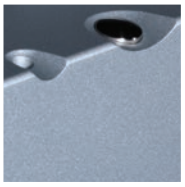
white paper

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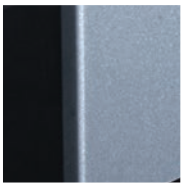



Phenotypic screening of a full compound library A reality or a pipe(line) dream?

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The search for new drugs is getting increasingly more difficult. This is due to a variety of factors, but the main ones are: the 'low hanging fruit' has already been identified and the regulatory hurdles to overcome are becoming ever more numerous and becoming increasingly more difficult to pass. This has resulted in a high rate of attrition, with many candidates failing very late in clinical trials due to safety/toxicity concerns as opposed to lack of efficacy, incurring enormous financial losses. This resultant decrease in R&D productivity has placed the pharmaceutical industry under immense pressure to find better ways to bring new drugs to market. Of the drugs that are approved, many are follower drugs. This is in stark contrast to the real goal of drug research which is to identify a first in class molecule. As a result, strategies for screening to identify more and better lead compounds are constantly evolving.

So why are there so few truly novel medicines coming through the pipeline? There are many possible answers, but a couple of interest are: is the screening pool from which we select compounds just too small, or not chemically diverse enough and are we just not using the best methods to select quality hit compounds?

HTS is very well established, though recent years have seen a steady focus towards higher content technologies and improved data quality for biological assays. The trend towards profiling smaller, more chemically-focussed compound libraries has placed increasing pressure on the relevance of the biological data generated and quality of the list of validated lead compounds. Such changes have in part led to an increasing application of so-called phenotypic, or more specifically, high content cell-based technologies. However high content analysis has remained in lower throughput laboratories and has yet to make the transition into full deck compound screening. With the availability of significant improvements in assay reagents, labware and readers, I believe it is now possible to run a cost-effective, robust approach to the screening of entire compound libraries in a phenotypic manner.

High throughput screening: counting compounds in or out?

During the 1990s, in an effort to screen more and more compounds in a reliable and consistent manner, companies turned to target based approaches. In this methodology, the target proteins are already identified and validated as having a key role in the disease area being investigated. This was made possible partly due to the Human Genome Project and in conjunction with increasing sizes of compound libraries, was heralded as the way forward for successful drug discovery. However, as noted by Szmkowski (Nature 2003), for the pharmaceutical industry, "the Human Genome Project has proved to be both a blessing and a curse". It is true that using this approach generated many more 'hits', but there was a greater failure in the clinic due to poor target validation early on in the drug discovery process.

Target based screening typically uses recombinant proteins or cells which overexpress the target of interest providing a consistent, sensitive model. The assays used were typically biochemical assays that were run in high throughput, were easily automated and were very reliable from day to day. Once hit compounds were identified, the effect of the compound needed to be checked in a biological system. During the 1990s, advances in combinatorial chemistry meant the compound libraries compiled by pharmaceutical companies became ever bigger, and consequently the number of molecules run in a primary screen became equally larger. A typical pharmaceutical compound library is now around 2-3 million compounds.

This entire approach relied on the panning for gold principle, that the more gravel you sifted through, the greater the chance of finding the nugget, in this case, the next blockbuster drug. The trouble with this approach is that if you are looking in the wrong place, or with the wrong tools, despite how much you look, you may never find your prize. When we take a retrospective look at those years, this was not always the best approach. Indeed, many 'hits' were identified, but once they went into biological systems, they then began to fail. These high attrition rates suggest that not all that glitters is gold.

Of course, this has always been the case, but the largest cost of drug discovery is not the initial high throughput screen, it is the stages after that where the costs really begin to escalate.

People often talk about screening as finding 'hit' compounds. Paradoxically, in my opinion, it is more about screening out the compounds that are no good - like the panning for gold analogy - so these compounds aren't pursued any further and money is not wasted during the later stages of drug discovery.

