Point of VIEW

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Swimming into the Future of Drug Discovery: *In Vivo* Chemical Screens in Zebrafish

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ABSTRACT In recent years *in vivo* chemical screening in zebrafish has emerged as a rapid and efficient method to identify lead compounds that modulate specific biological processes. By performing primary screening *in vivo*, the bioactivity, toxicity, and off-target side effects are determined from the onset of drug development. A recent study demonstrates that *in vivo* screening can be used successfully to perform structure–activity relationship (SAR) studies. This work validates the zebrafish as an effective model for not only drug discovery but also drug optimization.

The target-centric drug discovery paradigm predominantly followed over the past 50 years entails multiple iterations of *in vitro* biochemical and cell-based assays followed by *in vivo* studies in animal models and then ultimately trials in humans (Figure 1). This process typically takes 12–15 years before drugs reach the market. Less than 1% of developing drugs result in success, but the pursuit of many promising "failed" drugs can cost a company millions of dollars in R&D (*1, 2*). As a result, the discovery of new drugs from major pharmaceutical companies has declined while production costs have steadily increased. Prospective drugs can be terminated at any point during development due to lack of efficacy, adverse side effects, or excessive toxicity. Much of the failure comes at the level of animal testing where problems with *in vivo* absorption, distribution, metabolism, excretion, and toxicity (ADMET) are first assessed. Using *in vivo* animal models at the initial stages of screening can improve determination of ADMET from the start rather than after years of research and millions of dollars down the line. *In vivo* screening also simultaneously assesses drug selectivity and specificity in the context of a living organism. In an article in this issue, Hao and colleagues describe one of the first large-scale *in vivo* structure–activity relationship (SAR) studies (*3*). Dorsomorphin, a promiscuous hit previously identified in a phenotype-based small molecule screen for BMP (bone morphogenetic protein) inhibitors, was found not only to antagonize BMP signaling but also to abrogate angiogenesis in zebrafish embryos *via* VEGF (vascular endothelial growth factor) inhibition (*4*). Using *in vivo* SAR studies, Hao *et al.* generated two selective and potent inhibitors for BMP and VEGF signaling. This work demonstrates the ability to use an *in vivo* screening model for lead compound discovery and subsequent optimization.

Over the past decade the zebrafish has emerged as a vertebrate model amendable to large-scale forward genetic and chemical screens (*5*). Similar to classic invertebrate models, zebrafish develop extra-uterine, allowing for facile visualization of early embryogenesis and organogenesis. In contrast, zebrafish can be used to study the regulation of vertebrate-specific processes that affect disease and development. Many other advantages make zebrafish a particularly good model for high-throughput screening. High fecundity and small size permit the generation and storage of thousands of fish in a small space. Optical clarity of the embryo makes it possible to visualize a wide variety of phenotypes without killing or manipulating the embryos. Gross morphological changes are easily viewed with light microscopy, and the use of fluorescent transgenic fish allows for cell-type or pathway-specific visualization.

The process of embryogenesis involves the convergence of multiple signaling pathways. Forward genetic screens in zebrafish provide an array of mutants with specific

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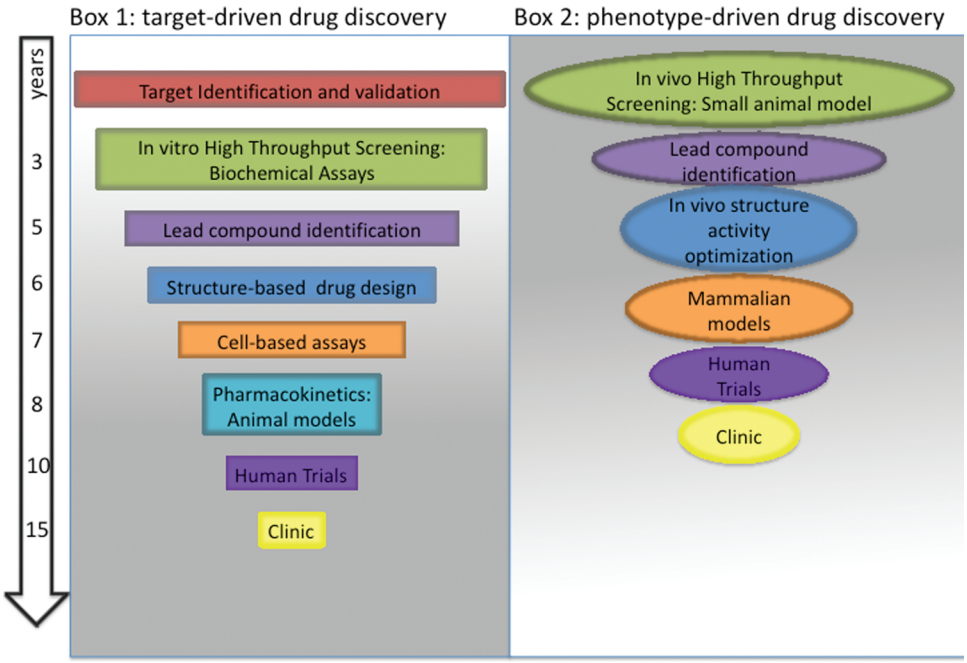

