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From phenotype to mechanism after zebrafish small molecule screens

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Abstract

Small molecules screens conducted with living zebrafish have become a commonly practiced technique for small molecule discovery. Embryonic and larval zebrafish exhibit an almost limitless range of phenotypes, from the cellular to the organismal. Consequently, small molecule screens can be designed to discover compounds modifying any of these phenotypes. The compounds discovered by zebrafish screens pose unique challenges for target identification, but the zebrafish also provides several powerful approaches for identifying targets and determining mechanisms of action. Four major approaches have been used successfully, including methods based on comparison of chemical structures, genetic phenocopy, pharmacologic phenocopy, and compound affinity. These approaches will continue to facilitate target identification for compounds from zebrafish small molecule screens, and more importantly, to reveal their mechanisms of action.

Introduction

In recent years, zebrafish screens have identified compounds affecting embryonic development, cardiac physiology, stem cell quantity, sleep, cancer cell differentiation, the cell cycle, regeneration, hair cell death, and fibroblast growth factor (FGF) signaling, to name just a few^{1–9}. Zebrafish screens complement more traditional in vitro and cell-based screens because they allow screening methodology to be applied to complex, organismal processes that would be difficult to reduce to an in vitro assay. From this perspective, zebrafish have been uniquely well suited for discovering modifiers of embryonic development, organ physiology, and behaviors. Nevertheless, even cellular phenotypes that could be studied with cultured cells may at times be appropriate for zebrafish screens, because some cell types can be generated more consistently in zebrafish embryos than in culture, where uniform cell identity can sometimes be difficult to maintain. Therefore,

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zebrafish small molecule screens are likely to continue to be useful tools for discovering compounds with novel bioactivities.

One often-cited challenge for phenotype-based small molecule screens is the difficulty of determining mechanisms of action for the compounds discovered. Although target identification remains one of the grand challenges for the field, the history of pharmacology is filled with outstanding target identification successes. Phenotype to mechanism studies have arguably provided some of chemical biology's most significant contributions, including discovery of the opioid receptors, the ryanodine receptors, the histone deacetylases, and mTOR (the mammalian target of rapamycin), among others. Therefore, while some scientists view the process of moving from phenotype to mechanism of action to be daunting, others view it as the most exciting and high impact activity within the field of chemical biology.

Although the central concepts of target identification have been around for decades, new developments (both technical and strategic) promise to facilitate the process of moving from phenotype to mechanism. Many of these developments can be applied equally well to small molecules discovered by zebrafish screens and to compounds discovered by other means. Nevertheless, this review will emphasize application of the developments to molecules discovered by zebrafish screens.

Target elucidation by structure

In principle, the binding partners of a small molecule should be predictable from the structures of the small molecule and targets alone. In practice, structural information is available for only a fraction of all potential targets in an organism, and calculating binding affinities for all potential targets is a daunting computational challenge. Nevertheless, as data accumulates about the targets of existing compounds, our ability to predict targets for novel compounds increases. This is particularly true for small molecules that share some structural homology with another compound for which binding information is available.

While there are several methods for predicting targets based on structural information, the method used most extensively for zebrafish-discovered molecules is the Similarity Ensemble Approach (SEA). SEA is a computational approach easily executed through an internet interface in which the structure of a novel compound can be compared to 167,000 organic molecules for which some target information is available¹⁰. Statistical comparisons analogous to those used for gene homology searching are used to identify structural similarities between the novel compound and compounds known to bind to a particular molecular target. In this way, targets of a novel compound can be predicted solely by structural comparison with existing, target-annotated compounds.

In a recent test of this approach, 681 hits from a large-scale zebrafish behavioral screen were examined by SEA¹¹. High confidence target predictions were generated for 86% (586/681) of the molecules. Although testing of all predictions was not possible, a subset of 20 predictions was tested using biochemical assays. More than half of the predictions (11/20) were confirmed at a biochemical level. Therefore, SEA can often generate testable hypotheses about targets for small molecules coming from zebrafish screens. Although these

hypotheses are not always correct, they represent an excellent starting point in moving from phenotype to mechanism of action.

