The power of combining phenotypic and target-focused drug discovery

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A fierce dispute has arisen between the supporters of phenotypic and target-focused screening regarding which path grants the higher probability of successful drug development. A chance to reconcile these two approaches lies in successful target deconvolution (TD) after phenotypic screens. But, despite the panoply of available *in vitro* TD methods, the task of matching a phenotypically active compound with a biomolecular target remains challenging. Consequently, this review details the latest developments of *in silico* techniques that expedite TD. Ultimately, the deconvoluted target allows us to reap the benefits of the phenotypic and target-focused approaches.

Introduction

During recent years, pharmaceutical drug development has encountered several challenges. Because healthcare systems only reimburse novel approaches that extend beyond the established standard-of-care, efforts are directed at finding first-in-class medicines with disease-modifying features. In contrast to the starting points for symptomatic treatment, the mechanisms of action for disease-modifying approaches are normally more challenging with regard to pharmacological intervention. Phenotypic screening (PS) can lend itself more suitably to the discovery of these new disease pathways, and the derived bioactives have typically established their efficacy in a pathologically relevant cellular model [1]. Accordingly, phenotypic approaches have gained new momentum [2–4], and the need for novel ways of TD is more important than ever.

Phenotypic versus target-focused screening

PS is typically defined as a target-agnostic approach to early drug discovery [5]. Given this rather wide-ranging definition, phenotypic approaches cover a broad variety of concepts. At one end of the spectrum, a phenotypic campaign can address a solitary signaling cascade that is believed to be pathophysiologically relevant. At the other end of the spectrum, PS could monitor a more complex cellular response to a test compound without any prior restriction of target space. Whatever the experimental particulars, PS attempts to recapitulate certain aspects of a broader disease context in a cellular *in vitro* system. The technical basis for high-throughput PS formats has improved over the past two decades through the development of: (i) sensitive high-throughput detection systems (Table 1); and (ii) more physiological *in vitro* model systems, for instance those derived from induced pluripotent stem cells (iPSCs) [1].

The success of PS-based drug discovery is founded upon the following three features: (i) the direct delivery of cellularly active hit compounds; (ii) the identification of novel targets or modes of action; and (iii) the option for polypharmacology [4]. Among these three advantages of the phenotypic approach, the direct access to small molecules with the desired cellular activity is particularly attractive. By contrast, target-focused screening (TS) produces many hit compounds that are only active on the biochemically isolated or cellularly overexpressed target protein for several reasons elaborated on below. A biochemical screen, for instance, does not always recapitulate the native environment of a target and thus has an element of artificiality; whereas a PS assesses a target in its physiological environment. In addition, a PS could also assess a target in a temporal fashion because it can investigate multiple functions that occur at different times in a cell. This is particularly the case with regard to PS for antiviral compounds where viral proteins often perform multiple functions that vary based on the point of time in the lifecycle [6].

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TABLE 1

Phenotypic screening techniques

Technique	Description	Pros	Cons	Refs
Reporter gene assay	A luciferase or fluorescent protein is genetically engineered into the model cell	Simple detection Typically large signal window High throughput	Need for genetic manipulation of model cell Artificial signaling event	[50,51]
High-throughput RT-PCR	A specific mRNA is measured in the crude lysate of the model cells	Very sensitive No genetic manipulation of model cells required	Quantified mRNA might not only be modulated by disease-relevant signaling	[1,52]
Alpha	Chemiluminescent proximity assay that quantifies a phosphorylation event or the concentration of a phenotypic marker protein	(Very) sensitive No genetic manipulation of model cells required	Quantified phosphorylation event or marker protein concentration might not only be modulated by disease-relevant signaling Need for specific antibodies	[53]
HTRF	Fluorimetric proximity assay that quantitfies a phosphorylation event or the concentration of a phenotypic marker protein	(Very) sensitive No genetic manipulation of model cells required	Same as for Alpha above	[54]
High-throughput MS	A mass spectrometric quantification of, for example, a secreted hormone or messenger protein	(Very) sensitive No genetic manipulation of model cells required	Restricted applicability (in general only up to a molecular weight threshold)	[55]
High-content screening	A fluorescence microscopic image is taken, then image-analyzed for morphological changes, protein translocations, among others	Effects that only occur in a subpopulation of the model cells can be studied separately Morphological changes or protein translocations can be used as readouts	Need for specific antibodies Labor-intensive set-up of bioassay and image analysis algorithm Occasionally labor-intensive assay protocols	[56]

