

Review

# Innovating cancer drug discovery with refined phenotypic screens

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Before molecular pathways in cancer were known to a depth that could predict targets, drug development relied on phenotypic screening, where the effectiveness of candidate chemicals is judged from functional readouts without considering the mechanisms of action. The unraveling of tumor-specific pathways has brought targets for molecularly driven drug discovery, but precedents in the field have shown that awareness of pathways does not necessarily predict therapeutic efficacy, and many cancers still lack druggable targets. Phenotypic screening therefore retains a niche in drug development where a targeted approach is not informative. We analyze the unique advantages of phenotypic screens, and how technological advances have improved their discovery power. Notable advances include the use of larger biological panels and refined protocols that address the disease-relevance and increase data content with imaging and omic approaches.

## The opportunity for phenotypic screens in cancer drug discovery

**Phenotypic screening** (see [Glossary](#)) is a systematic effort to identify chemicals or biologics that change a chosen readout in a living system. Strictly, the design of a phenotypic screen should be agnostic about the mechanism of drug action; moreover, awareness of the drug target is often viewed as introducing bias [1]. This type of drug screening has a long history in discovery science ([Box 1](#)) and flourished at a time when establishing causality between a chemical agent and an ensemble biological outcome was deemed to be sufficient for introducing a new therapeutic drug. Indeed, decades of clinical observation described cancer hallmarks [2] well before their underlying mechanisms were known, which is why phenotypic screens delivered the earliest cancer drugs [3–6]. Concurrent efforts across the research community to characterize the underlying molecular processes were justified by the expectation that target identification would accelerate anticancer drug discovery, eventually replacing the **black-box approach** of phenotypic screening. This forecast has been somewhat accurate because more novel drugs are now being delivered to the market by target-focused approaches than by function-first strategies [1,4]. Nonetheless, phenotypic screening in cancer drug discovery retains significant popularity because notable contributions continue to be made, including those highlighted in this review.

One reason for the continued use of phenotypic screening relates to the limitations of target-focused discovery. Success in target-driven strategies is contingent on understanding the molecular mechanism that links target inhibition to therapeutic outcomes. This is problematic because targets are typically put forward for testing before their complete cellular context has been established; consequently, many experiments are overshadowed by the lingering question of 'will the drug actually work?'. For example, drugging a preclinically verified target may not yield therapeutic outcomes if its molecular network manifests redundancy, feedback, or crosstalk, or is primed to undergo selection and acquire resistance [7–9]. It is plausible that a screen seeking improvements in overall outcomes, unshackled by molecular knowledge, is a more cost-effective platform for drug discovery. Another factor upholding the popularity of phenotypic

## Highlights

Phenotypic screens still produce novel drug discoveries, even though the emergence of the finer molecular details of cancer favors target-focused approaches.

Recent efforts to improve the design and power of phenotypic screens have addressed the persistent shortcomings of target-focused discovery, such as those related to heterogeneity, resistance, or redundancy.

Innovations in culturing patient-derived cancer cells in a tumor-relevant microenvironment have improved the disease relevance of findings.

Technological advances for high-throughput screening, ranging from imaging to omics, have increased the information content of readouts and strengthened discovery by phenotype-focused approaches.

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### Box 1. The origins of phenotypic screens

In many higher education institutions, physiology and pharmacology are grouped together for apparently didactic reasons, but this coupling is homage to a productive marriage that has delivered many breakthroughs in biomedical discovery science. Students learning about the discovery of acetylcholine receptors in muscles or of voltage-gated channels in axons are often awed by how pioneer-scientists were able to unravel the mechanisms behind muscle contraction or action potential propagation with the help of tubocurarine [99] and tetrodotoxin [100]. What is often overlooked is that these experiments did not benefit from 'off-the-shelf' pharmacology but were the product of laborious attempts to block biological functions using drugs available at the time, typically sourced from combatant or defensive organisms including microbes, plants, fungi, and animals. The chemical diversity of substances was at the mercy of evolution. These post-WWII investigations are examples of phenotypic screening because pharmacological agents were used to seek actions on readouts of biological processes in living tissue, even if the published reports emphasized the triumphant end-result over the tedious search for effective agents. Their motivation was to unravel mechanisms at a time when the notion of studying protein function seemed far-fetched. Decades ago, an effort to screen a panel of alkaloids from plants or toxins from snails would – sooner or later – identify a new process. Having exhausted the low-hanging fruit, phenotypic screens have since grown in terms of the panel of substances tested and biological models, but compatibility with high-throughput platforms has limited most research efforts to measurements of relatively simple readouts. Some classical examples range from a screen for substances that affect spindle formation in mitosis *in vitro* [101] to a chemical mutagenesis screen for mechanisms in the early development of mice [102].

screens is that technological solutions for delivering better data are more readily pioneered in function-first approaches because these short-circuit the need to adapt new methods to specific targets. Many enabling technologies, ranging from **microfluidics** to organoids, have sought applications in phenotypic screens, offering this discovery strategy a lifeline at a time of molecular-focused research.

The objective of this review is to articulate the opportunities that phenotypic screening offers in modern cancer research (Figure 1), with a focus on accessible readouts such as cell proliferation, **epithelial-to-mesenchymal transition (EMT)**, and angiogenesis. We first discuss the advantages of function-first approaches, and then describe recent breakthroughs that have empowered phenotypic screens to make new drug discoveries. A decade ago the proponents of phenotypic screening made recommendations for how to advance designs to ensure their longevity [1,3,10,11], notably how to increase discovery power, improve the disease relevance of biological models, and obtain **high-content data** using more refined readouts. Referring to recent drug discoveries, we evaluate the progress made in relation to these propositions and speculate on future trajectories.

### When target-focused screening is not feasible or desirable

Where a malignancy has no obvious target, phenotypic screening is the only realistic drug-discovery strategy. Triple-negative breast cancer (TNBC) lacks estrogen, progesterone, and human epidermal growth factor receptors that underpin successful therapies in its receptor-positive counterparts. This paucity of targets has turned attention to phenotypic screening, which discovered, for instance, tinengotinib using cell viability readouts [12]. The efficacy of the drug was attributed to broad-spectrum actions on kinases and would likely have evaded targeted approaches which prioritize selective inhibitors or assume their selectivity. Another cancer lacking druggable targets is osteosarcoma (OS). A recent screen of ~1000 substances for cell proliferation inhibition in eight OS cell lines identified thiazolidinone, then refined to (*R*)-8i that has nanomolar killing efficacy [13].