Target elucidation by genetic phenocopy

The phenotypes of thousands of genetic mutations have been described in zebrafish. These phenotypes represent a rich resource for connecting phenotypes to molecular mechanisms. The mechanisms of action of several small molecules have been determined by recognizing similarity between the small molecule-induced phenotype in zebrafish and a comparable mutation-induced phenotype. For example, Yu et al discovered a small molecule, dorsomorphin, that dorsalizes zebrafish embryos and causes loss of the ventral tail fin¹². This phenotype was virtually indistinguishable from that of the BMP receptor (*acvr11*) mutant *lost-a-fin*, suggesting that dorsomorphin inhibits BMP receptor activity. This hypothesis has been confirmed biochemically.

As other examples, the antiangiogenic compound TNP-470 was shown to phenocopy the *pipetail* (*wnt5b*) mutation in zebrafish. This observation led Zhang et al to hypothesize and confirm experimentally that TNP-470 inhibits noncanonical Wnt5 signaling¹³. Compounds that mimic the cardiac rhythm defect in *breakdance* mutants have been shown to inhibit the same potassium channel (*kcnh6*) defective in *breakdance* mutants¹⁴. Therefore, comparison to genetic phenotypes represents a viable means of predicting possible small molecule targets. As the number of mutant phenotypes increases and tools for comparing phenotypes improve, this approach is likely to become even more powerful.

Target elucidation by pharmacologic phenocopy

Phenocopy comparisons may also be made between new compounds of interest (for which the target(s) are not yet known) and “known” compounds whose biological activities have already been well established. These include compounds used clinically. If a new small molecule produces a specifically modified phenotypic state, then that modified state can be compared to the phenotypic states produced by various other small molecule treatments. Using clustering algorithms, small molecules can be arranged based on their phenotypic similarity rather than their structural comparability.

This approach is particularly amenable in the context of high throughput screening, since it involves screening libraries of known compounds in the same format used for the initial screen of unknown compounds. Several such libraries, of small molecules with known bioactivity, are already commercially and academically available. Therefore, although target elucidation by pharmacological phenocopy is limited by the availability of test data on known compounds, it is as easy to rapidly build a database of “known” phenotypes as it is to complete the original high throughput screen.

Recently, Kokel et al constructed a dataset of phenotypic modulations of the photomotor response (PMR) in embryonic zebrafish by 14,000 different small molecules¹⁵. Each complex behavioral chemotype generated by the high throughput screen was distilled into “barcodes” which were then hierarchically clustered by similarity. By clustering together phenotypes from both known compounds and those with no previously known bioactivity,

these investigators were able to generate testable hypotheses which lead to the identification of novel acetylcholine esterase (*PMR slow to relax*, STR) and monoamine oxidase (*PMR magnitude stimulant*, MAG) inhibitors.

Target elucidation by binding

Affinity-based approaches have historically been successful in identifying the direct binding partners of small molecules. These approaches use affinity for a small molecule to purify and identify its molecular targets. Affinity-based approaches do not generally require a priori assumptions about a compound's mechanism of action. Significantly, unlike some of the approaches described above, they can also identify binding partners for compounds that share no structural or phenotypic similarity with previously characterized compounds.

Most affinity-based approaches require that a small molecule be attached to solid support via a molecular linker. Structure activity relationship (SAR) studies can identify potential attachment sites for such linkers that do not interfere with the compound's activity. Once an attachment site is identified, the compound is linked to a solid support resin, enabling the resin to be used for affinity purification of binding proteins (or other macromolecules) from cellular lysates. Using an affinity-based approach, Jung et al recently identified heat shock protein 90 (hsp90) at the target of triazine compound S06, which was found to inhibit oral squamous cell carcinoma metastasis in zebrafish¹⁶.

Traditionally, affinity chromatography has been used to purify target proteins to a point where they can be visualized on a gel and excised for peptide sequencing. Within the past few years, however, new proteomic techniques have begun to greatly improve the process of affinity-based target identification. SILAC (stable isotope labeling with amino acids in cell culture) and iTRAQ (isobaric tags for relative and absolute quantification) are techniques that enable identification and quantification of a large number of proteins that bind preferentially to a small molecule of interest^{17, 18}. Using these techniques, one can identify all of the binding partners for a small molecule and estimate their binding affinities at once.

