

Discovery and use of small molecules for probing biological processes in zebrafish

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- I. Rationale for small molecule screens in zebrafish
 - A. The strengths and limitations of genetic screens
 - B. Advantages of small molecule screens
 - C. The potential for zebrafish-based drug discovery
- II. Assay development
 - A. General considerations for assay development
 - B. Examples of zebrafish small molecule screens
- III. Chemical suppressor and enhancer screens
- IV. Selections of small molecule libraries
- V. Screening methods
 - A. Handling embryos
 - B. Compound handling
 - C. Phenotype detection
- VI. Follow-up studies for active small molecules
 - A. Pathway interrogation
 - B. Affinity purification
- VII. Conclusions

I. Rationale for small molecule screens in zebrafish

A. The strengths and limitations of genetic screens.

Of all the virtues of the zebrafish as a model organism, its suitability for large scale screening is paramount. In no other vertebrate has it been possible to screen for mutations so readily and on such a scale as has been achieved using zebrafish. The earliest genetic screens captured the imaginations of many as wondrous mutant phenotypes were discovered, from the dramatically disrupted to the dramatically subtle (Driever et al., 1996; Haffter et al., 1996). In many cases, these mutants have allowed connections to be drawn between specific genes and their functions, especially for early developmental processes and organogenesis.

Despite the power and elegance of genetic screens, significant limitations prevent their more fruitful use in many circumstances. Most genetic zebrafish mutations discovered thus far are non-conditional, and there is not ability, generally, to modulate timing or dose of effect. Consequently, they are best suited for identifying the first developmental function of a gene but are less useful for studying later processes because early-onset effects may obscure, amplify, or complicate later-onset ones. For example, mutation of the sonic hedgehog gene in zebrafish prevents differentiation of medially located muscle pioneer cells (Schauerte et al., 1998; van Eeden et al., 1996). However, many other structures also fail to form in this mutant, and it is not clear from analysis of this mutant alone if *shh* is directly involved in muscle pioneer differentiation or if differentiation is prevented by the failure to form of some other structure, such as the lateral floor plate. In the most extreme cases, gene disruption is embryonic lethal at an early stage of development, making it impossible for traditional genetic screens to identify the role of that gene in any later process. In the absence of an allelic series, there is no information about the effect of different levels of gene product activity.

Additionally, genetic screens may fail to identify mutations in genes for which a functionally redundant isoform exists. In the case of the *shh* mutant *sonic-you*, the medial floor plate develops normally (Schauerte et al., 1998), seemingly contradicting the numerous biochemical and embryological studies that have demonstrated a requirement for hedgehog signaling during floor plate induction. The fact that *sonic-you* mutants form a medial floor plate is likely due to the midline expression of two compensatory hedgehog isoforms (*tiggy-winkle hedgehog* and *echidna hedgehog*) (Dodd et al., 1998). Thus, a genetic screen for medial floor plate inducers would have failed to identify *shh*, despite its role in floor plate induction. Functional redundancy of genes may be a more significant problem in zebrafish than in other model organisms given the apparent genome amplification that occurred in ray-finned fish prior to the teleost radiation (Postlethwait et al., 1998; Taylor et al., 2003).

Finally, the suppressor and enhancer screens that are valuable tools for identifying upstream and downstream components of genetic pathways in *Drosophila melanogaster*, *Caenorhabditis elegans*, and *Saccharomyces cerevisiae* have not been practical in the zebrafish (St Johnston, 2002).

B. Advantages of small molecule screens

Many of the limitations of traditional genetic screens outlined above can be overcome when genetic screens are complemented with small molecule screens. In fact, *Drosophila* and *C. elegans* are well suited for genetic screening but are not as tractable for small molecule screening because of difficulties in access of small molecules to tissues in these organisms. Zebrafish are amenable to both genetic and small molecule screening, and the ability to combine these approaches in zebrafish is particularly promising. Like genetic mutations, small molecules are a classical means of disrupting biological processes and serve to link genes or gene products with their molecular functions. This approach has different strengths and weaknesses from the genetic one, so chemical and genetic screens are complementary (see Figure 1). For example, small molecules are excellent conditional biological probes, can overcome gene redundancy, and facilitate suppressor and enhancer screens as described below. Furthermore, small molecule screens are generally simpler than are genetic screens.

1. Conditionality.

Most zebrafish mutations identified to date are non-conditional and have a fixed allele strength. In an effort to overcome these limitations, some screens for temperature-sensitive alleles have been performed (Johnson and Weston, 1995), but conditional mutants remain the exception. In contrast, small molecules are the ultimate conditional disruptors, allowing both the timing and dosage of pathway disruption to be regulated.

One elegant example of the use of a small molecule to complement genetic mutations is provided by studies of the hedgehog signaling pathway. The small molecule cyclopamine is an antagonist of the hedgehog effector *smoothed* and has been an invaluable tool for dissecting the roles of the hedgehog pathway in many developmental events (Chen et al., 2002). For example, cyclopamine was recently used to determine how three different muscle cell types (the muscle pioneers, the superficial slow fibers, and medial fast fibers) are specified in the zebrafish myotome. Wolff et al. demonstrated that all three muscle cell types are dependent upon hedgehog signaling and that the specific identity adopted by a particular cell is governed by the quantity and timing of the hedgehog signal received (Wolff et al., 2003). By treating embryos with cyclopamine at various times and doses, they were able to alter the cell types present in the developing myotome in a predictable manner and to discover the temporal and quantitative relationships between hedgehog and muscle cell specification. Such an analysis was only possible because of the temporal and quantitative control provided by cyclopamine.

