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Mini review

Zebrafish small molecule screens: Taking the phenotypic plunge

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ABSTRACT

Target based chemical screens are a mainstay of modern drug discovery, but the effectiveness of this reductionist approach is being questioned in light of declines in pharmaceutical R & D efficiency. In recent years, phenotypic screens have gained increasing acceptance as a complementary/alternative approach to early drug discovery. We discuss the various model organisms used in phenotypic screens, with particular focus on zebrafish, which has emerged as a leading model of *in vivo* phenotypic screens. Additionally, we anticipate therapeutic opportunities, particularly in orphan disease space, in the context of rapid advances in human Mendelian genetics, electronic health record (EHR)-enabled genome–phenome associations, and genome editing.

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1. Introduction

For much of human history, therapies for various ailments came about from astute phenotypic observations and serendipity [31]. For instance, the origins of digoxin, a cardiac glycoside currently in use for heart failure, can be traced directly to a traditional herbal remedy for dropsy made from the foxglove plant [37]. With the advent of modern biochemistry and molecular biology, drug discovery became dependent on the target-based approach to systematically screen for thousands and even millions of agents that modulate a particular biological target chosen based on a rational therapeutic hypothesis. In the decades that followed, an unprecedented number of new therapeutics have transformed modern medicine and pharmaceutical industry [21]. However, despite the disproportionate focus and funding on target based approaches for the past two decades, the pharmaceutical industry as a whole delivered fewer "first-in-class" drugs using this approach than using a phenotypic approach [56]. In fact, the cost, and the risks, of developing a new pharmaceutical entity have skyrocketed in the

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recent decades, with the costs of developing a new drug seeming to grow exponentially, a trend termed "Eroom's Law", to contrast with the Moore's Law describing exponential growth in computing power [51]. There are a number of reasons for this alarming decline in efficiency of pharmaceutical development. Obvious reasons include unforeseen off-target effects and toxic metabolites that result in deleterious effects in humans. While late stage failure in clinical trials captures headlines, a key reason for the sustained decline in productivity may lie in the earliest stages of drug discovery: specifically, poor target selection. For an industry grown around target-based discovery, picking a wrong target based on an invalid therapeutic hypothesis can be a death knell, a situation made worse by the fact that consequences might not be apparent until significant expenditure of time and effort. There are numerous causes of poor target selection, but chief among them appears to inadequate insight into human pathophysiology provided by *in vitro* and preclinical models [2,70].

Given the pitfalls of target-based screening, phenotypic screening has reemerged as an attractive alternative and complementary approach to drug discovery. As the name implies, this approach focuses on phenotypic perturbations – observable changes in complex biological function caused by small molecules – to identify chemical modulators of physiological or disease processes in a target-agnostic manner. The observed phenotype results from integration of all cellular pathway perturbations in the context of an active biological system, be it an individual cell or an entire organism. A phenotypic screen, by definition, identifies chemotypes that affect a biologically meaningful target or targets, including key nodes responsible for integrating cell pathways and behaviors. Importantly, since a phenotypic screen is conducted without regard to *a priori* knowledge of targets, it has the potential to discover new therapeutic targets, which may have greater impact at the systems level than established targets. Moreover, in contrast to target-based screens, a phenotypic screen permits discovery of compounds that affect a desired outcome *via* engaging multiple targets in a synergistic manner that may not have been otherwise anticipated. Indeed, recent studies have shown that polypharmacology is not necessarily deleterious, and that engagement of multiple targets can sometime be more effective for treatment of certain disease [50]. While a knowledge of the precise pharmacological target is traditionally considered essential, although not required by the FDA, to push a drug development forward; there is increasing willingness to be target agnostic provided there is a compelling biological rationale and an unmet medical need [32].

In contrast to traditional observational approaches, which were low-throughput and therefore depended on serendipity, the modern phenotypic screen combines the advantages of phenotype-based approaches with the latest high-throughput chemical screening capabilities. In this review, we will provide a brief overview of various models used in phenotypic screens, with a focus on zebrafish based screens, which has emerged as a powerful *in vivo* model amenable to high-throughput and high-content analyses, and a look to the future of phenotypic screening.