Phenotypic screening: physiological relevance versus throughput and cost

Following the relatively disappointing success of target-based screening, there has been a renewed interest in phenotypic screening as a discovery tool. It is a process that has come back into vogue in recent years. It stems from researchers dissociating the approach from the more recently traditional 'target based, or 'reductionist' approaches. The real benefit to running phenotypic assays is seeing what the compound actually does to a cell, not just to an isolated protein in a non-physiological environment.

Phenotypic screening has of course been around in one form or another for hundreds, or arguably, thousands of years. It stems from observing a response to an effector in a biological system. As Louis Pasteur said, "in the field of observation, chance favours the prepared mind". A good, well documented example of this is the vaccination for smallpox. In the late 18th century, it was a common notion within rural communities that people who had contracted cowpox, which was a relatively mild disease, were subsequently protected against the much more dangerous smallpox. Benjamin Jesty, a Dorset farmer (among others in Europe, notably, Peter Plett, a German teacher) tested this during an epidemic of smallpox in his town. He inoculated his wife and two young sons with cowpox, in a successful effort to immunise them against smallpox. It worked, and some 20 years later, Edward Jenner published similar work and was (erroneously) credited with the discovery.

Phenotypic screening brings a similar principle to drug discovery research. High Content Screening (HCS), also known as High Content Analysis (HCA) is now a standard approach to look at phenotypic changes within the cell in response to a compound. It is essentially an automated method that is used to identify a phenotypic change of a cell or whole organism cultured in microplates in response to a treatment. Normally, multiple features of each individual cell or organism are measured with one or more fluorescent dyes leading to the term High Content. It is this multi-parametric readout that underpins the true power of the approach. HCS enables phenotypic assays that can measure changes within a cell or movement between cells or permit the analysis of specific sub-populations of cells in a heterogeneous mix that would be difficult or impossible to perform with other technologies.

High content assays are amongst the most demanding assays to be run on a large scale since they involve using live cells, multiplexing of fluorescent dyes/proteins or probes and have multiple readouts. They can also be costly compared to target-based approaches due to the reagent requirements. The vast majority of HCS readers are based on automated microscopy. In the early days, these techniques, whilst powerful, were difficult to use, relatively slow and presented many barriers to gain a mass uptake within Pharma. Over the last 15 years, many of these issues have been addressed and it is fair to say that these readers have entered the mainstream R&D.

However, despite these advances, most high content imaging systems are relatively slow, compared to target-based screening methods. They are generally incompatible with high density (1536 well) microplates and still generate large data sets. Now, more sophisticated IT storage systems are available to archive these large amounts of data, but the IT infrastructure to handle the data effectively and crucially, to retrieve the data can still be a pain point. Another particular problem that has now arisen is that that whilst early phenotypic screens utilised generic cell lines (e.g. CHO, HEK, HeLa) for their ease of culture, modern screens have focussed on more physiologically-relevant cell lines, which now include primary cell lines, as well as induced pluripotent stem cells (iPS). The use of these cell lines is becoming increasingly more relevant with the growing importance of *in vitro/in vivo* correlation, or IVIVC. The desire to build assays in a "relevant" cell type to demonstrate IVIVC up front is becoming more pressing. These cell models are even closer to a true *'in vivo'* model, but the number of cells required to run a phenotypic screen can make the cost prohibitive.

For these reasons, high content screening has been predominately utilised more within the smaller scale phenotypic screening departments, looking at tens to possibly hundreds of thousands of compounds and has yet to be adopted into the primary screening environment.