Regarding target identification, PS allows us to explore novel druggable proteins, to seek out new mechanisms of action and to address signaling events that no scientist has investigated before. Agreeably, this places PS in an attractive position to deliver first-in-class compounds for unchartered target space, thereby crossing drug discovery's final frontier [3]. Different from target finding via a pooled RNA-mediated interference (RNAi) genetic approach with a flow cytometric readout, the small-molecule-centered PS assay is more flexible to keep some model cells in a physiological context such as a surface-attached neuronal network. In addition, in contrast to an RNAi genetic screen for target identification, compound-based PS will also engage targets that require stimulation or gain-of-function to produce an effect in the respective cellular model system.

Finally, PS offers the potential to address several targets in parallel [7]. The probability of a successful polypharmacologic approach increases if the respective targets share a related small-molecule binding site and/or if the engaged targets act synergistically in a disease-relevant network [8]. A classic example for a well conserved proteinaceous pocket is the ATP-binding site of the kinase superfamily. Thus, the archetypic nonselectivity challenge in regard to the paradigm 'one molecule – one kinase – one disease' could be turned into an advantage in the realm of polypharmacologic drug discovery. If a group of target kinases acts synergistically to cause a specific pathological outcome, a lower dose of a nonselective kinase inhibitor for this target group might suffice to produce the wanted disease-modifying result without triggering off-target kinase side-effects [8,9]. In the area of oncology in particular, there are several examples for such multikinase inhibitors serving as approved and marketed drugs [10]. However, the polypharmacologic approach hampers TD and, consequently, impedes the below-promoted combination of PS and TS.

In contrast to PS, the target-based approach enables a direct analysis of the interaction between ligand (i.e., bioactive test compound) and a presumably disease-relevant protein. Regarding the predefined target, biochemical and pharmacological techniques permit the distinction between orthosteric and allosteric modes of action as well as between competitive and noncompetitive binding. In addition, some of the biochemical finding techniques in TS campaigns provide access to small-molecular-weight ligands and fragments that possess potencies too low to be identified in a cellular phenotypic assay. If the target protein is suitable for X-ray crystallography, details of the ligand-binding site could be investigated and subsequently exploited by the medicinal chemist for ligand optimization. However, in addition, the direct interaction of the ligand with the aqueously dissolved target protein could be analyzed by techniques such as NMR, surface plasmon resonance or thermal shift assays. From some of these measurements, biophysical parameters of ligand–target binding such as the equilibrium dissociation constant or on- and off-rates could be derived.

While the SAR is typically very complex in a phenotypic assay, the optimization of a drug candidate compound in a target-based assay is focused on a singular ligand–target interaction. If the observed interaction is pivotal to the disease-relevant activity of the test compound, this unidimensional ligand–target concept is certainly an advantage of target-based drug discovery. However, these potential drawbacks of the phenotypic approach regarding a multifaceted SAR persist only for so long as the target of the phenotypically active compound remains obscure. Accordingly, although TD is no imperative process in post-PS drug development, the knowledge of the target might facilitate the understanding of the SAR, the chemical optimization of the drug candidate and target-associated safety considerations. As soon as TD is suc-

cessful after a PS campaign the above-described target-focused formats could likewise be applied to the chemical optimization of the original phenotypic hits. A first approach to TD can be carried out *in silico*, as described below.

