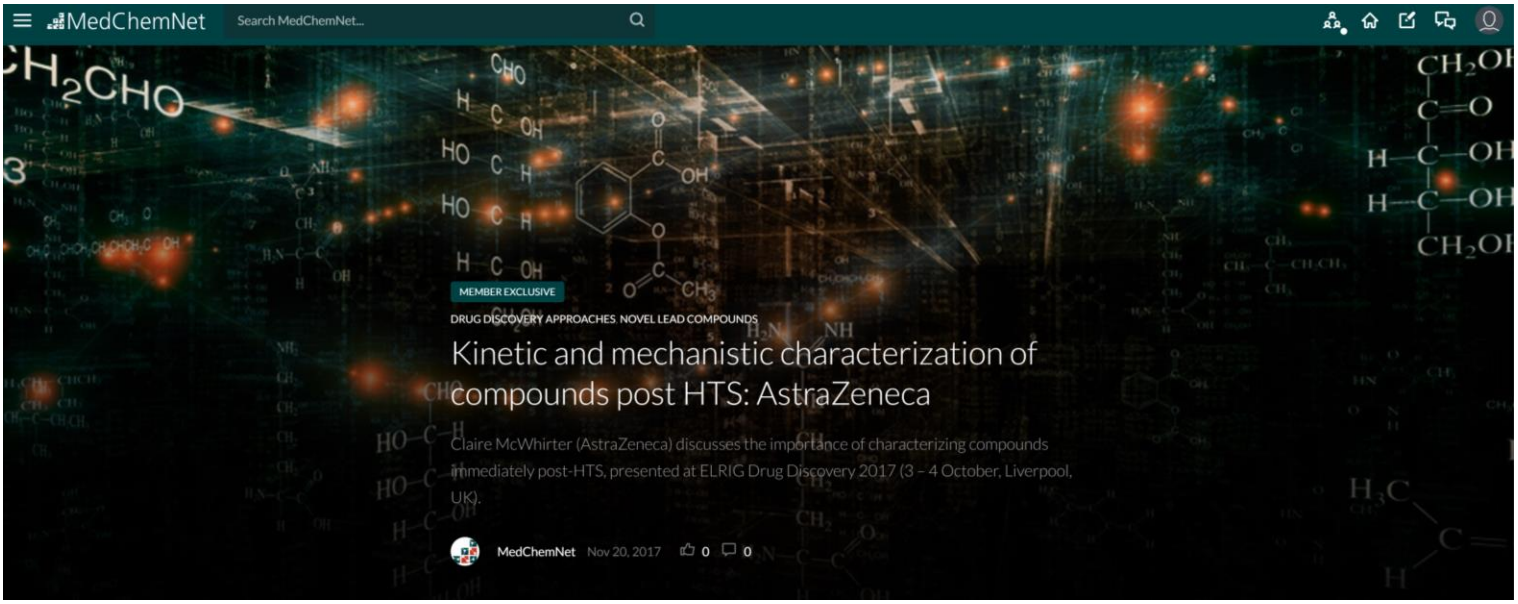

http://bit.ly/2AugVCf

Development of high-throughput assays for kinetic and mechanistic characterization of compounds immediately post HTS

Target-based drug discovery has traditionally used steady-state IC_{50} values to measure and rank compound activity. However, it is estimated that as many as 80% of new medical entities show non-equilibrium kinetics and there are numerous examples (such as FabI enoyl-reductase inhibitors) where *in vivo* activity has been found to correlate with drug target residence time rather than the steady state affinity.[1,2,3]

There is also a need for a detailed understanding of the desired kinetic properties for clinical efficacy as compounds such as anti-psychotic drugs may cause mechanism-based toxicity if the target is inhibited for an extended period. Similarly, for certain proteins such as b2-adrenoceptor agonists, compound rebinding and therefore association rate (k_{on}) may have more influence on clinical efficacy than the dissociation rate of the compound (k_{off}).[3,4]

The realization that compound kinetics can have a substantial effect on both clinical efficacy and safety has led to a recent update to EU guidelines for risk mitigation in first-in-human clinical trials. They must recognize not only the importance of on and off target binding affinities but also receptor / ligand occupancy and kinetics.[5]

Despite its importance, detailed kinetic and mechanistic characterization of compounds tends to be performed on a limited number of compounds during lead optimization as the assays required to carry out such kinetic characterization are often low throughput and labor intensive. There is, however, substantial interest in techniques that will enable more-detailed and comprehensive characterization of compounds earlier in the drug discovery process. This will enable the identification of compounds with differing mechanisms of action immediately after high-throughput screening (HTS), as well as to deconvolute changes in potency on going from enzyme to cell and *in vitro* assays which may be due to kinetics and / or compound mechanism. This has led to the development of plate-based kinetic techniques such as the high throughput kinetic probe competition assay and our own work on higher throughput continuous enzyme turnover assays for kinetic and mechanistic characterization of compounds.[6]