The small molecule concentramide was discovered in a zebrafish chemical screen (Peterson et al., 2000; Peterson et al., 2001) and has contributed to the biological understanding of the developmental patterning of the heart that is largely derived from genetic studies. One key developmental decision is the axial positioning of the two cardiac chambers, which are normally aligned along the anterior-posterior axis. In the heart-and-soul mutant, the ventricle forms within the atrium. When this pattern is established, and whether it is due to problems within the cardiac primordia or to cell polarity defects in the adjacent, degenerating brain, was not clear. Concentramide

mimics the cardiac effect, without causing cell polarity defects in the central nervous system. By adding concentramide at varying time points, it was possible to identify the precise stage at which cardiac chamber orientation is determined, which is the time (the 14 somite stage) when the bilateral cardiac primordia are preparing to fuse at the midline. In both concentramide-treated embryos and *heart-and-soul* mutants, fusion of the posterior end of the heart field is delayed. This delay prevents the rotation of the heart that transforms the concentrically-organized heart cone into a linear structure, with atrium and ventricle adjacent to each other on the anterior-posterior axis (Peterson et al., 2001). As in the case of cycloamine described above, the temporal control with which concentramide could be added was critical for dissecting the process of cardiac patterning, and use of the small molecule complemented analysis using existing genetic mutants.

2. *Redundancy.*

Zebrafish chemical screens have identified many phenotypes that are similar to those previously identified using genetic screens, but some of the small molecule-induced phenotypes are unlike any identified by genetic screening (Khersonsky et al., 2003; Moon et al., 2002; Peterson et al., 2000; Spring et al., 2002; Sternson et al., 2001). One potential explanation for this expansion of phenotypes is functional redundancy in the zebrafish genome. When multiple isoforms of a protein play overlapping roles in a biological process, mutation of one isoform may be insufficient to cause an observable phenotype. In contrast, a small molecule may bind to and inhibit multiple isoforms simultaneously, and thereby reveal the importance of those proteins in the biological process. As previously mentioned, the overlapping functions of sonic, tiggy-winkle, and echidna hedgehog genes in establishing the medial floor plate preclude them from being identified via genetic screens as regulators of floor plate induction (Dodd et al., 1998). A small molecule, however, may be capable of inhibiting all three hedgehog proteins and block induction of medial floor plate. In addition, small molecules can modulate both maternal and zygotic gene products and may also have non-protein targets, including lipids and nucleic acids, and therefore may disrupt processes not disruptable via traditional genetic mutations.

3. *Suppressors/enhancers.*

No genetic suppressor or enhancer screen has ever been reported in a vertebrate. Small molecule screens make it possible to identify suppressors and enhancers of existing mutations as described in section III.

4. *Ease.*

One final advantage of chemical screens is that they are much easier to perform than genetic screens. Whereas to reach any degree of saturation, genetic screens conventionally require large zebrafish facilities for the maintenance of thousands of zebrafish strains and lines, chemical screens typically require at most a few zebrafish lines. And, while the mutagenized fish used for genetic screens are often less fertile, the fish used for chemical screens can be selected in part for fertility.

C. The potential for zebrafish-based drug discovery

In addition to their utility for dissection of essential biological processes, zebrafish small molecule screens may be useful for discovering novel therapeutic compounds and drug targets. By modeling human diseases in zebrafish, it may be possible to screen directly for compounds that modify the disease phenotype. Compounds that ameliorate the disease phenotype may serve as lead compounds for drug development, and identification of the compound's protein binding partner may effectively identify novel drug targets for traditional drug discovery efforts.

Many zebrafish models of human diseases have already been developed and are reviewed elsewhere (Amatruda et al., 2002; Rubinstein, 2003; Shin and Fishman, 2002). The majority of these are single gene mutations that cause zebrafish phenotypes reminiscent of some aspect of human disease. In a number of cases where the genes underlying the human and zebrafish disease are known, orthologous genes are responsible for both conditions (Garrity et al., 2002; Roman et al., 2002; Xu et al., 2002). Recently, it has become possible to identify mutations in virtually any zebrafish gene by target-selected resequencing (Wienholds et al., 2002) or to 'knock down' the function of a gene using antisense morpholino oligonucleotides (Nasevicius and Ekker, 2000). Therefore, it should be possible to generate zebrafish models for many of the human diseases resulting from a known single-gene mutation. Therapies for many of these human diseases have not been developed because of the difficulty in predicting *a priori* which proteins should be targeted to reverse the disease phenotype. Significantly, unbiased screening in zebrafish may allow discovery of compounds that reverse the disease, even without knowing what protein is being targeted (MacRae and Peterson, 2003).

In addition to diseases caused by genetic mutation, it may be possible to discover novel drugs for treating infectious diseases. Several zebrafish models of infection have been developed, including models of tuberculosis and *Salmonella typhimurium* infection (Davis et al., 2002; Van Der Sar et al., 2003). Screening in zebrafish may allow assays to be performed on microbes that cannot be cultured outside of a whole organism. And by screening in the context of a whole organism, it should be possible to identify compounds with antimicrobial activity that have no undue toxicity to the host. Two of the infection models developed thus far use fluorescently-labeled microbes for infection (Davis et al., 2002; Van Der Sar et al., 2003), so the efficacy of a small molecule could be measured by quantitating the number of pathogens or by assessing survival of the host.

Will small molecules that reverse a disease phenotype in zebrafish have similar effects in humans? While that question has not been answered, it is clear that many drugs with known effects in humans cause analogous effects in zebrafish. For example, Milan et al. treated zebrafish with 23 drugs known in humans to lengthen the QT interval on the electrocardiogram, often a harbinger of arrhythmogenesis, an undesirable drug side-effect (Milan et al., 2003). Of the 23 drugs, 22 also caused an analogous prolongation of the cardiac cycle in zebrafish. Other drugs that have similar effects in humans and fish include angiogenesis inhibitors, vasodilators, opiates, cholesterol synthesis blockers, and anticoagulants (Langheinrich, 2003). Therefore, tissue access, drug binding sites, and pharmacodynamic effects seem to be generally well conserved between zebrafish and humans.

