HHS Public Access

Author manuscript

Nat Rev Drug Discov. Author manuscript; available in PMC 2023 June 01.

Published in final edited form as:

Nat Rev Drug Discov. 2022 December ; 21(12): 899–914. doi:10.1038/s41573-022-00472-w.

Phenotypic Drug Discovery: Recent successes, lessons learned and new directions

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Abstract

Many drugs, or their antecedents, were discovered through observation of their effects on normal or disease physiology. For the past generation, this approach was largely supplanted by the powerful but reductionist approach of modulating specific molecular targets of interest. Modern phenotypic drug discovery (PDD) combines the original concept with modern tools and strategies, and has re-emerged over the past decade to systematically pursue drug discovery based on therapeutic effects in realistic disease models. Here, we discuss recent successes, as well as consider ongoing challenges and approaches to address them. We also explore how innovation in this area may fuel the next generation of successful projects.

Introduction

Historically, new medicines were discovered through observation of their therapeutic effect on disease phenotypes either directly in humans as part of traditional medicine or in models of disease. With the advent of the molecular biology revolution in the 1980s and the sequencing of the human genome in 2001, the focus shifted to specific molecular targets. Since 2011, however, Phenotypic Drug Discovery (PDD) has experienced a major resurgence following the surprising observation that a majority in first in class drugs were discovered empirically without a drug target hypothesis between 1999 and 2008.[1] The modern version of this legacy strategy is defined by its focus on the modulation of a disease phenotype or biomarker rather than a pre-specified target to provide a therapeutic benefit.[2] Ten years in, PDD is maturing as a field, serving as an accepted discovery modality in both academia and the pharmaceutical industry as opposed to a transient fad. This

continued interest is rooted in notable successes in the past decade, including ivacaftor and lumicaftor for cystic fibrosis, risdiplam and branaplam for spinal muscular atrophy (SMA), SEP-363856 for schizophrenia, KAF156 for malaria and crisaborole for atopic dermatitis.

This is not to say that PDD approaches are a magic bullet to address issues with pharmaceutical industry productivity; the pros/cons of phenotypic screening need to be carefully balanced against molecular approaches for validated targets.[3] While PDD has been successful, many historical examples used highly complex disease systems (*in vivo* models and even humans) rather than cell-based screens and/or were the result of serendipitous discoveries (Figure 1),[4] and complex models are only now regaining prominence. Drug repurposing provides a compelling example of this state of affairs, with on the one hand well known examples of repurposed drugs based on serendipitous clinical observations (e.g. sildenafil, minoxidil, thalidomide, amantadine) and on the other hand a lack of approved repurposed drugs stemming from pre-planned screens of clinical compound collections.[5] This raises the critical question of how best to *prospectively* approach the discovery of novel drugs using phenotypic screening.

In addition to a renewed appreciation for the complexities of physiology and pharmacology, PDD challenges our assumptions in terms of what is druggable with unusual targets and mechanisms of action (MoA), including polypharmacology, and what is a drug with unexpected compound properties. While important hurdles remain in terms of target identification, a helpful step for safety derisking and the mapping of a clinical path, exciting opportunities are emerging for the application of functional genomics, machine learning/artificial intelligence and improved disease models.

Given the major differences between PDD and target-based drug discovery (TDD) and the new technologies that can now be brought to bear on phenotypic programs, the field is evolving at a rapid pace, with a need to establish and share best practices across industry and academia.[3,18–21] Although technical and cultural hurdles remain, here, we discuss how the renewed utilization of PDD has started to change the manner in which we conceptualize drug discovery and has proven to be an important testing ground for technical innovations in the life sciences. This perspective highlights the authors' collective thoughts on how PDD has influenced concepts related to drug discovery and ends with a discussion of the challenges ahead for maximizing the effectiveness of PDD.

Drug discovery concepts recently shaped by PDD

Expansion of "druggable" target space

The main driver for PDD stems from the disproportionate number of first-in-class medicines derived from this approach.[1] In contrast to TDD, which is based on an established causal relationship between a molecular target and a disease state, PDD relies on chemical interrogation of a disease-relevant biological system in a molecular-target-agnostic fashion. This empirical, biology-first strategy provides tool molecules to link therapeutic biology to previously unknown signaling pathways, molecular mechanisms and drug targets, as highlighted in the following examples.

- Hepatitis C is a liver disease caused by the hepatitis C virus (HCV), which infects 3% of the population and is estimated to cause 300,000 deaths worldwide each year.[22] In the past decade, the treatment of HCV has been revolutionized by the development of combinations of orally available direct-acting antivirals (DAAs) that inhibit HCV replication, and clear the virus in >90% of infected patients. Modulators of the HCV protein NS5A such as daclatasvir are a key component of these DAA combinations. The importance of NS5A, which is essential for HCV replication but has no known enzymatic activity, as well as its small-molecule modulators, were initially discovered using a HCV replicon phenotypic screen.[23]

- Cystic fibrosis (CF) is a progressive and frequently fatal genetic disease caused by various mutations in the CF transmembrane conductance regulator (CFTR) gene that decrease CFTR function or interrupt CFTR intracellular folding and plasma membrane insertion.[24] Target-agnostic compound screens using cell lines expressing wild-type or disease-associated CFTR variants identified compound classes that improved CFTR channel gating properties (potentiators such as ivacaftor), as well as compounds with an unexpected mechanism of action: enhancing the folding and plasma membrane insertion of CFTR (correctors such as tezacaftor and elexacaftor).[6,7] Notably, a combination of elexacaftor, tezacaftor and ivacaftor was approved in 2019 which addresses 90% of the CF patient population.[25]

- Inspired by observations that thalidomide effectively treated leprosy, modulated multiple anti-inflammatory cytokines, inhibited angiogenesis and showed activity in multiple myeloma,[26] the optimized analogue lenalidomide gained FDA approval for several blood cancer indications and has been highly successful (sales > $12 billion in 2020).[27–29] Significantly, the unprecedented molecular target and MoA of lenalidomide were only elucidated several years post-approval. Lenalidomide binds to the E3 ubiquitin ligase Cereblon and redirects its substrate selectivity to promote the ubiquitination and subsequent degradation of target proteins including the transcription factors IKZF1 and IKZF3.[30] Furthermore, this novel MoA is now being intensively explored in the development of further targeted protein degraders, dubbed ‘bifunctional molecular glues’.[31]

- Type 1 SMA is a rare neuromuscular disease with 95% mortality by 18 months of age. SMA is caused by loss-of-function mutations in the *SMN1* gene, which encodes a protein known as survival of motor neuron (SMN) that is involved in the formation and maintenance of neuromuscular junctions. Humans also have a very closely related *SMN2* gene, but a mutation that affects its splicing leads to exclusion of exon 7 and the production of an unstable shorter SMN variant. Phenotypic screens by two research groups independently identified small molecules that modulate *SMN2* pre-mRNA splicing and increase levels of full-length SMN protein.[32,33] Both compounds work by engaging two sites at the SMN2 exon 7 and stabilizing the U1 snRNP complex, [32,34,35] an unprecedented

drug target and MoA. One such compound, risdiplam, was approved by the FDA in 2020 as the first oral disease-modifying therapy for SMA.

Table 1 presents further recent examples of approved or clinical-stage compounds originating from phenotypic screens, including those where the affected cellular processes are well defined but the specific entity which binds the compound is a multi-component "cellular machine", a poorly defined molecular target. Taken together, these examples demonstrate how phenotypic strategies have expanded the "druggable target space" to include unexpected cellular processes (pre-mRNA splicing, target protein folding, trafficking, translation, and degradation), novel MoAs for traditional target classes (pseudo-kinase domain inhibition, allosteric kinase activation, masked covalent warhead), and revealed new classes of drug targets (e.g. bromodomains). They suggest that phenotypic strategies should be considered when no attractive target is known to modulate the pathway or disease phenotype of interest and/or the project goal is to obtain a first-in-class drug with a differentiated MoA.

Polypharmacology reexamined

With no restrictions in the available chemical and biological space other than those defined by the compound library and the disease model systems, phenotypic screening offers the opportunity to identify molecules engaging multiple targets in what is otherwise known as polypharmacology.[51,52] In this scenario, the intended effect of a compound depends upon a combination of targets (*on-targets*); however, these are not necessarily its full target signature that may include targets not required for activity (*off-targets*).

