REVIEW

Recent advances in phenotypic drug discovery [version 1; peer review: 2 approved]

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Abstract

There is a great need for innovative new medicines to treat unmet medical needs. The discovery and development of innovative new medicines is extremely difficult, costly, and inefficient. In the last decade, phenotypic drug discovery (PDD) was reintroduced as a strategy to provide first-in-class medicines. PDD uses empirical, target-agnostic lead generation to identify pharmacologically active molecules and novel therapeutics which work through unprecedented drug mechanisms. The economic and scientific value of PDD is exemplified through game-changing medicines for hepatitis C virus, spinal muscular atrophy, and cystic fibrosis. In this short review, recent advances are noted for the implementation and de-risking of PDD (for compound library selection, biomarker development, mechanism identification, and safety studies) and the potential for artificial intelligence. A significant barrier in the decision to implement PDD is balancing the potential impact of a novel mechanism of drug action with an under-defined scientific path forward, with the desire to provide infrastructure and metrics to optimize return on investment, which a known mechanism provides. A means to address this knowledge gap in the future is to empower precompetitive research utilizing the empirical concepts of PDD to identify new mechanisms and pharmacologically active compounds.

Keywords

Phenotypic drug discovery, first in class, empirical, PDD, Target, TDD

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Introduction

Drug discovery uses all available knowledge to identify biological assays and sources of compounds when initiating new efforts. Early remedies were identified empirically, assisted by serendipity and astute observation. As the understanding of medical science evolved, so did the desire for a more rational approach to invent new medicines. Prior to the genetic revolution, medicines were identified primarily by a "compound-first" approach. Pioneers in this era include Paul Ehrlich, who invented the first "magic bullet", salvarsan, for syphilis and African trypanosomiasis from chemical dyes[1], Sir James Black and Dr Paul Janssen, who emphasized starting with a "pharmacologically active compound"[2,3], and George H. Hitchings Jr., who highlighted the power of empirical, phenotypic screens when he stated in his 1988 Nobel lecture entitled "Selective inhibitors of dihydrofolate reductase" that "those early, untargeted studies led to the development of useful drugs for a wide variety of diseases and has justified our belief that this approach to drug discovery is more fruitful than narrow targeting"[4,5].

The genetic revolution with the resulting molecular view of biology led to the vision that new medicines would be discovered based on new understanding of the role of genes in disease. Since 1990, a "mechanism-first" strategy has dominated drug discovery and has led to the introduction of many new targeted therapies such as vemurafenib, a BRAF inhibitor for melanoma[6]. However, the cost of producing new medicines has far outpaced the ability of the industry to discover new ones. It is argued that there is a gap between the translation of new understanding of disease mechanisms and the invention of new medicines[7,8].

Addressing this gap in translation is a driving force for current drug discovery strategies. Two distinguishable strategies that can be loosely categorized as mechanism-first and compound-first are termed target-based drug discovery (TDD) and phenotypic drug discovery (PDD), respectively[9–12]. TDD is focused on a drug target, a gene product that provides a starting point for invention of a therapeutic which modulates its expression, function, or activity. PDD is defined as mechanism agnostic; the starting points are biological assays that use translational biomarkers as functional readouts. A knowledge gap for TDD is the uncertainty that occupancy of the molecular drug target translates to the desired safe clinical outcome and for PDD that the measured phenotype is clinically relevant.

Renewed interest in empirical drug discovery and its formalization under the name PDD came subsequent to an analysis of the discovery strategies for new molecular entities (NMEs) approved by the U.S. Food and Drug Administration (FDA) between 1999 and 2008[13]. This analysis determined that a majority of first-in-class small-molecule drugs were discovered empirically, whereas the majority of the followers were discovered using TDD. This report concluded that the mechanistic knowledge available when a program is initiated is often insufficient to provide a blueprint for the discovery of first-in-class medicines. This includes knowledge of not only the presumed drug target but also how that target translates to a specific, therapeutically useful phenotype—the molecular mechanism of action (MMOA)[10]. This knowledge gap was addressed empirically.

