Current and emerging trends in cell-based assays

In 2009 Drug Discovery World hosted a collegial-style roundtable discussion on the present and emerging developments in this exciting and now, fundamental area of drug discovery. The participants are recognised to be thought leaders in this field and represent the pharma, academia and vendor community. Although somewhat sketchy in places, this is a transcript of what was discussed in what was a lively and interesting debate.

PARTICIPANTS

Robert Jordan – Publisher & Editor in Chief, *Drug Discovery World*
Martina Bielefeld-Sevigny, PhD – Vice President and General Manager of Drug Discovery and Research Reagents, PerkinElmer
Arthur Christopoulos, PhD – NHMRC Senior Research Fellow and co-Director of the Drug Discovery Biology Laboratory, Monash University, Victoria, Australia
Richard M. Eglen, PhD – President, Bio-Discovery, PerkinElmer
David F. Mark, PhD – Senior Research Director, Roche Discovery Technologies, Hoffman-LaRoche
Kevin Pfleger, PhD – Research Associate Professor, Head, Molecular Endocrinology-GPCRs, Western Australian Institute for Medical Research (WAIMR) and Centre for Medical Research, University of Western Australia
Martin J. Valler, PhD – Group Leader, High Throughput Screening, Dept of Integrated Lead Discovery, Boehringer Ingelheim Pharma
Guido Zaman, PhD – Senior Director, GPCR and Kinases, Molecular Pharmacology Unit, Oss, Schering Plough

Robert Jordan (Chair): Today we're going to discuss the current and emerging trends in cell-based assays. I'd like to start off by looking maybe at screening strategy. We know that cell-based assays now play a fundamental part in a successful lead discovery strategy, and we know that functional approaches open new opportunities to novel targets and to produce results that are more biologically relevant in *in vivo* studies. So my question is, when should we use cell-based assays? Should they be in parallel to biochemical assays? Or indeed, can we leverage functional approaches to develop smarter assays that are more biologically relevant?

Arthur Christopoulos: How do you differentiate cell-based assays from biochemical, or biological-biochemical assays? Do we mean, basically, enzyme-based type direct on the target…?

David Marks: Or maybe you mean, for example, in GPCRs, a biochemical assay would be a receptor binding assay? And a cell-based assay would be a functional assay responding to a secondary messenger signal, or some other cellular response?

Robert Jordan: Yes. Absolutely.

Kevin Pfleger: So does cell-based have to be whole cell, or could it be membrane?

Arthur Christopoulos: I suspect membranes on the biochemical side of things. The other issue with that question is of course when you're talking

about tying a particular assay, whether it's cell-based or not, as a predictor of what's biologically relevant. I think the whole concept of biological relevance is still 'out there'. In terms of what's a predictor, no matter what we do, or what's going to be the desired therapeutic endpoint, I think in 99.9% of the cases we can't answer that.

Richard Eglen: I think if you look at biochemical versus cell-based assays, I think historically biochemical assays as mentioned in terms of ligand binding to a membrane that has the GPCR for example, gave very robust straightforward information. The problem was, to Arthur's point, that the cellular context was lost. And you lost a lot of the functionality in terms of the cell signalling pathway. And probably cell-based assays have started to merge, because you've got the whole functionality of the cell as it relates to the function of the target. Now that gives you some advantage in terms of amplifying the signal to measure it and you can get efficacy of the ligand versus the ability to block the receptor.

But it's also opened up some problems. To Arthur's point, what's the correct cell type to give you the right response? And how does this functionality mimic the physiological or the disease situation as it relates to drug discovery? David, I don't know if you want to answer that…?

David Marks: Yes, there's always the issue of whether an *in vitro* cell-based assay will truly mimic the *in vivo* response. I think, especially in the neuroscience area, they often have that problem – the fact that you get a response in that cell line doesn't necessarily give you an *in vivo* response. But back to what Robert was asking earlier, I think ideally you probably want to have a combination of an *in vitro* cell-based assay and a biochemical assay just to confirm that whatever you identify in a cell-based assay is really acting via the receptor and not some secondary response in an intracellular location. And alternatively, a pure biochemical binding assay may not work for certain G-coupled protein receptors, because for example, if you're looking for an agonist response, a binding assay doesn't really help you. I mean, you don't know if you have an agonist or an antagonist. So you may need the combination of both.

Arthur Christopoulos: I think there's no way around it now, in the sense that, for starters, the industry people can correct me if I'm wrong, but the cell-based assay has probably emerged upfront now. But the binding or the biochemical assay has got to remain as a validation step. And the second aspect, is and even one of each is insufficient nowadays, given the repertoire of behaviours of different ligands have been found to engender especially as a consequence of functional cell-based assays. Cell-based assays as Richard pointed out provide context, but the context can change from cell to cell. And on top of what we know now especially in some GPCRs, which have always been known to have lots of different possible coupling partners which can change with the cell type, that can do different things. So a series of ligands acting on the same receptor even in the same cell type can bias different types of signals. And so you have no choice other than to use multiple functional endpoints when trying to deconvolute those sorts of compounds. Because we don't know which is the right one. Is it the one that gives you arista, or is it the one that causes G-protein coupling to channel that's going to be therapeutically relevant?