Cancers with poor prognosis often require combinatorial therapies to eradicate all cells, but identifying an additional drug that would synergize with a primary strategy is challenging because it requires adequate knowledge of vulnerabilities in the molecular constellation, further exacerbated by heterogeneity. However, phenotypic screens can be upscaled to test large panels of drug combinations, as demonstrated by a recent study describing the efficacy of proteasome inhibitors in conjunction with platinum agents in slowing the growth of TNBC cells – an unexpected synergy [14]. Phenotypic screening is also preferred for testing naturally occurring compounds,

### Glossary

**AlphaFold:** a deep ML algorithm designed to predict the 3D structure of proteins from their primary sequence.

**Artificial intelligence (AI):** an evolving technology that aims to simulate human intelligence using machines.

**Black-box approach:** an approach for testing a system with no prior knowledge of its internal workings.

**Cancer Cell Line Encyclopedia (CCLE):** a collection of whole-genome, whole-exome, and RNA-seq datasets encompassing nearly 1000 human cancer cell lines.

**Cancer stem cells (CSCs):** self-renewable cells that are present in most types of liquid and solid cancers, and that contribute to tumor onset, expansion, resistance, recurrence, and metastasis after therapy.

**Chemoproteomics:** a method to identify the binding protein of a labeled drug.

**Combinatorial chemistry:** chemical synthetic methods that make it possible to prepare a large number of compounds in a single process.

**CRISPR/Cas9:** a laboratory tool used to change or 'edit' pieces of cellular DNA using Cas9 enzymes that are guided to a target nucleic acid sequence.

**Cuproptosis:** a form of cell death triggered by the accumulation of Cu in mitochondria.

**Deep neural networks (DNNs):** a type of AI that is used to deal with unlabeled and unstructured data, and that aims to mimic information processing in the brain.

**DNA-encoded libraries:** libraries of small chemicals each attached to a unique DNA sequence that acts as a barcode.

**Epithelial-to-mesenchymal transition (EMT):** a physiological process in which epithelial cells acquire the motile and invasive characteristics of mesenchymal cells.

**Extracellular vesicles (EVs):** cell-derived membrane-surrounded vesicles that carry bioactive molecules and deliver them to recipient cells.

**Genome-wide association studies (GWAS):** methods that compare the genomes from many different people to find genetic markers that are associated with a particular phenotype or risk of disease.

**High-content data:** recordings that contains a large volume of information.

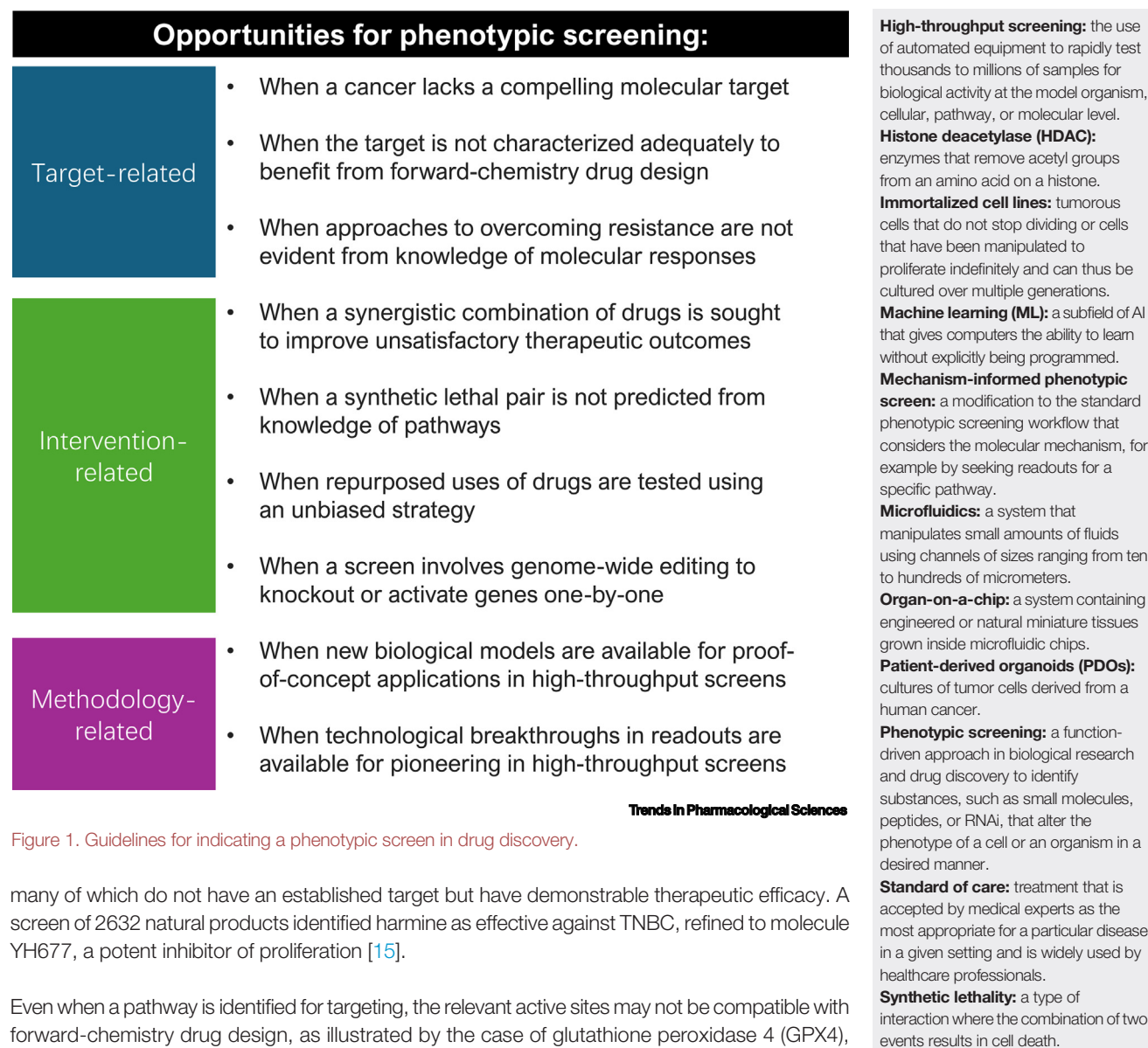


Figure 1. Guidelines for indicating a phenotypic screen in drug discovery.

many of which do not have an established target but have demonstrable therapeutic efficacy. A screen of 2632 natural products identified harmine as effective against TNBC, refined to molecule YH677, a potent inhibitor of proliferation [15].

Even when a pathway is identified for targeting, the relevant active sites may not be compatible with forward-chemistry drug design, as illustrated by the case of glutathione peroxidase 4 (GPX4), an appealing target in pancreatic cancer. Functional readouts of cytotoxicity from a panel of 12 pancreatic cancer cell lines identified A16, containing a novel sulfonyl-ynamide warhead, as having superior GPX4 inhibition [16]. Because this study was not agnostic about the target, it is classified as a **mechanism-informed phenotypic screen**, an increasingly popular approach that retains the hallmarks of a 'function-first' design but uses readouts of a specific molecular pathway.