We have used AssayQuant's PhosphoSens® flurogenic peptide system to develop high-throughput kinetic enzyme turnover assays which has enabled us to characterize the kinetics and mechanism of action of compounds immediately post-HTS. Whilst there are some commercially available continuous kinetic assays available for measuring kinase kinetics, we have found that they often require extensive method development, for example to find buffers that are compatible with both the protein of interest and the detection reagents or use complex instrumentation and separation techniques that are maintenance-intensive. PhosphoSens substrates are generated by standard solid-phase synthesis using Fmoc-Cys-Sox (+2 or -2 from the S/T or Y phosphorylation site), with flanking sequence on either side. Substrate phosphorylation and Mg^{2+} chelation create the dramatic increase in fluorescence. As it is the product which directly generates the assay signal, we have found them to be tolerant of a wide variety of assay conditions including variations in pH, selected metal ion cofactors, enzyme, substrate, and low to physiological (mM) ATP. This translates into the ability to develop kinetic assays quickly from existing HTS assays, thereby enabling the kinetic characterization of compounds immediately post-HTS.

Case study 1: Compound mechanism of action determination

Whilst the majority of approved small molecule kinase inhibitors are ATP-competitive active-site binders, there is substantial interest in identifying inhibitors that act through an ATP non- or

uncompetitive mechanism. The value of this new generation of kinase inhibitors is the ability to overcome difficulties with enzyme-to-cell drop off due to high physiological concentrations of ATP and poor selectivity due to a highly-conserved kinase active site. Importantly, these new drugs provide an alternative mechanism of inhibition to counter the resistance to ATP competitive active site inhibitors that frequently develops in cancer patients.[7]

To identify inhibitors that may be acting through an ATP non- or uncompetitive mechanism immediately post-HTS, we screened compounds in a standard IC_{50} assay at both K_M and 2 mM (physiological) concentrations of ATP. Compounds which did not display a decrease in IC_{50} in the high concentration ATP assay were further characterized using a PhosphoSens fluorogenic kinetic assay.

Compounds were assayed as an 8 x 8 matrix of varying ATP and inhibitor concentrations using the AQT0104 fluorogenic substrate. Initial rate data was fit to competitive, uncompetitive, non-competitive and mixed inhibition using Graphpad Prim v 7.0, with the best fit determined by AIC (figure 1).

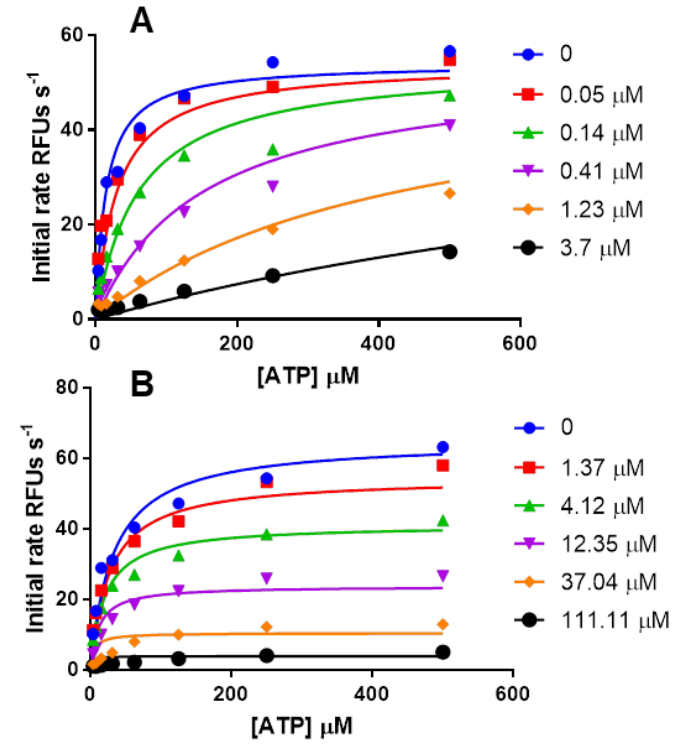

Figure 1: Example kinetic characterisation of kinase inhibitors A) ATP competitive inhibitor K_i = 44±6 nM.B) ATP uncompetitive inhibitor αK_i = 37±1 µM.

Case study 2: k_{inact} / K_I determination for covalent kinase inhibitors

Due to the clinical success of targeted covalent EGFR and BTK inhibitors such as afatinib and ibrutinib, there has been a resurgence in interest in developing irreversible covalent kinase inhibitors. Selective irreversible covalent inhibitors may have several advantages over reversible ATP competitive kinase inhibitors as the formation of an irreversible bond may help to overcome increased competition with ATP at the high concentrations of ATP found in cells, as well as giving prolonged pharmacodynamics.[8] The use of IC_{50} data to drive covalent drug-discovery projects is problematic as the IC_{50} is a composite of both the affinity and reactivity of the compound and is also dependent on the assay duration. Further compound characterization with kinetic assays is required to determine if any changes in potency are due to changes in affinity or reactivity of the compound.