David Marks: Another issue with cell-based assays is that you also have to be careful that you have the correct coupling of the G-protein, especially with GPCRs. We've seen instances where we have used promiscuous proteins to couple to a calcium response and then we find out that the compounds we've identified do not work when it's correctly coupled. So that's an issue too. You have to be careful.

Arthur Christopoulos: So how many people are using native cell expressors to screen, then? Endogenous expressors … that's still not that common, is it?

Martin Valler: No. We've had the same experience. The same target mated by two different coupling mechanisms and we've seen completely different results. But just to come back about the point about why we're really doing assays in the first place, I think there are an increasing proportion of assays that just can't be addressed sensibly by binding – and they can't be confirmed either by binding, because with the allosteric modulator class of targets, you're lucky to get a displacement of the ligands through such a binding. Maybe you might but in most cases you won't. You'll simply see a modulation of the functional activity, and the true ligand binding will be completely unaffected.

That's increasingly our direction, our rationale for doing more cell-based assays – projects that we're interested in functional readouts like allosteric modulators, positive or negative, and are less interested in straight inhibition.

David Marks: And I think it's also very important to have eventually a cell type that is the more appropriate cell type for the target. For example, with metabolic diseases, we always make sure that we use muscle or liver cells to check up on whatever we use as a surrogate cell during the screen.

Richard Eglen: If you look at the way kinases assays have gone, comparing and contrasting them to GPCRs – where with kinase assays, biochemical assays have been relatively successful. They've got the crystal structures of the kinases, they can now pick up allosteric regulators of kinases, as well as kinases interacting with other proteins. Is the fact that this is not yet here with GPCRs not a biochemical problem, it's that the technology is not here to do it biochemically with GPCRs. I mean the crystal structures are already starting to roll through for GPCRs. It's just that you cannot do it on a technical basis.

Guido Zaman: I think I agree with you. The major, the first thing about cell-based assays for GPCRs is they are resistant to purification. And radioligand binding assays have the disadvantage of creating radioactive waste. So I think cell-based assays work well for GPCRs but kinases purified by biochemical assays is a prime platform. Then again, if you have allosteric modulators which indeed can only pick up the cellular assays, in the second line, you always want to do binding assays to confirm specificity of your compounds, either with the radio-labelled variant of your allosteric modulator, or you may want to do disassociation experiments with natural ligands to confirm that it's allosteric.

David Marks: But we have also other binding assays that can be used in place of a required radiolabel… that can be labelled for direct binding, for purified proteins. And actually for GPCRs that might not be too far away. So that might be another method if you're concerned about radioactivity, and disposability. These binding assays even for GPCRs can be potentially developed quite soon.

Guido Zaman: I agree to a different path for binding, different tools. But also if you go back to kinases for instance, the traditional method is the enzyme activity assay. I think another advantage of biochemical assays is that you can do very precise selectivity determinations. I think binding maybe is the more traditional way of looking at GPCRs, but you can also look at binding with kinase assays. And if you start to think about this, you can go to totally new concepts while you look for instance, not for ATP competitor compounds, but look directly for allosteric modulators.

Martina Bielefeld-Sevigny: So for enzymes, then the other question for kinases, what's the substrate? Most of the time you're working with a peptide. How relevant is this really now in the cell context? Are there other co-factors that are really needed that modulate the kinase or proteins? So there still may be differences so that you have… but you still need both of the approaches to get to a good answer.

David Marks: I agree we still need cell-based assays ultimately to qualify your kinase inhibitors. And not only that, I mean, you mentioned about selectivity – you can do all kinds of selectivity screening *in vitro*. But how relevant that selectivity is in a cell setting is still questionable. Because it all depends on KM of ATP for your particular enzyme. And that could change the real, true selectivity profile in a cell. Because if you look at a compound that may have some activity at a certain ATP concentration *in vitro*, but in the cell the ATP concentration may be much higher, and that activity may not be relevant. Because ATP will compete out the activity of your compound *in vivo*, inside the cell. So I think it's still important and we find that it's very important to check to make sure that your compound, I mean look at selectivity, after you do the *in vitro* panel you also do some *in vivo* activity profiling of your compound in the cell to look to see if it is actually inhibiting the potential non-selective activity that you are worried about in the cell. And we've seen situations where we see good inhibition of a kinase, a non-selective kinase activity *in vitro*, but it really wasn't a problem in the cell.

Arthur Christopoulos: I want to get back to Richard's point, the whole kinases versus GPCRs thing to point out a couple of things. Yes, I think in the structural side of things, the kinases are ahead of the GPCRs, but as Richard said we're seeing structures starting to come out now. If you were to ask about an emerging trend, I would suggest that we will be seeing structural-based design on GPCRs in the next few years. There are actually companies based around that now. The second thing is, if we consider the targets, GPCRs versus kinases, kinases are very well developed along a certain path – because they're kinases. If you look at the GPCRs, I could be wrong, but I think they're far more promiscuous in what they do compared to kinases. I mean, a kinase phosphorylates proteins.

