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Phenotypic screens as a renewed approach for drug discovery

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The significant reduction in the number of newly approved drugs in the past decade has been partially attributed to failures in discovery and validation of new targets. Evaluation of recently approved new drugs has revealed that the number of approved drugs discovered through phenotypic screens, an original drug screening paradigm, has exceeded those discovered through the molecular target-based approach. Phenotypic screening is thus gaining new momentum in drug discovery with the hope that this approach may revitalize drug discovery and improve the success rate of drug approval through the discovery of viable lead compounds and identification of novel drug targets.

The goal of all drug discovery efforts is to develop efficacious and safe therapeutics to effectively treat human diseases. Modern drug development for a given disease usually begins with either target-based or phenotypic-based screening of a compound library. Well before molecular target-based drug discovery became popular, phenotypic-based screening strategies were the foundation of pharmaceutical drug discovery (Fig. 1). In the past 25 years, molecular target-based drug screening has become the main drug discovery paradigm used in both the pharmaceutical industry and in academic translational research centers. Recently, however, there appears to be renewed interest in reinventing phenotypic screens for lead discovery as a means of reenergizing drug discovery.

Molecular target-based screening

The foundation for a molecular target approach of drug development started with advances in pharmacology, as well as synthetic and medicinal chemistry beginning in the early 20th century. The wealth and depth of research performed in the 1950s and 1960s on enzymes and enzyme kinetics provided a method for precise calculation of a compound's potency (IC_{50} or EC_{50}) and efficacy (% maximal response) of an enzyme [1]. Hundreds of enzymes were discovered and purified during this period, later becoming important molecular targets of drug discovery [1]. The methodology of enzyme kinetics was extended to receptor pharmacology in 1970s [2], although the molecular entity of receptors was largely unexplored at this time. The progressive research in receptor pharmacology and the nature of druggability later made receptors the most popular targets for drug discovery [3,4]. Technological advances in molecular biology and genome science initiated a modern era of molecular target-based approach for drug discovery in the late 1980s. Recombinant DNA technology enabled the generation of new assays for a wealth of molecular targets, allowing rapid screens of large chemical libraries using purified recombinant proteins or engineered cell lines [5–8]. This, along with developments in combinatorial chemistry, assay miniaturization and robotic automation, greatly facilitated the emergence and rapid development of high-throughput screening (HTS) in the 1990s [9,10].

The molecular target-based approach for drug discovery, also called 'reverse pharmacology' or 'reverse chemical biology' [11–13], generally starts with target identification relevant to a disease of interest (Fig. 2a). Molecular targets are often discovered in basic research, with studies involving animal disease models and clinical observations of patient phenotypes. For example, an abnormal function of a specific protein, an aberrant signaling pathway, or a mutation in a specific gene can be identified in basic research with connection to a disease. Once a suitable target has been identified and validated, assay development is initiated, followed by HTS of chemical libraries to identify hits such as enzyme inhibitors or receptor antagonists against the target. The most active compounds, usually compounds making up one to three lead series,

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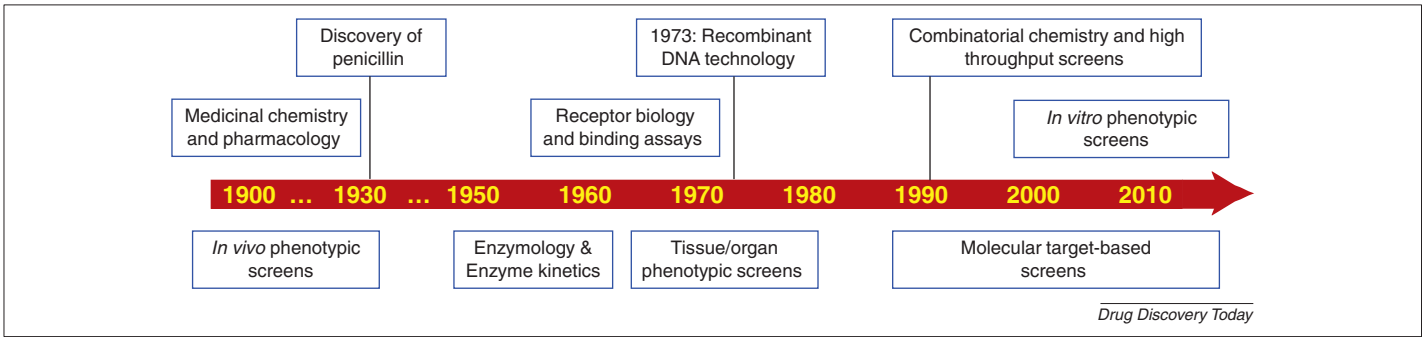