II. Assay development

A. General considerations for assay development

In designing an assay for a small molecule screen, two of the most important considerations are assay stringency and reproducibility. A stringent assay will score a small molecule as active only if it causes a phenotype that meets a demanding set of criteria. Obviously, an overly stringent assay may cause valuable small molecules to be overlooked, while an assay that is not stringent enough results in numerous false positives. In our experience, it has been preferable to err on the side of increased stringency, because pursuit of false positives and weakly active compounds can be time consuming. Fortunately, for most screens it is possible to assess the stringency of the assay using a small pilot screen of about 1000 small molecules and increase stringency if an undesirably high hit rate is observed. The optimal hit rate will vary from assay to assay but will likely be in the range of one in 100 to one in 10,000 molecules screened.

Variability between individual zebrafish can reduce assay reproducibility and lead to unacceptable numbers of false positives. Most reproducibility problems can be eliminated by placing multiple zebrafish embryos in the same well. Three embryos can be raised in a well of a 96-well plate for several days or in a well of a 384-well plate for about 1 day. By requiring that all embryos in a well exhibit the same phenotype, false positives can be eliminated. For example, even if a false positive phenotype is observed in 5 percent of untreated embryos, the probability of three out of three embryos in a given microplate well exhibiting the phenotype is about 1/10,000, which is an acceptably low rate of false positives for most assays.

Other factors such as the strain of zebrafish to use, the timing with which small molecules should be added, and the optimal means of imaging or measuring the results must be determined for each assay.

B. Examples of zebrafish small molecule screens

Most small molecule screens to date have used standard microscopic analysis of morphological defects in developing embryos (Figure 2). Lesions of the brain, eye, ear, skin, neural crest, blood, heart, vasculature, etc. have been identified (Khersonsky et al., 2003; Moon et al., 2002; Peterson et al., 2000; Spring et al., 2002; Sternson et al., 2001).

Additionally, small molecule perturbants of physiological function have been isolated using assays that take particular advantage of whole animal-based *in vivo* screens. For example, an assay has been developed that allows the heart rates of zebrafish larvae in 96- or 384-well plates to be determined automatically using a robotic microscope coupled with a digital video camera and image processing software (Milan et al., 2003). Such an assay can easily be used to identify novel compounds affecting cardiac physiology. Another assay uses a fluorescently quenched phospholipid substrate as a readout of *in vivo* phospholipase activity (Farber et al., 2001), which could be adapted for chemical screening to identify small molecule modifiers of lipid processing or other metabolic processes.

III. Chemical suppressor and enhancer screens

Forward genetic screens have provided novel entrance points into biological pathways, supplemented by suppressor and enhancer screens to identify additional upstream and downstream components of these pathways and to connect them with parallel pathways affecting the process of interest. While the use of modifier screens is widespread in invertebrate genetic models (St Johnston, 2002), genetic modifier screens have thus far been less facile in vertebrates. In part, this is due to the lack of balancer chromosomes and the resultant difficulty in phenotypic recognition of a suppressed mutant among its wild-type siblings. For example, in a cross of two fish heterozygous for both the original mutation and its suppressor, only 1/16th of the offspring would be expected to be homozygous at both alleles. Therefore, in a recessive suppressor screen, 3/16 of a clutch containing a fully penetrant suppressor would still exhibit the mutant phenotype, making it difficult to differentiate from the 1/4 mutant embryos expected in an unsuppressed clutch.

In contrast, chemical modifier screens are feasible because small molecules can be delivered easily and uniformly to all mutant embryos. This makes it possible, in theory, to suppress the mutant phenotype in all treated embryos and eliminates the need to quantify changes in mutant/wild-type ratios. This approach has been used to identify chemical modifiers of the *gridlock* mutation (Peterson et al., 2004). *Gridlock* is a recessive mutation that results in a dysplasia of the dorsal aorta that prevents blood flow to the trunk and tail 2 days postfertilization (Weinstein et al., 1995). By 4 days postfertilization, some homozygous *gridlock* mutants overcome the aortic dysplasia by forming collateral vessels that circumvent the blockage, and these individuals are often able to survive to adulthood. Forty pairs of homozygous *gridlock* adults were mated to generate homozygous *gridlock* embryos, which were arrayed three embryos per well in 96-well plates. Mutant embryos were then exposed to small molecules from a diverse chemical library and screened visually for aortic circulation to the tail.

After screening 5,000 small molecules, two structurally-related compounds were identified that completely reverse the *gridlock* phenotype and restore normal circulation to the trunk and tail (Peterson et al., 2004) (Figure 3). No adverse side effects are observed at effective concentrations, and treated embryos survive to adulthood. The compounds appear to function during angioblast specification and migration, and they also promote vessel formation in *in vitro* tubule formation assays using human cells. The precise mechanism of action of these *gridlock* suppressors is not yet known, but they may function in part by inducing expression of VEGF. Hopefully, like genetic suppressors, these small molecule suppressors will ultimately lead to the identification of novel components of the *gridlock* signaling pathway and enhance our understanding of how vasculogenesis is regulated in the developing embryo.

The search for chemical suppressors of the *gridlock* mutation was aided by the fact that breeding pairs of homozygous *gridlock* mutants could be generated. Screening for chemical modifiers of recessive lethal mutations is also possible but requires more embryos. Twenty-five percent of embryos from a cross of heterozygotes should exhibit the mutant phenotype. By placing 20 embryos in each well, the probability of

encountering a well without a homozygous mutant is 0.003. Therefore, in a screen for chemical suppressors of a recessive mutation, the rate of false positives is acceptably low, and a well containing 20 phenotypically wild-type embryos may be indicative of suppression of the mutant phenotype. A similar strategy has been employed to identify a small molecule suppressor of a recessive embryonic lethal cell cycle mutation (Stern and Zon, 2003).