Figure 1. Strategies for drug discovery. Box 1: The target-driven drug development pipeline takes 12−15 years on average before a drug is available in the clinic. Failed compounds drop out of development at every step of the process due to low specificity, efficacy, or toxicity. At each step of the process these failures accrue such that less than 1% of lead compounds result in a viable product. Box 2: Phenotype-driven drug development removes the need to identify a single protein for drug targeting and instead interrogates an entire pathway or tissue, while taking into account the environment of a whole organism. This strategy has the potential to save years and result in fewer failed drugs, as problems related to *in vivo* biology are caught early in the process.

phenotypes that correspond to perturbations in defined pathways. The signaling pathways utilized during embryogenesis are often defective in disease states, thus zebrafish embryogenesis provides an easy tool to interrogate a multitude of pathway-specific chemical modifiers that have therapeutic potential. For example, BMP signaling is defective in diseases affecting many tissues including bone, kidney, and a multitude of cancers and is essential for embryogenesis (*6, 7*). In development, the BMP signaling pathway controls the proper establishment of the dorsal-ventral (D-V) axis. Mutant zebrafish with defective BMP signaling display altered D-V axis formation with excessive dorsal tissues like the brain at the expense of ventral tissues like blood and muscle (*6*). An initial study executed by Yu and colleagues screened small molecules for those that could mimic the effects of BMP-defective zebrafish mutants (*4*). Over 7500 compounds with known bioactivity were screened, and one hit induced a reproducible dorsalized D-V axis phenotype. This compound, named dorsomorphin, is the first identified chemical inhibitor of BMP signaling. Its mechanism of action is inhibition of the serine-threonine kinase activity of Type I BMP receptors (BMPR-I), as measured by phosphorylation of the target substrates smads 1, 5, and 8.

As with most initial hits from a drug screen, dorsomorphin was a good inhibitor of BMP signaling but upon closer inspection was not the most specific or robust. *In vitro* kinase assays showed dorsomorphin only moderately inhibited the phosphorylation of smads 1, 5, and 8. Cuny *et al.* performed a SAR study using *in vitro* biochemical assays and identified LDN-193189, a derivative that showed higher potency for BMPR-I kinase inhibition and better pharmacokinetics in mice (*8*). This optimized compound was successfully used to treat a mouse model of fibrodysplasia ossificans progressiva, a congenital disorder of progressive and widespread postnatal ossification of soft tissue induced by constitutive activation of BMP receptor signaling, demonstrating the promise of combining zebrafish lead compound discovery, SAR functional studies, and mammalian modeling for drug optimization (*4*).

While this *in vitro* SAR study did uncover a better BMPR inhibitor, the experiments performed did not fully address off-target effects and bioavailability of a larger number of compounds, which could result in abandoning a potentially great *in vivo* drug that performs less than ideal in a biochemical assay. Many drugs that show great promise in biochemical assays often fall short during ADMET testing, while those that may have moderate biochemical activity are more specific and potent *in vivo* (*5*). In this issue, Hao *et al.* describe a large-scale *in vivo* SAR study on dorsomorpin, in which they identified specific and potent inhibitors of BMP and VEGF signaling (*3*). Using parallel library synthesis centered around the 3,6-disubstituted pyrazolo[1,5-*a*]pyrimidine core of dorsomorphin, 63 distinct compounds were generated, isolated, and tested for effects on D-V axis formation, angiogenesis, and overall toxicity. Three derivatives were highly selective for D-V axis defects, and one was specific for inhibiting angiogenesis.

