OPTIMISING PHENOTYPIC SCREENING

single-cell analysis versus 3D multicell analysis

In light of the high failure rate of compounds when they are subjected to clinical testing, we are seeing a renaissance in phenotypic screening in drug discovery. However, most phenotypic screening is based on the use of cellular assays and here we debate the advantages and disadvantages of single-cell versus 3D multi-cell analyses.

By Dr Richard M. Eglen

The phenotypic screening of novel drug candidates determines whether a small molecule (or biologic) exerts the desired pharmacology, either *in vitro* (isolated cells, organoids, tissues) or *in vivo*. This functional approach is 'unbiased' given that the molecular target, and therefore molecular mechanism of action (MMOA), is only determined following lead identification and preclinical optimisation. By contrast to phenotypic screening, target-based screening commences with a defined MMOA implicated in a specific disease pathology and utilises discrete compound libraries designed against this presumed molecular target. In this respect, target-based screening is a 'biased' approach. In general, phenotypic screening can identify 'first-in-class' compounds against novel targets, while target-based screening is optimal to identify 'best-in-class' compounds.

Despite the historical success of phenotypic screening techniques, target-based screening (often directly measuring the biochemical affinity between chemical compound and the biological target) has predominated compound screening campaigns. This is due to the efficiency of highly-automated and ultra-high throughput biochemical assay systems. By contrast, phenotypic approaches are comparatively inefficient given the high costs per sample and the low throughput assays frequently employed. Consequently, the approach is usually incompatible with the screening of large compound libraries. This is perhaps ironic, given that unbiased screening (eg phenotypic screening) should arguably be conducted against the broadest compound chemical library possible, with the goal of gaining the broadest range of lead chemical structures.

A common feature of both target-based and

phenotypic-based screening assays is the extensive use of human primary cells, often derived from the patient with the disease under investigation. Such cells, which can provide robust understanding of disease pathologies, are optimal for drug discovery, as they express the targets, disease-causing mutations and signal pathways involved in the disease pathology. The widespread availability of such cells has contributed to a renaissance in phenotypic screening, facilitated by the rapid development of robust, high throughput, high content cellular imaging systems. This is particularly relevant in assays directed at difficult drug targets as well as in diseases for which the molecular mechanisms are unknown. Furthermore, rapid technological advances in signal detection and microfluidic handling systems now enable phenotypic assays to be conducted using human cells in two mutually exclusive formats. The first is single-cell analysis, in which several analytical studies can be directed at a single-cell (genomic, proteomic, etc). The second is in multicellular populations, where aggregate responses are measured. In this format, the cells are cultured in three-dimensional assemblies (spheroid, organoids, tissues, etc) in a manner that more accurately resembles, or translates to, human tissues *in vivo*[1].

The question: phenotypic or target-based screening? Single-cell or multicell analysis?

Consequently, one can now pose the question of which assay approach, single-cells or multicellular complexes grown in three-dimensional structures, is optimal when conducting a phenotypic (ie unbiased) screening campaign. It is increasingly evident that both target-based and phenotypic screening share the same fundamental problem: the assay format's biology does not fully recapitulate the physiological or pathophysiological microenvironment of the cells *in vivo*, and thus the preclinical data may not adequately model the human clinical outcomes. Conversely, multicellular analysis, by definition, which deals with population responses, may obscure target sensitivity to compound pharmacology as well as the contribution of rare cells to the signal from the heterogeneous population. This question of whether phenotypic screening is best conducted by single or multicellular analysis is highly relevant when the disease target is incompletely defined, difficult to screen at, or simply 'undruggable' from medicinal, chemistry or biologic perspectives.

The goal of this brief review is, therefore, to address the use of single-cell as opposed to three-dimensional cell culture in the context of phenotypic screening. It is suggested that the use of both approaches can facilitate disease understanding to identify novel, validated drug targets. They can also identify novel biomarkers that drive patient stratification and help predict therapeutic responsivity. While the use of both approaches is optimal, it is surprising that few, if any, studies have been undertaken that compare data from single-cell to multicellular screens using phenotypic readouts.

One answer: single-cell analysis

The basic concept of single-cell screening is to enable a better understanding of assay responses from an individual cell, as opposed to measurement of population averages and the resulting heterogeneity of measurements from genomics, transcriptomic, epigenomic, proteomic and pharmacologic analysis. Screening for compound action at the single-cell level also reveals previously obscured individual cell-to-cell variations, frequently masked when whole population responses are taken.

The increasing use of single-cell analysis in screening demonstrates that many cell populations exhibit a level of heterogeneity, even among those cells of the same phenotype, such as cells growing within a single tumour. The ability to define this heterogeneity is important in understanding the complexities of polygenic diseases and their optimal treatment. Subtle, but significant, differences among patient cells are illustrated by the realisation that responses of individual cells to oncologic drugs cause the emergence of drug-resistant responses, even though only a small percentage (~0.3%) of cells possess the ability for tumour recurrence[2].