Although a heterogeneous population of embryos can be used for chemical modifier screens, homogeneous populations may be preferable, and additional methods of generating such homogeneous populations exist. Recently, germ-line replacement was used to create fertile, adult zebrafish whose germ cells were derived completely from a homozygous mutant donor (Ciruna et al., 2002). Because fish generated by this method produce clutches of uniformly mutant embryos, it should be possible to create breeding pairs that will produce homogeneous populations of embryos for almost any mutation. These populations would be ideal for performing chemical modifier screens. In addition, specific pharmacological inhibitors of many proteins exist, and treatment of embryos with these inhibitors often produces a reproducible phenotype. Treatment of large groups of embryos with an inhibitor, followed by screening for compounds that suppress the induced phenotype, may be another means of identifying novel components of the targeted pathway.

Chemical modifier screens have the potential to do for zebrafish what genetic modifier screens have done for invertebrate model organisms, namely moving beyond single gene discovery to the connection of multiple genes into functional pathways. And, given the numerous zebrafish disease models that have been developed (Amatruda et al., 2002; Rubinstein, 2003; Shin and Fishman, 2002), the ability to identify small molecule modifiers of disease phenotypes also presents exciting possibilities for discovering novel therapies and therapeutic targets.

IV. Selection of small molecule libraries.

When designing a zebrafish small molecule screen, selection of the compounds to be screened is one of the factors most likely to influence a screen's success. In a genetic screen, one attempts to optimize mutation rate and genomes screened in an effort to efficiently approach 'saturation,' or the identification of all possible mutations (Mullins et al., 1994). By analogy, a chemical library should be selected that contains a high proportion of biologically active compounds and approaches 'saturation' of chemical space. To have the greatest chance at modulating the activity of every protein in the organism, it is important to ensure that as many library members as possible possess physicochemical properties that are consistent with absorption and bioavailability. In addition, it is important to select a library that possesses as much chemical diversity as possible, rather than a library that is based upon a limited number of chemical core structures, or pharmacophores.

The principles that govern absorption, distribution, metabolism, and excretion (ADME) of small molecules have not been studied in detail in zebrafish. However, the effects of small molecule structural features on ADME have been studied extensively for

mammalian systems, and generalized principles for predicting the bioavailability of small molecules have been developed. Because these principles have been reviewed extensively (Lin et al., 2003; Lipinski et al., 2001; Poggesi, 2004; Yu and Adedoyin, 2003), they will not be covered here, except to say that such factors as molecular weight, hydrophobicity, and number of hydrogen bond donors and acceptors are considered to be predictive of a small molecule's absorption and potential for biological activity. Many of the collected and combinatorial libraries available have been designed to incorporate only molecules that fall within the accepted ranges for each of these measures (Oprea, 2000; van de Waterbeemd and Gifford, 2003). In addition, a number of chemical moieties have been shown empirically to cause non-specific toxicity, and molecules containing these moieties are excluded from some libraries (Llorens et al., 2001). Although the conventional predictors of bioavailability were not generated using data from zebrafish, it may be reasonable to presume that similar principles will govern bioavailability in zebrafish, and in the absence of data specific for zebrafish, libraries that have been tailored for use in mammalian cell-based assays are a reasonable starting place for whole organism screens in zebrafish.

One physicochemical factor that has been studied directly in zebrafish, albeit superficially, is the effect of a compound's octanol:water partition coefficient on absorption. The logarithm of the partition ratio between octanol and water ($\log P$) can be measured empirically or calculated for a given chemical structure and correlates with membrane permeability (Table 1). In a study of 23 drugs that lengthen a portion of the cardiac cycle in humans known as the QT interval, it was shown that 19 of 19 drugs with a $\log P > 1$ were absorbed from the water and 18 of them caused bradycardia in zebrafish, while the four drugs with a $\log P < 1$ caused bradycardia only after injection into the embryo (Milan et al., 2003). Therefore, $\log P$ seems to be predictive of absorption, and small molecules with $\log P$ values > 1 should generally be selected for zebrafish chemical screens.

Two main types of chemical library are available for use in small molecule screening—collected libraries and combinatorial libraries. Collected libraries are assembled over time and can contain natural products and/or synthetic compounds synthesized individually or in small groups. Their eclectic nature adds to the diversity of the library. The compounds in a combinatorial library are generally all synthesized in parallel through a series of synthetic reactions that join a small number of building blocks in various combinations to generate large collections of distinct molecules (Figure 4). The advent of combinatorial chemistry enables a single chemist to generate thousands of novel compounds simultaneously and has been used to generate compounds with interesting biological activities (Batra et al., 2002; Nicolaou and Pfefferkorn, 2001). However, caution should be exercised when using combinatorial libraries for small molecule screens in zebrafish. Despite the large numbers of distinct compounds contained in these libraries, many of the compounds are structurally related and share common core structures. Consequently, it is unclear whether these libraries are able to cover as much chemical space as collected libraries, even when they contain comparable numbers of distinct compounds (Lipinski, 2000). In our experience, combinatorial libraries have generated hit rates in broad screens for developmental defects that are

comparable to those of collected libraries but with fewer distinct phenotypes being observed. This observation is consistent with the idea that most combinatorial libraries possess less structural diversity than collected libraries.