The most promising BMP inhibitor was DMH1. This derivative was more potent than dorsomorphin or LDN-193189 with an effective concentration resulting in 100% of exposed embryos displaying the phenotype (EC100) of 0.2 μM compared to 2.5 or 3 μM, respectively. DMH1 was also more selective, displaying negligible toxicity compared to dorsomorphin or LDN-193189. DMH1 was completely selective for BMPR-I inhibition, showing no activity against TGFβR-I, AMPK, VEGFR, or PDGFR. In agreement with these *in vitro* results, DMH1 showed no effect on *in vivo* angiogenesis, even at high concentrations. The effect of DMH1 translated to human cell culture, blocking BMP-induced phosphorylation of smads 1, 5, and 8 and transcription in human HEK293 cells.

In contrast, DMH4 showed no effect on D-V axis formation but had an EC50 for disruption of embryonic angiogensis of 1 μM compared to dorsomorpin (5 μM) and LDN-193189 (20 μM). This compound had indiscernible toxicity regardless of dose, showing its high degree of specificity for angiogenesis. This effect was not restricted to fish embryos, as VEGF-induced endothelial tube formation of human venous endothelial cells in culture was abrogated by DMH4 exposure, suggesting the mechanism of action is likely *via* inhibition of VEGFR signaling. Supporting this notion, DMH4 was shown to have a low inhibitory concentration 50 (IC50) for VEGFR inhibition of 161 nM, compared to 3.5 μM for BMPR-I and 8 μM for AMPK using *in vitro* kinase assays. Of note, dorsomorpin, LDN-193189, and 6LP (another tested derivative) were found to be potent VEGFR inhibitors with IC50 of 25.1, 214.7, and 37 nM, respectively, but the *in vivo* EC50 for disruption of embryonic angiogenesis was 5, 20, and 0 μM, respectively, indicating reduced bioactivity of these analogues compared to DMH4. These results highlight the importance of performing *in vivo* screens to find the most relevant analogues for drug development.

The approach described by Hao *et al.* exemplifies the potential advantages of *in vivo* phenotype-driven screens in small vertebrates, like the zebrafish, at multiple steps of the early drug development stages in identifying the most bioactive and relevant compounds for subsequent investment of time and money. Chemical screening in zebrafish is an emerging field but is still in its infancy. A full understanding of the physiological and pharmacological similarities and differences between zebrafish and humans is not fully appreciated, and thus the predictive power of zebrafish ADMET into human ADMET is unclear. Several drugs utilized in human patients have been proven to work in zebrafish, indicating at least some degree of conservation (*9*). To circumvent this possibility of differences in ADMET in fish and humans, many zebrafish researchers use chemical libraries that are composed of small molecules with known bioactivity and mainly comprise already FDA-approved drugs (*3, 10*). Using these libraries, scientists can identify new therapeutic usage for old drugs, which can lead to a shortened time to human trials saving precious time and money. One example of a new use for an old drug is the newly uncovered ability of prostaglandin E2 (PGE2) to expand hematopoietic stem cells (HSC) *ex vivo* (*10*). Our lab observed that 16,16-dimethyl PGE2 (dmPGE2) can enhance HSC formation in zebrafish embryos. Murine marrow engraftment and human cord blood stem cell engraftment in NOD-SCID mice were both enhanced by pretreatment of transplanted cells with dmPGE2. A clinical trial for leukemic patients who are receiving a cord blood stem cell transplant is now being done on the basis of this work. This study demonstrates the value of zebrafish screening of known small molecules to expedite drug development. As many already FDA-approved drugs are used for off-label purposes, this strategy holds great potential. These positive "side" effects can be due to unknown usage of the target protein in other biological contexts or due to previously unknown off-target effects of the drug. *In vivo* SAR studies in genetically amendable models can distinguish these scenarios better and improve drug targeting.

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