In the quest for ever more selective drugs, polypharmacology has been traditionally associated with poorly optimized compounds prone to potential side effects due to the difficulty in tracking all the biological functions represented by *off-targets.* However at therapeutically relevant concentrations most, if not all, approved drugs are known to interact with multiple targets that often underlie side effects but can also contribute to clinical efficacy.[53–55] In fact, the simultaneous low potency modulation of several targets to achieve efficacy "by synergy" has been suggested as a strategy to minimize side effects.[56] One classic TDD example of unintended polypharmacology is imatinib, the first rationally designed kinase inhibitor approved by the FDA for the treatment of chronic myeloid leukemia's (CML) and other cancers, and currently in clinical development for recent-onset type I diabetes.[57] Initially regarded as an inhibitor of CML's BCR-ABL fusion protein,[58] imatinib also exhibits activity towards c-KIT and PDGFR receptor tyrosine kinases, among other targets, which are believed to contribute to its activity in several types of cancer.[59,60]

Multi-target approaches based on drug combinations, i.e. post-hoc polypharmacology by design, are well-accepted strategies for antiviral and oncology indications for which resistance to treatment can develop when only a single target is engaged.[61–64] Polypharmacological drugs are also commonly used to treat central nervous system and heart diseases for which single-target-based approaches have shown limited success and classical *in vivo* phenotypic models have long been used for drug discovery.[40,65,66] Generally speaking, multi-target drugs may be a better match for complex, polygenic

diseases with multiple underlying mechanisms often involving interactions with immune or nervous system components.

Phenotypic approaches have provided a number of drugs and candidate molecules that revealed *on-target* polypharmacology upon MoA identification. Representative examples include imipramine, a tricyclic antidepressant discovered in the 1950s with a prominent polypharmacological signature modulating some key CNS monoamine transporters and receptors,[67,68] topiramate, a neurostabilizer for epilepsy and migraine that engages a selection of neuronal receptors, ion channels and enzymes,[69] pemetrexed, a folate antimetabolite approved for mesothelioma and NSCLC that inhibits a combination of enzymes involved in folate metabolism and nucleotide synthesis,[70,71] molibresib, a first-generation BET inhibitor tested in phase 1/2 clinical trials for several cancers,[72,73] RG7834 evaluated for HBV infection,[74,75] SEP-363856, a clinical candidate for schizophrenia identified in an *in vivo* phenotypic screen[43] and new investigational compounds for CNS pathologies,[76] infectious diseases,[77] cancer[78] and metabolic disorders (Table 2).[79] Unsurprisingly, polypharmacology often occurs among proteins from the same family, sharing common structural domains or similar substrates or ligands (e.g. kinases, aminergic GPCRs, and BET domain proteins).

Drug combinations, engineered multi-targeted drugs and multi-specific antibodies represent simplified polypharmacology scenarios, typically combining two moieties with selectivity for each of the individual targets.[80–82] Engineering more complex polypharmacology into a single entity while balancing all other properties required by a drug candidate is a complex process that remains a daunting challenge, in spite of recent advances in molecular docking, computational technologies and artificial intelligence (AI).[76,82–84]

Phenotypic screening offers the possibility to identify hits with novel, unbiased polypharmacology signatures only limited by the target landscape of the model system and the number of activities potentially contained in each of the scaffolds available in the compound library.[85,86] An important practical consideration is the use of a gain-of-signal phenotype to help focus efforts on productive polypharmacology rather than cellular stress or cytotoxicity through targets unrelated to the biology of interest.[21,76] A first snapshot of polypharmacology signatures can be obtained using conventional selectivity panels.[87,88] These signatures can serve as starting points for a reverse engineering SAR-based process aimed at identifying and optimizing their *on /off-targets* balance, mapping correlations between activity in the phenotypic assay and in each of the individual targets.[76] An example of a systematic approach to map *off-targets* and increase selectivity for a phenotypic endpoint resulting from polypharmacology is provided by Tear *et al*, using hits from a phenotypic HTS screen to identify Trypanosoma brucei inhibitors.[89] Phenotypic endpoints integrate the contributions of polypharmacoloy *on-targets* in a single read out, while reduction of the *off-targets* footprint can be explored with support from SAR and, when available, reference compounds selective for the targets identified.[90]

On-target(s) identification efforts may be more productive after some compound optimization is undertaken to reduce the *off-targets* signature of initial hit compounds and its potential confounding effects. Additional support from chemical and functional

genomics tools at more advanced stages may also be required. Even if *on-targets* cannot be unequivocally identified, insight into the MoA and potential safety risks of candidate molecules can be obtained from the powerful phenotypic and molecular profiling platforms currently available using reference drugs as benchmarks.[91–94] Polypharmacology is unlikely to be limited to the proteome. In our experience, phenotypic screening using a chemical library containing highly selective legacy compounds from target-based drug discovery programs can provide hits (e.g. kinase inhibitors) whose phenotypic activity does not seem to rely in their bona fide targets alone.[90] This could be due to subtle polypharmacology resulting from a combination of lower potency activities or even involve interactions with non-protein targets like RNAs,[95] not detected in conventional protein-only selectivity panels but certainly potential targets of phenotypic screening hits.[96]

From a drug discovery perspective, phenotypic screening-derived polypharmacology can take advantage of increasingly robust phenotypic models combined with advanced profiling, omics and computational technologies to evolve from serendipity to a SAR-based reverse engineering approach that can minimize potential safety risks while preserving and eventually optimizing phenotypic activity to increase the chances of clinical success.

Drug “likeness” revisited: PDD successes with low molecular weight compounds

Modern phenotypic screening may also help expand the range of molecular properties displayed by new drugs. Specifically, the molecular weight of drugs has increased significantly over the past few decades in conjunction with the advent of TDD.[103] Historically, smaller molecules were discovered using phenotypic approaches, often using animal models (ibuprofen[104] MW 206, minoxidil[13] MW 209, memantine[12] MW 179) (Figure 1). These molecules were well within the broadly accepted criteria for fragments, such as MW<300. Contemporary examples include the discoveries of lacosamide (MW 250) using a model of epilepsy[11] and SEP-363856 (MW 183) for schizophrenia using a battery of CNS models,[42,43,98] along with the repurposing of MLR-1023 (MW 202) towards type 2 diabetes and NASH after testing in a diversified suite of in vivo models,[48,105] and of dymethyl fumarate (MW 144) for multiple sclerosis (Figure 1).[106]

The above examples raise a critical question. Why would fragment-sized molecules that would nowadays be looked at as weak hits in need of a large amount of potency optimization in the context of TDD programs be active in in vivo disease models and lead to similarly sized drugs after optimization? We hypothesize that several reasons might account for this unexpected pattern. First, fragment-sized molecules are known to deliver a higher fraction of valid hits when screened against specific targets due to their small size and larger number of orientations able to fit within a binding site.[107] As noted previously, polypharmacology is a frequent feature of PDD drugs and may also contribute here (e.g. SEP-363856 interacts with TAAR1 and 5-HT_{1A} receptors).[43,98] Finally, smaller molecules have documented advantages in terms of intestinal permeability, operating through paracellular spaces, while they present fewer options to metabolizing enzymes due to their smaller size.[108] While drug dosages and the corresponding exposures for many of these fragment-sized drugs may be high by the standards of larger molecules, they may still display high ligand efficiencies for their target(s) which allow them to be dosed safely.[109] In other words, the same high compound

concentration does not carry the same safety implications for molecules with MW of 200 vs 500 in terms of exposure.

An opportunity therefore exists to purposefully exploit this ability of phenotypic screening to tap into a chemical and pharmacology space poorly covered by TDD programs.[110] The recent discoveries of SEP-363856, MLR-1023 and lacosamide suggest that phenotypic screening using in vivo animal models can still be successful. Such success will depend heavily on the clinical relevance of the model, a controversial topic but one still worthy of exploration as will be discussed later on. Looking forward, a second option to consider would be to adapt this concept of screening fewer and smaller molecules in complex, multi-cellular organoid assays. Fragment libraries are known to cover a large pharmacophore space despite numbering only in the thousands.[107] Testing libraries of fragments or molecules in between fragments and standard compounds may potentially allow the coverage of a significant pharmacology space even with low-throughput, complex 3D assays.[110]

A related concept is the use of covalent fragments - a subset of fragments containing reactive moieties which can establish chemical bonds with proteins - to identify both novel targets and reveal chemistry starting points. Used in cell-based phenotypic assays, they are garnering recognition for their ability to access a greater slice of biology through the discovery of binding sites which may not be available to reversible compounds. Importantly, covalent fragments benefit from the strengths of both covalent targeting (sustained target engagement) and fragment-based screening (wide coverage of ligandable pockets with a small library).[111] For example, profiling of covalent fragments in primary human T cells revealed inhibitors of T cell activation operating by distinct mechanisms including the direct functional perturbation and/or degradation of proteins.[112] Notably, modulated targets included both previously liganded or unliganded proteins.

Target identification and project progression – a maturing discussion

Target identification for an active compound series is broadly accepted as being desirable to help derisk the project from a safety and clinical translation perspective. Whether it is absolutely necessary for compounds prior to entering the clinic has been the source of contentious debate over the years.[3] This discussion is now maturing. Target identification is usually viewed as leading to a simple binary outcome: either the target is identified or it is not (Figure 3A). Here, we wish to offer a different framework for discussing this important topic. First, it is important to keep in mind that target identification is only a means to an end, with that end being to obtain decision-making information for compound series on the path to and in the clinic. Target identification therefore represents only one option to meet this goal. Furthermore, identifying the molecular target does not equate to understanding the MoA of a compound series and may even be misleading in terms of program decisions. For example, the documentation of NS5A being the target of HCV drug daclatasvir did not rationalize its notable sub-nM cellular potency nor did it explain how efficacy can be obtained with a compound to protein stoichiometry lower than 1 to 1,000.[8] Similarly, the rational response to the identification of the ribosome as the target of a PCSK9 secretion inhibitor should have been to terminate the program as it raised the specter of significant safety liabilities through broad protein synthesis inhibition.