Another opportunity for phenotypic screens is where drugs have multiple therapeutic uses, but only one indication had received attention, often owing to historical precedents. Seeking alternative uses of a drug for new diseases is known as repurposing and is appealing because of an already established safety profile. However, repurposing has a track-record of unpredictability, and a target-agnostic approach is often mandatory. Li *et al.* screened a library of approved drugs for cytotoxicity in docetaxel-resistant prostate cancer [17]. This identified nicardipine, a drug previously indicated for angina, as effective in killing cancer cells, and later described the mechanism of action

as cell-cycle arrest and apoptosis. Recent efforts to repurpose the well-known drug thalidomide considered its molecular-glue properties which could be exploited for removing dysregulated proteins from cancer cells. Thalidomide, first administered without knowledge of the underlying mechanisms, is a cereblon E3 ligase (CRBN) modulator: to seek anticancer actions, Wang *et al.* combined large-scale synthesis of hundreds of thalidomide derivatives with a cell viability screen on a multiple myeloma cell line [18]. This direct-to-biology screening method identified derivative E14 as a potent molecular glue responsible for degrading two lymphoid transcription factors, IKZF1 and IKZF3, via CRBN–CRL4 ubiquitin ligase.

Historically, phenotypic screening is intertwined with pharmacology, but chemical drugs are not the only means to disturb a system to seek therapeutic responses. With gene-editing tools, such as **CRISPR/Cas9**, libraries of guide RNAs can be used to inactivate genes, analogous to the use of large chemical libraries to influence proteins. Gene editing overcomes the limitation that not every protein has a selective inhibitor, whereas essentially all protein-coding genes can be manipulated [19]. Genes essential for a cancer-relevant process can be targeted by gene therapy where no small-molecule drug is available. The accuracy of CRISPR phenotypic screening in breast cancer cells was demonstrated by the concordance between hits and risk loci identified previously in patients by **genome-wide association studies (GWAS)** [20]. In addition to a myriad of *in vitro* screens, CRISPR/Cas9 gene-editing technology can be applied *in vivo*. For example, E3 ubiquitin ligase COP1 was found to be essential for cytokine release and tumor infiltration by macrophages in TNBC mouse models, offering a means of potentiating immunotherapy [21].

### Innovations that address drug resistance

Whether a drug is discovered through a phenotypic or target-driven screen, a concern relates to the persistence of anticancer efficacy *in vivo*, where cancer cells have more time to respond to the presence of drug. Many cytotoxic drugs do not eradicate all cells, with the risk that the residual population will expand under drug selection. A consequence of the emergence of resistant phenotypes is that drugs deemed efficacious *in vitro* are not therapeutic in patients [22]. One strategy to identify resistance mechanisms at the screening phase is to ensure that the biological panel adequately copies the heterogeneity in human cancers, in other words is large enough to include potentially resistant cells. This imperative can be problematic for target-driven efforts because the choice of cells prioritizes those that operate the targeted pathway, possibly excluding resistant phenotypes. Given their unbiased approach, phenotypic screens are more likely to feature a representative panel of cancer samples for testing.

Three decades ago, in a bid to improve the power of distinguishing sensitive versus resistant phenotypes, the National Cancer Institute (NCI) introduced a panel of 60 human cell lines from nine human cancers. At the time this sample size was deemed sufficient to capture the range of genotypes that could lead to resistance. It later became evident that many screens had been underpowered to detect resistance in a minority of genotypes [3]. The number of cell lines needed to detect a resistant phenotype could be approximated from the binomial distribution (Box 2). In colon cancer, the prevalence of mutations ranges from ~70% in *APC* to ~40% in *KRAS*, 10% in *BRAF* and 5% for *CTNNB1*, but a resistant phenotype may be rare if it involves combinations of mutations. To detect resistance present in 5% of cells in a human cancer, a panel of 58 cell lines is recommended, but this threshold is rarely met. A notable cytotoxicity screen required a panel of >50 non-small cell lung cancer lines to show that *KEAP1* wild-type cells are resistant to complex I inhibition [23]. The **Cancer Cell Line Encyclopedia (CCLE)** project aimed to address the problem of inadequate power by introducing a panel of ~1000 lines [24,25]. This empowered researchers to make more informed decisions about including screening based on genotype, gene expression, or metabolite profile. Recent screens using

## Box 2. Powering cell line experiments

If proportion  $q$  of cancer cells have acquired drug-resistance, then the probability that a screen of  $N$  cell lines fails to detect any drug resistance is  $(1 - q)^N$ . Thus, the probability that at least one drug-resistant line emerges in the screen is  $1 - (1 - q)^N$ . Given that phenotypic screens represent a considerable investment, it is desirable to have a high probability of detecting resistance, say 95%. Thus,  $N$  can be calculated from Equation 1 (Figure 1):

$$N = \log(1 - 0.95) / \log(1 - q) \quad [\text{Equation 1}]$$

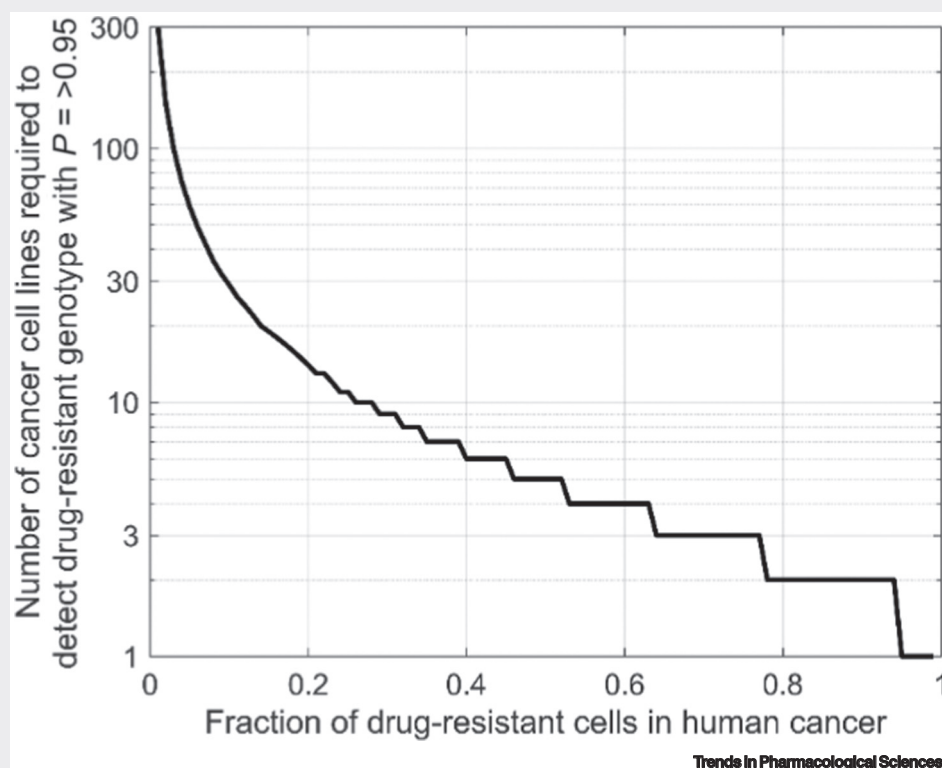


Figure 1. Prediction of the size of a cell line panel required for detecting drug resistance when the incidence of drug-resistant phenotypes in the human tumor is varied.