FIGURE 1

Evolution of drug screening and lead discovery.

are then confirmed and validated in orthogonal assays that are more physiologically related to the target. This is then followed by chemical optimization to characterize the structure–activity relationship (SAR) of the lead series and to enhance favorable absorption, distribution, metabolism, and excretion (ADME) and pharmacokinetic/pharmacodynamic properties of the compounds. In this paradigm, only a few lead compounds with a defined mechanism of action and demonstrated efficacy in disease models are able to move to preclinical drug development, toxicology studies, and hopefully, clinical trials. In the past 20 years, molecular target-based screening has become the major approach in early drug discovery. G-protein-coupled receptors (GPCRs), ion channels and enzymes are the most common and successful molecular targets for drug discovery [5–8]. It is interesting to note that all the biologics approved for treatment of human disease are target-based therapeutics [14]. In contrast to some small molecule compounds, biologics such as proteins (e.g. enzymes, antibodies), hormones, peptides, vaccines, and blood components are made through biological processes and their mechanism of action is dependent on a specific target.

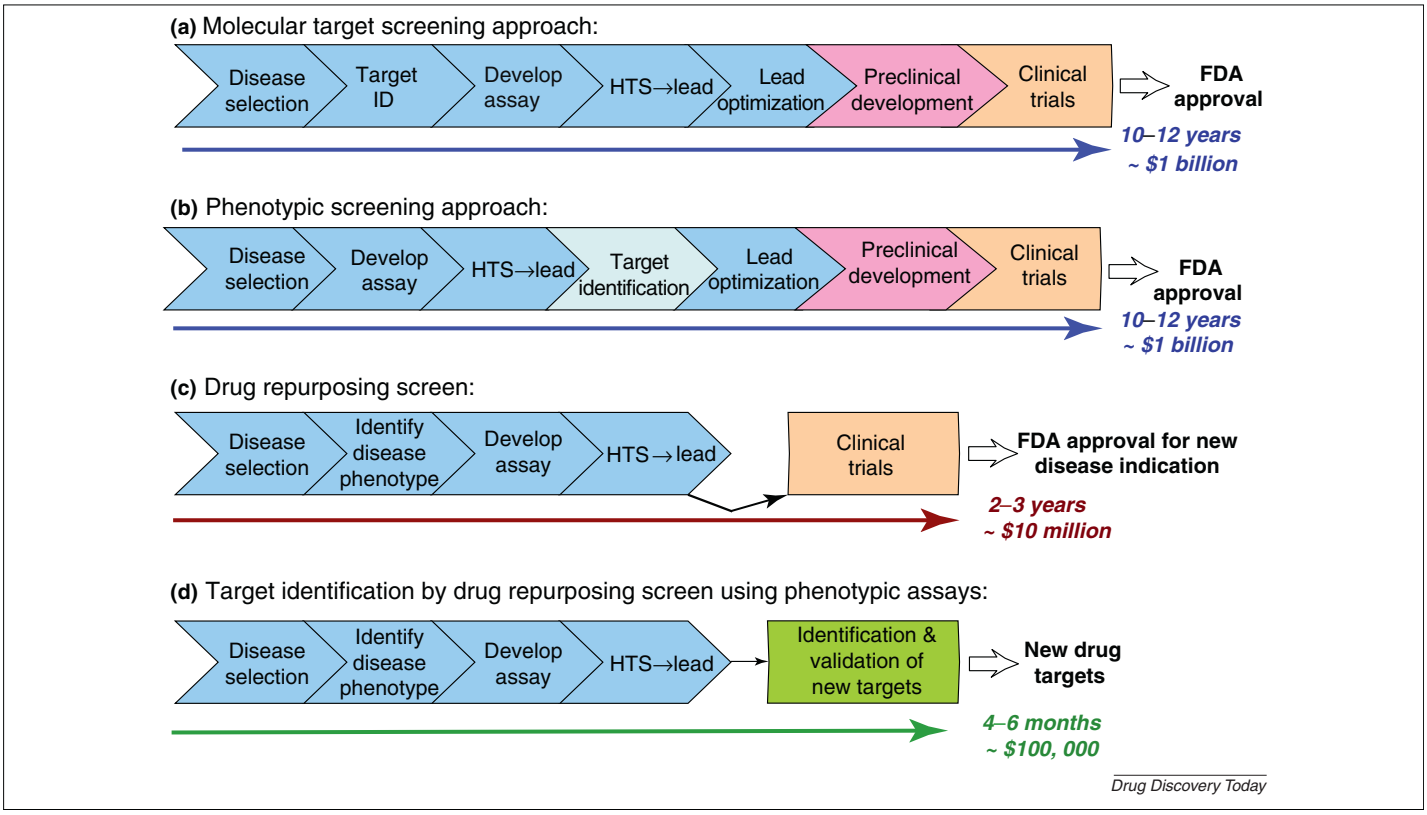

FIGURE 2

Comparisons of phenotypic-based screening and molecular target-based screening in drug discovery and development. **(a)** Traditional drug discovery usually takes 12 years and costs 1 billion dollars average to develop a drug. **(b)** The target does not need to be known for phenotypic based drug discovery and it may or may not be identified after lead discovery. **(c)** Drug repurposing screen using phenotypic assays has the potential for rapid drug discovery and development that may not need the prolonged preclinical drug development. The development time and cost in this approach can be much lower compared with traditional drug discovery. **(d)** Drug repurposing screens can also be used for new target identification because many active drugs have known mechanism(s) of action. The identified lead compounds that may not be used immediately as a drug for a new indication may point out a new target and direction for drug discovery.