However, further investigations including proteomics profiling revealed that its molecular MoA provided unexpectedly specific inhibition of the translation of the PCSK9 mRNA transcript due to the formation of a trimeric complex between the ribosome, the compound and the nascent PCSK9 polypeptide.[49,50] Another class of examples includes compounds exhibiting a unique phenotypic effect in their class due to degradation of the target (e.g. fulvestrant with ER)[113] or modification of its protein binding partners (e.g. DNMDP with PDE3A)[114].

As an alternative to target identification, we suggest that much actionable knowledge about the MoA can be acquired empirically (Figure 3B). Practically, cellular and in vivo assays can provide unbiased compound assessment with readouts relevant to efficacy and safety while mechanistic studies, now often employing omics approaches, may reveal information related to the MoA such as the specific biological pathways impacted by the compound.[115] Rather than the binary switch of identifying – or not - the target, a continuum of information can be obtained about a compound series during the course of a project which may lead to its progression to the clinic based on the accumulated confidence in safety and translation.

Target identification and mechanism-of-action profiling strategies

While finding direct binders to a small-molecule screening hit has the promise of delivering “a/the” target, it is not the complete picture when trying to deconvolute a phenotypic screen.[116–118] Affinity (or photo-affinity) enrichment combined with chemo-proteomics methods[119–123] or, more recently, Cellular Thermal Shift Assay (CETSA)[124] to identify the protein (or proteins) that bind to the small molecule hit are a way to understand mechanism of action (e.g. P2X4 as the target of autophagy inhibitor Indophagolin).[125] But this may not be sufficient, and as mentioned above for PCSK9 could even be misleading.

Developments in RNAi, and CRISPR-Cas9, have opened the ability to screen whole genome libraries, allowing gain of function and loss of function studies to be performed with high specificity.[126] Genetic perturbations, in combination with compound treatment, provide further mechanistic understanding of MoA and may lead to identification of the molecular target itself (e.g. NAMPT as the target of anti-leukemia agent STF-118804 and DHODH as the target of antiviral GSK983).[127–130] One large-scale effort is the Cancer Dependency Map that aims to systematically identify genetic dependencies and small-molecule sensitivities using massively-parallel compound screens in molecularly-barcoded cell lines using the PRISM method.[131,132]

More recently, molecular profiling methodologies that provide comprehensive information about biological changes resulting from a chemical perturbation have taken a prominent role in the follow up of phenotypic hits.[21] Examples of such large-scale profiling efforts can be categorized into measurements of gene expression, cell morphology or biomarker activity. The Connectivity Map has *gene expression* profiles of test and annotated compounds that can be used for signature similarity mapping.[133] An extension from that is the Library of Integrated Network-Based Cellular Signatures (LINCS),[91,134] an NIH Common Fund program that catalogs changes in cell lines in response to chemical, genetic, and disease perturbations. Cell Painting uses *morphological profiling*: quantitative data are extracted from microscopy images of cells to identify biologically relevant similarities and differences

among samples.[135–138] The BioMap panel profiles primary cell systems upon treatment (chemical or biological), reading out *biomarker activities* that are increased or decreased in comparison to vehicle control.[92,139,140] A key advantage provided by these technologies is the growing feasibility of testing numerous compounds (e.g. a list of top hits) rather than having to focus on a strictly limited number of compounds as with legacy proteomics strategies. Much of the value of these platforms is derived from the comparison of a hit's biological signature to a databank of signatures obtained with reference, annotated compounds. It may reveal a match or help construct hypotheses regarding its MoA. For example, Tapinaroff was matched to an AHR agonist using the BioMap panel, with its own AHR agonism validated in follow up studies.[141] Practically, molecular profiling is now becoming integrated in screening funnels and used in determining which hits and series will see further investment and investigation.[21]

These large-scale profiling methodologies, while higher in throughput and focused on pathway-level information, rely heavily on known compounds with similar phenotypes in the reference databases. Given that the purpose behind phenotypic screening is to discover new mechanisms of action, it may take time to build the knowledge base for these methods to live up to the expectation of a look-up table. However, many phenotypic screens are now guided by specific mechanistic information (mechanism-informed PDD as coined by Moffat et al).[142] This strategy provides a key biological framework to place into context the data generating hypotheses for the MoA of the phenotypic hits based on the tools described above.

Clinical development considerations for PDD-derived compounds

In the absence of target information, progressing a PDD-derived preclinical drug candidate into the clinic poses several challenges to development teams. Simply phrased, target identity provides valuable information both for derisking safety concerns and for predicting and monitoring efficacy. This section will present specific strategies and examples to address these hurdles.

A 'chain of translatability' — a molecular-level association between mechanisms which drive the original phenotypic assay, subsequent preclinical disease models, and is an inherent component of the human disease — is critical for a PDD program to succeed in the clinic.[3] For example, the HBV antiviral agent RG7834 lowers the secretion of non-infectious membranous particles containing the tolerogenic viral S antigen *in vitro* and therefore captures an essential (and prognostic) component of the disease in humans.[74,75,143,144] The programs for branaplam and risdiplam for spinal muscular atrophy (SMA) also provide a strong example of this concept. As noted above, SMA is caused by loss-of-function mutations in the *SMN1* gene, and both efforts originated from high-throughput phenotypic screening programs aimed at producing functional SMN to compensate by modulating the splicing of the almost identical *SMN2* gene to include exon 7 (this exon is normally absent, resulting in an unstable Δ7 protein).[9,32,33,145] These programs screened for a disease surrogate biomarker (inclusion of exon 7 in *SMN2* mRNA) of clear clinical relevance, as the subsequent clinical studies demonstrated. For instance, in a phase I healthy male volunteer single escalating dose study, treatment with risdiplam resulted in the intended

shift in *SMN2* splicing towards full-length *SMN2* mRNA, which further translated into a medically meaningful benefit in SMA patients in the SUNFISH (NCT02908685) pivotal clinical trial.[146] To summarize, clinical development may be feasible in the absence of an identified target but with a clear biomarker to monitor in humans based on a strong molecular MoA understanding.

On the safety front, target information, and the accompanying knowledge about its physiological expression pattern and role, is often used to focus attention on potential safety signals. These may be investigated early in the life of a TDD program to either derisk or terminate it rapidly. Regulatory guidelines do not require target information for safety evaluation though. Instead, they provide a list of required toxicology studies, to guide the choice of acceptable compound doses for human testing. Safety derisking was the topic of a workshop at the 2019 Keystone symposium on PDD with multiple themes and strategies emerging from these discussions.

First, safety considerations need to be incorporated as early as hit triage and validation in phenotypic programs. Effective methods include cytotoxicity counterscreens as well as molecular profiling to remove hits acting through frequent hitter and other undesirable MoAs.[21] Once the hit list has been trimmed to a few hits of interest, the use of active–inactive compound pairs has proven helpful to either increase or decrease confidence in a given compound series and its cognate target/MoA. These molecular tools can enable the identification of specific biomarkers and signatures of the MoA of interest while also allowing the assessment of adverse reactions that are not directly related to the compound mechanism of action (analogous to “off target” adverse effects in target-based drug discovery). This approach was followed to gain confidence in the MoA of the original PCSK9 secretion inhibition hit, R-IMPP.[49] As often with screening hits, this hit was rather weak and promiscuous, raising concerns about further investment. The fact that its enantiomer, S-IMPP, was observed to be similarly promiscuous yet was inactive in the PCSK9 secretion assay suggested the R-IMPP series acted through a specific molecular target rather than through broad cell stress or injury (Figure 4A). Conversely, this strategy was used to justify the termination of a series of CFTR correctors being developed for cystic fibrosis.[147] Here, following the observation of severe in vivo toxicity following chronic dosing of a lead molecule, a closely structurally related but inactive analogue was similarly tested in vivo. The inactive compound was well tolerated with similar exposure levels, suggesting that toxicity was more likely MoA-related rather than compound-related (Figure 4B).

Additionally, researchers can access more complex phenotypic characterizations and map compound-specific profiles to reference collections in zebrafish[149] or in human primary cell-based disease systems such as Biomap, validated with fingerprints generated using clinically approved drugs.[150,151] While these systems do not fully recapitulate the range of safety issues which may be observed in humans, they still provide an opportunity to detect some multiorgan liabilities while allowing the testing of significantly larger number of compounds or series. Finally, in vivo toxicology studies, which include testing of multiple compound doses in two separate animal species, constitute a key step and the subsequent determination of a no-observed-adverse-effect level (NOAEL) guides the choice of compound doses for

human testing. Here, ensuring that the two species chosen display modulation of a disease biomarker or biological signature associated with the series MoA is important to maximize the value of these studies prior to entering the clinic.

