KINASES – old dogs and new tricks

application of new assay technologies to shed light on kinase drug discovery

The human kinome comprises 518 known protein kinases and more than 20 lipid kinases. Nearly all aspects of control within a cell are modulated by reversible phosphorylation of proteins, mediated by protein kinases.

By Dr Gary Allenby

Abnormal phosphorylation is a cause or effect of many diseases including cancer and inflammatory diseases, hence many pharma and biotech companies continue to undertake research and development in this area believing it to be of clinical importance. While we can congratulate ourselves on our success in identifying more than 37 kinase inhibitor compounds that have made it to patients and more than 250 drug candidates that are undergoing clinical evaluation, arguably we have only scratched the surface of kinase biology. A seminal paper[1] published in *Science* in December 2017 provides an interesting insight into drug discovery within the kinome space and, to use the space analogy, helps us to realise that at best we have only explored our own kinase solar system, let alone our galaxy and what lies beyond in kinome space.

Within the paper, Klaeger et al used a chemical proteomic approach (using kinobeads) and quantitative mass spectrometry to characterise the drug discovery space of 243 clinical kinase inhibitors that are either approved drugs or have been tested in humans across a wide variety of protein kinases. Interestingly, although many kinase inhibitors are claimed to be both potent and selective, the majority are not, resulting in failures in the clinic and obstacles to the use of these compounds in basic research as tools to investigate kinase biology. Selectivity does not appear to correlate with clinical efficacy or safety. Some compounds such as rabusertib, a very selective CHEK1 inhibitor, show exquisite selectivity (rabusertib was developed by Eli Lilly but dropped due to its apparent lack of activity in any clinical setting). Other examples of selective inhibitors include capmatinib for MET and lapatinib for EGFR. However, many more inhibitors are less selective, targeting more than 100 kinases simultaneously and making it impossible to attribute a specific inhibitor activity to a particular clinical outcome. Of the 243 compounds examined in this paper, approximately 15% were reasonably selective, but selectivity decreased rapidly to a class of almost non-selective compounds such as midostaurin and XL-228, interestingly with approved and non-approved clinical candidates at both ends of this selectivity spectrum.

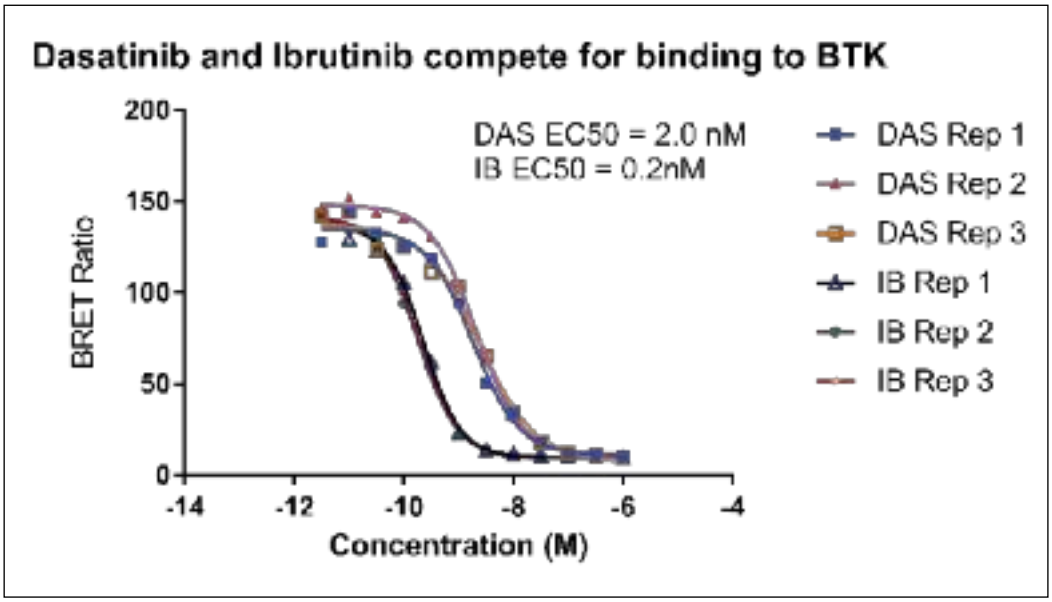

Figure 1
Binding of Dasatinib (DAS) and Ibrutinib (IB) to Bruton's Tyrosine Kinase (BTK) Nanoluc. HEK293 cells were transiently transfected with a Nanoluc-BTK construct. Cells were incubated with a fixed concentration of tracer for two hours then treated with DAS or IB as a dose response. The degree of loss of luminescence signal is proportional to the binding of DAS or IB to the BTK receptor. Each curve is a separate replicate on the same 96-well plate