CCLE datasets included a study of drug resistance in pancreatic adenocarcinoma (PDAC) cell lines that correlated growth inhibition with transcriptomic data [26], and a study of muscle-invasive bladder cancer that used CCLE to select a balanced panel of basal, luminal, and mesenchymal-like lines to profile growth inhibition by **histone deacetylase (HDAC)**, CHK, and MDM2 inhibitors [27].

Once a panel is sufficiently comprehensive, the odds of identifying a drug-resistant cell line are favorable. This offers opportunities for phenotypic screens to find druggable vulnerabilities that would otherwise be hidden in smaller screens lacking resistant phenotype representation. A recent example sought new drugs for castrate-resistant prostate cancer by using a two-tiered approach to screen a panel of drugs for inhibition of a chemoresistant cell line and compare with the responses of a sensitive line. Their phenotypic screen identified bromocriptine as an inducer of cell-cycle arrest and apoptosis in docetaxel-resistant cells only, revealing a hidden vulnerability [28].



In lieu of larger cell line panels, resistance mechanisms can be discovered efficiently by gene editing in the presence or absence of drug to determine which knockouts alter responses. A genome-wide CRISPR/Cas9 screen, performed with and without EGFR or ALK inhibitors, identified a small group of genes (*MCL1*, *BCL2L1*, and *YAP1*) whose targeting sensitized lung cancer and colorectal cancer (CRC) to cytotoxic EGFR/ALK therapies [29]. In a prostate cancer study, a CRISPR/Cas9 screen performed in the presence or absence of an androgen receptor blocker linked *BRAF* with therapy resistance by using cell proliferation readouts [30]. A cell viability screen that combined CRISPR-knockout with CRISPR-activation in pancreatic cancer cells identified ABCG2, an efflux pump, as a resistance mechanism to gemcitabine, 5-fluorouracil (5-FU), irinotecan, and oxaliplatin [31]. The aforementioned discovery strategies are effective when resistance can be pinpointed to a few genes. This becomes problematic when resistance is the product of network activities where the elimination of one specific gene is insufficient for sensitization.

An innovative approach to address resistance is to push cells into a less malignant state, rather than aiming for eradication. This type of experiment is compatible with phenotypic screening because the underlying mechanisms are highly speculative. In the case of acute myeloid leukemia (AML), where cytarabine plus daunorubicin therapy does not eradicate all cancer cells, an emerging concept is to stimulate the differentiation of leukemic blasts into more mature, less malignant states. In the absence of molecules to target, Jose-Cullere *et al.* used the myeloid marker CD11b as a readout to identify pro-differentiation drugs. This effort identified compounds with trisubstituted imidazo[1,2-*a*]pyridines that induce differentiation in AML cell lines, regardless of mutational status [32]. Another effort to influence differentiation used rhabdomyosarcoma (RMS) cells, where a chromosomal rearrangement commonly produces a pro-oncogenic fusion protein PAX3-FOXO1 that arrests differentiation. Sroka *et al.* found that loss of nuclear factor Y produces a similar outcome to the loss of PAX3-FOXO1 [33].

### Innovations that address the failure of preclinically promising drugs

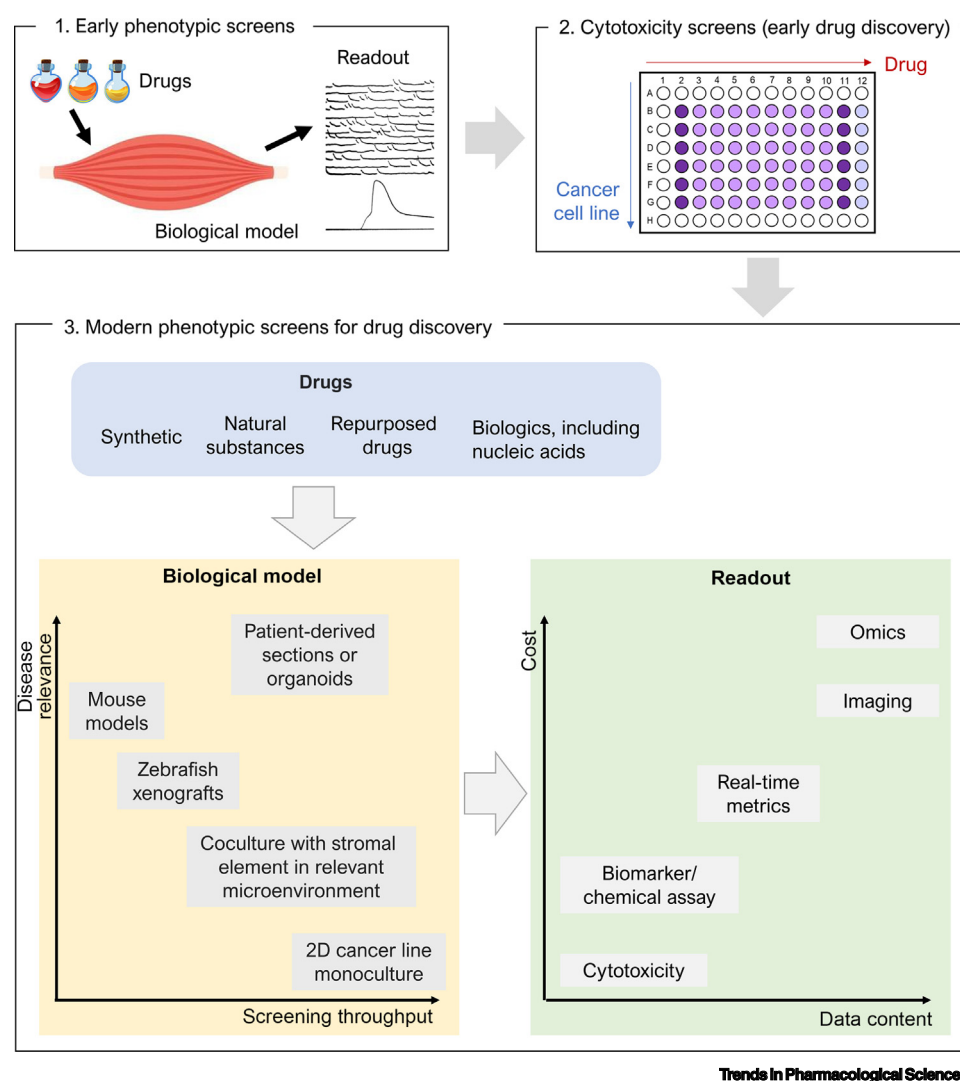
Some preclinically successful drugs fail in the clinic because a therapeutic effect is not achievable within a tolerable range or because the killing effect *in vitro* is not replicated in patients. A proposed solution to supplement efficacy *in vivo* is to seek synergistic interventions, known as **synthetic lethality**. Predicting effective combinations for synergy is difficult because it is contingent on understanding complete signaling systems. To short-circuit this, innovative designs of phenotypic screens have been successful in identifying synthetically lethal combinations. Notably, Kim *et al.* used a panel of eight lung cancer lines that included phenotypes differing in nicotinic acid phosphoribosyltransferase (NAPRT) expression [34]. Their screen found that compound A4276 was selectively lethal against NAPRT-negative cells. A4276 was determined to be an inhibitor of nicotinamide phosphoribosyltransferase (NAMPT), thereby establishing a synthetically lethal pair, NAPRT/NAMPT, for effective killing. Phenotypic screening was also used to seek synthetic lethality in SF3B1-mutant AML, wherein iron/sulfur transport is abnormal. Using a library of 10 000 compounds applied to 56 AML samples, Moison *et al.* found that copper ionophores selectively killed mutant cells, exposing their vulnerability to a non-canonical death process called **cuproptosis** [35].