Drug candidates derived from PDD can and have transitioned to the clinic in the absence of knowledge of the molecular target.[1] These include for example, 1) Lenalidomide (2005 approval for multiple myeloma) with its MoA being elucidated in 2014,[30] 2) Lacosamide (2008 approval for epilepsy) with information with its likely complex MoA still under investigation,[11,152,153] and 3) RG7834 which recently entered phase I clinical trials while target identification efforts had yet to succeed.[74,75,154] Howeverin the absence of target information, it is necessary to identify surrogate disease biomarkers that translate effectively to human patients. Similarly, specific strategies can help evaluate safety risks for a given series and the associated MoA. An additional consideration is the unmet medical need and clinical landscape for a given indication as an absence of well validated targets may provide further impetus to progress a compound into the clinic in the absence of target information.[3]

Looking Forward

Surrogate Phenotypes for phenotypic screens

At times, phenotypic screening can present a conundrum. It is obviously valuable for diseases that do not have well validated therapeutic targets and which may be correspondingly less well understood or characterized. However, disease knowledge is essential to design a phenotypic assay with a relevant in vitro or in vivo biological system, stimulus and readout and establish the required Chain of Translation.[3,18] In much the same way they are being used to define the MoA of a small molecule, high-dimensional profiles such as gene expression and cellular morphology could be used to define a surrogate disease phenotype as the readout of the phenotypic assay.[155] Instead of a chemical compound, the perturbagen in this case is the disease itself with the screen aimed at reverting the system from diseased to healthy state.

Gene expression profiling has been used to define disease states, such as those caused by genomic alterations in cancer. For example, high-throughput mRNA profiles were used to cluster alleles found in lung adenocarcinoma based on their functional impact, a precursor to therapeutic strategy for variants of previously unknown significance.[156] As indicated previously, the LINCS program is designed to create a network-based understanding of biology by cataloging changes in gene expression that occur in response to a perturbagen or disease state.[134] A goal of the program is to develop a computational framework to discover therapies on the basis of restoring perturbed pathways and networks to their normal, healthy states. A recent study used high-throughput drug screening combined with in silico analyses of *existing* transcriptomic datasets to identify a compound capable of reversing pulmonary arterial hypertension (PAH) in vivo.[157] The authors note that their studies could be further improved by generating LINCS gene expression signatures using vascular cells rather than cancer cells. The promise of these methods, however, is still in its infancy, as shown by elegant work done by Alvarez et al.[158] Here, the authors combined gene expression profiling and several computational algorithms to define master regulator proteins, for gastroenteropancreatic neuroendocrine tumors (GEP-NETs) and then

conducted transcriptome analysis of GEP-NET-derived cells, perturbed with a library of 107 compounds. Conceptually the method showed that compounds capable of inverting the coordinated activity of tumor-checkpoint master regulators can effectively destabilize tumor cell state. However, validation of two drugs predicted to induce patient-specific master regulator collapse was inconclusive. The authors provide an in-depth discussion of the reasons why that could be.

As discussed previously, cell morphological phenotypes, including shape, size, intensity, and texture of cellular compartments have been shown to change in response to perturbation - be it a small molecule or disease associated alleles. Already, the LINCS portal has incorporated such data from Cell Painting[136] and the Drug Repurposing Hub[159] reporting Cell Painting data for 1,571 compounds (92% of them mapped to a human protein target or assigned a mechanism-of-action label).[135] These data could be used to define phenotypes reversed by drugs of known MoA.

Additional methods to computationally compare and visualize drug and disease gene expression profiles are used to define reversal of disease phenotypes. A ranking system, the Reverse Gene Expression Score (RGES) provided a systematic way to connect disease gene expression with drug-induced expression profiles.[160] Integrating data from TCGA (https://tcga-data.nci.nih.gov/), LINCS,[91,134] ChEMBL[161] and CCLE[162] allowed the researchers to show that drugs with efficacy in cancer cells had enhanced potency to reverse disease gene expression compared to ineffective drugs.[160]

Analogous to gene set enrichment analysis (GSEA), Nassiri et al developed a cell morphology enrichment analysis to assess the association between transcriptomic alterations and changes in cell morphology underscoring that the interdependence between transcription and cell morphology can be linked to disease state, in this case looking at cell morphological changes in a human bone osteosarcoma cell line.[163]

The repositories of high dimensional profiling datasets mentioned in this section already exist in the public domain. For drug discovery, the challenge of how to best use these data to define surrogate disease phenotypes that can be reversed as an indication of therapeutic efficacy remains as one of the next big hurdles for the field. Some pharmaceutical companies have started to use this approach as a screening platform. Starting in 2013, Recursion Pharmaceuticals may be the first industrial effort to use a surrogate disease signature via cell painting to screen for reversion of a surrogate disease signature. Their main focus is on drug repurposing aimed at rare monogenic disorders.[164]

Artificial intelligence and PDD

The application of artificial intelligence is well accepted in multiple domains of drug discovery and development (for a comprehensive review see [165]) including drug design,[166] protein folding,[167] chemistry,[168] *in silico* toxicity prediction,[169] and drug repurposing.[170] The exponential pace of application of this methodology relies on the ability of machine learning (ML) algorithms to identify patterns and learn from their association with certain parameters such as potency, selectivity, etc.

A query of the published literature on PubMed using the keywords “deep learning”, “artificial intelligence” and “phenotypic” or “drug discovery” results into two rather different class of papers: 1) classifiers applied to a large collection of compounds or chemical structures and 2) classifiers applied to phenotypic assay derived features. The vast majority of the published works belong to the first category (graph-based or deep learning-based with or without transfer learning from different training datasets) to a large collection of compounds or chemical structures with associated pharmacology data generated in previous screens. The classifier is not applied therefore to phenotype-derived features but to chemical structures in order to come up with potential novel scaffolds that in turn can become substrate for experimental validation. A prototypical example is the DeepMalaria study, where a graph-based model was trained on antiplasmodial hits from a GSK dataset to predict *Plasmodium falciparum* growth inhibition (and mammalian cell cytotoxicity) aiding in the rational selection of scaffolds as input for further investigation.[171] To overcome the difficulty of low training data, transfer learning was used from an unrelated dataset. Those molecules were then subsequently validated in a phenotypic assay. Though the AI classifier is deployed on chemical structures prior to the phenotypic effort, that approach can still contribute to PDD as demonstrated by the identification of potent candidate antimalarial agents.[171]

Conversely, beyond the AI hype, efforts to deploy the classifier on the phenotype-derived features are still rare even though this is clearly a tantalizing application of this technology capable of revealing patterns hidden in what is apparently chaos to a casual observer. PDD relies by definition on phenotypic pattern changes to identify and optimize molecules with little or no knowledge of the biological target or MoA.[172] Characterization of drug-induced perturbations at the cellular level (e.g., Cell Painting[136]) has shown that subcellular feature metrics can be used to cluster and classify compound and gene perturbations.[138,173,174] ML is particularly useful in instances when the feature space is not well defined, and could therefore be remarkably enabling to PDD. Leveraging a large cell painting data set (126,779 morphological profiles induced by 30,616 compounds) for instance,[138] Hofmarcher et *al* demonstrated that convolutional neural networks operating on raw images are able to extract morphological changes of cells from images, outperforming a traditional image-processing pipeline based on segmenting cells and subsequent feature extraction.[175] Interestingly, application of different dyes to cells may not be necessary as other authors have shown that even bright field images can be used to train an algorithm that can discriminate specific phenotypes.[177]

There are also other opportunities for the integration of ML with PDD. For example, phenotypic screening and ML can be combined to extract target information from promiscuous compound collections such as unselective kinase inhibitors. In this area, studies showed that, even while using promiscuous kinase inhibitors, it was possible to deconvolute the kinase dependencies of active molecules and identify the kinase combinations whose inhibition delivered the desired outcomes of increased neurite outgrowth or breast cancer cell death.[76,178]

Two studies highlight the transformative potential of the application of ML to PDD with bacterial phenotypic fingerprinting and the repurposing of high-throughput images.[94,176]

Though others have deployed deep learning to antibiotics discovery,[179] Zoffmann et *al*[94] using a combined high-content imaging and genomic approach in conjunction with a ML-powered dataset analysis effectively narrowed down, compared and predicted compound MoAs. In that study, application of ML could therefore define, in the feature space, compound 'archetypes', enabling chemists to proceed in their compound optimization efforts while constantly monitoring how such modifications affected the MoA of analogues — *a major* difference with traditional PDD. Janssen researchers brought this concept to the next level, integrating the high-throughput imaging, normally used to read out a handful of morphological features documenting a single biological process, with ML to establish a proof of concept that images from a given cellular assay can support activity prediction across a spectrum of biological assays.[176] Specifically, they were able to predict compound activity against two different targets using cellular morphology information extracted from an unrelated imaging screen, increasing hit rates by >50 fold.