More generally, a small cell population within the larger heterogeneous population often dominate the assay response being measured[3]. Specifically, next generation sequencing (NGS) technologies are classically used to genomically characterise bulk cell populations. However, NGS is increasingly being used to focus on characterising single-cells. Transcriptomic and epigenomic analysis of single-cells can reveal novel biological pathways masked by the heterogeneous nature of large cell populations. Single-cell RNA sequencing (scRNA-seq), for example, reveals complex and rare cell populations, uncovers regulatory interactions between key genes, and tracks the trajectories of distinct cell lineages during development[4].

Historically, screening for novel compounds using single-cell responses has been limited both

Table 1: Drug screening using organoids[10]

CANCER TYPE	ORGANOID TYPE	LIBRARY	# COMPOUNDS TESTED	# CASES TESTED	ASSAY CONDITIONS
Colorectal	CSC-derived	Target-known inhibitors + chemo drugs	83	19	With 2% BME in culture medium on BME
Breast	CSC-derived	EGFR/AKT/mTORC pathway inhibitors	6	28	With 2% BME in culture medium on BME
Gastric	CSC-derived	Approved anti-cancer drugs	37	7	On 50% Matrigel
Bladder	CSC-derived	Target-known inhibitors + chemo drugs	50	11	With 2% Matrigel in culture medium
Liver	CSC-derived	NCI-approved oncology drugs set VIII	129	5	In Matrigel
Various	CSC-derived	Chemo drugs and targeted agents under clinical development	160 (single) + 120 (combination)	4	2D culture of organoids for screening
Ovarian	CSC-derived	Target-known inhibitors + chemo drugs	22	10	With 2% Matrigel in culture medium on Matrigel
Colorectal	CSC-derived*	Target-known inhibitors + chemo drugs	8	19	In Matrigel
Endometrial	CTOS	Target-known inhibitors	79	5 (2 hit drugs evaluated in 12 CTOS lines)	Without matrix
Colorectal	CTOS	Target-known inhibitors	71	1	Without matrix
Colorectal	CTOS	Target-known inhibitors + FDA-approved drugs	2427	2 (15 hit drugs evaluated in 30 CTOS lines)	Without matrix

** Prepared by CTOS method; CSC = cancer stem cells; CTOS = cancer tissue-originated spheroid; BME = basement membrane extract*

by economics and technical shortcomings in assay throughput and sensitivity. This is principally due to the high equipment costs and throughput required to characterise large cell numbers in very small volumes. Nonetheless, recent advances in automated liquid handling, coupled with precision microfluidics, are beginning to mitigate these shortcomings, as they provide increased sensitivity, economy of scale and precise automation. Indeed, microfluidic engineering is now foundational in manipulating single-cells for assays and analysis[5]. Part of the reason for this is that the handling of extremely small volumes and low concentrations of target molecules that is required when working with individual cells necessitates precise manipulation, high precision of assay conditions and highly-sensitive signal detection. Specifically, microfluidic chips are being combined with several analytical techniques to assess cell morphology and a range of cell behaviours, including growth dynamics, migration, proliferation, differentiation and apoptosis[6].

Another answer: multicell analysis

While single-cell analysis can provide an unprecedented view of pharmacology and physiology from a single rare cell – unmasked by heterogeneous population responses – each cell in the assay must be isolated from the microenvironment. A significant disadvantage of this approach, therefore, is that single-cell pharmacology *in vitro* may differ from that present in a larger population, particularly in an architecture that is not three-dimensional (3D), where cell:cell contact is critical. In contrast, 3D cell assembles, including spheroids and organoids, provide a much more physiologically-relevant context for compound screening. Indeed, 3D cell culture has been rapidly adopted in compound identification and optimisation, as they better model *in vivo* human physiology and pathophysiology. Many techniques are now in routine use and recent reviews emphasise the use of 3D cultures as preclinical models as a common practice in both drug discovery and fundamental disease research[7].

To take a recent example, ultra-high throughput screening (uHTS) using 1536-well microtiter plate formats was used to study cellular spheroids against compounds targeting a critical KRAS kinase mutation involved in several cancers[8]. Previously, directly targeting oncogenic group RAS mutations had been challenging due to the enzyme biology and the complexity pathways involved, including downstream effectors and upstream regulatory networks. As importantly, confounding factors associated with uHTS in 2D adherent monolayer cell cultures resulted in false assay negatives, with many active compounds being undetected. In contrast, uHTS using 3D spheroid culture allowed the identification of Proscillaridin A as a selective inhibitor of cells harbouring the oncogenic *KRas*^{G12V} allele. Importantly, Proscillaridin A identified by the 3D screening platform was not identified by standard 2D culturing methods.