The formalization of PDD to a strategy or discipline was subsequently proposed by Eder and coworkers in analysis of first-in-class NMEs from 1999 to 2013[11]. In this work, phenotypic screening was defined as testing of a large number of (in most cases randomly selected) compounds in a system-based approach using a mechanistic agnostic assay. Using this different definition, Eder and coworkers found that PDD contributed to much fewer discoveries. Despite the difference in conclusions, these authors proposed "the goal will be to screen phenotypically in an efficient and effective manner and to combine phenotypic screening sensibly and productively with target-based drug discovery"[11].

PDD has the potential to be much more than random screening in complex systems as defined by Eder and coworkers[11]. PDD has the opportunity to create further value by providing a strategy to address mechanistic knowledge gaps at any level. This was the thinking and rationale behind the earlier analysis by Swinney and Anthony[13].

Following this report in 2011, there has been a resurgence in phenotypic screening in industry as well as academia. The major focus has been to develop more disease-relevant assays and to identify new medicines with novel mechanisms of action. An informative recent report by Haasen and coworkers document the lessons from 5 years of phenotypic screening at Novartis from 2011 to 2015 and show a dramatic increase in the percentage of phenotypic screens. Among the many lessons and trends the authors documented was an increase in more disease-relevant models using iPS and primary human cells and the use of small-scale screens in flexible lead discovery strategies[14].

PDD has identified game-changing medicines with novel mechanisms, including medicines for spinal muscular atrophy (SMA), cystic fibrosis (CF), and hepatitis C. The molecular basis of SMA entails inappropriate exon splicing of SMN2 RNA. "Black Box" cellular assay screens by two research groups independently identified small molecules which correct SMN2 RNA splicing and increase levels of SMN2 protein[14–17]. One interesting point here is the Novartis and Roche SMA screens were conducted with simple cell-based reporter gene assays. It was the mechanistic understanding of the disease that enabled constructing a disease-relevant but simple HTS, demonstrating that PDD does not have to be complex when models are mechanistically accurate. Target-agnostic compound screens using cell lines expressing wild-type or disease-associated CFTR variants identified compound classes which improved CFTR channel gating properties (potentiators) and enhanced the folding and plasma membrane insertion of CFTR (correctors)[18–21]. Identification of the pivotal NS5A direct-acting antiviral class utilized a target-agnostic cell-based hepatitis C virus (HCV) replicon assay; isolation and sequencing of drug-resistant

mutations identified the molecular target, NS5A, a protein with unknown biochemical activity[22].

These mechanisms highlight the utility of PDD's empirical screening to bridge mechanistic knowledge gaps. While the role of empiricism to identify new targets is self-evident, its contribution to the identification of specific MMOAs that translate target binding and occupancy to a specific response is underappreciated. Knowledge of a target does not always provide the molecular details required to predict a specific therapeutic response. This is exemplified in the specific molecular action of aspirin as an anti-thrombotic agent. The molecular target of aspirin is cyclooxygenase, and the molecular mechanism is irreversible inhibition of prostaglandin formation in platelets[23]. Binding to the target is required, but not sufficient, to explain the activity of aspirin as an anti-platelet drug. The differentiating action is the inability of platelets to synthesize new enzyme owing to their lack of a nucleus. This results in a pharmacodynamic effect that will last the lifetime of the platelets (lifespan 8–10 days). Both the MMOA (irreversible binding) and the physiological context (lifespan of platelets) are critical to the therapeutic action.

Many of the medicines that were invented, starting with a target-specific assay, required an additional empirical phenotypic assay to prioritize the actives and identify candidates with functional efficacy. The discovery of Gleevec, a c-abl kinase inhibitor that works through stabilizing the kinase inactive state[24,25], PARP inhibitors such as olaparib[26,27], which trap the PARP to damage DNA, and maraviroc, a CCR5 inhibitor for HIV infection that stabilizes a unique conformation of the receptor that does not bind to the virus[28,29], all required empirical assays to identify molecules with effective molecular mechanisms. John Moffat coined the term "mechanism-informed PDD" (MIPDD) to account for the need to use empirical assays to identify MMOA in target-based strategies[30]. It is also worth noting that PDD can be of value to identify followers and best in class with differentiated molecular mechanisms to the same targets.