Richard Eglen: So you mean it's function? It's a transducer.

Arthur Christopoulos: Yes, I mean it's function. GPCRs, they're promiscuous. They need partners that can change, a lot. And the coupling partners, even in the given cell type can vary a lot, and the same GPCR can simultaneously couple here, there, and everywhere. And also in terms of the ligands that they recognise, I think other than maybe drug transporters, GPCRs probably recognise a larger diversity of ligands than any other type of protein. Moreso than kinases. So there are extra challenges there as well, associated with that promiscuity, both on the outside, and on the inside. These things then move along.

David Marks: I will challenge that, naturally, since kinases have the same promiscuity if you look inside the cell. Kinase pathways branch off and overlap a lot. So in terms of signalling of the kinase in the cell, and how it behaves in different cell types, it is also different.

Arthur Christopoulos: Oh, I agree, I'm not denying that. If you look in fact at the genome, there are more kinases than GPCRs. Am I wrong?

Richard Eglen: There are 118 kinases.

Arthur Christopoulos: Are there? So about the same. So, they are prevalent, and probably they are coming up as drug targets, and maybe they're up there with GPCRs.

David Marks: But actually there are many more GPCR drugs developed. Based on GPCRs more than kinases. So kinases are actually early, even though it seems like it's very well developed in terms of its assays.

Arthur Christopoulos: Yes, but there were all those … I mean that recent Hopkins work that was suggesting kinases are along the road. But in terms of the level of function, kinases are serving a well-defined purpose. Whereas GPCRs, we're still trying to find out what these things are doing. And in terms of targeting – put it this way: do we think there will be more allosteric ligands for kinases, or GPCRs? They both in theory should be both allosterically targetable.

Guido Zaman: I think in GPCRs there are more examples, but in kinases there are now more and more examples appearing. I think the traditional kinase technologies, we fished out more ATP competitors.

David Marks: Because that was easy to do. A lot of chemists are not really focused on allosteric kinases. A biologist would be more interested in that. And actually now there's been more and more of that.

Arthur Christopoulos: Actually, that's a good point. Let me ask this question. Is that then because of the switch in the way people screen for kinases?

David Marks: Yes.

Richard Eglen: I think the way we see it as an assay provider is that there is definitely the thinking that assays for GPCRs are like looking into the lamp-post – that is, you do the assay that works because that's where the light is, as opposed to maybe that's what the GPCR actually does physiologically. If you consider to Arthur's point that GPCRs are probably part of a signal cell, as part of a functioning network, then probably what needs to be developed are systems that would relate to other parts of this network. And you need a cellular context to do that. So beta arrestin, beta arrestin independent signalling, for example, GPCRs signalling through kinases. I think that the kinases are starting to be worked out more quickly, because people are putting them in the intracellular context.

David Marks: That's true, if you look at a map of the kinase.

Arthur Christopoulos: What's the substrate for a kinase?

David Marks: There are at least two substrates for a kinase.

Arthur Christopoulos: That's why I'm asking. I'm trying to get us on the same...

Guido Zaman: A protein and an ATP.

Arthur Christopoulos: A protein and an ATP. So ATP doesn't vary. But the protein varies dramatically.

David Marks: The ATP concentration in a cell doesn't vary, that's true. But the context can vary a lot, depending on the concentration of substrate and other accessory matrix proteins that often the kinase can bind to. It could modulate its activity quite a bit.

Richard Eglen: So I guess the point is that intracellularly, or even on the tyrosine kinase receptor of the membrane, there are a lot of proteins that

are allosterically regulating the kinase per se. So I would say allosterism at kinases is more normal as opposed to exceptional. Whereas allosterism with GPCRs is starting to become better understood.

Arthur Christopoulos: I think allosterism is the norm with just about everything.

Martin Valler: But we shouldn't forget the reason why GPCRs are good targets is because although they may have multifunctional responsibilities within the cell and downstream, upstream the ligands are very specific, and can be defined pretty much in advance. The best thing of course is the target is on the outside of the cell, so we don't need cell penetration for the drugs. And that is an overriding benefit. So that is the key thing singling out GPCRs as a promising class of targets.

Richard Eglen: I just want to say, are all of the functional assays, the cell-based assays, are they surrogates to investigating the ligand binding to the receptor, in terms of discovering drugs? Or are there other avenues for discovering new drugs that GPCRs act on other parts of the GPCR-mobilised pathway? In other words, if you could really measure ligand binding, at the biomolecular event, is that really what you're looking for? Or are now people looking for drugs that act at the GPCR driven pathway?

David Marks: I don't think there are a lot of people actually targetting the GPCR pathway itself, because you may lose specificity there. Because the pathway proteins, when you look at them, are the same. While the area where that's different is the receptor itself. So that's where you can have selectivity. Once you go past the receptor, it is difficult to find selectivity.

Richard Eglen: I was wondering about the protein-protein interface, the arrestin interface, dimerase interfaces...

Robert Jordan: Well that's a good question, because one of the questions I was going to ask is on the dimer side. I wanted to ask whether any of you have enabled or planned to enable GPCR dimer assays? And do you think they will be the source of the next generation of targets?