TABLE 1

Comparison of target based and *in vitro* phenotypic screens for lead discovery

Features	Target-based screening		*In vitro* phenotypic-based screening	
	Advantage	**Disadvantage**	**Advantage**	**Disadvantage**
Molecular target of a disease	Known	Have to know	Do not need to know	Unknown
Screening throughput and assay	Higher; relatively easy to set up	Assay may be less biologically relevant	Medium or low; biologically relevant	Could be low; could have higher cost
Mechanism of action of lead compound	Known at onset, which can accelerate preclinical drug development	Limited possibility of identifying a new mechanism	Multiple targets and signaling pathways can be targeted; may involve native biological targets and complexes	Unknown at onset
Methods for confirmation of lead compound	Direct binding assay, modeling, X-ray crystallography, or other biophysical methods	Need to be confirmed in cell- based and phenotypic assays with native targets and complexes	Can move to *in vivo* study quickly	Target identification may be required; which can be complicated and time consuming
Methods for SAR optimization	Readily available and direct		Additional assays may need to support SAR	May need to develop a more targeted assay
Disease relevance of lead compound	Direct if it is relevant	Drug target may not be disease-relevant, as lack of human efficacy found in late-stage clinical trials	Usually disease relevant; may target more complex diseases	
Hypothesis limitation of lead compound [77]		Limited by the hypothesis, simple	Less hypothesis-restricted	

There is no doubt that molecular target-based screening has some distinct advantages over phenotypic screening (Table 1). For example, a molecular target and its related screening assay are often vital in guiding subsequent chemical optimization of lead compounds and necessary to fully characterize the SAR. Additionally, knowledge of a molecular target can help guide toxicology studies during preclinical development. Biomarker development, which is critical for evaluation of drug effects in animal disease models and clinical trials, may also be facilitated by the known molecular target and its signaling pathway.

