to carry out its function on its substrate is then assessed under in vitro conditions with and without the small molecule.

2.6.2.4 Computer docking simulations: If crystal structures of targets are available, computer modeling can be performed to study the possibility of small molecules binding to their targets. Molina et al. (2009) use this method in their analysis of Dusp6 inhibition by BCI. Since the crystal structures of Dusp catalytic sites exist (Almo et al., 2007; Jeong et al., 2006; Stewart, Dowd, Keyse, & McDonald, 1999), Molina et al. could ascertain the probable binding site of BCI to Dusp6 with a program called ORCHESTRAR (Tripos).

3. DISCUSSION/CAVEATS

3.1 BIOLOGICAL RELEVANCE OF ZEBRAFISH SCREENING

Multiple phenotypes can be observed in zebrafish chemical screening. One can observe behavioral changes such as sleep/wake patterns or a movement response to light. In addition, one can also observe changes in gene expression either overall or in specific tissues due to chemical action. Such observations are not possible in cell-based or biochemical screening platforms. Zebrafish screening allows for exploration into the behavioral effects of small molecules, something only whole organism screens can achieve. In addition, gene expression changes are observed in vivo so this reflects accurately the biologically relevant action of the molecules. In zebrafish screening, there is no doubt that small molecules exert an effect in the context of a multicellular organism with active metabolism. This cannot be said for cell-based or biochemical platforms where further in vivo testing is required to

confirm biological relevance. Compound toxicity or side effects are also not readily apparent in cell-based screens, unlike in zebrafish screens (Zon & Peterson, 2005).

Zebrafish chemical screening accounts for the biological response of cell niches. This allows one to conclude that phenotypic changes resulting from the chemical are relevant in a multicellular environment. Conversely, traditional biochemical and cell-based screens only indicate chemical activity on a specific target or cell type, ignoring the interactions of the cellular niche and the metabolic activity of the whole organism. Some chemicals undoubtedly exert phenotype change due to modulation of the surrounding cells, rather than the cell type in question. Also, phenotypic change can occur by modulation of a wide range of cell types. In vivo screening can elicit hits that in vitro screens cannot pick up. One might therefore observe different sets of hits and/or unexpected outcomes when comparing in vivo and in vitro screens. Hits from the zebrafish screen are more biologically relevant since phenotypes are due to chemical action on the whole organism instead of on one protein or one cell type.

Drug effects in humans are largely conserved in zebrafish; so zebrafish are ideal for human therapeutic discovery. Data from previous zebrafish chemical screens have shown a high degree of conservation between mammals in zebrafish, in terms of drug effects and toxicities. Cardiotoxicity screening has shown a high degree of correlation between humans and zebrafish (Milan, Peterson, Ruskin, Peterson, & MacRae, 2003). In addition, screens to discover novel neuroactive and psychotropic drugs detected known human drugs with similar effects on zebrafish (Baraban et al., 2013; Kokel et al., 2010, 2013; Rihel et al., 2010; Wolman et al., 2011). Furthermore, 50–70% of chemicals in a zebrafish cell cycle screen show similar effects when tested in a mammalian cell culture assay (Zon & Peterson, 2005). As such, zebrafish screening is very relevant when applied to novel human therapeutic discovery.

3.2 SCREENING TECHNOLOGY CAVEATS

Compound concentrations are fixed per screen; so some compounds that should be classified as hits may be missed because they are not effective at the screening concentration. Others might cause toxicity at the screening concentration and are also ignored, even if they might be nontoxic and effective at lower doses. In addition, certain phenotypes are not amenable to high-throughput screens due to specific scoring requirements.

3.3 HIT DETECTION CAVEATS

Hit rates from all zebrafish studies range from <1% to 70% (Fig. 4). This can be influenced by scoring system and zebrafish biology. As mentioned previously, there are three different scoring types: morphological, behavioral, or cell state alteration. Using a different scoring type can lead to vastly different results. Also, human judgment plays an important role in what is scored as a hit. Since it is difficult to find two or more individuals with identical interpretation of scoring phenotype, human error introduces more variability into the screen.