Kevin Pfleger: Yes, I think that dimerisation and allosterism are the two main thrusts where the field is going to be going to. I think that as has been said here, yes we can look at a lot of drugs where GPCRs are on the market. But actually most of those drugs target a very small subset of GPCRs. There are a lot of GPCRs for which there aren't targets, I'm sorry, for which there aren't drugs. Is that because the assays are not picking anything up or, as Richard said, because we're just looking under the lamppost? I think we need to be looking for assays that can appreciate whether you have signalling through different G-proteins, signalling through beta arrestin, signalling when you modify with other co-factors, such as another GPCR. And I think GPCRs shouldn't be put up on a pedestal. At the end of the day, they're proteins. So if you have an interaction between proteins, they will change each other's confirmation. So when you're looking for ligand binding interaction, whenever you have an interaction at the intracellular surface, they will be changing confirmation of the receptor. So assays that are for example based on promiscuous G-proteins, you'll be pulling out ligands that are specific for a promiscuous G-protein stabilised receptor because the G-protein actually does affect the confirmation of the receptor. So you will be pulling out a subset of ligands, if you're looking specifically for ligands that are going to certain G-proteins you might miss them. If they're specifically going to arrestins you might miss them. And the other point as well is that a lot of the drugs – the problem with drugs – is not finding the compounds, it's finding compounds that don't have side effects. So if we're looking for selective compounds that don't have side effects, perhaps we need to identify specific coupling partners and exclude others? Maybe we need to be looking at drugs that actually couple to multiple partners, but exclude a subset. Maybe you have two pathways that are good and one's bad, so you exclude the one that's bad? We need to be a little bit cleverer about how we actually measure these endpoints. And perhaps we need to be doing multiple endpoints.

David Marks: But the issue is really do we know what is the relevant receptor confirmation or receptor complex that we should be using for any particular disease phenotype that we want to treat?

Arthur Christopoulos: In most cases the answer is no.

David Marks: Right, if that's the case, there's nothing that can be done to try to take that approach. So first we have to have a much better understanding of whether dimers, or heterodimers of receptors really have physiological relevance in

a certain physiological pathway. That's still a lot of questions there.

So until we can understand that and I'm talking about GPCRs, we can't really think about developing an assay based on that.

Kevin Pfleger: But the point that I would make there is at the moment we're going round and round in circles. Because you're absolutely right. We don't know whether these heterodimers are relevant. So we need to actually look into animal models, to see whether these heterodimers are relevant. But we can't do that until we have tool compounds that can look at those heterodimers. So at the moment the field is in a state where it's hard to identify heterodimers specifically because we don't have the compounds.

David Marks: But you have agonists, you have natural ligands. So why can't you use the natural ligands to investigate that?

Kevin Pfleger: The natural ligands for a heterodimer?

David Marks: The natural ligand for...

Martin Valler: ...that encourages the formation of the...

Kevin Pfleger: Right, if you speak to a lot of people they would suggest that heterodimerisation is constituative, and the heterodimers form in the ER and traffic up together.

Arthur Christopoulos: The problem with the natural ligand is that it will behave one way if the monomer is there. And if you co-express the receptors, it will behave a different way. Is that due to the heterodimer formation, or is that due to the crosstalk between the two receptors in an intracellular manner? One of the difficulties is interpreting that.

David Marks: One of the problems is that you don't try to investigate that first with a natural ligand or ligands, because with a heterodimer you may have two natural ligands... if you don't try to investigate the relevance of that interaction first, and you start screening for compounds, that will work on that heterodimer, then how are you going to know how to interpret those new compounds.

Arthur Christopoulos: I would suggest that most people looking at any dimer situation, they must be looking at natural ligands for their receptor, or how else would you screen? Unless you're screening for surrogate agonists, otherwise you're screening against the natural ligand, right?

David Marks: Yes. But one thing is that so far there's still not a lot of information about the relevance of these heterodimers or dimers, even using a natural ligand.

Richard Eglen: Do you think, David, that as the technology becomes reduced to practice to screen at dimers, that people will do it anyway because what have they got to lose? And they can find compounds, to Kevin's point?

David Marks: But the problem potentially is that what Kevin is also saying, if you screen in that context, you may be missing a lot of other things outside of that context.

Richard Eglen: Oh, I'm not saying they do this only, I say they're doing this in addition.

Arthur Christopoulos: I think part of it is... I hope we've come to the tail end of the dimer hysteria phase, for want of a better way of putting it. Where everyone and anyone's... if you stick two things together, you can find something in a recombinant cell and call it dimerisation. Now I think there are guidelines that are starting to emerge that are more strict along the lines of criteria and expectations, of which Kevin is more aware of than I am. Including native tissue relevance, physiological, pathophysiological, all of that. So the bar has been set very high now in saying that a dimer in a particular heterodimer is particularly relevant for a given condition. And I think probably what we're going to see is a lot fall by the wayside that don't meet this criteria, and there are a few that will emerge. Having said that, it might imply – and again, I'm not a dimer expert – that they are going to end up in the minority. But the targets you find are probably real. I don't know.