It has been recognized recently, however, that target-based drug discovery may have its limitations. Recent analysis has revealed that high attrition rates in Phase II and III clinical trials are mainly due to lack of drug efficacy along with other factors [15,16]. Although the lack of drug efficacy in late stage drug development can be the result of multiple factors, including poor correlation of animal models with human diseases and genetic variation of patient populations, invalidated targets for disease is a significant factor for many failed drug candidates. Additionally, the numbers of validated druggable targets currently available for drug development are seemingly more limited than previously thought [8,17]. A recent review of FDA approved drugs indicated that there currently exist only 435 effective drug targets although the success of human genome program has revealed a total of approximately 20,000 human genes that encode approximately 500,000 proteins [8]. Thus, identification of new drug targets from the human genome remains an unmet biomedical research goal.

The success of target-based screens used for drug discovery has also recently come into question. Swinney and Anthony [14] analyzed the first-in-class small molecule drugs approved by the FDA between 1999 and 2008 and found that 28 of them were discovered using a phenotypic screening approach compared to 17 drugs discovered by a molecular target-based approach. This surprising discovery has contributed to growing interest and reconsideration of phenotypic screens for drug development in both pharmaceutical industry and academic research centers, with a hope that newly increased application of this traditional approach can rejuvenate early-phase drug discovery and improve the success rates in late stage drug development (Fig. 1).

Phenotypic screening in drug discovery

Today, the main application of cell-based phenotypic assays is to screen large compound libraries, composed of 0.4–2 million compounds, to identify lead compounds for drug discovery projects. Historically, drug discovery was phenotypic by nature – with new drugs either accidently found, as in case of penicillin, or through designed bactericidal screens to discover additional antibiotics [18]. The phenotypic screening approach for drug discovery is also called 'forward pharmacology', 'classical pharmacology' or 'forward chemical biology' [11–13] and the molecular mechanism and protein target can remain unknown even after the drug's activity and efficacy are determined. Generally, a characteristic associated with the disease is exploited to develop a cell-based assay for a modern phenotypic screen (Fig. 2b). Compounds are then screened in the phenotypic assay to identify active lead compounds that ameliorate the disease phenotype, exemplified by selectively killing cancer cells [19], eliminating pathogens in culture [20], or reducing lysosomal cholesterol accumulation in Niemann Pick disease type C patient cells [21].

The phenotypic screen is usually more physiologically relevant and less artificial because intact cells and native cellular environment are used. Primary hits identified in the phenotypic screens can potentially target different types of proteins (receptors, enzymes, transcription factors, among others) and even different

signaling pathways. Lead compounds can be further selected from the hits with or without knowledge of the target, although identification of the target can facilitate the SAR study. The phenotypic screen in this 'forward pharmacology' process enables lead discovery for many diseases in which a drug target has not been identified and/or validated. Therefore, this approach can have a useful role in drug discovery for many rare diseases which tend to be understudied, and with most lacking an effective drug therapy. Phenotypic screening can also be applied to the discovery of novel drug targets, which may prove useful for common neurological diseases such as Alzheimer's and Parkinson's diseases, for which there have been many failures of target-based drug candidates in clinical trials.

Recent retrospective analysis has found that many drugs approved by the FDA (especially those the in 1970s) have an unknown mechanism of action or an unknown target [22]. Not surprisingly, many of these early approved drugs were discovered using phenotypic screens and were approved by regulatory agencies before their precise mechanism of action or protein targets were identified. A famous example of this is aspirin (acetylsalicylic acid) for which it took almost 100 years to determine the mechanism of action and molecular target [23]. Calcium channel antagonists [24] including 1–4 dihydropyridines (nifedipine, nicardipine and nimodipine), verapamil and diltiazem were found and developed using phenotypic screens involving smooth muscle relaxation, vasodilatation and reduction of high blood pressure [25,26]. The precise mechanism of action for the treatment of hypertension and other cardiac indications was not clear when the first of these drugs were approved in the 1980s. They originally had the generic name of 'calcium blockers' [27], while the first L-type calcium channel that these drugs act on was cloned in 1987 [28]. Ezetimibe (Zetia), a cholesterol absorption inhibitor, was also discovered in an animal model with a high cholesterol diet [29,30]. It received FDA clearance in 2002 as a cholesterol lowering drug without a known molecular target [31], which was reported later to be the NPC1L1 cholesterol transporter [32]. Even today, regulatory agencies around the world will approve a new drug without requiring the precise mechanism of action or a molecular target, as long as the drug is efficacious and safe for patients. It should be noted that in-depth characterization of the drug properties including mechanism of action and molecular targets can aid in the design of improved next-generation compounds with reduced adverse effects.