In addition, variability in screening results is observed when similar chemicals are applied at different stages of development. There are a number of possible entry sites for small molecules into the fish. In embryos, chemicals permeate through the chorion and uptake can

occur through the epidermal layer or through the digestive system. In the larval and adult stage, entry points are similar with the inclusion of uptake through gills. The epidermal layer of the larvae is much less permeable than that in developing embryos, so one can surmise that the distribution of compound uptake by each of the aforementioned means is different depending on the stage of development. This distribution affects the action of small molecules since differing chemical modifications occur when compounds are subjected to varying cellular environments. Compounds that enter the digestive tract are subject to first-pass metabolism that often alters their chemical properties. Compounds that permeate through the epidermis or that are administered intravenously typically undergo little chemical change. The biological activity of certain chemicals requires activation of these compounds by chemical modification in vivo. On the other hand, the biological activity of certain other chemicals is abolished by in vivo chemical modification. The influence of the whole organism biological system can alter screening results and lead to inconsistencies that require further study.

Biological organism screening variability is also affected by penetrance. The genetic background of the fish exerts a considerable influence on screening results and can affect confirmation assays. Even in wild-type strains, genetic variability leads to different levels of drug penetrance in different clutches. Often, one can look for consistent penetrance percentages to determine true hits.

SUMMARY

Zebrafish chemical screening is very useful for therapeutic and bioprobe discovery. It provides a medium- to high-throughput manner of assessing the phenotypic effects of small molecule libraries on an in vivo system. This allows for toxicity, pharmacoproperties and effects of compounds to be studied in a complex biological system, taking into account metabolism and cell–cell interactions. Also, zebrafish provide a wide variety of scoring phenotypes which can be adapted to specific study aims. In addition, compound library choices are abundant and although the largest library used so far was ~160,000 compounds, technological advances can potentially increase this number. Scaling up embryo generation to a larger scale should also not be problematic.

However, there are some caveats to note when screening zebrafish. Using juvenile/adult fish restricts the throughput since fish at this stage take longer to accumulate in large numbers. Also, juvenile/adult manipulation is more challenging. Variable hit rates are observed in screens due to compound libraries used, human error, scoring type, genetic penetrance, and fish developmental stage. Target ID is another challenge in zebrafish screening. The complexity of the whole organism means that traditional target ID methods are not ideal, and the main target ID method is candidate-based inference.

Overall, zebrafish chemical screening is an indispensable tool in therapeutic discovery. The “low hanging fruit” of drug discovery has already been taken and the focus is now on “undruggable” targets such as transcription factors and protein–protein interactions. Traditional cell-based and biochemical drug discovery screens are no longer efficient in finding therapeutics to “undruggable” targets. Also, these methods do not consider in vivo

drug interactions, which could result in unwanted side-effects. Zebrafish screening has the added advantages of assessing drug toxicity at an early stage of drug development (Zon & Peterson, 2005). Also, any drug processing by metabolism is taken into consideration. Furthermore, pharmacokinetics and pharmacodynamics can be studied in the fish. Screening in zebrafish can also discover drugs that modulate the cell niche, rather than the target cell-type directly. This type of drug target would not be detected in cell-based and biochemical screens, which focus on a specific cell-type or protein target. With the aforementioned advantages, whole organism screens are undoubtedly the next step forward in chemical screening for therapeutic discovery.

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FIGURE 1.
iSpawn breeding cage.

FIGURE 2.
Frequency of screening plate format.

FIGURE 3.
Concentration (μM).

FIGURE 4.
Percent hit rate (log10 scale).