David Marks: I think that if, in any particular system, or target, if there is information where a heterodimer is relevant, I'm sure we will use it as a way for screening. But until there is demonstrated relevance to a certain heterodimer, I'm not sure a lot of companies would want to screen in two or three different assays. If you have a heterodimer, that means you have to screen against three different assays.

Arthur Christopoulos: I think I mentioned this in

before, we don't in particular. The key criteria for me is reproducibility. If different labs can't get the same results given a similar target, similar approaches and similar cell types, then you've got to wonder, do the stars have to align and do the waves have to be just right to see what someone else says they saw with a dimer? It's a matter of how much time and effort am I going to put into something like that.

Guido Zaman: Yes I think I agree with what Kevin said, that the proof is in the physiological relevances and there you need very sensitive technology to make use of labelled compounds, labelled antibodies to look at native tissues. We know that in overexpression systems and depending on the technology that you can use here, you can see various combinations, combinations that are sometimes less specific, but then the next question is, if indeed, this changes this heterodimer, does this change the pharmacology of the natural ligands, and if it does not change the pharmacology, then it's not very interesting.

Martin Valler: In the natural situation, that may be true, but for intervention, for drug finding, that could yield new information, new binding sites, new modalities for those compounds. So for drug discovery that kind of interaction is extremely relevant.

Guido Zaman: That's true.

Martin Valler: I agree fundamentally this underpins this whole concept that we need morphological systems to do our testing, and if we say the real answer is, we go from our compound that we find to an animal model, well, that's not my definition of high throughput screening. We need our high throughput systems to be as relevant as possible, and informative and not only to just represent potential dimerisation, but they also need to represent the signalling pathways. In all those areas, we've got a long way to go. I see the dimerisation is only one aspect of that. The question is, do we have the relevant signalling pathways going on, do we have the relevant expression levels, do we have the assay systems to detect relevant drug effects in a system where maybe the receptor is extremely low expression.

David Marks: Even for GPCRs, there are really two different types of drugs that you're looking for. The issues that we've been discussing is really only relevant for if you're interested in agonists. If you're interested in antagonists, it really doesn't matter if it's a heterodimer or a dimer, as long as you're interfering with one of the ligand interactions with the receptor.

Kevin Pfleger: It also depends how you define antagonists.

Martin Valler: There's an increasing tendency toward inverse agonists.

Arthur Christopoulos: Depending on the same receptor constant, whether it's a mono or a dimer, the same receptor, the same ligand it could be an inverse agonist on pathway A, a positive agonist on pathway B, neutral for pathway C, in the same cell type. And then, a ligand is a ligand, maybe we should come up with a new term called 'affinist' – anything that has affinity. I'm not really proposing we do that.

What does this mean? I have no idea what this means other than a headache for the screening, it means you've got to have multiple ligands for the one target, for the one library unless you can predict where... I mean the interesting thing is that you may find the compounds cluster. If you do a large scale screen you may find compounds cluster. This cluster will give us inverse agonism here, positive agonism there. If this cluster gives us only inverse agonism, then you might take a small subset of those, and they're the ones you take forward for a more detailed approach.

David Marks: Yes, that's another issue that you brought up, that is, even for antagonists there could be inverse agonism. In the past, that really hasn't been investigated very much. So I think that moving forward, that should be more interesting, looking at how to do that.

Arthur Christopoulos: There's an element of aestheticness, you know what I mean? A mechanistic... you don't like not understanding how these things tend to work but there is an element of empiricism when you're screening a large number of compounds, and you can see... you can fingerprint compounds in these sorts of profiles without necessarily knowing all the mechanisms and that may be sufficient to take things forward. In other words, compounds that make this go up, this go down, and this do nothing, may work *in vivo*, whereas compounds that go in that direction don't work *in vivo*. I don't know why, but I already know they work *in vivo*, then I'll spend time trying to work out why.

blank

Martin Valler: Those sort of efforts will certainly be supported by the structural biology, the structural studies of binding. It's not going to be overnight, but stepwise, people are going to come forward and say, this class of compound, will interact with this residue, and we can do pharmaco modeling on this class of compounds, and we know this receptor is involved, and this class is somewhere else, and on that basis you could separate potential mutual agonism, inverse agonism, allosteric modulation...

David Marks: But ultimately, you still need the cell based assay to tell you that. Without the cell based assay, you have all the binding assays, all the structures you want, you still don't know what the interactions give you.

Martin Valler: In the ideal case, you just go, you just put all the one million, two million compounds straight into some inflammation model in some animal, it's not realistic is it?

Richard Eglen: Do you think where people are with dimerisation, is where people screening for allosteric modulation was ten years ago, it's a reflection of the maturity of the field of knowledge in the way in which you do it? There were clearly allosteric modulators known since the Fifties, at GPCRs, it's just that as the field developed, they became legitimate targets for new avenues of drug discovery, and then all the molecules that had this mode of action assigned to them. Because I think now, screening for allosteric modulators of GPCRs in a cell based assay is even a legitimate activity in HTS.