Target deconvolution *in silico*

The route toward TD can be prepared upfront by screening compounds with known biological activities. The annotated targets of the hits can serve as first hypotheses for TD. The development of these annotated libraries started at least 15 years ago [11]. In the meantime, many design approaches have been published and several chemical vendors offer annotated compound sets for phenotypic screening. Often, these collections contain launched drugs and published tool compounds put together from literature and public databases. A recent review has been published by Jones and Bunnage [12].

Pharmaceutical companies can make use of their historic screening data like the BioProfile of Boehringer Ingelheim [13]. In this context, Novartis published different ways to put together biodiverse screening libraries based on HTS data [14] and based on internal and external dose–response data [15]. Generally, over recent years, extensive information has been gathered connecting small molecules with biological activity and probable target proteins. Examples for respective public databases include DrugBank [16], ChemBank [17], ChEMBL [18], PubChem [19] and PBBind [20]. Mok and Brenk [21], for instance, describe the generation of an ion-channel-focused screening library from ChEMBL. The Broad Institute recently published a dataset of manually curated compounds with annotated targets from literature and created the online Drug Repurposing Hub where the compound annotations can be analyzed [22].

Instead of taking starting points for TD from annotated compound libraries, target hypotheses for hits in PS can be generated by target predictions. In this context, ligand-based similarity searches with various descriptors can be used to derive hypotheses from known activities of the most similar compounds. Structure-based methods like pharmacophore searches and docking can be applied for the same purpose. Finally, the broad knowledge about biological activities can also be used to train models with different machine-learning techniques. These provide target hypotheses for compounds so far not annotated. The review by Koutsoukas *et al.* [23] lists a variety of these methods. Some of the techniques can be accessed via public search engines such as Similarity Ensemble Approach (SEA) [24], SwissTargetPrediction [25] and TarFisDock [26]. Successful target predictions have been published, for example by Cabrera *et al.* [27] and Schneider *et al.* [28].

Target hypotheses from *in silico* methods must be made more stringent, for example by testing other compounds that hit the annotated or predicted targets. Several hits in PS from different structural classes linked to the same target point to valid hypotheses. These can be further confirmed by analyzing whether the corresponding target candidate genes are expressed in the cells used for the screen and by pathway analyses before starting the *in vitro* TD. Of course, *in silico* TD is limited to targets for which active compounds are known but it offers chances for drug repurposing and for the detection of new links between diseases and known targets. Pathway analyses can lead to additional upstream and/or downstream target hypotheses. A challenge in using annotated libraries and target predictions is that the hypotheses rely on the quality of data used for the annotations and model building. *In silico* target hypotheses can also be built for weak hits, whereas *in vitro* TD typically requires hits with higher potency as described in the following section.

Deconvolution of targets *in vitro*

Although *in silico* techniques can narrow down the target space for a phenotypic hit or can even suggest a specific target, the *bona fide* interaction between a compound and a target protein needs to be proven *in vitro*. For this purpose, different approaches (Table 2) have been developed. Affinity purification is a classic technique to isolate specific target proteins for a phenotypically active compound. The small molecule of interest is immobilized onto a solid matrix, then used to fish the target protein out of a cellular lysate [29]. Subsequently, the pulled down proteins could, for instance, be identified via mass spectrometry (MS). Other deconvolution formats make use of a chemical reagent with three functionalized side-chains. In the case of the so-called TRICEPS, for instance, the first prong of this molecular trident covalently binds to the phenotypic hit, the second prong enables the covalent attachment of the trident to the target protein and the third prong provides a molecular handle to extract the trident-labeled target from a cellular lysate and to identify it by MS [30,31].

In a variation of the above-mentioned strategy, the target can be identified from a library of recombinantly overexpressed proteins. This approach is of particular interest if the target protein is of low abundance when endogenously expressed. One example of such a protein expression library is the phage display technique. In this case, a library of cDNA sequences is fused to a gene encoding phage coat protein so that each phage particle exposes several copies of one unique heterologous protein on its surface. Affinity pull-down of phages bound to the compound and subsequent sequence analysis led to the identification of target candidates [32]. An alternative to the phage library, a so-called yeast three-hybrid (Y3H) system, could also be employed. For this purpose, the phenotypic hit must be conjugated with a small-molecule 'bait' so that it can bridge between the bait-capturing transcription factor and the hit-capturing target from a cDNA library [33]. Because phage display and Y3H are prone to some artifacts, for instance related to protein overexpression, both techniques are less frequently applied to TD than the above-described affinity purification.