Despite all of the new advancements in medical research and the exponential growth in new medical knowledge, there is still a large knowledge gap in the rational identification of mechanisms and the corresponding therapeutics. Arguably, difficulties in linking a molecular target to a pathophysiological state (target validation) may contribute to the probabilities of success for transition from phase 2 to 3 and phase 3 to approval ranging from 32.4–48.6% and 50–59%, respectively[31,32]; in addition, a lack of therapeutic efficacy is associated with >50% phase 3 failures[31], a clinical research phase where issues with toxicity and target engagement have been typically eliminated.

Recent advances addressing PDD uncertainties and risk

There has been much progress in recent years to better describe and understand the uncertainties associated with PDD and provide strategies and processes to manage the risk. Below we highlight some of the recent reports addressing important issues. It is beyond the scope of this short review to address these in detail.

Compound source

A major unresolved question for PDD, as well as other strategies, is the source of compound libraries that will provide the precursor to the medicine. Many different approaches are used, including efforts to increase the chemical diversity, focus on hypothesis-driven libraries, increase biological diversity, and identify undesirable compounds to minimize unwanted mechanisms. The libraries are designed to balance chemical tractability, chemical diversity, and biological target coverage[33]. These authors note that there are two main design principles that underpin an ideal small molecule screening subset for a phenotypic screen: diversity and tractability. There is also increased use of genetic-derived compound libraries (si/shRNA, CRISPRi/a, cDNA) and libraries of biologically active molecules[34].

Disease models, biomarkers, and translatability

The choice of biomarkers and disease models and the likelihood of their translation from the bench to the clinic are foundational to the success of PDD strategies[12,35]. Moffat and coworkers highlighted the importance of the chain of translatability, a qualitative measure of the reliability of the endpoint of an *in vitro* assay to translate successfully to the clinic[12]. The high translatability of assays for infectious diseases and epilepsy—death of infectious organisms and efficacy in animal models of seizure, respectively—have provided many first-in-class medicines[36–38]. Perhaps not surprisingly, disease areas with high success in PDD reflect more relevant disease models[13].

Accordingly, the choice of components and conditions for *in vitro* assay development is critical for the successful outcome of a phenotypic approach[12,39–41]. Independent analysis of biopharmaceutical R&D concluded that small increases in the predictive validity of screening and disease models can offset large changes in brute-force efficiency[42]. Based on this analysis, Scannell and Bosley suggested that the rate of creation of disease-relevant models may be the major constraint on R&D productivity and much of the decline in R&D efficiency may be caused by the progressive exhaustion of predictive disease models of clinical utility[42].

The development of *in vitro* assay systems and analytical methods has expanded significantly over the last decade. Assays using immortal, often tumor-derived, cell lines grown in conventional two-dimensional cell culture conditions are unlikely to fully recapitulate the physiological context of many pharmacological systems[41]. Technical advances to construct better *in vitro* mimetics of *in vivo* biology include the use of primary cells, patient-derived cells, and iPS-derived cells, 2D versus 3D cell culture conditions, 2D/3D co-culture systems, and organ on a chip and microfluidic technologies. Unfortunately, the complexities of biology preclude a one-size-fits-all solution; each approach with their strengths and weaknesses must be individually considered (reviewed by 39,40) with the goal of optimizing issues related to assay enablement, testing capacity,

operational costs, and clinical relevance of the assay. In principle, the chain of translatability of a cellular system should be benchmarked against multiple aspects of the human disease state[43].

Mechanism of action and target ID

Identification of the mechanism and validating a target are important and challenging. There are many different approaches and recent reports to describe these approaches[44–46]. What is clear is that no one approach works for all drugs and there can be a continuum of mechanistic information[44–46]. It is also clear that some medicines are approved with unknown mechanisms and identification of the target is not a criterion for approval. Also, as noted above, the molecular mechanisms of many medicines are not identified until long after approval. However, it is generally agreed that it is easier to move a program forward when the mechanism is known. An important question when following up PDD is when to pursue deconvoluting the mechanism. Since this can be a very time- and resource-consuming process, it is recommended that deconvolution should wait until a robust, reliable candidate has been identified[12,14,47].

Safety issues: what is the safety risk without knowing the mechanism of action?