Arthur Christopoulos: There are two compounds that have made it to the clinic on the basis of allosteric modulators. I'm not following all the trials, but I know of at least 14 from various Phase II-plus trials. Yes, that's a legitimate approach, though there are still significant challenges.

Richard Eglen: I'm just thinking about your point about assigning large clusters of compounds to modes of action. I think that was one of the approaches, to screen compounds that had a particular mode of action, subsequently assign a mechanism.

Arthur Christopoulos: We're doing that now on a small scale. We've got allosteric ligands that now engender stimulus bias in endogenous agonist of the GPCR... And we've got examples of things where one potentiates multiple pathways, we've got another one that potentiates these pathways but inhibits those pathways in the same receptor, in the same cell type. What does an enhancer of ERK, and an inhibitor of calcium mean? I don't know, but I'm going to stick them in a model, and I'm talking about receptors so we're looking for neuropathic pain. We're going to stick it in the animal and find out. We have clusters, we just have to find out how the signals work. I can't get beyond that. But I couldn't have gotten there without the cell based assays in the first place. And multiple cell-based assays. I'd love to see one multiplex, whether it's high content, or however they're going to do it, when you see everything going up or down.

Kevin Pfleger: Just to throw an idea out there – how does physiology work? Do we, are we really looking at receptor systems that have evolved over millions of years, where you hit it with an endogenous agonist, and you get a cascade? Or do we have such intricate evolution where you hit it with a ligand, there is a cascade, but there's also an opposite effect. Because then you can get homeostasis. So you're looking at different pathways, which actually in the body, give you this balance. And then in pathologies, you can actually get an upset of that balance. And you need to re-set that modulation. So if you're just hitting it with a sledgehammer, and you block the receptor, and just shut it down, maybe you're actually removing the body's own homeostatic side to its evolved function.

Richard Eglen: I think that's a very good point. We see an increased amount of cell based assays using primary cells, or even induced pluripotent stem cells, because people are looking for doing the screening in the situation that mimics the disease, and not do screening in normal tissue, because of that point. Now that point I made about kinases is exactly where kinases have gone. People want to do screening in cell types from tumors that have the kinase receptor mutation.

Arthur Christopoulos: That makes sense. If you can take a diseased tissue from the patient, and screen on that, that's your ideal, right?

Kevin Pfleger: I mean you want a drug, ideally, that somebody who has normal physiology can take, and it won't do anything. But if they've got a disease, like cancer, they take it and they have a beneficial effect.

Guido Zaman: But you were also probably also referring to the mechanism of receptor desensitisation.

Arthur Christopoulos: We have an allosteric modulator that potentiates just about every pathway we looked at, but it potentiates internalisation better. So now the question is, when you take this modulator into an animal, and you see a lack of *in vivo* efficacy, is that because it's switching things off better than it's switching things on? So that's a new headache to consider.

Guido Zaman: And do you study that in primary cells?

Arthur Christopoulos: We do. We've done it in both.

Guido Zaman: Do you think it will make a difference in the end?

Arthur Christopoulos: I don't know, because this is still a relatively new issue in some regard, because on top of this – not the one I just mentioned – but we also have species of variance in the allosteric pockets. And so now the next question is, if you find a compound from whatever cell based screening you use, then you've got to take it into a preclinical animal model, and if it fails in the preclinical animal model again, is it because the target or the compound's wrong? Or is it because they're right, but because the pocket in the species is different, and we've found that too, with the same modulator.

Guido Zaman: Even without allosteric modulators, with even [autosteric], we have species differences.

Arthur Christopoulos: Yes, and that's been very well known, and I think a lot of people have forgotten that the same thing could apply to an allosteric site, if not even more so because it doesn't have the pressure to conserve?]. So how many *in vivo* models have failed not because the compounds aren't good, but because of lack of activity. So if we seduce the animal with a little bit more of an agonist, all of a sudden we see activity. So to me that says the compound can work, provided there's enough tone, and provided it's the right sort of agonist. Because the problem with allosterics of course, is that if you change the agonist from the autosteric site, you change the natural allosteric direction. That's called probe dependence. That's another headache. So all these things are converging, happening at the same time. So our approach now, whenever we are serious about a target, or a series of molecules, we actually screen at the cell-based level, on the different species as well, that we intend on moving to an animal model.

David Marks: Yes, we do the same thing as well.

Martin Valler: Because otherwise you could end up with a compound that's basically good, but just not developing, because your validated animal models just don't work.

Richard Eglen: So do you think then there's a case to take receptor pharmacology back 20 years, and if I look at the holistic approach, that is, either look at changes in the cell phenotype, as a response to a pathway, or look at some cellular change many of the label free technologies are directed at... and to hell with the pathway, tell me what the cell does, and combine all these different mechanisms...

David Marks: But the problem is you have to figure out how each class of molecules is going to work. You're going to end up by finding a lot of different things, that may not be acting on the receptor, maybe somewhere else, and how are you going to figure that out?

Arthur Christopoulos: No matter what you have to validate the target.