Further development of physiological and disease-relevant assay systems

Whether a phenotypic drug discovery project succeeds or fails depends on the inherent strength of the "chain of translatability", discussed above, that links the primary phenotypic assay at the outset to patient efficacy at the end, often with an animal disease model in between.[3] An analysis of the biopharmaceutical industry by Scannell and Bosley suggests that the decline in R&D efficiency may be caused by the progressive exhaustion of the most predictive disease models, and that the rate of creation of new disease relevant models may be a major constraint on R&D productivity.[180] Notably, the authors also conclude that small increases in the predictive validity or translatability of disease models can offset large differences (i.e. orders of magnitude) in assay throughput.[180] Taken together, the continued development of realistic disease-relevant assays with validated clinical translatability is of critical importance to future PDD efforts.

Fortunately, advances in diverse disciplines such as stem cell biology, functional genomics, bioengineering/microfabrication, and instrumentation/data analytics have converged to provide an expansive experimental pallet to develop potential disease relevant assay systems. Technology advances include but are not limited to: platform approaches to model organisms,[181] high capacity in vivo mammalian pharmacology,[40,182] the use of high fidelity Cas-9 methods to modulate gene regulation/structure,[183,184] access to novel cellular systems such as primary cells, patient derived cells and induced pluripotent stem cell (iPSC)-derived cells,[18,185–187] the use of mono or co-culture[188–190] systems in 2D or 3D cell formats,[191,192] and integration of microfabrication/bioengineering advances to provide micropatterned cell culture surfaces[193], 3D matrices/microfluidic systems[187], and organ on a chip[194–197] capabilities.

The complexity and number of experimental variables pertinent to the design of disease relevant biological models are significant.[18,185,186,198,199] Unfortunately, recapitulating all aspects of the relevant patient biology in a model is at best an aspirational goal. More realistically, research usually focuses on reproducing specific disease features deemed to be essential for model value. Even so, these are usually complex systems, and therefore require significant development, optimization and validation efforts.[18,185,186] As with

molecular target validation,[200] disease models should be considered translational only after concordance is established between discovery and clinical phase data. This represents a high hurdle which is obtained only late in a project's lifecycle.

Although relevant cell types and culture conditions are necessary to develop physiologically relevant models, their use alone is not sufficient to guarantee disease relevance.[199] Critically, the chain of translatability of a cellular system should be benchmarked against multiple aspects of the human clinical condition such as morphology, multi-omics characterization, and pharmacological responses. The recent development of an in vitro model of non-alcoholic fatty liver disease and non-alcoholic steatohepatitis (NASH) illustrates this key point.[196,201] This model uses 3D co-culture of primary human hepatocytes, Kupffer cells, and hepatic stellate cells which display disease-relevant tissue morphology, biomarker expression/secretion, transcriptional profiles and responsiveness to obeticholic acid, an advanced clinical compound which improves the histological features of NASH but has yet to secure FDA approval.[196,201] Similarly, oncology models utilizing 3D organoids can be frequently derived from patient biopsies. Patient-derived organoids (PDOs) retain aspects of the original patient tumor such as histopathology, biomarker protein expression, and genomic features (copy number variations and mutational landscapes).[202–205] Significantly, PDOs derived from gastrointestinal and pancreatic cancers show diverse ex vivo responses to standards of care but closely match the clinical chemotherapeutic responses of specific patients [203,204] and ex vivo responses of rectal cancer PDOs to chemoradiation treatment correlate with the clinical responses noted in individual patient tumors.[202,205]

In the era of big data, integration of real-world patient records from large populations (e.g. UK Biobank, FinnGen) and their omics data may also be used to help both build and validate model systems. Utilizing an in vitro phenotypic screen monitoring alpha-synuclein gene expression as a Parkinson's disease (PD) model, Mittal et al identified βB2-adrenergic receptor modulators in a drug repurposing screen. Significantly, the in vitro model was subsequently validated by analysis of 4 million patient records which correlated the use of a β2AR agonist or antagonist with a reduced or increased risk of developing PD, respectively.[206] Alternatively, the Tumor Profiler Study seeks to deliver patient specific treatment recommendations by integration of a patient's real world clinical data with high-resolution multi-omics profiles and ex vivo drug response of the patient tumor within a clinically relevant turnaround time.[207]

Animal models of human disease are an important component of preclinical drug discovery. While nowadays they are used mostly to validate modulation of specific molecular targets, models utilizing mammals were a mainstay as primary phenotypic assays in drug discovery until the late 20th century and led to the discovery of therapeutic agents for indications such as epilepsy, gastric ulcers, hypertension, inflammation and pain.[11,13,69,104,105] With the advent of the 21st century, this approach to drug discovery was all but ruled out at larger pharmaceutical companies.[105]

As the need for phenotypic assays with high translational potential becomes ever clearer, perhaps it is time to reconsider the wholesale abandonment of in vivo models as first line phenotypic screening systems. While whole organism models may be necessary to

more fully recreate the disease state for certain complex or multi-organ indications poorly described by in vitro systems, even those including native cells and 3D architecture, the development of valid in vivo translational models is certainly not trivial however.[180]

Importantly, in vivo disease models have recognized issues with human disease translation due to factors including methodology, systematic data review, and critical disease specific disparities.[208–210] Lack of species translation constitutes another significant hurdle as exemplified by the failure in clinical trials of flavonoid DMXAA despite promising efficacy in preclinical models. Following the identification of its target, the disconnect was traced to the selective activation of mouse STING over its human ortholog.[211] Overall, ethical, cost, translatability and throughput considerations combine to place a high bar on the development and use of suitable in vivo models.

Efforts to enhance their translational value include the development of a “Mouse Hospital” and co-clinical trial concept, where *in vivo* preclinical mouse models and early clinical studies are closely aligned for *in vivo* testing of drugs in order to mitigate translational issues.[212,213] Similarly, comparative expression profiles of genetic mouse models of Huntington’s disease (HD) correspond well with patient profiles, particularly for mRNAs that are decreased in HD striatum[214] and thus increase the predictive validity of HD mouse models.

Advances in disease models may also encompass aspects of data acquisition and analysis rather than improvements in the model organism per se. SEP-363856 is a novel psychotropic agent with a mechanism of action which is independent of D2 and 5-HT2A modulation employed by legacy agents such as chlorpromazine. It was identified by SmartCube phenotypic screening — an automated system to capture digital video of various domains of mouse behavior followed by algorithmic data reduction to approximately 2,000 features and supervised learning to derive drug class signatures or behavioral barcodes based on mice treated with compounds validated for specific therapeutic indications and marketed drugs.[40,41,215] The resulting drug class/behavioral signatures are high dimensional/high content and are likely to capture drug-induced aspects of behavior which may not be apparent in conventional manual, stand-alone mouse models.

While screening throughput is a significant hurdle in such in vivo systems, it is incorrectly thought to be an insurmountable barrier. Historical success stories document that hits could be identified as part of smaller, often hypothesis-based and pharmacophore-informed in vivo phenotypic screens.[11,104] As discussed earlier, the success observed with very small molecules in legacy and recent screens may be rationalized by several key features collectively leading to increased odds of success.[42,108] Finally, the in vivo profiling of 1,000 analogs which led to the discovery and development of SEP-363856 indicates that a reasonable throughput may still be obtained.[43,215] Alternatively, CRISPR sgRNA technology using Cas9 mice enables genetic screens to identify pathways and nodes modulating some disease phenotypes of interest, especially in the field of oncology.[216,217] Models based on lower order organisms such as Zebrafish also offer an opportunity for higher throughput but at the cost of being potentially further removed translationally.[218]

The inherent multi-disciplinary nature of disease model enablement and the iterative process to optimize and correlate discovery and therapeutic endpoints for these assets presents a major hurdle to the biopharmaceutical research community. Notably, much of the expertise in these diverse disciplines resides in academia whereas the end users and experience to identify/prioritize disease indications for model development to address unmet medical needs reside largely in the biopharmaceutical sector. Additional barriers to the development of new disease model systems include uncertainty and time constraints imposed by grant support of academic research along with lower appetite for fundamental research with long- or uncertaintime horizons in the for-profit sector. The development of physiological and disease relevant models may thus benefit from a non-profit, pre-competitive research organization,[185,186] similar to consortia developing probe compounds to pre-competitive molecular targets.[219–222]

Concluding remarks

PDD has demonstrated its potential by identifying drugs, targets and MoAs that in many cases would have been impossible to discover using target-based approaches.[1 3,4,5 223] This strategy offers a path to novel therapeutics when molecular information about disease pathophysiology is lacking, providing access to the untapped “dark biological matter” represented by the proteome and any other biomolecule and cellular process underlying disease.[4,6] The choice of compound library and the clinical translatability of the phenotypic model are essential for the success of PDD. [5,7,8]

Key aspects highlighted here include the discovery of new MoA only accessible to PDD, the need for phenotypic models that can better recapitulate the pathophysiology of complex diseases (for example, integrating immune or nervous system components), the opportunities offered by polypharmacology and the advantages of using libraries with smaller-than-conventional molecules. Increased uptake of bioactivity profiling and MoA characterization approaches applied to efficacy / safety assessment and the increasingly powerful computational technologies have become essential to extract the full potential of PDD.