A pathway that is appealing to target but attempts in patients have had poor outcomes is EGFR–RAS–MAPK. This cascade is frequently deregulated in CRC and targeted strategies, for example against mutant KRAS, gave good results *in vitro*, but these were not recapitulated in human trials because a cytostatic phenotype emerged. Mertens *et al.* [36] approached this challenge by screening >400 substances for an effect on switching EGFR–MAPK-inhibited cells to a cytotoxic phenotype using high-content imaging. This screen identified microtubule-targeting agents, such

as vinorelbine, which have an established niche in oncology but a novel use in conjunction with EGFR–MAPK inhibition.

### Improving the disease relevance of biological models of cancer

Because phenotypic screens rely on functional readouts, the biological models producing these outcomes must adequately resemble the human disease (Figure 2). Cancer cell lines have been the workhorse of phenotypic screens because of their compatibility with *in vitro* culture and global dissemination that encourages sharing and benchmarking [37]. However, **immortalized cell lines** are merely a surrogate of the human disease. Efforts to address this have involved adding relevant components to the microenvironment, for example by coculturing with stromal cells or



Trends in Pharmacological Sciences

**Figure 2. Evolution of phenotypic screens.** (1) Early screens were motivated by basic-science questions about how biological systems work. (2) The first phenotypic screens in cancer drug discovery used small panels of cell lines tested with a selection of drugs for readouts related to cell growth or death (i.e., cytotoxicity). (3) Modern phenotypic screening can benefit from large libraries of substances and a range of biological models to suit the desired throughput and disease relevance. Readouts have expanded beyond cytotoxicity screens to include data-rich methods. With increasing information content, screens need appropriate data management and analysis pipelines.

mimicking the physicochemical milieu. The most recent advances have focused on making these enhancements compatible with **high-throughput screening**. A coculture of CRC cells with immune cells was implemented as a platform to screen 1280 FDA-approved small molecules for repurposing using image-based quantification of GFP fluorescence [38]. This determined that pitavastatin had a potent anticancer effect involving a proinflammatory response, highlighting an unexpected effect of a statin. A method developed by Zhao *et al.* harnessed dendritic cells (DCs) for a genome-wide CRISPR/Cas9-based screen [39], which is noteworthy because DCs are more difficult to drive into proliferation than T cells.

Compared to the stroma, replicating the chemical environment of tumors is simpler, provided that accurate and exhaustive measurements are available. To that end, guidelines for best practice must be adhered to [40]. Modeling solute gradients is a challenge but becomes possible through the ingenious use of microfluidics and imaging [41]. The significance of non-uniformity is that it may reveal direction-sensitive responses such as chemotaxis or cell polarity. In terms of the physical framework for cell growth, various substrates have been developed by microfabrication. For example, Martewicz *et al.* produced micropillars for osteosarcoma SaOs2 cells and imaged cell movement to determine nuclear deformability, a prerequisite for metastasis [42]. A phenotypic screen was appropriate because the regulation of nuclear deformability is poorly understood, hence no obvious targets emerge. This screen found that HDAC2 is important for nuclear deformability [42].

Although generating a complete *in vivo* milieu in a culture dish for high-throughput screening remains aspirational, a tantalizing solution is to use xenotransplantation in zebrafish. In this model, human cancer cells grow in an organism that is compatible with culture-dish screening platforms [43]. Grissenberger *et al.* grew Ewing sarcoma cells in zebrafish larvae and applied a combinatorial drug screen, a feat that is restricted to only the most robust types of screens, and that is certainly not feasible with mice. This imaging screen identified killing efficacy of irinotecan combined with either MCL1 or BCL-X<sub>L</sub> inhibition [44].

Even in the most disease-relevant microenvironments, there are oncogenic processes that cell lines cannot capture because of their developmental stage. An example is acinar ductal metaplasia (ADM) that is relevant to pancreatic cancer. Atanasova *et al.* prepared pancreatic organoids from wild-type and *p48<sup>cre/+</sup>* mice for imaging [45] and found that class I HDAC inhibitors apicidin and FK228, and the histone methyltransferase inhibitor chaetocin reversed ADM at concentrations below their cytotoxic thresholds.

Notwithstanding these milestones in improving the disease relevance of cell line studies, accurate modeling of malignancy ultimately requires patient samples, but these are challenging to implement in high-throughput screening. *Ex vivo* tissue slices accurately recapitulate the architecture and composition of human cancers, and pioneering efforts have been made to maintain these for long enough to screen drug responses. A notable effort by Voabil *et al.* used pancreatic tumor fragments to study PD-1 blockade using cytokine readouts [46], and a method by Xing *et al.* was able to preserve tumor-like features for a week *ex vivo* [47].

