Drug discovery for challenging targets

What is a challenging target?

What constitutes a challenging target depends on what you want to measure. While there are many available methods in biophysics, what we usually want to know is: are our components engaging the target? We want to know something about affinities, kinetics, mode of action or thermodynamics. We need combinations of these data to build up a meaningful characterisation that can be used to predict how components will really interact. Good and meaningful data will guide the development of the drug, and how data are collected is, therefore, of vital importance. It must be collected in an environment that closely resembles human physiology, because if compounds are not active there, they will ultimately never become drugs.

This need for physiologically relevant environments puts high demands on our biophysical assays. To be considered a good assay, we need to get three important factors right.

Reagents

Reagents should allow you to perform your assay optimally while also mimicking the biological environment as closely as possible.

Sensitivity

Highly sensitive measurements help us to gather more data which can be used for understanding binding interactions.

Relevance

The entire assay must serve to predict a binding event that could really happen in the patient. Using truncated proteins in sub-optimal conditions decreases the chances of finding a compound that will eventually become a drug.

Drug screens often require a high throughput, meaning one of these three pillars is sacrificed. It is very common to conduct high-throughput screens far away from their biological context, for example when measuring membrane proteins that are not embedded in a membrane. This means that there is a lot more downstream work required to cross-validate any results that are generated.

Challenging targets make it even more difficult to have the right reagents, sensitivity and relevance in one assay. In short, challenging targets are difficult to characterise using current technologies.

Challenging targets have suffered from a lack of attention

Challenging targets have been somewhat neglected in R&D. Since the 2000s many new drugs have been developed for simple targets such as kinases. However, over the same period of time far fewer drugs have been developed for GPCRs, one of the most challenging target types.

What can be done to redress this imbalance? How can we use new technology to understand the neglected and challenging targets?

Single molecule methods for surfaces

The workhorses of biophysics and label-free biosensing are the surface-based methods. The target is immobilised on the surface and our compound is flushed over it, generating a signal. However, if our target cannot be easily immobilised, or if it is simply not very active on a surface, then it can be impossible to get the right reagents, sensitivity and relevance all in one assay. This then leads to many downstream assays being required to cross-validate any potential targets.

However, single molecule methods can help to overcome these challenges. To see how, it is important to understand the difference between single molecule and ensemble methods. In the case of ensemble methods for surface-based biosensing, the targets are sitting on the surface that the reagents are flushed over. This means that at the very beginning of the experiment there's almost no compound on the target. This is reflected in typical data sets from these setups, where there is a lot of binding at the beginning and almost no release.

After this, there is often little more to see from ensemble data as the line goes flat, suggesting that nothing is happening. Of course, this is not the case: when the compound becomes saturated the binding and release is happening in equilibrium, and there is constant release and re-binding. This is impossible to see with ensemble methods, but single molecule methods highlight the release and binding kinetics very well.

Another limitation of some surface-based methods is the requirement that the target on the surface must be immobilised in such a way that it will be able to interact in a natural way with various compounds. At InSingulo, we have developed a simple yet effective solution; we immobilise known binders to the surface and allow the targets to be free in solution. This gives additional benefits when it comes to challenging targets such as membrane proteins, which can be embedded in liposomes, therefore preserving their natural function.

Even though the binders are surface bound they can have a range of affinities and still produce a signal in our assay. We can work with nanomolar and micromolar binders using a technique called TIRF microscopy. As soon as a liposome containing a target binds to the surface it shows up as a flash under the microscope which is recorded and counted.

The number of binding events are directly proportional to the amount of free target in solution, which means that it is possible to deconvolute the data continually to give more information about the binding and dissociation of targets from the surface.

Characterising your compound

So, if you add a compound that can bind to your target, and this inhibits the binding to the immobilised tool compound, what will happen? Well, because the number of binding events over time are directly proportional to the free concentration of target in solution, they will decrease. That means what was a straight line before will deflect downwards to reach a new equilibrium, and this allows us to calculate two things. Firstly, the ratio of the two slopes tells us the inhibition level, allowing a dose response curve to be created. Secondly, the speed of the transition between phases tells us something about the kinetics, equivalent to the Kobs in SPR measurements.