A survey of the scientific and patent literature (ChEMBL, SciFinder and PubMed) revealed that many of the 243 clinical kinase inhibitors examined in this study are poorly characterised in terms of their selectivity. The process of assessing selectivity is both costly (often outsourced to CROs) and time-consuming and therefore not often accomplished in a comprehensive manner during drug development. Rarely is a complete profile of activity of the compound studied in detail across many kinases at many compound concentrations. In an effort to bring a degree of clarity to this area, Klaeger et al profiled the activity of numerous inhibitors in a dose-dependent manner on many kinases at near thermodynamic equilibrium in material isolated from cellular lysates using a kinobead approach. This binding data enabled the development of a new selectivity metric termed CATDS (concentration- and target-dependent selectivity), a metric capturing aspects of both target binding and drug mechanism of action. "CATDS measures the reduction of the binding of a particular protein to kinobeads at a particular compound concentration relative to the summed reduction of binding of all proteins at that concentration". CATDS values close to one or zero indicate either selective or non-selective compounds respectively. The group has published this data in an interactive database called ProteomicsDB (www.proteomicsdb.org) or as a PDF on ProteomeXchange (www.proteomexchange.org). This represents a great resource for kinase hunters and candidate drug reprofilers.

The kinobead approach is a competition assay followed by a 'pull down' then analysis of bead bound material using mass spectrometry. Briefly, beads labelled with kinase binding compounds are incubated with cell lysates in the presence of test compounds at different concentrations to allow competition to occur between the test compound and the bead bound compound for binding to the kinase derived from the lysate. Kinase captured by the bead is precipitated, eluted from the bead and analysed by LC-MS/MS. Specific compound binding to a specific kinase will sequestrate that kinase, preventing it from binding to the bead and thereby decreasing the amount of that kinase being pulled down in the assay. Non-specific compound binding will generally inhibit the binding of kinases to the beads and result in a more non-specific pull down result. A disadvantage of this technique is that it utilises the promiscuity of the ATP binding pocket to pull down kinases and therefore has limited use as this approach is aimed at Type I compounds binding active enzyme.

More traditional screening assays designed to detect compound binding to protein kinases tend to be biochemical in nature using recombinant kinases, either full length or partial sequence. While these assays can give highly accurate, reproducible binding kinetics, they fail to predict how a kinase may function inside a cell. Differences in localised ATP concentration inside the cell, the use of truncated kinase domains, absence of appropriate cellular co-factors and differences in kinase activation states can result in the development of compounds that appear potent in a biochemical assay but this activity

does not translate to cellular activity. Biochemical assays often do not recapitulate the physiological complexity of kinase biology encountered inside an intact cell, be it a normal or diseased cell. There is therefore a need to examine the properties of kinase inhibitors in a more physiological cellular environment rather than an isolated biochemical system.