The current challenge is how to rationally combine these key aspects to prospectively “industrialize” phenotypic drug discovery. Given the exponential growth in this area, we are confident that increased application of ML (and deep learning in particular) will contribute to the effective implementation of PDD (Figure 5). Our vision is that industrialization of phenotypic drug discovery coupled with the extensive experience accumulated after more than a decade of “modern” PDD practice will contribute to a more productive drug discovery process that seamlessly integrates target and phenotype-based approaches.[223]

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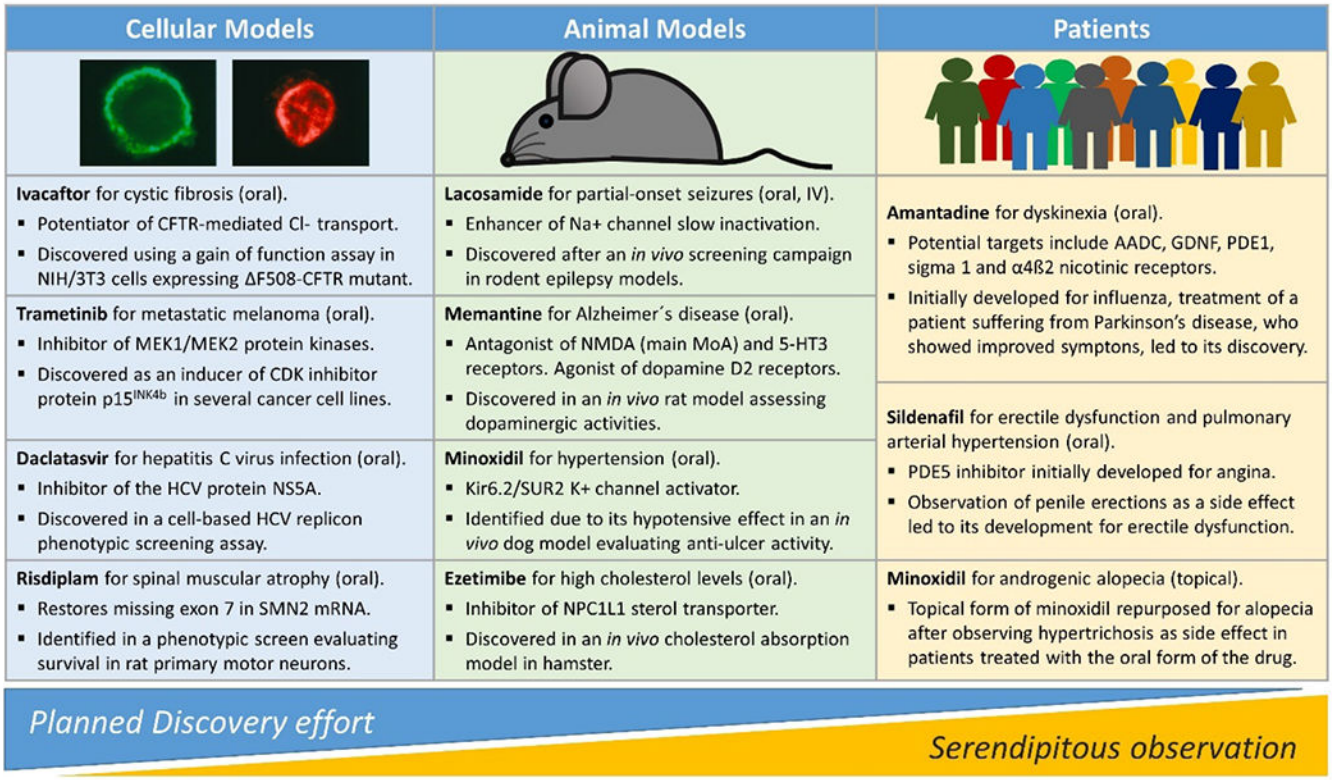


Figure 1:

Modern PDD strikes a balance between planned discovery and serendipity. Approved drugs are listed based on the original phenotypic assay which first connected the compound series or the drug itself to the disease. Notably, while all discoveries from cellular screens represented the outcome of planned efforts, it was unexpected clinical side effects in patients which led to compound repurposing. References: ivacaftor,[6,7] , daclatasvir,[8] risdiplam,[9] trametinib,[10] lacosamide,[11] memantine,[12] minoxidil,[13,14] ezetimibe,[15] amantadine,[16] sildenafil.[17]

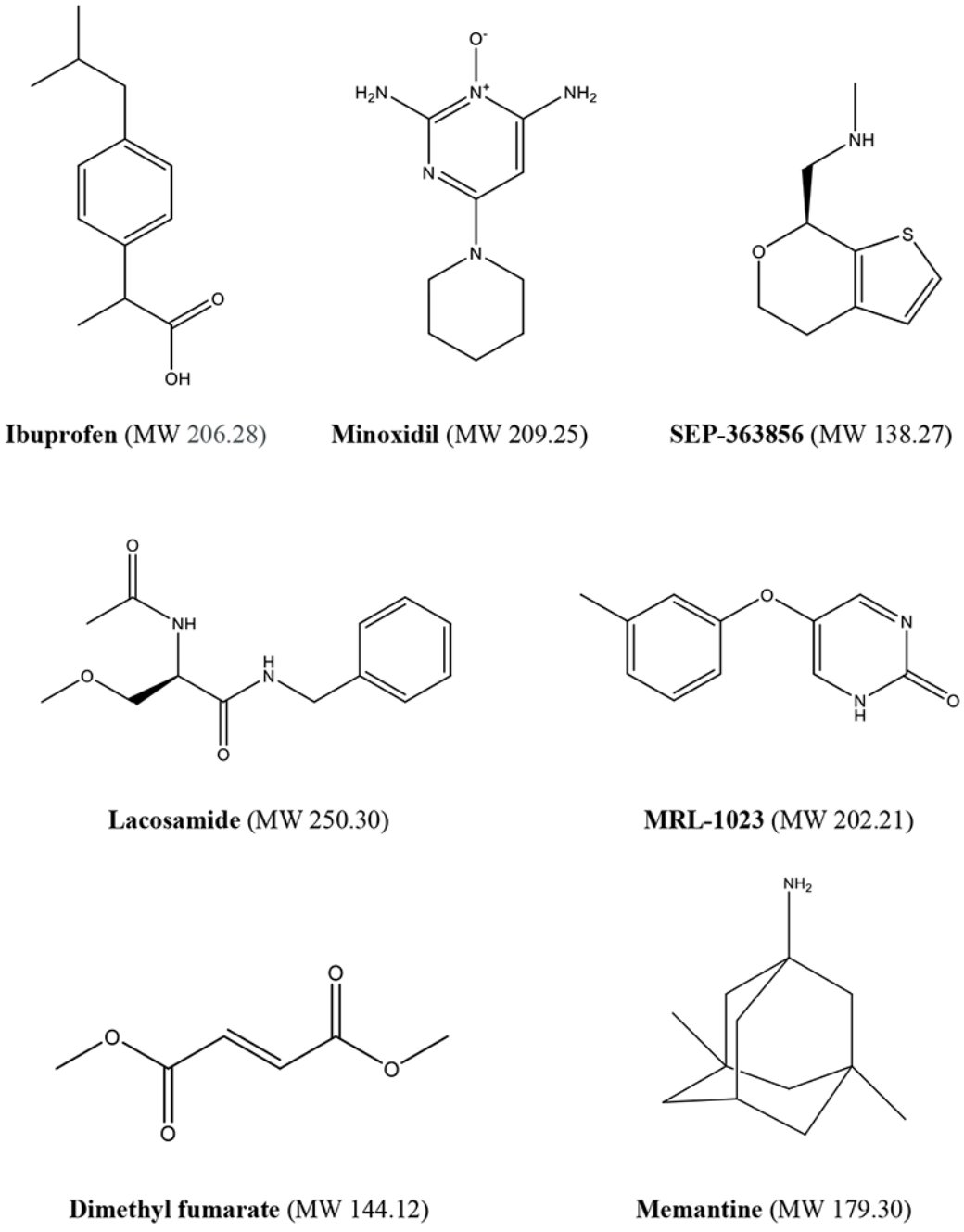


Figure 2.
Low molecular weight clinical candidates and drugs derived from phenotypic approaches