This is a universal concern for actives from empirical screens. Paradoxically, toxicity screening in phenotypic assays is a well-accepted part of drug discovery, utilizing cell-based assays, tissues, and animals. Haasen and co-workers stated that early toxicity assessment is very important: that is, test the toxicity of the hit series as well as known cytotoxic compounds in various cells to understand the relevance of a potential toxicity finding in the project context[14]. Platforms such as BioMAP which use well-characterized primary cells have been developed to assist in the evaluation[48].

Surrogate phenotypes, artificial intelligence, and deep learning

We have stressed the advantages of using phenotypic endpoints which translate to the clinic, the chain of translatability[12]; however, not all disease states are associated with defined sets of disease markers. In these situations, high-dimensional profiles composed of gene expression profiles or cellular morphology features are envisioned to define surrogate disease phenotypes where reversion of the "disease state" profile to a "wild-type" or "normal" representation is an indication of therapeutic efficacy (see 49 for review).

Cell painting is a surrogate phenotype approach where cellular morphology is measured by fluorescent labeling of eight cellular compartments and subsequent automated high-content imaging analysis of ~1,500 features per cell[50,51]. The resulting cellular morphological profile reflects general changes in cellular state following chemical[52,53] or genetic perturbation. Morphological changes in cell state can cluster structurally similar compounds or can identify functionally similar but structurally distinct molecules[54] and have been used to deconvolute the mode of action of phenotypic actives working through non-protein targets[55].

These profiles as well as compound selection have been shown to be facilitated by artificial intelligence and machine learning. Kraus and coworkers report using deep learning approaches that combine deep convolutional neural networks with multiple instance learning (MIL)[56] to facilitate the evaluation of morphological changes. They introduced a new neural network architecture that uses MIL to simultaneously classify and segment microscopy images of cell populations[56]. Stokes *et al.*[57] have provided proof of concept that deep learning can facilitate the identification of phenotypically active compounds. Using a novel method to describe chemical structures and iterative rounds of deep learning based initially on 2,335 diverse molecules and their ability to inhibit the growth of *Escherichia coli*, a predictive model was developed and subsequently used to analyze over 107 million molecules from diverse libraries. Subsequent filtering of molecules with high prediction scores but low Tanimoto similarities to known antibiotics identified 23 for testing, eight of which displayed growth inhibition in at least one of five bacterial species (*E. coli, Staphylococcus aureus, Klebsiella pneumoniae, Acinetobacter baumannii, and Pseudomonas aeruginosa*). Significantly, two compounds showed broad-spectrum activity with low Tanimoto similarity to known antibiotics and low predicted toxicity in humans. It will be very interesting to follow whether the expansion of this approach will generalize to biological systems with more complex disease phenotypes.

Scientific and portfolio risk of PDD versus TDD

Overall, the TDD strategy is a linear process with well-defined and tractable technical milestones[47]. Typically, targets representing known "druggable" proteins are selected by their association or "validation" with a particular therapeutic indication. Enablement of primary screens is typically low risk and is based on previous industrial experience with members of the molecular target class. TDD flow schemes are principally concerned with enhancing primary target potency/efficacy, achieving biochemical selectivity, and demonstrating cell-based activity upon target engagement, tasks which are informed and generalizable from prior experience with the target class. With these assets in place, a drug discovery team can optimize lead compounds for biopharmaceutical properties and safety to provide a drug candidate. Although challenging, discovery and optimization of advanced leads/clinical candidates by TDD follows a process, with predefined milestones and established cycle times. Uniformity of the TDD process provides easily defined metrics for project support and portfolio prioritization decisions, which mitigates perceived risk.

This process is strongly dependent on the validity of the target and is most effective for followers/best in class and monogenetic diseases including many cancers, where a target as well as MMOA are well validated and genetics help identify patient populations for clinical studies, thereby increasing the chance of downstream success[58]. This process is not as efficient for first-in-class medicines for complex diseases in which the target and MMOA are difficult to pinpoint.