A limited TD across a large portion of the kinome is enabled by the nano bioluminescence resonance energy transfer (NanoBRET) format, measuring energy transfer from the fusion protein of a target kinase candidate and bioluminescent NanoLuc to a fluorophore-labeled ATP-binding-site ligand (tracer). Using a set of NanoBRET assays with target candidates from a panel of 178 kinases, the displacement of the tracer by a phenotypic hit permits the assignment of a specific target [34]. If the compound target is a protein complex the above approaches relying on protein overexpression might not succeed because they rely on the interaction with a single protein.

All of the above-described formats require a chemical derivation or immobilization of the phenotypic hit, whereas the following approaches for TD do not. For instance, an expressed protein library can be screened for targets using a combination

TABLE 2

***In vitro* TD techniques**

Technique	Description	Pros	Cons	Refs
Affinity purification	Hit is immobilized onto a solid matrix, then used to fish the target protein out of a cellular lysate	•Target is fished from physiological cellular context	•Chemical tagging of hit required	[29]
LRC-TRICEPS	Molecular trident: (i) binds hit, (ii) binds target, (iii) molecular handle to extract target from a cellular lysate	•Target is fished from physiological cellular context	•Chemical tagging of hit required •Only extracellular target epitopes detectable	[30,31]
Phage library	Matrix-immobilized hit is employed to pull phage particles with compatible surface proteins out of an expressed phage library	•Physiologically low-abundance targets can be detected	•Chemical tagging of hit required •Target nonphysiologically expressed on phage	[32]
Y3H system	Hit conjugated with 'bait' bridges between bait-capturing transcription factor and hit-capturing target	•Physiologically low-abundance targets can be detected	•Chemical tagging of hit required •Hit must penetrate into yeast	[33]
Nano BRET	Hit disrupts energy transfer from Nluc-tagged kinase to fluorophore-labeled probe	•Physiologically low-abundance targets can be detected •No chemical tagging of hit	•TD so far limited to 178 kinases •Target nonphysiologically expressed in fusion with Nluc	[34]
SEC-MS	Hit passes faster through SEC column when bound to target from expressed protein library	•No chemical tagging of hit	•TD limited to expressed protein library •High protein concentrations required	[35]
CETSA	Hit stabilizes target protein against thermal denaturation	•No chemical tagging of hit	•Thermodynamic stabilization of target must be sufficient	[36,37]
DARTS	Hit stabilizes target protein against proteolysis	•No chemical tagging of hit	•Thermodynamic stabilization of target must be sufficient	[39]
SPROX	Hit stabilizes target protein against denaturation and oxidation	•No chemical tagging of hit	•Thermodynamic stabilization of target must be sufficient	[41]

of microplate-based size-exclusion chromatography (SEC) and MS [35]. When the phenotypic hit binds to the cognate target protein it passes through the SEC column in the exclusion volume. Other techniques for target identification rely on the ligand-based thermodynamic stabilization of the target proteins. For instance, a widely used assay format investigates how a small-molecule ligand modulates the apparent melting temperature of a target protein. This thermal stabilization of a protein conformation in the presence of a ligand could also be monitored in living cells [36] using a technology known as cellular thermal stability assay (CETSA). The cells are first treated with the phenotypic hit, then heated to denature and precipitate cellular proteins, followed by cellular lysis and a separation of denatured protein aggregates from the soluble protein fraction. While unbound proteins denature and precipitate at elevated temperatures, ligand-bound proteins remain in solution. When CETSA is combined with MS in so-called thermal proteome profiling, it does not only enable the verification of a previously proposed target candidate but it enables an unbiased, proteome-wide interaction mapping for a phenotypic hit [37,38]. Another assay for measuring protein stabilization in the presence or absence of a ligand is the drug affinity responsive target stability (DARTS) format [39]. More specifically, DARTS relies on a ligand-based protection of the target protein against proteolysis. In a variation of the DARTS technology, limited proteolysis (LiP) was combined with MS for proteome-wide small-molecule mapping (SMap) of binding sites [40]. Similarly, the stability of proteins from rates of oxidation (SPROX) format investigates the ligand-mediated stabilization of a protein against denaturation and oxidation [41].