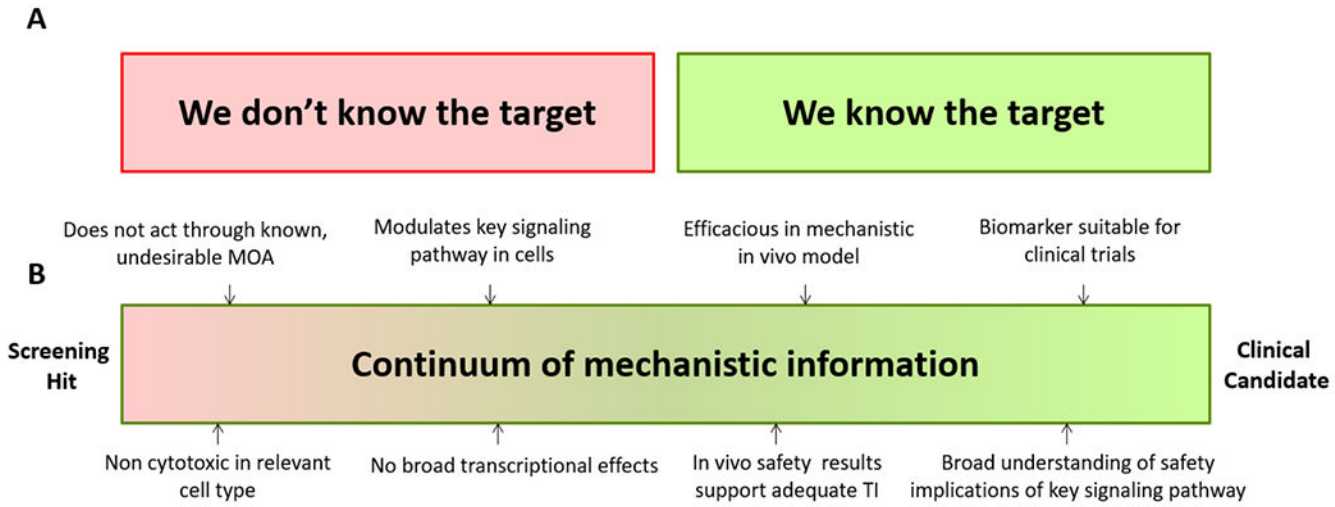


Figure 3.

Target identification is sometimes perceived as necessary and having a simple binary outcome: the target is identified or it is not (Panel A, legacy thinking). We suggest instead that a continuum of information can be accessed which may address the true end goal of target identification, helping obtain sufficient confidence in safety and translation to support progression into the clinic (Panel B, emerging thinking). TI: Therapeutic index.

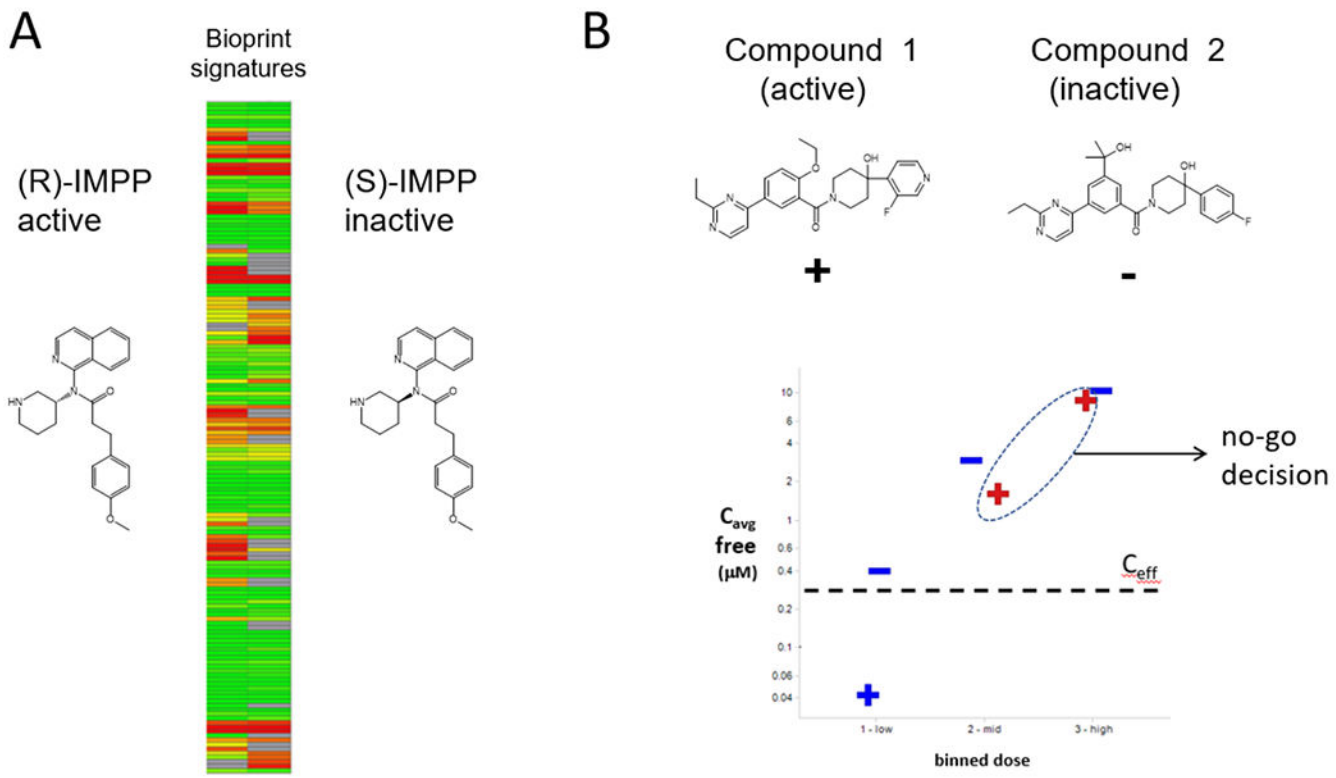


Figure 4.

Utility of active / inactive compound pairs to address safety questions for compound series with unknown target(s) or MoA(s). A) Profiles in the BioPrint pharmacology panel[148] of PCSK9 secretion inhibitor (R)-IMPP and its inactive analogue (S)-IMPP. Figure adapted with permission from ref [49] B) In vivo toxicology results following multi day dosing of of cystic fibrosis lead Compound 1 (+) and its inactive analogue Compound 2 (−). Blue coloring indicates the compound was tolerated while red lettering indicates it was not. C_{ave}: average *in vivo* concentration, C_{eff}: predicted *in vivo* effective concentration for Compound 1.

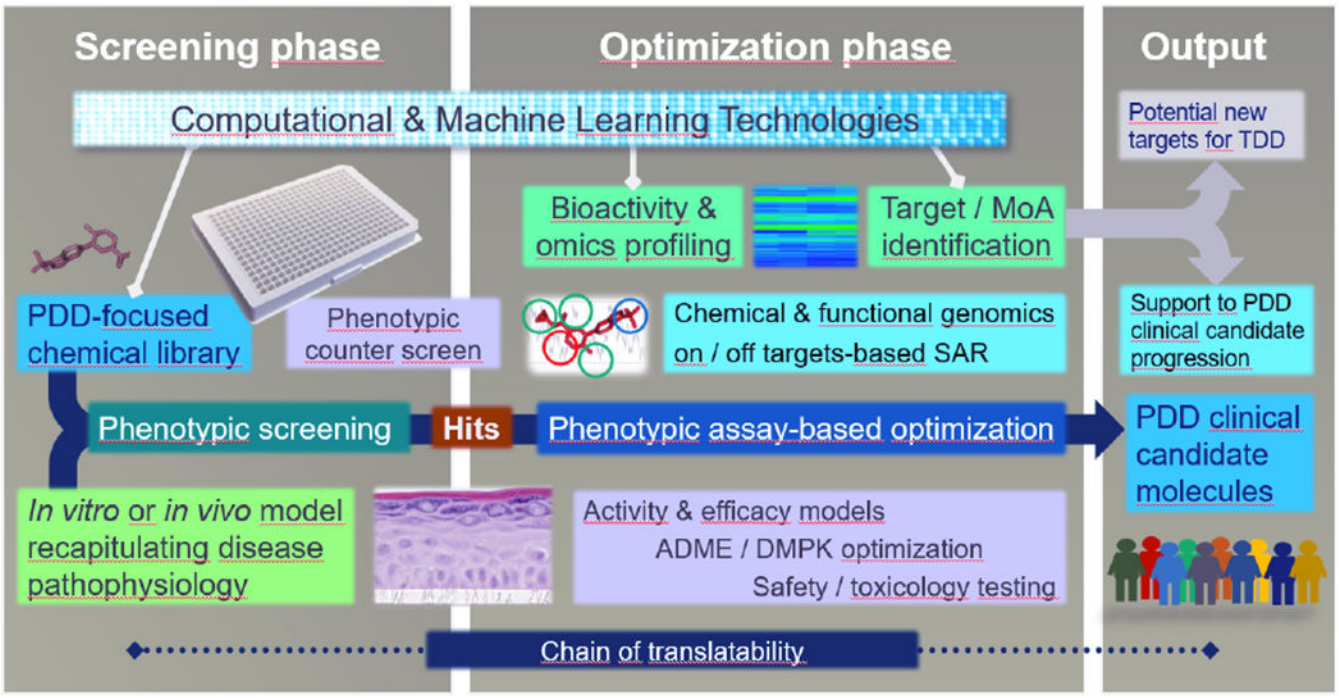
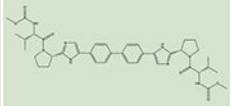
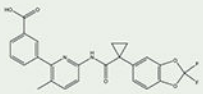
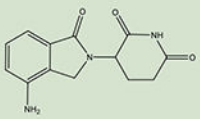


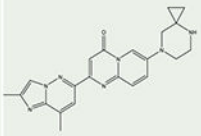
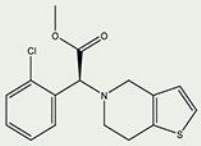
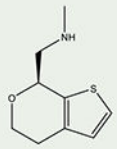
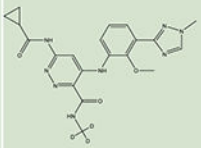
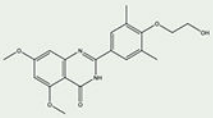
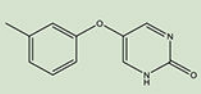
Figure 5. Schematic overview of an industrialized phenotypic drug discovery process.