Biology is digital, pharmacology is analogue…

In a cell, it costs 'money' (ATP) to phosphorylate proteins. This process is thermodynamically expensive, therefore it is unlikely that a cell will generate concentrations of phosphorylated proteins. A cell will manufacture a protein, phosphorylate it, transmit the signal and await a digital return signal – a feedback loop. If no feedback signal is forthcoming then the cell will manufacture and phosphorylate another protein and continue this process until the system returns a digital stop signal. Cellular kinase pathways are constantly switching on and off in this digital manner controlling cell functions. Proteins are dephosphorylated and rephosphorylated or degraded and remade. Each phosphorylation event is a digital signal, on or off, one or zero. However, in pharmacological we think in terms of concentrations of proteins and concentrations of compounds. Who hasn't run a Western blot to correlate a cellular response to a change in concentration of a specific protein only to find the correlation was poor or non-existent? As demonstrated in this paper, digital signals are much more complex. The authors analysed the phosphoproteome (all phosphorylated proteins) of BT-474 cancer cells after treatment with the EGFR/HER2 inhibitors lapatinib, afatinib, cancertinib, dacomitinib and sapitinib to a depth of approximately 15,000 phosphorylation sites. Analysis revealed a large number of statistically significant regulated phosphorylation events for each drug with more than 200 common phosphorylation events across all five compounds. Arguably this 'shared' commonality profile between the compounds could equate to the 'therapeutic footprint' of these compounds represented in BT-474 cells, but what about the other 14,800 phosphorylation events, what do they tell us? Furthermore, the experiment literally represents a snap shot in time and drug concentration, the cellular environment at the exact moment in time when the experiment ended and the cells were lysed.

When developing compounds in the pre-clinical phase of a kinase drug discovery programme, both biologists and chemists tend to drive structure-activity relationships (SAR) through league tables of kinetic binding data in an attempt to increase the potency of compounds and drive selectivity through affinity. This is followed by testing of a few chosen compounds in a selectivity panel of representative members of kinase families tested at selected doses of compounds to give a generalised selectivity profile. Subsequently, cellular penetration *in vitro* is followed by drug disposition, pharmacokinetics and metabolism in a well plate based assay followed by *in vivo* studies. Techniques such as Surface Plasmon Binding (SPR) are often used to study the interaction of compound and kinase. In addition, biochemical kinase activity assays are

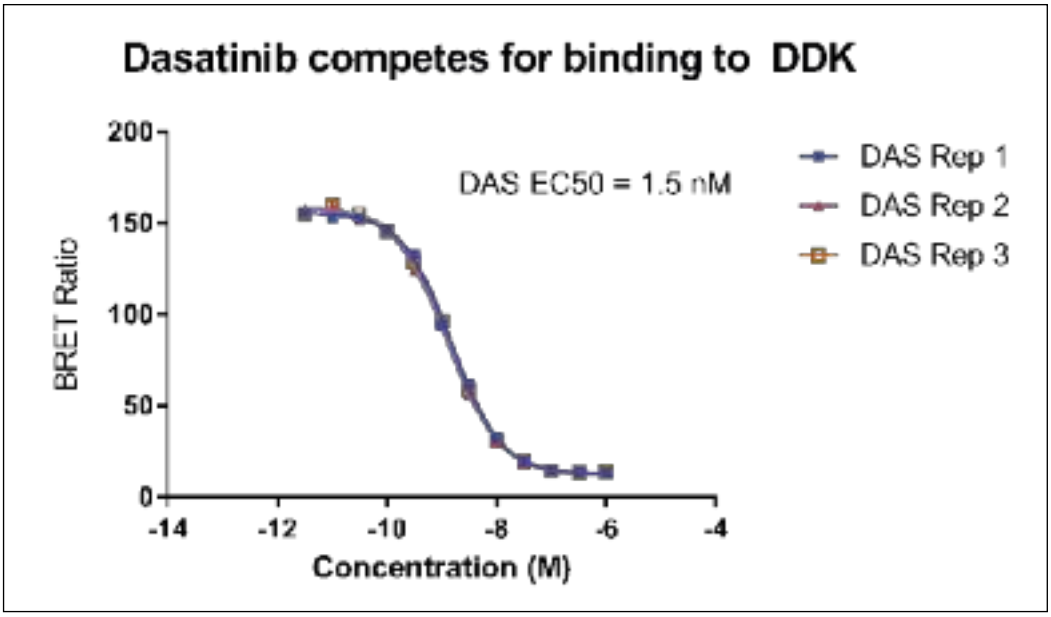

Figure 2
Binding of Dasatinib (DAS) to Dbf-4 Dependent Kinase (DDK) Nanoluc. HEK293 cells were transiently transfected with a Nanoluc-DDK construct. Cells were incubated with a fixed concentration of tracer for two hours then treated with DAS as a dose response. The degree of loss of luminescence signal is proportional to the binding of DAS to the DDK receptor. Each curve is a separate replicate on the same 96 well plate. Ibrutinib was also tested but showed no competition with the tracer on DDK (data not shown)