Possibly the most significant development over the past 5 years is the use of **patient-derived organoids (PDOs)** which resemble tumors, albeit without an immunological component, but can be studied *ex vivo*. Towards the end of the 2010s, biobanks of breast [48], CRC [49], gastrointestinal [50], and bladder [51] cancer organoids have been refined for use in drug screening platforms largely based on cytotoxicity readouts. Organoids have now been grown with stromal cells [52] showing, for example, how the presence of fibroblasts among pancreatic cancer can influence



drug sensitivity and promote EMT phenotypes. Ebisudani *et al.* established lung cancer PDOs from sputum and blood, in addition to lung tissue, and used 43 organoid lines to screen for responses to the WNT3A and R-spondin [53]. This library, that was claimed to capture all histological types of lung cancer, associated WNT independence with EGFR gain-of-function mutations. Taking an unbiased approach, the authors determined that the loss of NKX2-1 sensitized cells to WNT-targeted therapy. Recent drug discoveries using PDOs included a biobank of 31 genetically distinct human pancreatic cancer lines that enabled large-scale drug screening (1172 compounds) for cytotoxicity, including those intended for repurposing [54]. Among the hits were emetine and ouabain that showed new properties in perturbing responses to the hypoxic tumor microenvironment. A recent screen of 335 repurposed drugs applied to CRC organoids used a two-stage screening strategy to shortlist drugs by cytotoxicity and then determined their concentration–activity curves. This identified 34 drugs with anticancer actions, which were then characterized transcriptomically in terms of differentiation induction, growth inhibition, metabolism inhibition, immune response promotion, and cell-cycle inhibition [55].

Unlike 2D cell line monocultures, where simple cytotoxicity screens can give useful information, readouts of PDOs are often more subtle and rely on imaging. A commendable study by Betge *et al.* obtained 5 million CRC organoids and performed image-based profiling of the response to >500 small molecules. This phenotypic screen was sufficiently powered to determine that IGF1 signaling relates to organoid size, and LGR5<sup>+</sup> stemness relates to cystic versus solid morphology [56].

The investment in making PDOs encourages efforts to generate large datasets, such as RNA/DNA sequencing. To justify the costs, the choice of drugs to test must be made rationally, even if the targets are unknown. A powerful tool for this analytical problem is therapeutically guided multi-drug optimization that combines cytotoxicity and biochemical assays [57]. Ramzy *et al.* sequenced the transcriptome of CRC PDOs and used a computational model to determine the most appropriate therapy on a case-by-case basis. This pipeline successfully identified four low-dose synergistic combinations, including regorafenib (VEGFR2/TIE2 tyrosine kinase inhibitor), vemurafenib (B-RAG kinase inhibitor), palbociclib (CDK4/6 inhibitor), and lapatinib (HER/NEU and EGFR pathway inhibitor), which outperformed the **standard of care**, FOLFOXIRI (folinic acid, 5FU, oxaliplatin, and irinotecan).

For some cancers, such as CRC, lung, and pancreatic, PDOs will 'compete' with cell lines for phenotypic screens, at least in the short term while the costs of generating organoids are factored. By contrast, some cancer types have few or no suitable cell lines, making PDOs necessary. Notable beneficiaries are rare cancers such as leiomyosarcoma (growth-inhibition screen) [58] and desmoplastic infantile ganglioglioma (imaging screen) [59] for which drug discovery is impeded by small market size. Screening a library of anticancer drugs found that a leiomyosarcoma PDO line responded to bortezomib, dasatinib, mitoxantrone, and romidepsin [58]. A new method for growing PDOs from RMS needle biopsies preserved cancer heterogeneity and provided a much-needed platform for phenotypic screening by imaging and cytotoxicity [60].

Methods for establishing PDOs are developing at a rapid pace, driven by the promise of replicating inter- and intra-patient heterogeneity for precision medicines. Protocols for challenging cancers, such as the GliExP platform for gliomas, can enable a small-scale imaging screen of ~30 drugs within days of resection to identify the most suitable drug for the malignancy [61]. Conditionally reprogrammed PDOs (CR-PDOs), first formulated in 2017, can offer the most personalized drug screening platform by growing patient-derived tissues under bespoke culture conditions [62]. This system allows epithelial cells to expand and propagate clonal heterogeneity. When applied to a drug

screen, imaged responses in colorectal [63] and pancreatic cancer [64] CR-PDOs showed good concordance with patient profile. These breakthroughs could address concerns about relevance and heterogeneity, and provide access, cost, and reproducibility that meet the strict criteria for drug discovery.

### Implementing innovative readouts linked to cancer hallmarks

Historically, the most common measurement in phenotypic screening used cytotoxicity assays (Figure 2). This choice is justified in terms of the clinical imperative to kill cancer cells and remains popular with modern screens, largely because low costs allow significant upscaling of drug and cell panels. For example, clofarabine identified from a panel of 1700 substances was found to kill all 23 bladder cancer cell lines [65]. Affordable commercial sources, such as the Tocriscreen library of 1280 substances, have been used to identify new drugs for medulloblastoma, for which only seven drugs are currently approved [66]. Furthermore, modern chemical synthesis allows greater diversity and enabled the discovery that  $\alpha$ -substituted thiocarbohydrazones have superior anticancer properties against leukemia and breast lines [67].

Notwithstanding the value of the killing efficacy of a drug, emphasis on cytotoxicity can obscure the interpretation of phenotypic screens because it measures an outcome but offers little insight into prior events. This is problematic when various mechanisms lead to the same cytotoxic outcome, and could group unrelated drugs indiscriminately into a single category. More refined readouts of upstream events could improve drug discovery by assigning more precise actions to drugs. A good example is AMG900, canonically an inhibitor of aurora kinase B (AURKB), which is presumed to produce anticancer effects by disrupting the cell cycle. However, an image-based screen for tracking the distribution of AURKB revealed that AMG900 disrupts the localization of the enzyme and thus affects cell cycling [68]. Moreover, its 2-phenoxy-3,4'-bipyridine moiety was found to disrupt AURKB localization without inhibiting its enzymatic activity. Aside from demonstrating a new mechanism of action, this study highlights the 'noise' introduced by cytotoxicity readouts. A subsequent mechanism-informed phenotypic screen that used imaging produced a more refined derivative, 4-(4-methylthiophen-2-yl)-N-(4-(quinolin-4-yloxy)phenyl)phthalazin-1-amine [69], a feat not possible with cytotoxicity readouts.

The most suitable readouts for drug discovery should directly gauge the hallmarks of cancer (Figure 2). EMT is a particularly challenging phenotype to capture accurately for high-throughput applications. Organic electrochemical transistors, which offer real-time monitoring of cell coverage and differentiation state, have been implemented in a phenotypic screen to demonstrate that heparin is an effective inhibitor of EMT triggered by **extracellular vesicles (EVs)** released from breast cancer cells [70]. A related technological breakthrough is 'electrical imaging', whereby impedance measurements using a fine array of electrodes capture key features of living cells in real time without labeling or fixing [71]. Genetic instability is another cancer hallmark that lacks suitable readouts for high-throughput screens. Addressing this, a flow-cytometric method was developed to detect double-stranded breaks in DNA and identified a potent agent, DDA-1, from a library of 9600 compounds [72].