Animal-based phenotypic screens

Historically, isolated tissues or animal models were involved in phenotypic screening, as described briefly above. In the past 10–20 years, many disease models of several small animals including *Caenorhabditis elegans*, zebrafish, *Xenopus laevis*, and *Drosophila melanogaster* have been developed and applied to compound screening to achieve relatively high screening throughput [33]. The phenotypic screens using *in vivo* model systems can provide rich information on compound absorption, distribution, metabolism and toxicity in addition to valuable efficacy data in a disease model. Although the throughput of compound screens in rodents or large animal models is limited, the screening capacity with *C. elegans*, *D. melanogaster*, Zebrafish and *X. laevis* has been improved by using 96-well plates [34–38] although it is still significantly lower throughput compared to cell-based assays. One disadvantage to screening with *in vivo* models is that potential lead compounds with properties of poor drug absorption, quick metabolism, limited cell membrane permeability and toxicity may not be active in the primary screens. The poor relevance of some animal models to human diseases, due to species differences and other reasons, can contribute to failures in the late stages of drug development. Therefore, cell-based phenotypic screening seems more suitable for primary compound screens to identify physiological and disease relevant lead compounds for drug development.

Cell-based phenotypic assays

With advances in new assay technologies, the throughput of phenotypic screening has greatly improved in the past ten years (Fig. 2). Robotic screening platforms and highly sensitive detection systems have been developed which allow phenotypic assays to be miniaturized and used to rapidly screen large chemical libraries. In contrast to the lack of cellular content of many molecular target-based assays using purified recombinant proteins, cell-based phenotypic assays offer additional biological complexity, with the cellular milieu of interacting proteins and signaling networks, while still maintaining the capacity of HTS. Cell-based phenotypic assays usually use primary human cell lines, immortalized cell lines (primary or engineered), or, more recently, specific cell types differentiated from induced pluripotent stem cells (iPSCs) derived from patient or normal human cells (Table 2).

TABLE 2

Examples of cell types used in phenotypic screens

Disease	Cell type	Assay type	References
Primary cells			
Thyroid cancer	Thyrocytes	TSH responsive proteins	[79]
Cystic fibrosis	Bronchial epithelial cells	Electrophysiology	[80]
Immortalized primary cells			
Respiratory papillomatosis	Tumor cells	Cell viability (ATP content)	[19]
Cystic fibrosis	Bronchial epithelial cells	Electrophysiology	[81]
Engineered cell lines			
Huntington disease	PC12 expressing HTT Q103-GFP	Protein aggregates (GFP)	[43]
SMA	U2OS expressing SM2-luciferase reporter	RNA splicing (luciferase)	[82]
Human cells derived from stem cells			
Familial dysautonomia	Neural crest precursors	RT-PCR	[63]
NSC proliferation/differentiation	Neuroepithelial-like stem cell line	Cell viability (ATP content)	[65]

Cell viability assays, cell signaling pathway assays, and disease-related phenotypic assays are three types of cell-based phenotypic assays commonly performed in lead discovery. These assays can be miniaturized to a robotic screening platform and have higher screening throughput. Active compounds are identified that confer a change in a cellular phenotype such as killing pathogens or cancer cells, activating or inhibiting a signaling pathway, or normalizing a phenotypic change associated with human disease. Additionally, other types of phenotypic assays are also available including autophagy, apoptosis, cell cycle analysis, cell infection, cell motility, cell secretion, cytoskeletal rearrangement, nuclear translocation, receptor internalization and neurite outgrowth.