PDD can be complementary to TDD for first-in-class medicines (Figure 1). Development of physiologically relevant

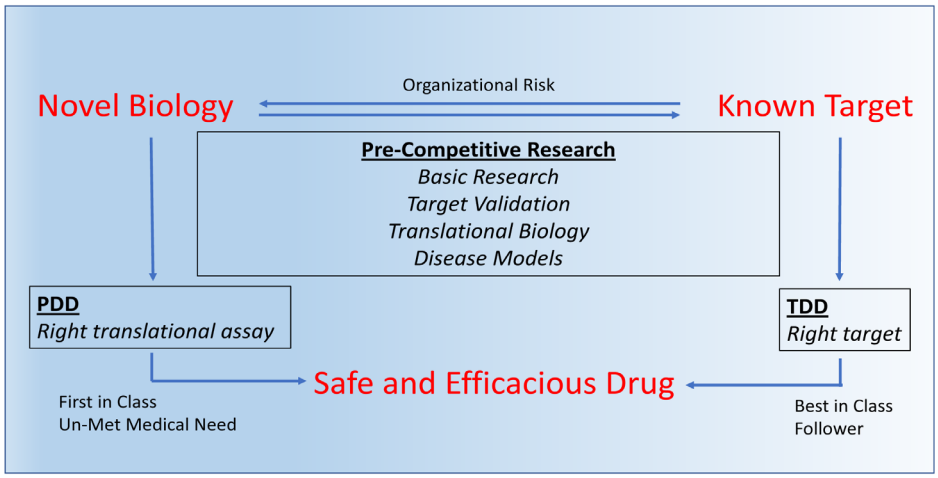

Figure 1. Phenotypic drug discovery (PDD) complements target-based drug discovery (TDD). PDD uses empirical, target-agnostic lead generation to identify pharmacologically active molecules and novel therapeutics which work through unprecedented drug mechanisms. A significant barrier in the decision to implement PDD is balancing the potential impact of a novel mechanism of drug action with an under-defined scientific path forward, with the desire to provide infrastructure and metrics to optimize return on investment, which a known mechanism provides. A means to address this knowledge gap in the future is to empower precompetitive research utilizing the empirical concepts of PDD to identify new mechanisms and pharmacologically active compounds to explore disease biology and de-risk pharmaceutical R&D.

in vitro disease models are foundational to PDD. As a result, PDD assays are frequently very complex multifactorial cellular systems[39,40] which tend to be unique to the disease model and contrast significantly to TDD assays, which are generally more standardized and process friendly. PDD flow scheme development is frequently dynamic and utilizes the results of pilot screens and project progression to reveal unwanted cellular processes and signaling pathways, which in turn requires modification of flow schemes to identify undesirable phenotypic mechanisms. Phenotypic actives representing distinct chemical clusters can, in principle, be working through diverse mechanisms; as a result, *in vivo* proof of concept data are frequently desired early in the LO phase to confirm/establish linkage between the *in vitro* and *in vivo* systems. These and other factors reviewed by Moffat *et al.*[12] indicate that PDD projects tend to front load resources and introduce uncertainty in PDD project milestones and progression metrics, which increases perceived risk.

Future uptake of PDD

PDD has contributed to a disproportionate number of first-in-class medicines with novel molecular mechanisms and the development of commercially successful therapeutics for unmet medical needs (HCV, CF). The upsides of PDD are hard to ignore; in the last 10 years, PDD usage has grown from an estimated <10% to 25–40% of the project portfolio of select companies[14,47,59]. Although difficult to quantitate, utilization of PDD does not appear to be uniformly embraced in biopharma. AstraZeneca and Novartis have published that PDD, broadly defined, contributes 25%[47] and up to 40%[14] of their respective discovery portfolios; in contrast, other pharmaceutical companies utilize "PDD lite" for focused investigation of new biology for established targets, have discontinued their use of PDD, or have yet to utilize PDD.

Organizations must balance the potential up-sides of identifying innovative game-changing therapeutics from PDD to the more streamlined mechanism-first approaches. It will be difficult for PDD programs to compete against TDD projects in organizational structures optimized for mechanism-based approaches, timelines, and risk profiles.

Expanding on previous efforts and consortia to develop probe compounds to specific molecular targets[60–64], we and others[39,40] support the notion that the global health community should establish a pre-competitive, non-profit center of excellence for disease model development and PDD (Figure 1). In collaboration with venture philanthropy groups like the Chan-Zuckerberg Initiative, the proposed PDD center of excellence will deliver mechanistically novel disease-specific pharmacological tool compounds and advanced leads to academic and biopharma collaborators for disease model exploration and de-risking of drug discovery R&D.

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