Multicellular organoids are self-organised, three-dimensional tissue cultures often derived from adult stem cells or induced pluripotent stem cells (iPSCs). They can replicate a degree of organ complexity to a greater degree than seen with single-cells, 2D culture and simple 3D cultures such as spheroids. Furthermore, as organoids are cultured using cells from a specific individual, they can be used to predict individual patient responsivity to a specific drug or a specific dose. Related to this is the advantage that cancers and many other diseases can be modelled *in vivo* using biopsy samples and be subsequently used for compound screening (**Table 1**). As an example, a recent study presents data for more than 80 tested compounds, registered or in clinical evaluation, that are screened against a colon cancer organoid 'biobank' and demonstrate the utility of a high throughput approach[9].

Conclusion

The high failure rate of preclinical compounds in clinical study remains a key problem in modern drug discovery. Innovative technologies will only be adopted if they can improve efficiency and, ultimately, the probability of clinical translation. In drug discovery, the renaissance in phenotypic screening, particularly against the non-classical, previously 'undruggable' drug targets and/or signalling pathways, provides novel opportunities for a new generation of medicines.

However, most phenotypic-based screening, and, to a large extent, target-based screening, is based on the use of cellular assays, increasingly with primary stem cells from the individual patient. Should the cell-based assay, therefore, be predicated on single-cell techniques, or multicellular techniques? Single-cell analysis is critical to understanding cell:cell variability and the impact of measuring responses based on population-average measurements. Interrogating individual cells, as opposed to measuring the average population response, enables meaningful understanding of the interactions and contributions from low numbers of cell subpopulations in the organoid or tissue. By contrast, the growing adoption of 3D cultures is also increasing clinical predictability of compound

pharmacology (and ADME/toxicology) of novel compounds.

Evidently, both single-cell and multicellular analysis have their advantages and disadvantages in drug development and in compound screening, especially when used to phenotypically assess compound pharmacology and where there is little *a priori* knowledge of the molecular target(s). Currently, techniques for both single-cell and multicell assay screening are rapidly advancing, as are imaging and multifluidic approaches that are already impacting phenotypic assays. There are few, if any, studies directly comparing the two approaches, a deficit in the field that should be addressed given the growing establishment of cell and tissue biobanks from patient cell populations, and their subsequent use in compound screening and profiling. DDW

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References

1 Friese, A et al. The convergence of stem cell technologies and phenotypic drug discovery. Cell Chemical Biology. 2019 26:1050.
2 Gao, D, Jin, F, Zhou, M and Jiang, Y. Recent advances in single cell manipulation and biochemical analysis on microfluidics. Analyst 2019, 144:766-781.
3 Heath, JR, Ribas, A and Mischel, PS. Single-cell analysis tools for drug discovery and development, Nat. Rev. Drug Discov. 15 (2016) 204-216.
4 Hwang, B et al. Single-cell RNA sequencing technologies and bioinformatics pipelines. Experimental & Molecular Medicine Volume 50, 2018.
5 Luo, T et al. Microfluidic Single-Cell Manipulation and Analysis: Methods and Applications. Micromachines. February 2019.
6 Craig, F. Microfluidics – driving innovation and streamlining single cell analysis. Drug Discovery World. https://www.ddw-online.com/enabling-technologies/p322392-microfluidics-driving-innovation-and-streamlining-single-cell-analysis.html.
7 Kaushik, et al. 2018 Stem Cell 2018 36:1329.
8 Kota, S et al. A novel three-dimensional high-throughput screening approach identifies inducers of a mutant KRAS selective lethal phenotype. Oncogene. 2018 37:4372-4384.
9 Takahashi. 2019, Ann Rev Pharmacol. Toxiol., 559:447
10 Kondo and Inoue. Cells 2019 8:470.

Collecting phenotypic screening data. While single-gene experiments share the same general experimental workflow and factors critical for success, functional genomic screens generally need more stringent assay validation and optimization before beginning. Phenotypic read-outs from RNAi and CRISPR-Cas9 screening can be performed on equipment that is standard in many laboratories such as plate readers. To optimize phenotypic screening process, we used a chemical genetic screening approach by building a small-molecule library with prior knowledge of activity-based protein profiling. The "positive hits" result from the screen will be small molecules with known protein targets. This makes downstream deconvolution process (i.e., determining the relevant biological targets) less time-consuming. phenotypic screening | The new Tecan blog. Hereâ€™s where we take a closer look at the research and development stories, trends and developments shaping the diverse areas in which our customers and partners work.Â By Simon Fogarty. If youâ€™ve decided you need to incorporate phenotypic screening into your discovery program and you know that one of the new generation of automation platforms is the way forward, what factors should influence your choice? Automation, miniaturization, cell based assays and 3D cell culture.