Genetics can also be employed to identify the protein targets of small molecules. For instance, overlapping phenotypic endpoints between small-molecule hits and RNAi-based effects enable the assignment of the compound effects to a particular gene expression level [42] or could at least narrow down the potential target space. In this context, novel genetic techniques such as CRISPR/Cas9 [43] come into play and allow us to investigate a PS hit in a control model cell, where the respective disease-relevant pathway has been switched off.

A more indirect way to address TD is to examine the broad range of effects of a phenotypic hit on gene expression. The transcriptomal response to a test compound can be investigated, for instance, by next-generation sequencing [44]. Likewise, translational or post-translational changes after test compound administration can be analyzed by proteomics in combination with MS. Monitoring the effect of a phenotypic hit regarding the transcriptome or translatome might not directly deliver a target assignment. It does, however, generate a broader understanding about the signaling chains or intracellular networks that the test compound interferes with. Along those lines, a phenotypic hit could be analyzed in a panel of bioassays that recapitulate various cellular signaling pathways [45].

All of the above target-fishing technologies work better with high-affinity ligands, so that TD is more likely to succeed with chemically optimized compounds than with primary phenotypic hits. However, even with a potent hit compound, the TD techniques enumerated here will, in all likelihood, only deliver target candidates. Secondary assays of target engagement are required to validate the presumed ligand–target assignment [46]. Recent advances in the areas of *in silico* and *in vitro* TD have greatly

improved the probability of successful target identification [47]. Nevertheless, a considerable risk remains that the direct biomolecular target of a phenotypically active drug candidate cannot be identified.

Why phenotypic approaches can fail

One of the major reasons why phenotypic approaches do not succeed is a failure in TD. Several drugs (e.g., pirfenidone) have reached the market in ignorance of the target [48], but the probability of success is reduced when biochemical data and respective SAR information are lacking. Another fundamental challenge in the context of a PS campaign is that the primary hit list typically contains a high portion of compounds that (i) are unselective, (ii) follow an unwanted mechanism of action or (iii) rely on a system or technology artifact. Regarding point (i), the issue of unselective hits (e.g., kinase inhibitors targeting the ATP-binding site) represents a particular challenge. But it can also be difficult to deconvolute between two closely related non-kinase proteins that contain highly homologous small-molecule binding sites. With respect to (ii), the unwanted mechanism of action, the phenotypic assay could, for instance, monitor an endpoint of several converging signaling cascades, which are not all disease-relevant. With regard to (iii), system or technology artifacts, an antiapoptotic compound or a compound-driving cell proliferation could show up as a false positive, if the readout of a phenotypic assay corresponds to the increase of a biomarker protein or mRNA. By contrast, if the readout of a phenotypic assay is linked to a signal decrease a toxic or antimitotic compound could feign disease-relevant bioactivity. Accordingly, hit selection that is solely based upon potency is doomed to fail, and a solid hit triaging strategy is required to sort the wheat from the chaff. However, the ultimate success of the hit triaging scheme depends on how well the phenotypic model system recapitulates the significant features of the disease and how well the secondary control assays restrict the assay readout to the appropriate signaling pathway. In this context, human iPSC-derived model cells, for instance, have broadened the basis for proper *in vitro* mirroring of disease-relevant biological systems, but there is still much room for improvement, for example by using 3D cell culture or mixed cell culture. The closer the *in vitro* model resembles the actual *in vivo* setting the higher is the predictivity of the phenotypic assay for *in vivo* efficacy. But, of course, physiological meaningful and complex *in vitro* formats are much more demanding to design. A comparable list of challenges exists in the field of target-based drug discovery as elaborated on in the next section.