A chemical library designed for phenotypic screening (e.g. with smaller MW compounds) and a disease-relevant in vitro or in vivo model system capable of providing sufficient throughput, are combined in a phenotypic screening campaign to identify hits whose optimization, characterization and progression to clinical phases can take advantage of the current plethora of omics, profiling and computational approaches (including machine learning). Target or MoA information is used to support the progress of clinical PDD candidates. One additional possibility, if targets are identified, is their potential use as starting points for new TDD programs. Chain of translatability is shown to represent the molecular association between the mechanisms driving the phenotypic assay, the preclinical disease models, and human disease. Counter screen refers to an assay aimed at verifying the selectivity of the hit molecules versus other unintended phenotypic endpoints. SAR: structure-activity relationship, MoA: mechanism of action.

Table 1:

Phenotypic Origins of Approved Drugs and Clinical Phase Molecules

Drug or Clinical Candidate	Structure	Indication	Phenotypic Screening Strategy	Mechanism of Action	Development Phase
Daclatasvir (Modulators of NS5A are components of all anti-HCV drugs)		Hepatitis C infection	Target agnostic viral replication screen[23]	NS5A identified as molecular target, HCV replication inhibition, MoA unknown	Launched
Lumacaftor (component of Orkambi along with Ivacaftor)		Cystic Fibrosis	Mechanism agnostic cellular screen to enhance CFTR function[7]	Correctors enhance the folding and plasma membrane insertion of CFTR; novel MoA[36]	Launched
Lenalidomide		Multiple myeloma and other hematologic malignancies	Functional cellular assays and off label observational studies in patients[27,28]	Alters protein substrate specificity of E3 ubiquitin ligase Cereblon; novel target class and MoA[30]	Launched

Risdiplam		Spinal Muscular Atrophy	Mechanism agnostic cellular assay to correct SMN2 RNA splicing[33]	Engagement and stabilization of SMN2 exon 7 and U1 snRNP complex; novel target class and MoA[33-35]	Launched
Clopidogrel (prodrug of active metabolite responsible for activity).[37]		Cardiovascular disease	Anti-platelet activity identified using a battery of in vivo and ex vivo rodent models screened to explore anti-inflammatory activity.[38]	Active metabolite selectively and irreversibly blocks platelets P2Y12 ADP receptors.[39]	Launched
SEP-363856		Schizophrenia, Psychosis	Automated in vivo behavioral models, the "SmartCube System"[40-42]	Positive Phase 2 results mediated by novel non-dopamine GPCR mechanism; novel MoA[43]	Phase 3 (Schizophrenia) Phase 2 (Psychosis)
Deucravacitinib		Psoriasis and other autoimmune conditions	Kinase biased compounds tested in cellular assay monitoring IL-23 signaling pathway[44]	Positive Phase 3 results; Novel MoA; Allosteric inhibition of Tyk2 kinase through catalytically inactive pseudokinase domain[44]	Phase 3 (Psoriasis), Phase 2 (Other indications)
Compounds from multiple companies: Apabetalone (RVX-208) shown as an example of the most advanced clinical candidates		Adverse cardiovascular events in type 2 diabetes; Oncology various tumor types	Initial compounds identified with mechanism agnostic cellular assay, selective upregulation of ApoA1[45,46]	Bromodomain proteins identified as novel drug target class for epigenetic gene regulation[45,46]	Phase 3 (Type 2 diabetes), Phase 2 (Oncology)
MLR-1023		Type 2 Diabetes	Battery of in vivo models; effective with in vivo glucose tolerance test, oral delivery[47,48]	Positive Phase 2 results; Allosteric activation of Lyn kinase, novel MoA[47,48]	Phase 2 (Type 2 Diabetes)

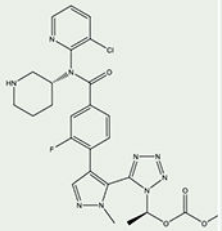
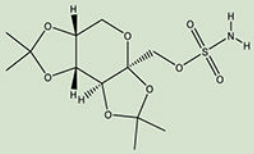
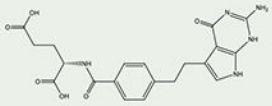
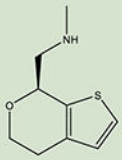
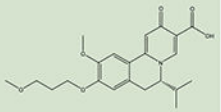
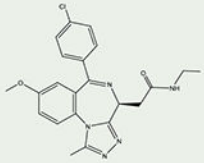
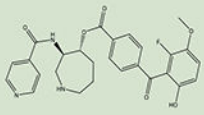
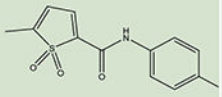
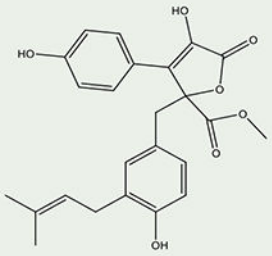
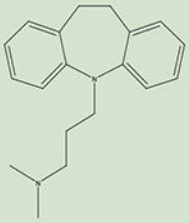
PF-06815345		Dyslipidemia	Mechanism agnostic cellular assay for inhibition of PCSK9 secretion[49]	Novel target and MoA; inhibition of PCSK9 translation via ternary complex of compound, PCSK9 amino terminus and ribosome[49,50]	Phase 1 (Terminated)

Table 2.

Representative examples of polypharmacology among marketed drugs, clinical candidates and investigational compounds. Generic name, code name or identifier from the original reference is indicated for each compound. For investigational compounds representative examples have been selected from the corresponding referenced publications.

Drug or compound	Structure	Indication	Phenotypic Screening Strategy	Mechanism of Action	Development Phase
Topiramate		Epilepsy, migraine, obesity	Assessment of anticonvulsant activity in a mouse MES model[69]	Modulator of GABA-A and AMPA/kainate glutamate receptors, voltage-gated sodium and R-type calcium channels, and several isozymes of carbonic anhydrase.[69]	Launched
Pemetrexed		Several cancers	Inhibition of growth in a panel of mouse tumors and in human tumor xenografts[97]	Inhibitor of Thymidylate synthase, DHFR, GARFT and AICART enzymes.[70,71]	Launched

SEP-363856		Schizophrenia, Psychosis	Evaluation in in vivo behavioral models[40-42]	Not fully elucidated: suspected agonist of TAAR1 and 5-HT1A receptors.[43,98]	Phase 3 (Schizophrenia) Phase 2 (Psychosis)
RG7834		HBV infection	Screening for inhibitors of HBsAg secretion in the human hepatoma cell line HepaRG[74]	Inhibitor of PAP-associated domain-containing proteins 5 and 7 (PAPD5 and PAPD7).[74,75]	Phase 1
Molibresib (I-BET762)		Several types of cancer	Cell based screen for upregulators of an Apo1-luciferase reporter gene.[72]	Inhibitor of BD1 and BD2 bromodomains shared by BRD2, BRD3, BRD4 and BRDT paralogs of the BET family of epigenetic proteins.[72]	Discontinued for cancer (evaluated in phase 1/2 trials)
RO0480500-002		CNS axon repair	Screen for inducers of neurite growth in primary rat hippocampal neurons using a library of protein kinase inhibitors[100]	Inhibitor of PKC, ROCK, S6 kinases, PKG1 and PKX.[76]	Investigational compound
11426026 shown as representative compound		Anti-infective (tuberculosis)	Activity against non-replicating M. tuberculosis[101]	Inhibitors of M. tuberculosis CTP synthase PyrG and PanK kinase.[77]	Investigational compound

Butyrolactone I		Metabolic disease	Induction of adiponectin in human bone marrow mesenchymal stem cells (hBM-MSCs)[79]	Inhibitor of CDK1, CDK2, CDK5. PPARγ receptor partial agonist. [79]	Investigational compound
Imipramine		Depression	Antidepresant activity serendipitously identified evaluating potential antipsychotic effects of anti-histamine compounds in schizophrenia patients[102]	Not completely understood: Inhibitor of serotonin (SERT) and norepinephrine (NET) transporters, antagonist of histamine H1 and 5-HT_{2A} GPCRs among other receptors and enhancer of BDNF receptor TRKB function[67,68]	Launched