2. Phenotypic screening modalities

Modalities of phenotypic screens can be broken into two components: the biological model and the assay outputs. These two factors must be considered prior to any screen. A number of model systems have been used in phenotypic screening, ranging from single cells, to organoids and whole organisms.

Cell based screens vary in scope of potential readouts from a simple cell viability assay to complex cell behavior analyses. At the simple end of the spectrum, most screens for potential anti-cancer agents are cell viability assays using established cancer cell lines [53]. At the complex end, Lum and colleagues have screened small molecules in HCT116 human colorectal cancer cells using multiplexed luciferase assays and dot blotting to monitor multiple pathways simultaneously [25]. By assessing multiple pathways in a quantitative manner, they were able to collapse the cellular phenotypes elicited by individual compounds into a "fingerprint." Traditionally, determining mechanism of action (MOA) can be laborious, however; such an approach provides mechanistic insights by clustering compound induced "fingerprints" to those obtained from an siRNA library [24]. Cell based screens have also been conducted in an image based analytics paradigm. Peppard and colleagues identified novel autophagy regulators in HeLa cells expressing LC3 (microtubule-associated protein light chain3)-GFP (green fluorescent protein) fusion protein as an autophagy readout. LC3 is normally cytosolic, however during autophagy is recruited to autophagosomal membranes, which manifest as GFP granules in this read out. When nutrient starved cells are treated with lysomotropic agent hydroxychloroquine (HC), which inhibits the lysosome, LC3-GFP degradation by autophagy is blocked. Using HCS imager *Incell 3000*, a 250,000 compound screen was conducted to identify inhibitors of the formation of autophagosomes, which was thresholded as *<4 GFP granules* [42] Notably, the authors validated this assay with wortmannin, a known inhibitor of autophagosome formation and used this as a positive control to set the threshold.

While most cell based screens have been conducted in established cell lines grown in simple monolayers or suspension, investigators have developed 3-D organoid models of tumor cells, with the aim of developing an *in vitro* model that is more relevant to human tumor biology, including the role of metabolically quiescent tumor stem cells and the effect of hypoxia gradient within solid tumors. For instance, Walsh and colleagues have developed a model of spheroids derived from primary human tumors, utilizing intrinsic fluorescence properties of FAD and NADH called optical metabolic imaging (OMI). OMI has previously been shown to serve as an early endpoint biomarker for drug response [60]. Using this technique the authors carried out a screen for small molecules that altered metabolic activity of tumor spheroids [61].

In the past few years, human induced pluripotent stem cells (hiPSCs) have emerged as a promising human biological platform for phenotypic screening. Since their initial description less than a decade ago, researchers have created iPSC models of a myriad of human diseases using patient-derived iPSCs [58]. For example, Burkhardt and colleagues have generated hiPSC from ALS patients and demonstrated that neurons differentiated from these hiPSCs exhibit TDP-43 aggregation, a pathological hallmark of ALS. Using an image-based screen based on TDP-43 aggregation in neurons generated from ALS hiPSCs, they discovered that known small molecule inhibitors of the Na^+/K^+ ATPase, GSK3 and CDK could ameliorate this phenotype, providing supporting not only for prior studies that have implicated these proteins as potential ALS therapeutic targets but also the use of patient-derived iPSCs for drug discovery [4].

Cell based screens, while providing an inexpensive, quantitative and high throughput platform for phenotypic screening, suffer from several disadvantages. Despite advances in engineered tissue constructs, cultured cells do not exist in a native biological context and lack critical tissue interactions and paracrine factors which clearly play an important role *in vivo*. Compound liabilities such as poor metabolic stability, suboptimal bioavailability and undesirable off-target as well as on-target effects are not recognized early on during the primary screen. Such issues can be addressed from the start with *in vivo* chemical screening of living organisms and whole animals. Thus far, large-scale *in vivo* phenotypic screens have been conducted in various model multicellular organisms ranging from nematode such as *C. elegans* to vertebrates such as zebrafish.

For instance, Petraschek and colleagues have performed a small molecule screen for compounds that affect aging in the nematode. From this screen, they identified 60 compounds that increase *C. elegans* lifespan without obvious deleterious effects. Concordant with existing genetic models of aging, over half of the hit compounds increased the animal's resistance to oxidative stress [71]. Importantly,