Small molecule libraries can be obtained from numerous commercial and non-commercial sources. Commercial sources include ChemDiv, Sigma-Aldrich, Chembridge, Peakdale, Maybridge, Bionet, CEREP, and Microsource Discovery Systems. These companies sell libraries as dry compounds or as predissolved stock solutions in microformat plates. In addition, many academic institutions and research organizations have acquired chemical libraries that are made available at minimal or no cost to affiliated researchers. See <http://iccb.med.harvard.edu/index.htm>, <http://www.hts.wisc.edu/Index.htm>, <http://www.medicine.mcgill.ca/biochem/htsfacility/>, and <http://www.hts.ku.edu/index.shtml> for examples. Significantly, the National Institutes of Health has also made the collection and distribution of small molecule libraries a major priority. Libraries have been created by individual institutes including the National Cancer Institute, which manages a collection of over 100,000 compounds. Furthermore, one of the major initiatives of the NIH Roadmap is to “offer public sector biomedical researchers access to small organic molecules which can be used as chemical probes to study cellular pathways in greater depth.” (nihroadmap.nih.gov). Initial libraries generated through this initiative and made available to researchers will contain at least 500,000 compounds. In short, numerous small molecule libraries are available to zebrafish researchers interested in performing chemical screens.

V. Screening methods

A. Handling embryos

For most zebrafish chemical screens, embryos should be placed into multiwell plates prior to addition of small molecules. This task is much easier to perform prior to hatching, even if the screen design calls for addition of compounds to larval stage zebrafish. In a basic developmental screen, 96-well plates are pre-filled with 200 μ L embryo buffer per well using a multichannel pipettor. Phenylthiourea (0.003%) can be added to prevent pigmentation if desired, and antibiotics (160 U/mL penicillin, 160 μ g/mL streptomycin) can be added to reduce bacterial contamination if infection becomes problematic. Three embryos are added to each well manually using a glass Pasteur pipette attached to a manual pipette pump. Hundreds of embryos can be drawn into the pipette at one time and dispensed by gently touching the pipette tip to the surface of the buffer in a well. Surface tension will draw one embryo out of the pipette and into the water without significantly changing the liquid volume in the well. With practice, one person can fill several 96-well plates with embryos in one hour. For many screens, the rate limiting step will be embryo production or phenotyping, not arraying embryos into plates, in which case this manual method should be adequate. However, if manual arraying becomes limiting, a robotic system for arraying zebrafish embryos into 96-well plates has been developed and is available commercially from Union Biometrica.

B. Compound handling

Small molecule stock solutions are prepared in DMSO at a concentration of about 5 mg/mL and are stored frozen in polypropylene 96- or 384-well plates. After thawing the

plates, 100 nL of stock solution should be transferred into the assay plates containing embryos and embryo buffer. This is most reliably performed using a pin transfer robot, but arrays of stainless steel (V&P Scientific, Inc.) or polypropylene compound transfer pins can also be used to transfer small volumes manually, 96 wells at a time. If a researcher does not have ready access to a pin transfer robot or is only screening a few plates per day, it may be more convenient to perform dispensing for several weeks of screening at once. Compounds can be dispensed into plates containing only 50 μ L embryo buffer per well. The plates can then be sealed and frozen at -80 °C for several weeks. On each screening day, the plates are thawed, and for each well, the 50 μ L embryo buffer containing 100 nL of compound stock solution is transferred to the assay plate holding the arrayed embryos using a multichannel pipettor. This approach is more convenient for laboratories that do not own compound handling robots and does not appear to significantly reduce the stability of most compounds.

It is generally not possible to screen all compounds in a library at a full range of concentrations, and so a screening concentration must be selected that is most likely to enable identification of active compounds. Screening at concentrations that are too high can lead to high rates of non-specific toxicity and death, which obscures interesting results and leads to identification of weakly active compounds. Screening at concentrations that are too low may cause potentially interesting leads to be missed. We have generally used a screening concentration of about 2 μ g/mL, a dose that produces an acceptably high hit rate in our assays but only identifies compounds potent enough to facilitate follow-up studies and efforts to identify protein binding partners.

C. Phenotype detection

Morphological phenotypes can often be detected by direct observation with a dissecting microscope. Use of screening plates with round-bottom wells facilitates observation by keeping embryos in the center of the well and preventing optical distortion caused by proximity to the side of the well. Observation from below the well is also possible, but most inverted microscopes do not offer stereo views of the embryo. Although the human eye remains the most adept tool for detecting subtle morphological changes, automated screening systems and pattern recognition software can be used to detect changes, especially quantitative changes, in zebrafish morphology or physiology. For example, the Discovery-1 screening system (Universal Imaging Corporation, Figure 5) has been used to automatically screen through plates of zebrafish embryos and detect subtle changes in heart rate that would be difficult to identify visually (Milan et al., 2003). Many additional screens requiring minimal human intervention can be envisioned, particularly as more fluorescent markers of morphology and physiology are developed for the zebrafish.

VI. Follow-up studies for active small molecules

The full benefit of identifying a genetic mutation comes after the mutated gene and mechanism of action have been identified (Talbot and Schier, 1999). Similarly, the full benefit of identifying phenotype-inducing small molecules comes after the molecular target and mechanism of action are identified. This process allows a phenotype to be converted into molecular understanding of the affected process. Two general approaches

have been used successfully to assign mechanisms of action to small molecules. The first approach involves testing whether candidate genes participate in a small molecule's mechanism of action. This 'pathway interrogation' approach includes hypothesis-based experiments to test the effect of a small molecule on a single gene and also genome-wide analyses of the effects of the small molecule on many pathways simultaneously. The second approach is to use affinity between the small molecule and its biological binding partners as a means of purifying and identifying the compound's molecular target. This 'affinity purification' approach does not require a priori assumptions about a compound's mechanism of action and includes such techniques as affinity chromatography and expression cloning.