Perceived barriers to primary phenotypic screening

As Table 1 shows, there are distinct differences which relate to challenges when moving from target based screening to phenotypic based screening. As a result, compromises are made when deciding on the best screening path to take.

	phenotypic	target
mechanism	cellular	molecular
assay dev	complex	simple
throughput	slow	fast
running costs	high	low
data analysis	complex	simple
infrastructure	expensive	cheap
labour skills	specialised	general

Table 1. Comparison of phenotypic-based assays compared to target-based assays

Typically, large full deck compound screens are run in a target based assay screen, or a phenotypic screen is performed on a much smaller targeted library subset. In the current drug discovery environment, a chemist would ideally want all the compounds available in the library to be screened to get as much information as possible, but the phenotypic biologists want smaller subsets screened due to the very real concerns they have about technical limitations and costs. For the Project Director, a compromise has to be struck. Full deck target based screen to cover all the compounds, or a smaller subset screened phenotypically to gain detailed, physiological relevant data?

The library subset approach is designed to capture all the areas of the chemical space within a particular company's compound library set. However, I believe that this approach is fundamentally flawed as these library subsets have been selected based on target-based information, which drug discovery is moving away from.

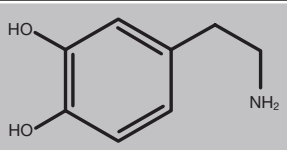
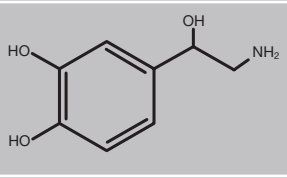
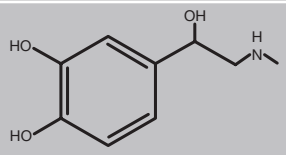
	structure	function
dopamine		Dopamine has a key role in motor control, motivation, cognitive control. Often prescribed for heart failure, or shock, or as the precursor, L-Dopa for Parkinson's disease.
noradrenaline		Noradrenaline is a vasoconstrictor, increasing the tension of vascular smooth muscle via activation of the α-adrenergic receptor Often prescribed to those with severe hypotension.
adrenaline		Adrenaline is secreted in response to stress, increasing heart rate and blood pressure. Often prescribed for patients that have anaphylactic reactions

Table 2. Similar compounds structures exhibit very different phenotypic responses

So using the data generated from historic target-based screens to select subsets for phenotypic screens runs a large risk of missing good quality hits - or selecting poor compounds for next stage interrogation. Just because a compound is in a similar chemical space to another molecule, doesn't necessarily mean that it will exert the same phenotypic response, *in vivo*.

A good example to demonstrate this is the catecholamines, dopamine, noradrenalin and adrenaline, which only differ by the addition of a single functional group, but exert very different physiological responses (see Table 2).

So is there a different solution to the conundrum posed? What if a full deck compound library could be screened phenotypically at primary throughputs? What would the requirements for that need to be in order to be implemented? Is it just a pipe dream, or can it become a reality?

Full deck phenotypic screening requirements

Target-based screening was adopted partly because it is easy to automate, fast, reproducible and relatively inexpensive to run very large compound sets. So what are the major requirements of running a full deck phenotypic screen? There are a number of basic requirements as listed in Table 3 that are required to enable a full deck phenotypic screen, but there are a number of other considerations as well.

From an automation perspective to get the required throughput, say to screen a compound library of 3 million compounds, the assays must be able to be run in 1536-well plates. This means that cells, dyes, drugs must be able to be plated into a 1536-well plate. In fact, this is now less of a challenge than it once was. Many liquid handling systems are able to do this very effectively with minimal negative effect on cell viability.

Another limitation that is becoming an issue for HTS is the cost of cells. To run a primary screen, as many as $1.5e^9$ cells may be required. This is expensive even with standard cell lines, but with the phenotypic trends moving ever towards more and more physiologically relevant models, primary cells and even more recently iPS cell lines are becoming increasing utilised incurring prohibitive costs. To make this a more viable option for HTS, requires the use of approximately 100 cells per 1536-well plate.