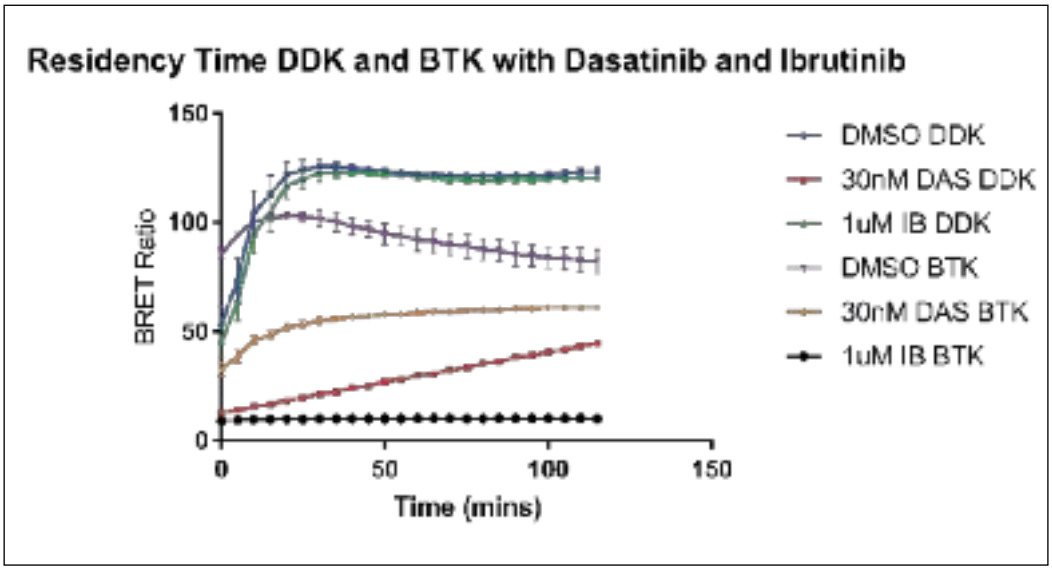

Figure 3
Bruton's Tyrosine Kinase (BTK) and Dbf-4 Dependent Kinase (DDK) Nanoluc constructs were transient expressed in HEK-293 cells in 96-well adherent format. Cells were treated for two hours with Dasatinib (DAS) and Ibrutinib (IB) (30nM and 1uM) then washed to remove the compounds. Respective tracer for each kinase were added and the rate of dissociation of the compounds measured every five minutes. Data represents mean +/- standard deviation for n=4 replicate wells per treatment

often run using radiolabelled material in the presence of physiological levels of ATP. More recently, techniques such as affinity-based chemoproteomics use cell lysates as a source of kinases followed by treatment with compound then LC-MS analysis of material bound to the kinobeads. Cellular kinase-based assays include cellular thermal shift assays (CETSAs) that do not require cell lysis during compound incubation but are dependent on the compound stabilising the kinase protein prior to heat treatment and analysis. However, CETSA does not provide a quantitative measure of drug affinity. Other assay technology providers have kits designed to detect kinase activity in cell lysates isolated from pre-treated cells. These provide a snap shot in time of activity inside the cell at the point of lysis but do not provide kinetic data.

All of the above assay formats generate data on binding, either reversible or irreversible, and potency of a molecule against the kinase of interest either as a recombinant protein or within a cell lysate, either before or after treatment of the cells with a fixed concentration of compound. None of these approaches can determine the duration of time the compound was in contact with the target kinase. Consider you are part of a kinase project development team and were presented with the option of choosing between two compounds that bind the target kinase, one compound has binding affinity of 1nM and residency time (eg the duration the compound occupies the binding site of the target kinase) of, say, 10 minutes, versus a second compound with binding affinity of 100nM and residency time of five hours. Both have equivalent cellular penetration and DMPK properties, which one would you choose to develop? My choice would be the compound with the longer residency time. Why? In a digital world the inhibition of the digital signal for a longer duration may have a more profound effect on the kinase pathway than simply the binding affinity. In this digital world what are the implications for selectivity versus affinity versus residency time? Is residency time and selectivity all part of the same puzzle? For example, if a kinase inhibitor is relatively non-selective but has a residency time favouring the inhibition of the target kinase does this make it a better drug? This target engagement profile defined by both the on-rate (binding) and, equally important, the off-rate (residency) of the compound, for both the target kinase and other kinases inside and outside the target kinase family may represent a more clinically efficacious measurement of the activity of the compound. Furthermore the target engagement profile may, in part, explain the mechanism of action associated with off target safety concerns.