Richard Eglen: I think it's emerging. Let me give you some examples. We are working with groups that are looking for HIV infection and they're doing this... with human cells, disease-relevant cells, and they screen for changes in the cell phenotype. And they have then defined six or seven different cell phenotypes, and then defined to those discrete mechanisms of action. And then they've gone off to their chemists and done SARs around mechanisms of action on each of these phenotypes. But they did such a broad net on the initial screen, they found all of these leads, and the challenge was to ascribe them to a mode of action for the SAR, so it wasn't finding the leads that was the problem, it was the chemistry of the follow up activity for the therapeutic.

David Marks: What I was saying earlier is that we've done a small screen using a label free type approach, compared to a FLIPR approach, and we found that there's only a one percent overlap of the hits. So you could take that to mean that with the label free approach you find a lot of other things but we don't know how it works.

Richard Eglen: Or even if it's a hit.

Martina Bielefeld-Sevigny: Generally it could be said that there are only a few GPCRs that are successful drug targets. There's only a certain group around the drug targets, or don't we have the methodology to detect that they are? Or did we target in the past always the easy ones? And the complex ones, we don't just have the methods to find them?

Kevin Pfleger: I mean the chemo guys are a good example. They're not aware of any compounds, everybody's looking at them...

Martina Bielefeld-Sevigny: So GPCRs can really come back as drug targets. We really see that it's going down and why is it going down?

David Marks: I don't think it's ever gone down, based on our own experience internally. It's never gone down. We've always had GPCR targets every year.

Martin Valler: For us it's a major part of the pipeline and it's remained so for some time.

Arthur Christopoulos: The 30 percent that everyone quotes of drugs on the market attributed to GPCRs they're almost all Family A. All these others, and there's still quite a number of Family A's that haven't been tapped, but there's no shortage of GPCRs to drug, I think.

David Marks: I still think a lot more work on Family B as well.

Robert Jordan: Talking about the vendors. Do you think it's feasible, or even warranted, to develop a universal cellular assay platform for GPCRs around one technology? As some vendors seem to be suggesting it's possible to do.

Richard Eglen: Speaking as a vendor... I'll get my retaliation in first. Our perception at least is you can have what are promoted as universal assays but I suspect, as something David said earlier, is that many people want to take a battery of approaches to screening that either narrow things down to a discrete pathway, or a different mode of measuring the interaction, and then do the cross-calibration. Because quite clearly now as one develops separate panels of assays, one is finding different libraries of compounds emerging as leads. And the question is, are these all new varieties of leads, or are they leads at all? So that needs to be decided. I still remember the days when measuring change in muscle contractions was a readout for GPCRs.

Robert Jordan: That's a pretty good readout.

Richard Eglen: It's not bad! They've found some very successful billion-dollar drugs. The point is, now of course, one has to deconvolute how did it modulate that particular physiological response to its mode of action? Which is why I raise things like the label free. Are you looking at some holistic change that maybe we're not too worried about how it's doing it as long as it does it. So I would say at least from our perspective we're developing a range of assays for a range of different systems seems to be what people want instead of a universal cure type of technology. Maybe when there's crystal structures, maybe when there's docking type things developed, that may change some things, but perhaps not yet.

David Marks: You have the crystal structures. I would argue that still doesn't change the need for GPCR assays that are coupled to different pathways, so you can measure its activity through different pathways – you still need that to understand what changes in the structure interaction mean in signal transduction.

Richard Eglen: I mean, Guido, you do a range of assays in your setup?

Guido Zaman: Yes, we prefer to screen with primary assays for physiological relevant readout, and still an artificial readout can be very useful in the primary realm, but then we are going to confirm and test particular lead compounds in a range of signals and possibilities. With respect to label free technology, I also think for now, this can be placed when you have a lead series that you know modulates a certain target but you want to confirm it for physiological relevance, then that indeed does what you want it to….

Martin Valler: Is there any advantage to having multiple assays as well? At this stage, nobody's got the full answer. It would be very risky to say this is the one assay that will deliver... the general feedback is do you want more information in this situation which is becoming complex not less complex and every day you get different ideas and feedback. I think people want more information, not less.

Kevin Pfleger: I say you've got two ways to go with

that. You can either do multiple assays in parallel, or you can go to multiplexing where you've got assay systems that can be run on the same cells, which have different components in them.

Arthur Christopoulos: Let me ask, how common is one versus the other? I suspect that multiplex is not that common?

Guido Zaman: Multiplexing I think it's not always easy, because you have different kinetics of the assays, sometimes you have in the same cell line you have different DMSO sensitivity, for instance, we have done beta arrestin cyclically in one cell line that had a different DMSO sensitivity... because you use a second line, it's not essential to do it multiplexed. You can do it separately. There are many examples of screens, primary screens that other companies do, where you use multiplexing, and have the second signal as the internal control.

David Marks: We're not doing multiplexing. We find that trying to do multiplexing is too complicated in terms of assay development, and making sure that you don't have interference against one and the other. I say it's simpler to run two assays.

Martin Valler: Same with us. The assay development has to be optimised enough for one readout. To optimise for two, there's a danger maybe you miss something in the middle.

Arthur Christopoulos: What about high content? Do you guys do high content work?