What happens when you change the concentration?

You will get a dose response curve and bilinear extrapolation from Kobs over the concentration, and the association constant can be extracted. With the Kd and the association constant you have the full kinetics. This represents a significant improvement when compared to other methods that require you to immobilise the target. So, without immobilising the target, we are able to measure the affinity and binding kinetics of unlabelled compounds with the target in solution.

This is an important step for drug discovery with challenging targets. If SPR fails for a challenging target, most of the other assay methods available can only be performed in solution. Critically, this will not give kinetic data to evaluate the interactions properly. Now, we have a method that can.

How to get your reagents to a challenging target like a membrane protein

When you're working with challenging targets, the main question is: how do you get your reagents to your target? Membrane proteins are particularly difficult on account of problems with purification. To counteract this, we have developed a method where purification is not required. We take cells that over-express our target protein and then induce them to shed small vesicles that are ultimately made from the cell membrane itself. These vesicles can then be collected, purified, and labelled, but the important thing is that the target is still embedded in its native membrane, which it never left. This means that you can be more confident than ever that the target interactions that occur using this method will closely represent the natural physiology.

This is just one method; we can also express proteins and add them to a more tightly controlled vesicle system. The choice depends entirely on the experiment and the target. Our assays are also performed, well by well, in 384-well plates, meaning you do not have to continually regenerate the sensor surface in order to perform additional experiments.

Case Study: Beta-2 adrenergic receptor

We took the beta-2 adrenergic receptor, over-expressed it in CHO cells, and made vesicles that could interact with the surface. Then we added different inhibitors, which caused a reduction in binding over time. Antagonists and agonists were also tested, as these can be differentiated in our assay. More information on this can be found in our preprint publication [1].

Summary of how dISA can help with challenging targets

First of all, dISA maintains the membrane environment. For the biggest group of challenging targets, GPCRs and membrane proteins, the main problem is that you have to remove the membrane. This means that it is difficult to make reagents work and also collect data that will ultimately be relevant to what will really happen physiologically.

You can also get full kinetics. That is something you don't get with lots of the back-up methods that you would usually use if you can't do label-free biosensing. We have excellent signals for low molecular weight compounds, because what we count in our signal is how many binding events happen on the surface, and what reduces the number of binding events on the surface. If you were to do a fragment screen on a GPCR you would get the same signals as you would with an antibody screen. With many label-free methods the signal is proportional to the mass of the compound making lower molecular weight compounds difficult to detect. However, this is not a problem with dISA.

We are also very flexible when it comes to the target, working with membrane and soluble proteins alike. For example, soluble proteins that are challenging could be intrinsically disordered proteins, because they tend to aggregate. However, once you tether them to a liposome they become extremely stable in the solution.

Our single molecule sensitivity also gives us two key advantages. Firstly, we cannot increase the levels of sensitivity because, as there is no half-binding event, we are already at the natural limit of what we want to observe. Secondly, you get counting data that enables you to do different statistical and stochastical analysis. You can now do model selection in an unbiased way; just ask the analysis software, "Is it a one-step or a two-step binding," and you will get an answer based on data, and not just a gut feeling on which fit looks better. You can get a hard number that tells you which model to select, and you do the same for each measurement.

The dISA method is also not really influenced by DMSO. For some methods that measure the refractive index close to the surface, high DMSO content is a problem.
The fact that the dISA assay is in a microplate means that we are not constrained by microphoretics and liquid handling. We use a liquid handling robot which can easily build up complex protocols to tailor your experiments.

If you're interested in trying dISA please get in touch with us, and we will quickly be able to determine if your experimental idea would qualify.

References

1. Single-molecule Dynamic In-Solution Inhibition Assay: A Method for Full Kinetic Profiling of Drug Candidate Binding to GPCRs in Native Membranes
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