Perhaps the most promising readouts relate to next-generation imaging because this offers high-content data. Automated approaches have enabled cell segmentation and classification [73]. A phenotypic screen of 90 FDA-approved drugs found that trametinib and copanlisib below the cytotoxic threshold inhibit the uptake of breast cancer-derived EVs into lung fibroblasts [74]. Cell painting offers great potential for phenotypic screening because of the richness of the data generated by labeling various cell compartments with spectrally distinct probes. Using this approach, a phenotypic screen of 20 000 substances identified copper ionophores as

modulators of the phenotypic composition of esophageal adenocarcinoma cells [75]. With the increasing availability of spectrally resolvable biocompatible dyes, multiplexing will become more accessible for phenotyping, but a potential problem relates to the autofluorescence associated with many drugs. Nao *et al.* showed that time-resolved microscopy can effectively address this spectral inference, and implemented the technology to screen 1456 FDA/European Medicines Agency (EMA)-approved drugs for actions against uracil DNA glycosylase, an example of a mechanism-informed phenotypic screen [76]. This effort identified stibogluconate as an effective agent in prostate cancer.

Imaging has a major advantage where cell viability is not an accurate gauge of therapeutic outcome. For example, residual cells that survive chemotherapy are often enriched in mesenchymal-like properties that confer metastatic potential. Thus, a drug that eliminates all but these mesenchymal cells could be erroneously considered to be effective in viability screens but lack any long-term benefits. This concern is pertinent to TNBC patients with claudin-low, mesenchymal-like tumor subtypes. By imaging morphological landmarks in breast organoids, a screen of epigenetic modifiers found that class I HDAC inhibitors and bromodomain inhibitors reversed EMT [77]. This study was notable for leveraging **deep neural networks (DNNs)** that use raw images to extract features and seek similarities against control treatment – a feat that cannot be objectively accomplished by visual inspection.

Developments in microscopy, microfluidics, and microfabrication have enabled progress in **organ-on-a-chip** approaches to study more complex aspects of tumor biology, such as angiogenesis, that cannot be replicated on simple growth substrates. Soragni *et al.* developed an organ-on-chip assay for angiogenesis, which enabled screening of >1500 kinase inhibitors for antiangiogenic properties, far exceeding the throughput of animal studies [78]. In addition to 53 hits with known antiangiogenic properties, the study found 44 new compounds. A more complex microfluidic system called OrganoPlate included stromal components with breast cancer cells to assess invasiveness, and identified 30 compounds with potential therapeutic applications against metastatic phenotypes, albeit in the early phases of *in vitro* growth [79]. Implementing microfluidics to sort cells by phenotypes such as deformability or adhesive properties can enrich a population of cells with desired properties. Good long-term prognosis often relies on the elimination of **cancer stem cells (CSCs)** [80], but their low abundance in most preparations makes therapies difficult to test. To that end, a microfluidic mechanical sorter has been designed to extract lung cancer CSCs and perform drug screening by cytotoxicity assays [81]. Another microfluidic system called nuPRISM separated nuclei for rapid profiling of nuclear proteins such as  $\beta$ -catenins using a flow-cytometric system [82], and similar refinements can enable automated separation of cellular or subcellular components for detailed analysis.

### Concluding remarks and future perspectives

At the turn of the century it was debatable whether the growing depth of knowledge about the molecule mechanisms of cancer would lead to a complete takeover of drug discovery by target-driven approaches at the expense of phenotype-driven methods. Since 2020 the FDA has issued 226 approvals for oncological drugs, of which 21 related to drugs originally discovered through a phenotypic screen, often decades earlier (Table 1). Nonetheless, phenotypic screens maintain a strong presence in ongoing drug discovery because they address some of the limitations of target-driven discovery, have responded to calls for improvements (Figure 3), and are likely to feature in future drug-approval decisions. At present, our molecular understanding of cancer is inadequate for predicting the best targets to overcome drug resistance, for finding repurposed uses of available drugs, or for seeking combinatorial or synthetically lethal pairs. In these instances, phenotypic screens offer an expedited route to drug discovery. Notable efforts

### Outstanding questions

Is it reasonable to predict that molecular mechanisms will soon be characterized to a level that provides excellent predictive power, such that target-driven approaches are better informed?

Will chemical synthesis of drugs become rate-limiting for biological screening, and will screening efforts move away from small-molecule inhibitors?

Is there an acceptable compromise between the disease relevance of biological models and the technical constraints of high-throughput screening?

Will access to patient-derived organoids be sufficient to support large-scale screens and ensure robustness and reproducibility?

Considering the cost of large-scale animal studies, are improvements to the *in vitro* microenvironment adequate for modeling human cancers, or is the use of refined animal models unavoidable?

If high-content readouts become cost-effective and widespread, will analysis require advanced computational methods, such as AI and ML?

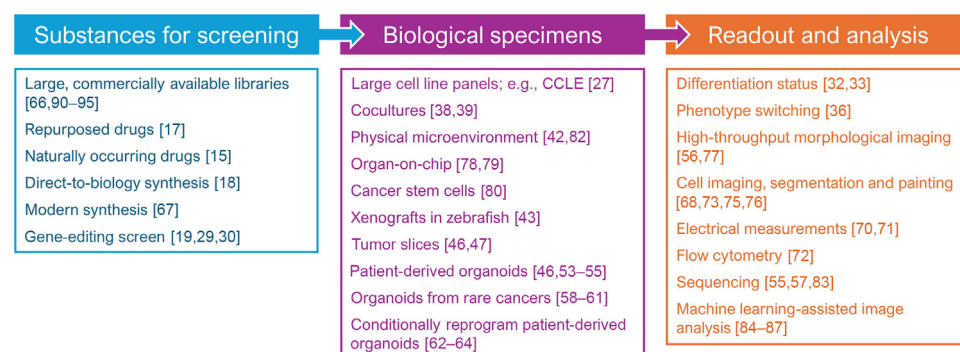
How can the community improve the curation of datasets obtained from phenotypic screens to encourage their reuse and spread the costs of screening through increasing data longevity?

Is there a role for phenotypic screening in discovering mechanisms of signaling and regulation outside the primary objective of drug discovery?

Table 1. List of FDA-approved drugs or drug combinations (2020–2024) that include at least one biologically-active substance discovered by phenotypic screening, indicated in bold type<sup>a</sup>