Target engagement parameters are fundamental to drug efficacy. It is the affinity and residence time at primary and secondary target kinase that can often underlie the therapeutic potential of lead candidates. However, it is debatable if target potency or binding kinetics are most relevant to establish intracellular SAR for a lead series of compounds. Drug efficacy is significantly influenced by drug residency time – ie the associative on rate (kon) and dissociative off rate (koff) of the compound interacting with the target kinase. It would be desirable to perform target engagement in live cells in a kinetic manner, using full-length kinase pro-

teins without disrupting the cell membrane. It would also be desirable to evaluate other kinases in a similar manner to generate a profile of kon and koff for compounds to determine residency time selectivity and add value to the decision process.

With recent advances in assay technology it is now possible to study target engagement and residency time in live cells in a kinetic manner[2]. By using a bioluminescence resonance energy transfer technology (NanoBRET) designed to generate light (photons), coupled with a cell-permeable energy-transfer probe (fluorescence tracer) that reversibly binds the active site of the kinase, the interaction of the tracer with the binding site of the kinase can be examined in real time in live cells. Cells are transiently tansfected with a construct composed of the full-length kinase cDNA coupled to a small (19KD) Nanoluc® luciferase cDNA. When the cells are treated with the Nanoluc substrate, photons are generated and detected at 460nm. On administration of the fluorescence tracer to the cells, this molecule binds the kinase, bringing the tracer into close proximity with the Nanoluc generated photons and resulting in BRET. The fluorescence signal is detectable at 600+nm and the result can be expressed as a ratio of the 460 to 600+nm signal. Binding of test compounds to the kinase can be examined by incubating the tracer at a fixed concentration with the cells, followed by various concentrations of a test compound, to observe the decrease in the BRET signal as the compound competes the tracer from the kinase. Residency time can also be studied by first incubating a fixed concentration of test compound with the cells, then washing the cells to remove the compound from the well followed by the addition of a fixed concentration of tracer. The rate of dissociation of the compound can be detected as an increase in BRET signal over time as the tracer competes for binding to the kinase. By using this approach both binding and residency time of test compounds within the native environment of the cell can be determined in a kinetic manner. Furthermore, the addition of the same concentration of compound to other residency time experiments using other Nanoluc kinase constructs allows for the development of a 'picture' of both binding and residency time of the compound in cells and may add value to the selectivity versus non-selectivity question for many of the 243 clinical kinase inhibitors.

Comments...

I applaud the authors of this paper for shedding some light on drug discovery within the human kinome. There is overwhelming evidence to suggest that mutations in kinase proteins result in modifications of phosphorylation that in a clinical setting manifest themselves as cancer and inflammatory disease. With 37 kinase inhibitors in the clinic and more than 243 being developed, it is clear that the scientific community recognises the potential to alleviate these diseases with both small molecules and biologics to target kinases. New and innovative technologies, when combined with past and current technologies, can help to add value to historic data and shed light on our understanding of how compounds can be developed in the future. In addition, it is also possible to repurpose past clinical candidates that have failed by revisiting their potential using some of these new technologies, such as residency time. It is possible that focusing on residency time in addition to binding affinity and selectivity may lead to a better understanding of kinase biology and better clinical candidates. So let's 'boldly go' into kinase space using all the technologies available to us. DDW

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References

1 Klaeger, et al. Science 358, eaan4368 (2017).
2 Huwiler, et al. Genetic Engineering and Biotechnology News, Vol. 38, No 2 (2018).