Table 1

Phenotypic Readouts

Screening Type	References	Phenotype	Fish
Morphology	Cao et al. (2009)	Polycystic kidney disease (PKD)	PKD mutants
	Colanesi et al. (2012)	Pigment cell patterning and number	Mifta mutant
	Das et al. (2010)	Body axis/cardiac defects	Wild type
	Hao et al. (2013)	Embryo dorsalization	Wild type
	Ishizaki et al. (2015)	Pigmentation and notochord defects	Wild type
	Jin et al. (2013)	Survival in presence of organophosphates	Wild type
	Jung et al. (2005)	Pigmentation	Wild type
	Khersonsky et al. (2003)	Brain and eye	Wild type
	Mathew et al. (2007)	Fin regeneration	Wild type
	Milan et al. (2003)	Heart rate	Wild type
	Moon et al. (2002)	Microtubule disruption	Wild type
	Nishiya et al. (2014)	Presence of eyes in 6-bromoindirubin-3'-oxime-treated zebrafish	Wild type
	Oppedal and Goldsmith (2010)	Fin regeneration	Wild type
	Padilla et al. (2012)	Toxicity in developing embryos	Wild type
	Peal et al. (2011)	Atrioventricular heart rhythm	tb218 mutant
	Peterson et al. (2000)	Multiple organs	Wild type
	Peterson et al. (2004)	Coarctation	Gridlock mutant
	Sachidanandan et al. (2008)	Multiple organs	Wild type
	Sandoval et al. (2013)	Morphological defects in the embryo	Wild type
	Spring et al. (2002)	Multiple organs	Wild type
	Torregroza et al. (2009)	Body axis/cardiac defects	Wild type
	Truong et al. (2014)	Toxicity	Wild type
	Williams et al. (2015)	Embryonic morphology	Wild type
	Yu et al. (2008)	Dorsal-ventral axis	Wild type
	Wong et al. (2004)	Cardiac defects	Wild type
Cell state	Alvarez et al. (2009)	Angiogenesis	fli1:EGFP
	Asimaki et al. (2014)	Normalization of natriuretic peptide levels	Plakoglobin mutant; nppb: luciferase
	Becker et al. (2012)	Modifiers of hypertrophic cardiomyopathy signaling	nppb:luciferase
	Clifton et al. (2010)	Lipid absorption	Wild type
	Evason et al. (2015)	Liver size	fabp10a:pt- β -cat; fabp10a: EGFP
	Gallardo et al. (2015)	Migration of labeled lateral line primordium	cldnb:EGFP
	Gebruers et al. (2013)	Inducers of ectopic tail formation	Wild type; cmlc2: eGFP
	Gut et al. (2013)	Activated fasting-like energy state	pck1:Luc2; cryaa:mCherry
	Gutierrez et al. (2014)	Death of Avian myelocytomatosis virus oncogene cellular homolog (MYC)-expressing thymocytes	rag2:Myc-ER; rag2:dsRed2; mitf mutant
	Hong, Peterson, Hong, and Peterson (2006)	Coarctation	Gridlock mutant