Date	Oncological indication	Drug	In combination with
02/03/2020	Multiple myeloma	Isatuximab-irfc	<b>Pomalidomide</b> 2004 [103]
30/03/2020	Extensive-stage small cell lung cancer	Durvalumab	<b>Cisplatin</b> 1972 [104]
15/04/2020	Low-grade upper tract urothelial cancer	<b>Mitomycin</b> 1957 [105]	
14/05/2020*	Kaposi sarcoma	<b>Pomalidomide</b> 2004 [103]	
15/06/2020*	Metastatic small cell lung cancer	<b>Lurbinectedin</b> 2010 [106]	
07/07/2020	Myelodysplastic syndrome	<b>Decitabine</b> 1968 [107]	
01/09/2020	Acute myeloid leukemia	<b>Azacitidine</b> 1964 [108]	
26/02/2021*	Multiple myeloma	<b>Melphalan</b> 1955 [109]	
01/07/2021	Leukemia and lymphoma	<b>Asparaginase Erwinia chrysanthemi</b> 1963 [110]	
09/07/2021	Multiple myeloma	Daratumumab and hyaluronidase-fihj	<b>Pomalidomide</b> 2004 [103]
23/11/2021	Malignant perivascular epithelioid cell tumor	<b>Sirolimus protein-bound particles</b> 1984 [111]	
20/05/2022	Juvenile myelomonocytic leukemia	<b>Azacitidine</b> 1964 [108]	
25/05/2022	Acute myeloid leukemia	Ivosidenib	<b>Azacitidine</b> 1964 [108]
02/09/2022	Locally advanced or metastatic biliary tract cancer	Durvalumab	<b>Gemcitabine</b> 1990 [112] and <b>cisplatin</b> 1972 [104]
14/08/2023	Uveal melanoma with unresectable hepatic metastases	<b>Melphalan</b> 1955 [109]	
14/09/2023	Project Renewal [113]	<b>Temozolomide</b> 1986 [114]	
27/10/2023	Nasopharyngeal carcinoma	Toripalimab-tpzi	<b>Gemcitabine</b> 1990 [112] and <b>cisplatin</b> 1972 [104]
31/10/2023	Biliary tract cancer	Pembrolizumab	<b>Gemcitabine</b> 1990 [112] and <b>cisplatin</b> 1972 [104]
13/12/2023	High-risk neuroblastoma	<b>Eflornithine</b> 1980 [115]	
13/02/2024	Metastatic pancreatic adenocarcinoma	<b>Irinotecan liposome</b> 1988 [116]	
07/03/2024	Unresectable or metastatic urothelial carcinoma	Nivolumab	<b>Gemcitabine</b> 1990 [112] and <b>cisplatin</b> 1972 [104]

<sup>a</sup>An asterisk (\*) indicates accelerated approval. Drugs in bold font were discovered by phenotypic screening rather than by target-driven discovery. Numbers in italics indicate the year of first phenotypic screen. Data from: <https://www.fda.gov/drugs/resources-information-approved-drugs/oncology-cancer-hematologic-malignancies-approval-notifications>.



Trends in Pharmacological Sciences

Figure 3. Advances in phenotypic screening highlighted in this review. See also [15,17–19,27,29,30,32,33,36,38,39,42,43,46,47,53–64,66–68,70–73,75,76,78–80,82–87,90–95]

have been made to improve the disease relevance of biological models by bringing microenvironments closer to the *in vivo* setting and adapting these to high-throughput experiments. In parallel, improvements have been made to data content and precision (Figure 2). Although only 10% of phenotypic screens have used omic readouts because of prohibitive costs, this space is likely to witness growth facilitated by **machine learning (ML)** and **artificial intelligence (AI)**. The richness of obtainable data can be illustrated by the pharmaco-proteogenomics approach that generated multi-omic data for a patient-derived liver cancer-like organ biobank [83]. The depth of data stored in images will increase but its value is gauged in terms of interpretation. Where visual inspection of images cannot be objective, ML algorithms using DNNs have been successful in classifying compounds according to pre-defined responses or in clustering outcomes by similarity [84–87]. Some applications of AI are not primarily aimed at improving phenotypic screens. **AlphaFold** [88] is likely to benefit target-driven screens by providing detailed predictions of protein structure. As proof-of-concept, AlphaFold identified a novel drug (ISM042-2-048) against a novel target (cyclin-dependent kinase 20) in hepatocarcinoma cancer despite no prior experimentally determined protein structure [89]. Crucially, this workflow generated data in 1 month, demonstrating the power of AI but also posing an existential threat to phenotypic screens which so far maintain a niche where the targets are unknown or cannot be exploited for drug design.

Although developments in readouts and analysis are ongoing, libraries as large as 300 000 compounds are on standby for testing (e.g., <https://hts.scripps.ufl.edu/facilities/compound-libraries/>). These include natural and synthetic substances [90–92] developed through **combinatorial chemistry** and **DNA-encoded libraries** [93–95]. It is tempting to imagine phenotypic screens running tens of thousands of substances, but a more urgent priority is to increase the diversity of biological models such that they replicate heterogeneity, and to make allowance for drug combinations and concentration–response curves. To meet the demands for larger panels of cells and drug libraries, and the imperative for more complex readouts, phenotypic screens will need to capitalize on automation. This process began with the evolution of plate readers and is now involving microfluidics to regulate experimental conditions, separate or assemble tumor components, or deliver material for rapid and more readouts.

A recurring critique of target-driven discovery is that many drugs are not as selective as anticipated, either because new targets emerge in patients because of the complete cellular ecosystem or adaptive responses, or because drugs change chemically to gain access to new molecules. However, phenotypic screens can suffer from low specificity, because the same readout may reflect multiple, unrelated mechanisms of action. This scenario would group drugs by similarity, and potentially lead to the erroneous conclusion that they share a mode of action. This problem can be avoided by acquiring multiple readouts concurrently to increase the specificity of the test, but target discovery is ultimately warranted, for example using **chemoproteomics** [96]. It is likely that more phenotypic screening efforts will culminate in target discovery as chemoproteomic studies become more affordable.

Although phenotypic screens are now synonymous with drug discovery, their role in unraveling the fundamental biology must not be ignored. Seeking functional responses to endogenous substances can identify regulatory and signaling mechanisms that cancers may exploit to gain a survival advantage. For example, using large panels of CRC lines, we described a mechanism (respiration) that enables survival at low pH, a chemical signature of the tumor microenvironment, and identified a marker (CEACAM6) of acid-resistant cells [97,98]. With a growing list of signaling molecules involved in auto-, para-, neuro-, or endocrine signaling, the case for deploying phenotypic screening for basic science discovery is compelling.



It is debatable whether drug discovery will be overtaken by more molecularly informed approaches (see [Outstanding questions](#)). The definitive test of phenotype- versus target-driven discovery lies with clinical outcomes, but, even if the latter makes a claim to successful therapies, it is likely that the drug had been identified at some point as a substance-of-interest through an earlier phenotypic screen. Both drug-discovery strategies stand to benefit from advances in techniques that are better at replicating the modeled disease and providing data that are more informative, specific, and meet ever-increasing high-throughput aspirations. However, the unpredictable relationship between molecular pathways discovered in cells and their manifestation at higher levels of organization adds an element of risk to target-driven discovery pipelines. For this reason, phenotypic screens are first in line to benefit from technological breakthroughs. Although these innovations find their applications in high-throughput screens, urgent matters must be addressed by the community. First, there is a need to improve reporting standards of phenotypic screens to improve searchability and transparency. Second, database curation must improve data accessibility and encourage information reuse because the growing investments in screening will need to be justified through data longevity.

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### Declaration of interests

The authors declare no conflicts of interests.

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