A. Pathway interrogation

Pathway interrogation experiments aim to identify the molecular pathways affected by a particular small molecule. These experiments may or may not identify a compound's specific molecular target, but they serve to generate mechanistic insight and new, testable hypotheses about how a compound functions. They include:

1. Expression profiling/Quantitative PCR.

Altering a biological process with genetic mutations or small molecules may result in transcriptional changes, which can be detected by quantitative PCR for small numbers of genes or by global analyses using microarray transcriptional profiling (Brown and Botstein, 1999). By identifying genes that are up- or down-regulated upon treatment with a particular compound, it may be possible to generate testable hypotheses about the compound's mechanism of action. In yeast, transcriptional profiles from drug-treated yeast match profiles from strains in which the drug targets have been deleted (Kuruvilla et al., 2002; Marton et al., 1998). Microarrays for zebrafish expression profiling are also becoming readily available, including cDNA microarrays (Clark et al., 2001; Lo et al., 2003; Ton et al., 2002), spotted oligonucleotide microarrays, and Affymetrix chips. These tools may facilitate the elucidation of small molecule targets, although examples in zebrafish have not yet been reported.

One problem with transcriptional profiling in general is that it is often difficult to separate immediate responses from secondary or adaptive changes. For example, transcriptional changes in embryos with a vascular defect may be complicated by growth inhibition or cell death. Fortunately, with chemical compounds, a treatment time course can be performed to identify the precise time window during which the compound must be applied to have the desired effect, and thereby refine the window for expression profiling. Transcriptional changes observed under these conditions should better reflect rapid alterations in the primary target pathway, rather than downstream responses or adaptations.

2. Overexpression/knockdown experiments.

Gene overexpression and knockdown experiments in whole zebrafish embryos can be used to test specific hypotheses and to identify epistatic relationships between the small

molecule targets and other pathway members. In these experiments, a gene with a hypothesized role as a mediator of a small molecule mechanism of action may be overexpressed or its expression may be ‘knocked down’ by injecting 1-4 cell stage embryos with mRNA encoded by the gene or with antisense morpholino oligonucleotides, respectively. Methods for such experiments have been well described (Nasevicius and Ekker, 2000; Nusslein-Volhard and Dahm, 2002). When upregulation of a gene is hypothesized to mediate a small molecule’s effect, knockdown of that gene might be expected to block the small molecule from exerting its normal effect. Conversely, overexpression of the gene might be expected to mimic the small molecule’s effect. For example, the *gridlock* suppressor GS4012 has been shown to increase expression of the VEGF gene (Peterson et al., 2004). Consistent with a role for VEGF in GS4012’s mechanism of action, overexpression of VEGF is sufficient to suppress the *gridlock* phenotype.

Using the logic of epistatic analysis, genes that function upstream of a small molecule target would not normally be expected to modify the effect of a small molecule. Overexpression and knockdown experiments will undoubtedly be important techniques for establishing mechanisms of action for the small molecules under study, just as they are important for assigning functions to genes and genetic mutations.

B. Affinity purification

Affinity purification experiments seek to identify the specific protein targets to which a small molecule binds. These experiments are unbiased and do not require *a priori* assumptions about a compound’s mechanism of action. They include:

1. Biochemical purification.

Small molecules can be used as affinity probes for biochemical purification of their molecular targets. One classical approach is to radiolabel small molecules and use the radiolabel as a means of following target proteins through the steps of a biochemical purification. Proteins from a cell or embryo lysate treated with the small molecule are fractionated using standard, sequential biochemical purification techniques including ammonium sulphate precipitation, gel filtration, and ion exchange chromatography. Fractions containing target proteins are identified by the presence of the radiolabel. The purified target protein is then identified by tandem mass spectrometry. Several examples of the successful use of this strategy have been described XXX. A small molecule identified in a zebrafish chemical screen can be readily radiolabeled, and several companies will perform custom radiolabeling of compounds on a fee-for-service basis.

2. Affinity chromatography.

The second biochemical approach is to perform affinity chromatography using a matrix coated with the small molecule of interest. Prior to affinity chromatography, a site for attachment of a chemical linker must be identified. Variants of the compound containing modifications at various potential linker attachment points are purchased or synthesized and then tested for efficacy and potency. Once an attachment point is identified that does not interfere with the compound’s activity, a linker (e.g. aminocaproic acid) is appended to the compound. The linker is then covalently attached to a solid support resin such as

Affigel (Biorad), directly or via biotin, following the manufacturer's instructions. A negative control matrix is also synthesized by attaching the linker to the compound of interest at an inactivating site, when possible, or by attaching linker alone to the Affigel. By performing a time course experiment it is usually possible to identify a developmental window during which a compound must be present to cause the expected phenotype. A lysate is prepared from zebrafish embryos of the appropriate stage and exposed to the affinity and control matrices. Non-binding proteins are washed away, and tandem mass spectrometry is used to identify proteins that bind specifically to the affinity matrix. Several excellent references describe this process in further detail (Brown et al., 1994; Chen et al., 1999; Chen et al., 2002; Crews et al., 1994; Harding et al., 1989; Khersonsky et al., 2003; Kwok et al., 2001; Liu et al., 1991; Miller et al., 1990; Shimizu et al., 2000; Taunton et al., 1996).

Many small molecules are known to bind to several targets. As a result, affinity purification may identify proteins that bind a small molecule but are not responsible for its biological effect. This problem can be minimized and the likelihood of identifying the compound's target can be increased by ensuring that the small molecules studied are potent. Compounds with EC50s in the high micromolar range have been used successfully for target identification studies (Nguyen et al., 2003), but generally compounds with such low potency are unsuitable for affinity-based target identification. Small molecules with EC50s in the low micromolar or nanomolar range are more likely to produce successful target identification efforts. For this reason, setting the original screening concentration in the low micromolar range is helpful to ensure that compounds identified can be used for follow-up experiments. Even very potent compounds may bind to proteins that are not responsible for producing the phenotype of interest. Therefore, the function of any protein identified by affinity purification should be confirmed by performing gene knockdowns of the identified targets using antisense morpholino oligonucleotides (Nasevicius and Ekker, 2000). In this way, it should be possible to identify which proteins are relevant to the process being studied, even if multiple binding partners exist.