Why target-focused approaches can fail

The attrition of drug candidates in clinical development is mostly due to a lack of efficacy [12]. Currently, many small-molecule drug candidates reaching this stage have been identified in a target-focused manner. Consequently, the respective compounds have typically been optimized by investigating the SAR of molecular alterations with regard to one or only a few pivotal target-centered bioassays. Therefore, the new chemical entity (NCE) candidates that have passed through these SAR-driven optimization cycles will usually display a high affinity and a high functional efficacy with regard to the targeted biomolecule. Moreover, the target itself has normally been validated and shown to be of disease relevance. So, how can it be that these biophysically flawless target modulators are inefficacious in human patients? Unfortunately, there is an abundance of possible reasons.

In a physiological cellular environment, the target can, for instance, be in a functional complex with other proteins. In such a protein complex, the target and the small-molecule binding site can be conformationally altered or even orthosterically blocked by a proteinaceous binding partner of the target. By contrast, in the artificial recombinant model cell the drug target could be stoichiometrically out of balance with its physiologically interacting proteins and other cellular biomolecules. In addition, following the law of mass action, the heterologous overexpression of proteins in a target-centered bioassay can produce artificial protein complexes with target conformations that are irrelevant to the disease situation. Furthermore, the efficacy of signal transmission for an intracellular phosphorylation cascade will be dramatically modified if a physiologically limiting signaling protein is more highly expressed in the SAR-driving bioassay; or there might be poorly understood redundant pathways that readily overcome the effect of a targeted compound.

In another imaginable example, the disease-relevant signaling cascade might only be correctly assembled in the physiological target cell, for instance in a specialized subcellular region like the neuronal synapse. At this point, the supporters of TS might argue to carry out the target-focused drug discovery in a physiological model cell. This is indeed a potential way forward. Typically, however, a bioassay to demonstrate direct target engagement in a natural cellular model is challenging to establish. Instead, one could monitor a downstream signaling event dependent on this target. Hereby, one reenters the phenotypic realm of a target space that is restricted to a particular signaling cascade and associated crosslinked or modulatory signaling events. In general, TS is more hypothesis-driven than PS. This focus on an assumed target, which restricts the pharmaceutical line of attack to a single biomolecule, might not sufficiently reflect a more complex disease biology.

Concluding remarks

Various statistical analyses as to the origins of drugs approved by the FDA demonstrated that phenotypic strategies have provided a significant contribution to the discovery of first-in-class small-molecule drugs [2,5]. Likewise, target-focused pharmaceutical research can present a remarkable track record of bringing NCEs to the market [49]. Consequently, why not take the best out of both worlds? Thus, whereas some scientists consider TD after PS as a 'nice to have', this review aims to emphasize the benefits that phenotypic NCE research can draw from a deconvoluted target. But, despite all of the above-described advances in TD, the assignment of biomolecular targets to phenotypic hits remains a daunting task. However, the probability of TD increases if *in silico* deconvolution techniques are applied. Once the target for a phenotypically active compound class has been identified, the target-focused and the PS-type assay formats suitably complement each other (Fig. 1). The target-centered biochemical or biophysical assays are more suitable for lead optimization. They illuminate the molecular mode of action for test compounds and enable the quantification of binding and kinetic constants, whereas the PS-