Cell viability assay

The cell viability assay is one of the most common phenotypic assays performed and has multiple assay formats. Active compounds are identified that kill cancer cells or exogenous pathogens including bacteria, fungi, protozoa, and parasites. The assay principle of different cell viability assays involves mitochondrial activity, cellular metabolism or the activity of enzymes associated with viable or dead cells. The AlamarBlue assay has been used in mammalian cell lines as well as in bacteria, yeast and protozoa [39] and involves a cell permeable profluorescent dye (resazurin) that is reduced to a fluorescent product (resorufin) upon oxidization in mitochondria of viable cells. The colorimetric MTT assay is commonly used to assess compound cytotoxicity that also relies on mitochondrial metabolic activity in viable cells in which 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) is reduced to a product with a purple color [40]. Additionally, cell viability can also be assessed by the release of intracellular enzymes upon cell death such as the lactate dehydrogenase (LDH) assay and protease release assay [43,44], or by quantitating fluorescent dyes intercalated into DNA, such as Hoechst dye (cell membrane permeable; live cell stain) and propidium iodide dye (membrane impermeant; dead cell stain) [41]. The ATP content assay is a newer addition that measures the ATP levels in live cells and is a more robust with better signal-to-basal ratio for HTS compared with the MTT, AlamarBlue and DNA dye assays [42].

Signaling pathway assay

Signaling pathway assays are generally considered to be partially phenotypic, as a known signaling pathway such as a GPCR, nuclear receptor, MAPK/ERK, transcriptional or ubiquitin–proteasome pathway is targeted. The signaling pathway assay links a complex network of protein–protein interactions to transcriptional activation and expression of a reporter gene (e.g. luciferase, beta-lactamase or enzyme complementary coupling) or fluorescence protein (GFP and YFP), which produces a measurable luminescence or fluorescence signal [45–47]. Targeting all proteins and components in a pathway is the main advantage of a signaling pathway assay. Active compounds identified from signaling pathway-based screens may interact with molecular targets at any point or multiple points, in the pathway, an advantage that is not achievable in the single target-based drug discovery approach [48,49].

Disease-related phenotypic assay

Many diseases are characterized by cellular phenotypic changes relative to healthy cells, such as morphological changes, or differences in protein translocation, expression, activity, or function. For example, expression of long CAG trinucleotide repeats in the mutant *HTT* gene in Huntington's disease is cytotoxic and results in cell death, which can be detected by a cell viability assay [43,50]. In Niemann Pick disease type C, lysosomal cholesterol accumulation in patient cells is a characteristic disease phenotype that can be measured by a filipin staining assay [21,51].

There are many examples of phenotypic assays that measure cellular morphological changes associated with disease cells and a compound's effect on normalizing those changes. Examples of these assays include neurite outgrowth assays for Alzheimer's and Parkinson's disease, measurements of aberrant cytoskeletal structure for myopathy and CNS pathologies [52], and nuclear morphology for cellular apoptosis associated with many diseases [53]. Recently, high content screening has been broadly applied to measure phenotypic morphological changes [54], such as visualizing neurite outgrowth using an antibody specific to β-tubulin, fluorescent dye-tagged phalloidin for the actin cytoskeleton, and Hoechst dye for assessing nuclear morphology. Additionally, intracellular localization of a GFP-tagged protein of interest has been used to analyze protein expression levels or translocation of the tagged protein to subcellular compartments and structures. High content screening assays require an automated fluorescence imaging system and quantitative software analysis of the resulting fluorescence-based images, which may limit its use to a well-equipped central laboratory or core facility.

Many diseases are associated with an altered activity or expression level of certain proteins due to disease status or genetic mutation, resulting in dysregulation of important cellular signaling pathways or functions. Measurements in DNA content, nuclear morphology and protein levels involved in the cell cycle can be used for screening of cell cycle modulators, such as mitotic inhibitors [55,56]. Bioluminescence resonance energy transfer (BRET), fluorescence resonance energy transfer (FRET) or protein fragment complementation assays can be used to identify compounds that inhibit or enhance the intracellular protein–protein interactions that may be altered in a disease [57]. Reporter gene assays can be used to probe for changes in cell signaling pathways [58].