VII. Conclusions

In the last 15 years, the zebrafish has become established as a powerful tool for genetic experimentation. In contrast to the other mainstays of metazoan genetics—*Drosophila*, *C. elegans*, and mouse—it has proved equally amenable to chemical screens. Whether the goal is discovery of conditional probes for basic research or discovery of therapeutic targets and lead compounds, the zebrafish enables high-throughput assessment of the biological effects of small molecules. Several challenges remain, particularly in the area of identifying small molecule targets. However, the ability to discover specific, interesting, and useful compounds using zebrafish is clear, and zebrafish-based small molecule screens can provide a novel, accessible approach for launching projects, both in pathway dissection and drug discovery.

Figure legends

Figure 1. A comparison between genetic mutations and small molecules. Although mutations and small molecules can both be used to disrupt gene functions, they differ in their conditionality, specificity, and the ease with which they are discovered. See text for details.

Figure 2. Examples of small molecule-induced phenotypes. Zebrafish screens have identified small molecules that affect the ontogeny of the fins (a), otoliths (b), heart chambers (c), body axis (d), notochord (e), and eye (f). Arrowheads mark the locations of the pectoral fins (a), otoliths (b), and notochord bulge (c).

Figure 3. Chemical suppression of a genetic mutation. Fluorescent microangiograms show the circulation pattern in *gridlock* embryos untreated (a) or treated with a *gridlock* suppressor compound (b). The structures (c) and dose response curve (d) of *gridlock* suppressors identified by zebrafish chemical screening.

Figure 4. A split-and-pool approach to combinatorial chemistry. Small molecule libraries for zebrafish chemical screens can be generated by splitting synthesis beads (circles) into multiple reaction vessels and attaching different first monomers (squares) to the beads in each vessel. The beads are then pooled and split again into separate vessels, where different second monomers (triangles) are attached to the first monomers in each vessel. With a relatively small number of monomers and split-and-pool cycles, thousands of distinct small molecules can be synthesized.

Figure 5. The Discovery-1™ Screening System. Automated imaging can increase the throughput of zebrafish screens for chemical modifiers of morphology or physiology.

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Table 1. The relationship between logP and absorption in the zebrafish. Drugs with logP values greater than 1 were absorbed, while drugs with logP values less than 1 were not absorbed. Where available, experimental logP values were used. Asterisks indicate values calculated using KowWin software (Syracuse Research Corporation).

small molecule	logP	absorbed
pentamidine	< -1.5	NO
sotalol	0.24	NO
procainamide	0.88	NO
NAPA-HCl	0.99*	NO
E-4031	2.56*	YES
valproic acid	2.75	YES
erythromycin	3.06	YES
cisapride	3.09*	YES
ketanserin	3.29	YES
quinidine	3.44	YES
droperidol	3.50	YES
flecainide	3.78	YES
fluoxetine	3.82	YES
ibutilide	3.82*	YES
haloperidol	4.30	YES
amitryptiline	4.92	YES
chlorpromazine	5.41	YES
thioridazine	5.90	YES
pimozide	6.30	YES
tamoxifen	6.30*	YES
astemizole	6.43*	YES
terfenadine	7.62*	YES
amiodarone	7.80	YES

Figure 1.

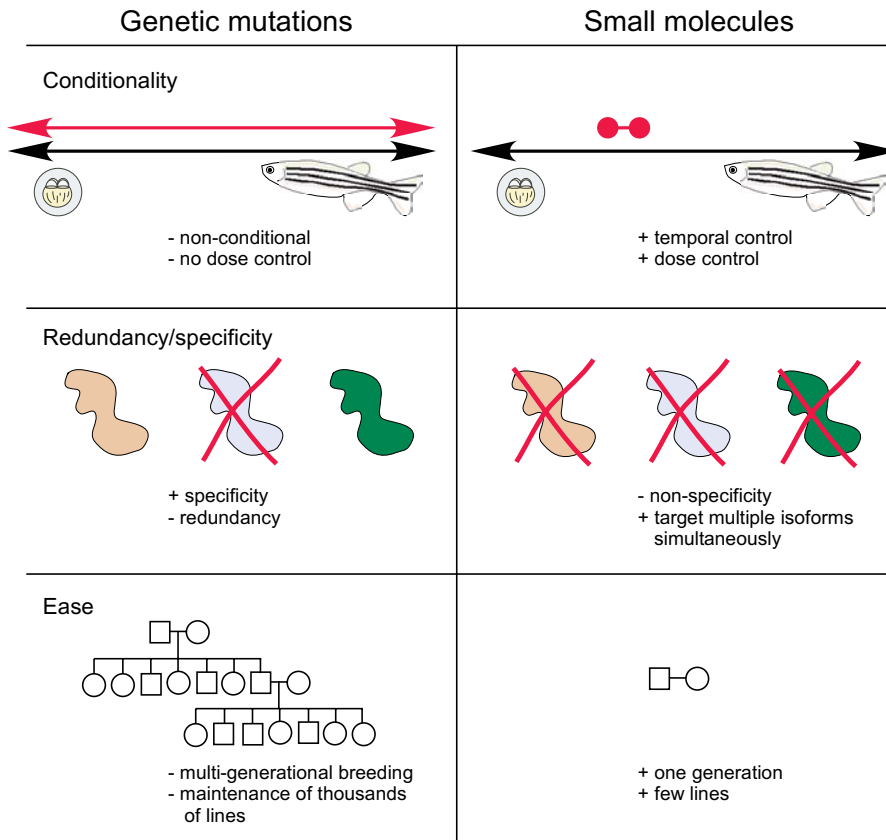


Figure 2.