Guido Zaman: Yes, also on a smaller scale, and particularly now we have these alternatives which are compatible with HCS technology. It's not necessary anymore to look at beta arrestin with high content.

Kevin Pfleger: So the reason you're not doing multiplexing at the moment is that the compatibility of the assays isn't very good. If you could get good compatibility of assays to multiplex, is that something you'd want to do?

Guido Zaman: Yes, if somebody has developed it as such, yes. And I know from the literature that R&D companies have completed a screen on two readouts at the same time.

Kevin Pfleger: So essentially you need assays that are not cytotoxic, and not affecting your cells too much, and therefore don't interfere with the other assay.

Martin Valler: On multiple levels of optimisation, the behaviour of compounds, the behaviour of DMSO, incubation times, signal levels, the readout modality itself, the switch between readers to get the second readout. On multiple levels, in our hands, it hardly ever works. It's not easy to do a multiplex assay. It takes twice as long in development, and the screening is generally quicker than the development process.

Guido Zaman: We didn't spend too much time on it because we didn't think we could gain too much time with it.

Kevin Pfleger: Maybe someone else needs to do the development, and then you guys...

Martin Valler: Yes, that's also a wish, that somebody can develop to your target for you.

David Marks: In automated systems, we sometimes run two parallel assays at the same time, instead of running a multiplex assay, or duplex assay.

Richard Eglen: One of the things to your point that Arthur mentioned about imaging that data that's emerging, we're seeing people look at imaging as a precursor to a lot what we've talked about. The field of HCS at induced pluripotent stem cells, to find small molecules that control the differentiation then as a prelude to finding authentic cell types. It seems to be rapidly emerging, and that's where small molecules, stem cells, and imaging are coming right together. And I think that will evolve into science to help provide cell types that may answer some of the points we've made here today, where you are now in a series of cell lines that have authentic human relevance in reproducible fashion that may answer some of these questions. There's clearly a couple of years before that happens, the question is finding these things and controlling their state of differentiation. Now that may be an activity best done in a series of university labs, or in big pharma, I don't know, but it's clearly something that's going on.

Richard Eglen: Just a general question mainly to you guys in pharma. Do the compounds come from general libraries? Or from enriched, substructural searched libraries? Is there a chemistry virtual screening step before dive into it?

David Marks: It depends on what kind of family class you're talking about. With GPCRs, I don't think anybody really has a good idea how to make

a GPCR specific like that. It's just too diverse. But kinases are different. The kinase... it's really just the ATP binding site type libraries.

Richard Eglen: So there's no allosteric-rich fragmented library or something like that?

David Marks: No.

Arthur Christopoulos: Having said that, I'm aware of at least one or two instances where colleagues in pharma have said to me they've gone back and enriched a focused library for allosteric modulators for a given target.

David Marks: Oh, for one target, yes. Not for general kinases, no.

Arthur Christopoulos: And if you could do that, I think there's something wrong with your concept.

Martin Valler: For us, after the primary screen we go back and identify classes which could be enriched by external purchasing of compounds, but I'm sure the compound collection gradually assumes and increases the GPCR relevance.

David Marks: Because screening is really not a bottleneck at this point, we really don't try to restrict our screen to a very small selective class. Just for an example, we have seen a number of leads come out of what we have classified as a traditional kinase library. We find very good GPCR antagonists out of this. So we wouldn't restrict ourselves to non-kinase libraries when we screen for GPCRs, so we wouldn't want to miss those.

Martin Valler: Exactly, it's not a bottleneck anymore, and we want the full data picture at the beginning to really pick the best. And the best is really the core structural types, the phenotypes, the class of the compound, but of course to go further, it's not just only the binding or activity, it's also the developability of the compounds, they have to have a synthetic advantage and can we actually make the compound and variations.

Robert Jordan: There's been a lot of hype recently about assay-ready, cryo-preserved frozen cells, aliquots... are there any downsides to that approach, do you think?

Guido Zaman: I don't think so. What we've done is carefully examine these cells, and look at what the quality was, and in many cases, they really seem to work well. So the pharmacology was fine, the background was fine, and you could use your own cryopreserved stocks, or in one case we have also bought stocks from a frozen cell providers. But we find it doesn't work for every receptor or every assay, probably because it's related to that receptor, sometimes we need to grow the cells a few days before we use them, or it doesn't really work, or we don't have the time to optimise it. But it's common to use them in our organisation, not just for HTS, but also for head and lead optimisation. You can use special cells for a very long time because projects take a long time and you want to have consistent results.

David Marks: For selectivity, I think that's really an advantage to use cryopreserved cells.

Martin Valler: We've taken as our default strategy for assays, initial analysis can we produce frozen cells do they provide the same data as cultivated cells and in most cases that is the case. So that is default. The quality advantage is the batches are the same, we can control with a fair degree of confluence, and the quality control is better from day to day. We employ them predominantly for primary screening.

Richard Eglen: The only thing that may come up on the horizon is if the world used screening in primary cell lines, what would it mean for primary cell lines to cryopreserve them, some of the more esoteric cell types are a bit more finicky. **DDW**