Screening Type	References	Phenotype	Fish
Author Manuscript	Kitambi, McCulloch, Peterson, and Malicki (2009)	Angiogenesis	fli1:EGFP
	Kong et al. (2014)	Craniofacial morphology	Wild type
	Le et al. (2013)	Modified RAS activity/Dusp6 expression	hsp70: HRASG12V
	Li et al. (2015)	Imaging of fluorescent stem cell grafts	Casper
	Liu et al. (2013)	Migration of leukocytes to wound	zlyz:EGFP
	Liu et al. (2014)	Rescue of cardiac function	Myl7:EGFP
	Molina et al. (2009)	Dusp6 expression	dusp6:EGFP
	Murphey et al. (2006)	Cell cycle	crb mutant
	Namdar, Reinhart, Owens, Raible, and Rubel (2012)	GFP expression in hair cells after ablation	pou4f3:gap43-GFP
	Nath et al. (2013)	Survival of cyanide exposure	Wild type
	Nath et al. (2015)	Biochemical measurement of glucose	Wild type
	North et al. (2007)	Hematopoiesis	Wild type
	Owens et al. (2008)	Hair cells	Wild type
	Paik et al. (2010)	Hematopoiesis	cdx4 mutant
	Ridges et al. (2012)	Selective leukemia toxicity	p56lck:EGFP
	Rovira et al. (2011)	Number of fluorescent β -cells in pancreas	Tp1:hmgbl-mCherry; pax6b: GFP
	Saydmohammed, Vollmer, Onuoha, Vogt, and Tsang (2011)	Fibroblast growth factor signaling, dusp6 expression	dusp6:EGFP
	Shafizadeh, Peterson, and Lin (2004)	Hematopoiesis	gata1:EGFP
	Stern et al. (2005)	Cell cycle	crb mutant
	Tran et al. (2007)	Angiogenesis	VEGFR:GRCFP
	Tsuji et al. (2014)	In vivo cell cycle indicator technology	ins:mAG-zGeminin
	Wang et al. (2010)	Angiogenesis	flk1:EGFP
	Wang et al. (2015)	Number of fluorescent β -cells in pancreas	ins:PhiYFP-2A-nsfB; sst2: TagRFP
	Weger, Weger, Nusser, Brenner-Weiss, and Dickmeis (2012)	Glucocorticoid signaling reporter	AB.9 GRE:Luc
	White et al. (2011)	Neural crest	Wild type
	Xu et al. (2010)	Angiogenesis	fli1:EGFP
	Yeh et al. (2009)	Leukemia (AML1-ETO)	hsp:AML1-ETO
Behavior	Baraban et al. (2013)	Inhibition of convulsive behaviors	Nav1.1 mutant
	Kokel et al. (2010)	Photomotor response	Wild type
	Kokel et al. (2013)	Photoactivation of motor behaviors	Wild type
	Rihel et al. (2010)	Rest/wake	Wild type
	Wolman et al. (2011)	Habituation to acoustic startle	Wild type

Table 2

List of Chemical Libraries

Library	# Chemicals	Times Used
LOPAC library (Sigma–Aldrich)	1,280	14
Prestwick chemical library (Prestwick)	1,280	9
DIVERSet (ChemBridge)	10,000	14
Spectrum collection (Microsource)	2,560	12
Biomol ICCB known bioactives (Enzo Life Sciences)	472	10
NINDS custom collection II (Microsource)	1,040	4
FDA-approved drug library (Enzo Life Sciences)	640	2
Johns Hopkins drug library		2
NatProd library (MicroSource Discovery Systems Inc.)	800	2
InhibitorSelect 384-well	160	1
Protein kinase inhibitor library I (EMD Millipore/Calbiochem)		
Actiprobe library (TimTec)	10,000	1
NIH clinical collection (Evotec)	719	1
Chemistry and marine natural product libraries (University of Utah)		1
Screen-Well kinase library (Enzo Life Sciences)	80	1
Screen-Well phosphatase library (Enzo Life Sciences)	33	1
GSK published kinase inhibitor set (PKIS)	376	1
International drug collection (MicroSource Discovery System)	400	1
Maybridge screening collection (Fisher Scientific International)	53,000	1
US drug collection (MicroSource Discovery Systems)	1,360	1
Molecular screening Centers Network	100,000	1
Nuclear receptor ligand library (Enzo Life Sciences)	74	1
Phosphatase targeted (ChemDiv)	15,000	1
Small molecule library from Vanderbilt HTF	160,000	1
Natural products library (University of Strathclyde)	5,000	1
TocrisScreen mini library	1,120	1
ToxCast EPA phase I chemicals	293	1
Diversity set (NCI)	1,593	1
Neurotransmitter library (Enzo Life Sciences)	661	1
Ion channel ligand library (Enzo Life Sciences)	70	1
Orphan ligand library (Enzo Life Sciences)	84	1
Screening Committee of Anticancer Drugs library		1
Serotonergic ligand library (Enzo Life Sciences)	79	1
InhibitorSelect 96-well protein kinase inhibitor library II (EMD Millipore)	80	1