The plates have to be screened both quickly, (sub 5 minutes per plate) to hit the throughput requirements, but also analysed within a consistent timescale. This is important because many systems don't have consistent plate read times when there are low cell numbers in the well. It may depend on how many cells are in the well, therefore affecting the number of field of views required, and the time taken to capture enough events. To fit into a fully automated HTS process, you ideally need to know that each plate will take, say for example, 4 minutes, 35 seconds to read, for every plate in the run.

It is much more difficult to manage an automated process if plate 1 is analysed in 5 minutes 42 seconds, whilst plate 3 is analysed in 9 minutes 27 seconds. An additional requirement is that the resulting data files must be manageable. When obtaining data from 2-3 million wells, you can't afford large data files, or else the IT requirements become unsustainable. Finally there is the idea that the biology of the assays are simply too complicated to be run on a full deck screen.

So, looking at these requirements, are throughput and complexity of the assay the main barriers to running a full deck phenotypic HTS? I think that these are certainly perceived to be the biggest challenge, although I question whether that perception is really true? The biology itself can be limiting certainly, but only in certain projects. Phenotypic screening has been around long enough to prove that it is a robust technique. Many phenotypic assays are still looking at quite basic readouts: cell viability (live dead/apoptosis), reporter gene expression, cell cycle, angiogenesis formation. These are simple assays to run.

	phenotypic
throughput for 1536-well plate	fast - minutes per plate
time	consistent - must know how long each plate will take
file size	low - for millions of compounds
ease of use	simple
data analysis	on the fly - immediate results
plate format	must be capable of 1536-well plate
cell counts	low

Table 3. Main requirements for a full compound library phenotypic-based assay

Once cells and compound are added, the reader simply has to count cell number, or fluorescent dye intensities. Other assays can be much more complex, neurite differentiation, receptor internalisation for example. The more complex the assay, the less compatible it is with running it in a true HTS environment. But it is still true that the vast majority of assays are relatively simple to run and are therefore compatible. As for throughput, it is true that many typical imaging systems do not fulfill the criteria for a HTS manager to run in a full deck screen as shown in Table 3. So maybe rather than fitting a square peg in a round hole, the best way to run full deck screening assay is not to use a standard microscope-based system, but to think, and take a different approach.

How can full deck screening be run today?

Laser-scanning imaging cytometers (LSICs), such as the acumen Cellista®, are compatible with primary full deck compound library screening campaigns. They combine the object-recognition capabilities of a CCD/CMOS microscope based systems with the fast read speeds of bulk fluorescence readers (typically sub 5 minutes per plate). These read times are equivalent for 96-, 384- and 1536-well plates, as scanning is performed on an area and not well basis. It can scan and analyse over 400,000 wells/day, with a standardised plate read time, suitable for fitting in with automation workflows for a wide range of phenotypic assays. It also has the added advantage of producing very small file sizes, so the IT requirements are the same as for target-based screening. These features mean that the acumen Cellista is ideally placed for practical high-throughput, full deck phenotypic screening.

Summary

The only barrier to running full deck phenotypic screening today is, I believe, the willingness and belief to implement it and accept it as a key area of drug screening and to implement it. Of course it is true that there are examples of certain projects that may not be compatible with this approach, but that doesn't mean we should avoid benefiting from the success of the many, just because of the incompatibility of the few. The costs are higher than traditional target-based screening approaches, but it is surely a much more cost effective way forward in the long run than performing easier, cheaper assays up front and then pursuing poor compounds which ultimately fail at later stage drug development. As I mentioned before, in my view, screening is really about excluding the compounds that are not useful, so time and money later on is not wasted on them. It is not necessarily about finding the hits. Maybe it is time to turn the screening scenario on its head and instead of screening in a target-based way initially, then using a phenotypic response to back up the data, we should screen phenotypically and follow up with target-based information to determine the mechanism of action

In the end we do have the ability today to phenotypically screen our entire compound libraries and potentially find new and better drugs, earlier and cheaper. The real question is, do we have the desire and the belief in the technology that exists, to make it a reality today?

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