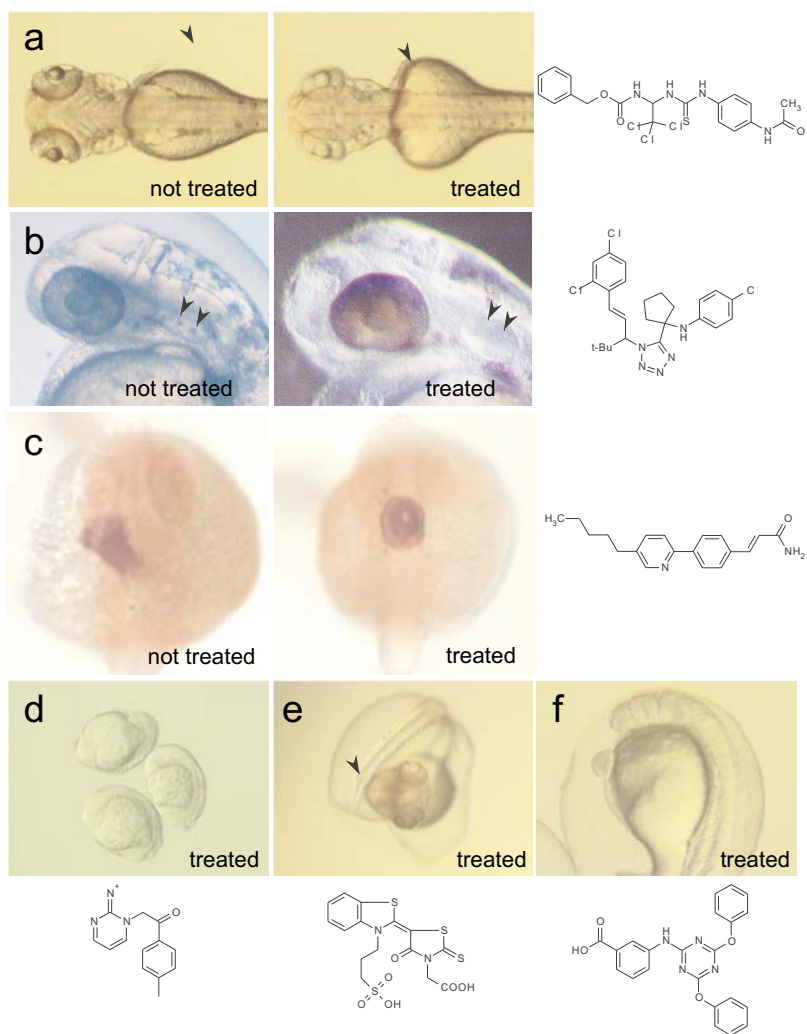


Figure 3.

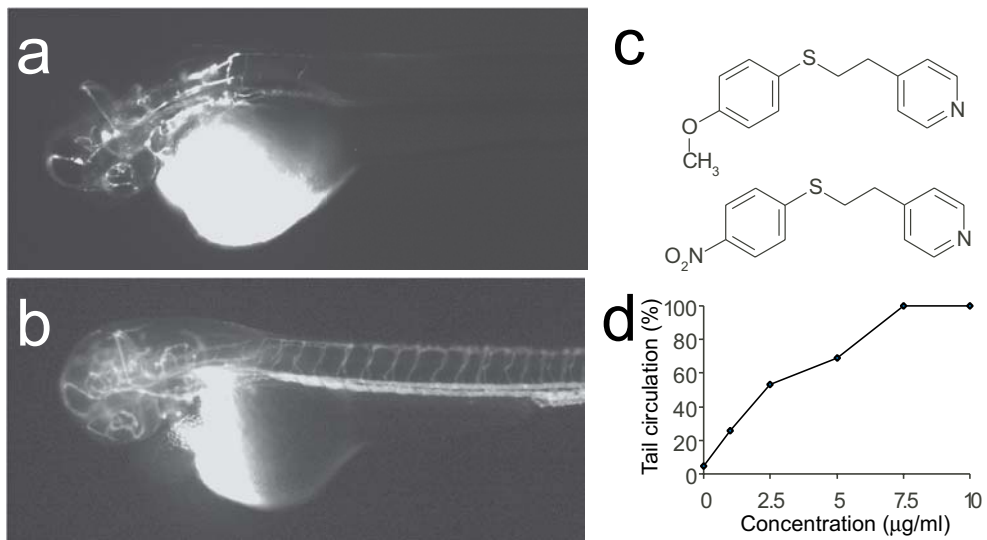


Figure 4.

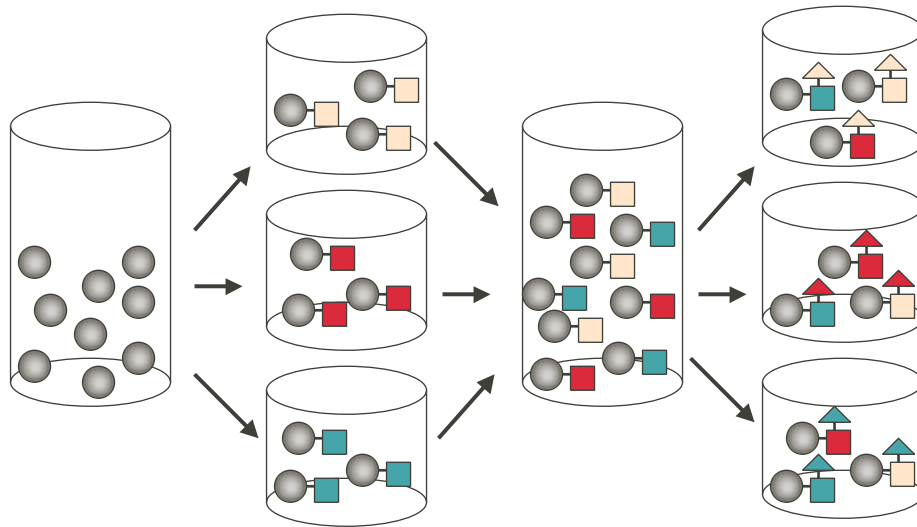


Figure 5.