We have developed a kinetic assay using the PhosphoSens fluorogenic peptide system to support an early-stage covalent kinase drug discovery project. In order to support the medicinal chemistry strategy for the project, the assay was used as a weekly screen to quickly determine how changes in structure were affecting the affinity and reactivity of covalent inhibitors from several compound series. All compounds were assayed as an 11-point, 1.5-fold serial dilution with a top concentration approximately 10 x the initial estimates of K_I. The reaction was initiated by the addition of enzyme to a mixture of ATP, peptide substrate (AQT0001) and compound in assay buffer. Using GraphPad Prism, the individual progress curve data for each inhibitor concentration were fit to a single exponential. A secondary plot of k_{obs} vs [I] was used to determine k_{inact} and K_I (figure 2).

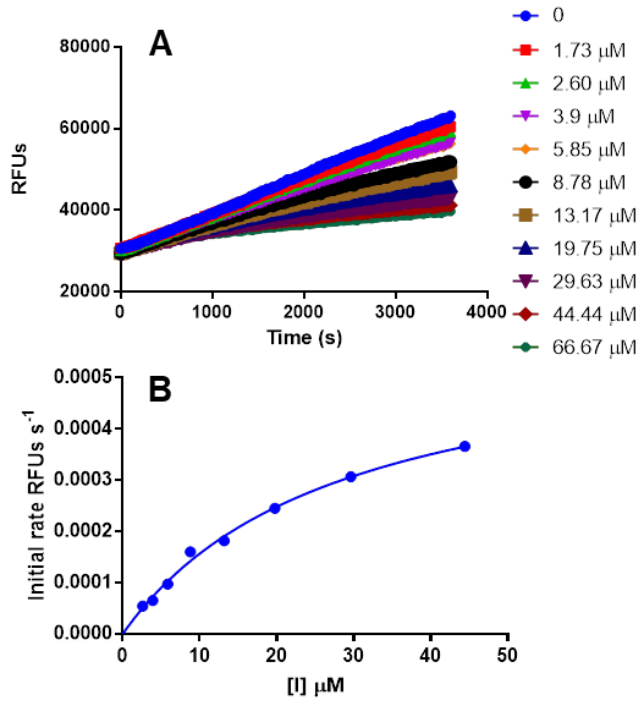

Figure 2: Example k_{inact} / K_I determination A) example time-course data.. B) secondary plot of k_{obs} vs [I] to determine the affinity (K_I)and reactivity (k_{inact}) of a covalent compound.

High-throughput kinetic assays using FLIPR Tetra®

The two case studies described above used a conventional fluorescence plate reader which reads one well at a time with a cycle time for a full 384-well plate of approximately 1.5 mins. Both this and the requirement to start the reaction and begin collecting kinetic data relatively quickly limited our assay throughput to one compound per run for mechanistic characterization and 2–4 compounds per run for covalent inhibitor studies. To help alleviate this bottleneck in data collection, we have developed the PhosphoSens kinetic assays to run on the Molecular devices FLIPR Tetra (figure 3).

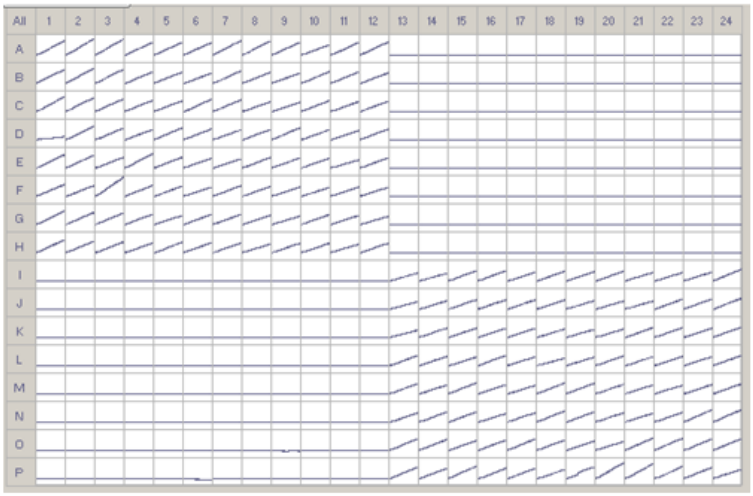

Figure 3: FLIPR Tetra max / min plate Max / min plate responses show consistent signal across the plate.

The FLIPR Tetra offers two key advantages over our existing screening workflow; its 384-pipettor head enables sample liquid transfer to all 384-well simultaneously and it is equipped with a CCD based camera that collects data from all wells at the same time. Collectively, its simultaneous pipette and read function has increased the covalent assay throughput from two compounds to thirty compounds per run. In principle, the assay can be further miniaturized and adapted to FLIPR Tetra configured with a 1536-pipettor head which would lead to an additional increase the assay capacity.

Conclusion

The development of high-throughput kinetic assays such as PhosphoSens continuous enzyme turnover assays means that kinetic characterization of compounds can be supported in the numbers required to identify compounds with differing MoA or slow kinetics in high-throughput. This will facilitate deconvolution of changes in potency on going from enzyme to cell and *in vitro* assays which may be due to kinetics and / or compound mechanism and allow series with differing mechanisms to be identified and developed in parallel immediately post-HTS.

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Further reading

Assayquant: http://www.assayquant.com/

FLIPR Tetra: https://www.moleculardevices.com/systems/flipr-tetra-high-throughput-cellular-screening-system

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