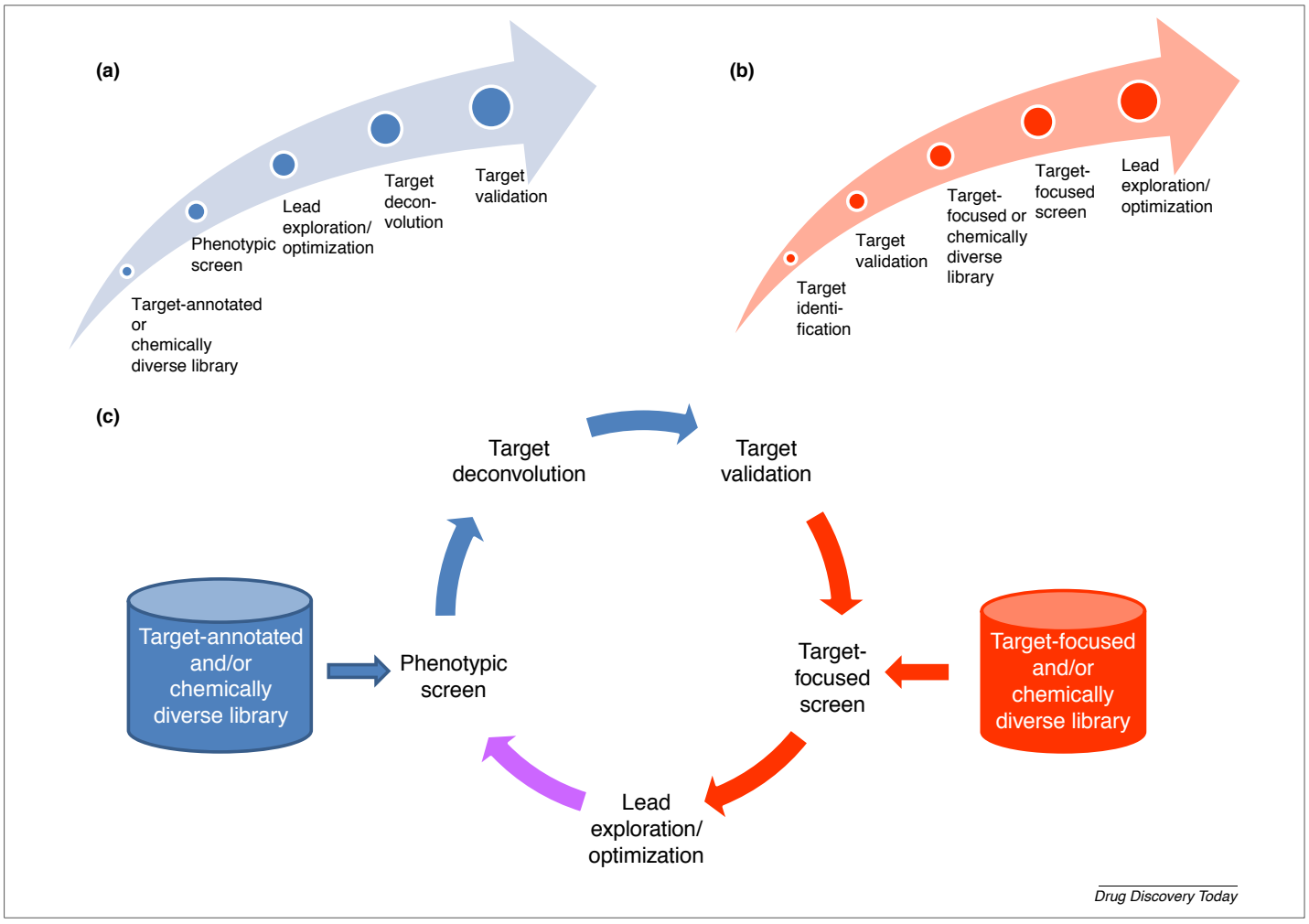

FIGURE 1

Workflows of lead discovery **(a)** for phenotypic screening, **(b)** for target-focused screening and **(c)** for a combined approach.

style assays in disease-relevant model cells help elucidate the test compound activities in a physiologically relevant context. Thus, a chain of translatability can be established that ranges from the biochemical drug effect to the *in vivo* drug efficacy. Gaps in the understanding of the disease mechanism are typical causes for clinical drug failures in the context of target-focused approaches. By contrast, the phenotypic approach assumes less and agnostically enables more routes of drug activity. Therefore, PS can disclose unexpected modes of action, thereby providing phenotypic assays with the potential to better address the unappreciated complexity of many diseases.

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