Application of primary cells and human cells derived from stem cells

Although recombinant cell lines and immortalized primary cells are commonly used in phenotypic screens to identify lead compounds, largely because they rapidly proliferate and can be expanded for the generation of large quantities of cells needed for HTS, primary human cells and patient derived cells are more desirable for phenotypic screens because of their biological insight and disease relevance. Primary human cells have been used in compound screens that are more biologically relevant for drug discovery [59]. However, limited availability of large amounts of cells and cell types has prevented the broad application of isolated primary cells in lead discovery. Embryonic stem (ES) cells and induced pluripotent stem (iPS) cells are capable of being differentiated into expandable progenitor cells that can be further differentiated to many types of mature cells such as neurons, cardiomyocytes and hepatocytes for drug screens [59,60]. In addition, the capability of generating iPS cells from a patient's skin,

blood or other cells allows establishment of disease models using patient cells that have better pathophysiological relevance to human disease [61,62]. While the methods for stem cell differentiation including the differentiation efficiency, scale-up, reproducibility and cost effectiveness are still being improved, several pilot compound screens using stem cell differentiated progenitor cells have been recently reported. High throughput screens of smaller compound collections have been performed using precursor cells derived from patient or normal stem cells, including neural crest stem cells (from iPSCs with familial dysautonomia, *IKBKAP* expression) [63], neural progenitor cells (from normal iPS cells, Wnt/β-catenin signaling) [64], neuroepithelial-like stem cells (from normal iPS cells, cell proliferation and viability) [65], and neurons (from ES cells, AMPA glutamate receptor) [66]. Additionally, several other types of human cells derived from stem cells have also been used to assess drug efficacy and evaluate compound toxicity for a small set of compounds [67,68].

Phenotypic screening to identify new indications and new targets of approved drugs

The second application of a phenotypic assay is to identify new indications of known drugs – an application that is particularly useful for diseases without an effective therapy. An approved drug collection has recently been established at our center [69] and has been used to identify lead compounds for new applications in different diseases including Giardiasis [20], NF-κB signaling [70], Niemann Pick disease type C [21], Chronic Lymphocytic Leukemia (CLL) [71], Chordoma [72], adrenocortical cancer [73] and thyroid cancer [74]. Similarly, a smaller collection of approved drugs has become available (http://www.nihclinicalcollection.com/) that has been used to identify lead compounds for the CaV1.3-selective L-type calcium channel and a lithium mimetic project [75,76]. The identification of new applications for approved drugs can save time and resources in drug discovery and development, while reducing the risk of failure in early clinical trials [77,78]. This approach is particularly useful in attempts to identify potential therapies for the vast number of rare diseases, as well as neglected diseases, in which it is imperative to find an effective drug quickly at the lowest possible cost (Fig. 2c). Additionally, phenotypic screening of approved drugs may lead to the identification of new drug targets, because of the known pharmacological properties of the drugs on a specific enzyme, receptor or protein. The information obtained from the phenotypic screen can be used for a new drug development program once the new target is validated (Fig. 2d).

Concluding remarks

It has been recognized that there is a genuine need for more biologically relevant screening platforms for drug discovery that may lead to the identification of high quality lead compounds. The new phenotypic screening assays should have great potential to meet this challenge as they are usually much more biologically and/or disease relevant. While the screening throughput and disease relevancy of animal models still needs to be improved, the new cell-based phenotypic screens including those utilizing primary cells and stem cell derived human cells have recently emerged for lead discovery in early drug discovery in parallel to the molecular target-based screening approach. The application of using differentiated cells derived from patients for phenotypic screening assays can greatly expand the types and numbers of cell-based disease models. Therefore, phenotypic screening using newly developed cell-based disease models may lead to a new era of lead discovery and contribute to development of personalized medicine.

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