It is increasingly clear that most small molecules bind to more than one target. In some cases, only one target contributes to the compound's primary mechanism of action, but in others, it is the combined activity at multiple targets that is responsible for a compound's activity. Through their ability to simultaneously identify multiple binding partners for a small molecule, techniques like SILAC and iTRAQ offer a more realistic (albeit complex) view of how a small molecule interacts with proteins in a native biological context. Of course, multiple targets can mean ambiguity about the actual mechanism of action, and demonstration of binding to a putative target does not assure that the target is involved in the compound's mechanism of action (MOA). Therefore, the function of a protein identified by any affinity purification technique must be confirmed by an orthogonal technique, such as knocking down the putative targets with antisense morpholino oligonucleotides¹⁹ or mutating the corresponding gene using zinc finger nucleases or TALENs^{20–24}.

Beyond target to mechanism of action

As highlighted above, there are several viable approaches for elucidating the direct binding targets of a small molecule. The process remains challenging, but technical advances are making it easier and easier. Although the importance of identifying direct binding partners should not be overlooked, there is some risk of placing too much emphasis on it. For example, some reviewers and editors will spurn any paper describing a small molecule without a clear binding partner, regardless of how much mechanistic insight it provides. And yet, small molecules can provide invaluable mechanistic insight even in the absence of a clearly defined molecular target. For example, the effect of the small molecule ryanodine on muscle contraction was described in the 1940s²⁵, but the identity of the ryanodine receptor wasn't reported until 1989²⁶. In the intervening decades, hundreds of studies used ryanodine to probe the biology underlying muscle contraction. These studies revealed many of the fundamental mechanisms of muscle contraction. By the time the ryanodine receptor was cloned, its role in releasing calcium stores from the sarcoplasmic reticulum was already documented in astonishing detail. Thankfully, reviewers and editors appreciated the value of the mechanistic studies performed with ryanodine in the forty years prior to the cloning of the ryanodine receptor.

Conversely, it should not be assumed that identifying a binding partner for a small molecule ends the quest to understand its mechanism of action. Much additional work is typically required to understand how a specific binding partner contributes to a compound's mechanism of action. Additionally, many small molecules have multiple binding partners, and it is often the combined activity at multiple targets that is responsible for a compound's activity. Therefore, it is important to avoid a single-minded focus on identifying the target of a small molecule, and to focus instead on understanding its mechanism of action.

Conclusions

High throughput assays for complex phenotypic readouts are becoming commonplace for good reason. The two main causes for drug failure are lack of efficacy and toxicity. The small molecules currently fed into the clinical trial pipelines are often identified in oversimplified cell-based or *in vitro* assays, which do not always demonstrate efficacy in whole organisms and may cause unacceptable side effects when metabolized or exposed to organ systems. Small molecules identified in complex, physiological systems, such as the zebrafish are more likely to have improved ADMET (absorption, distribution, metabolism, excretion and toxicity).

While it is true that complex phenotypic readouts can engender difficulty in determining mechanisms of action for the compounds discovered, they also allow simultaneous screening across a broad set of potential targets. Many disease states cannot be reduced to a single *in vitro* or cell-based system owing to their complex and often poorly understood etiology. Therefore, performing small molecule screening on a "black box" system where the precise mechanism of action is unknown can also be an advantage.

Fortunately, there exists an expanding arsenal of approaches for target elucidation “*post cribrum*” (after the screen). These include direct binding approaches, phenotypic comparisons between chemotypes and genotypes, between chemotypes themselves, and target predictions based on chemical structure. Many of these have already been successfully applied to zebrafish screens.

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Figure 1.

High throughput phenotypic screens using whole organisms are now possible and becoming more widely used due to the small size, high fecundity and transparency of zebrafish (*D. rerio*). The compounds discovered by these screens pose unique challenges for target identification but also provide several powerful approaches for identifying targets and determining mechanisms of action.

Table 1

Once a chemical of interest has been identified using a high throughput phenotypic screen, there are several ways to move from phenotype to identification of a biological target and mechanism of action.

Mode of Identification	Basis for Comparison	References
Structure	chemical structure of interest ↔ known chemical structures and their known pharmacology	Keiser et al. 2007 Laggner et al. 2011
Genetic Phenocopy	chemical phenotype of interest ↔ known mutant phenotypes and their genetic basis	Langheinrich et al. 2003 Yu et al. 2008 Zhang et al. 2006
Pharmacological Phenocopy	chemical phenotype of interest ↔ known chemical phenotypes and their known pharmacology	Kokel et al. 2010
Binding	pulldown sequence of interest ↔ known genetic sequences	Jung et al. 2011