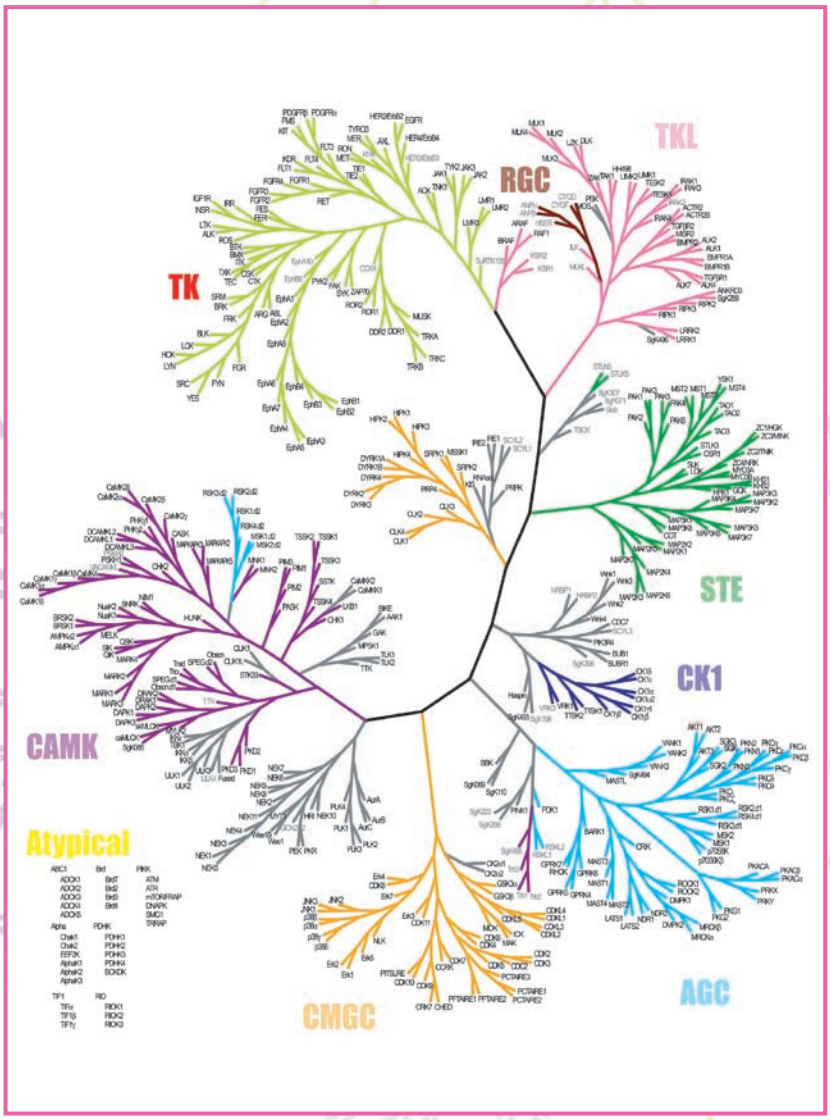
Kinase Assay Development

A Strategy for Comprehensive Kinase Profiling for Drug Discovery



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518 human protein kinase genes have been identified in the human kinome [1]. It is well known that protein kinases are key elements in intracellular signaling pathways that control many physiological processes. Furthermore, it has been demonstrated that the activity of protein kinases are altered in several human diseases such as cancer and autoimmune disorders. In order to develop good therapies based on small molecule inhibitors while avoiding unanticipated side effects, it is crucial that the targeted kinase is specifically and selectively modulated. Profiling techniques, in which the same molecule is assessed across a broad panel of kinases, is an accepted method to evaluate the specificity and selectivity of drug candidates. For assaying kinase activity, there are multiple strategies available. In this article, I introduce the platform technology of the mobility shift assay (MSA), for profiling multiple kinases that Carna Biosciences has adopted to use in our kinase profiling service.

Kinase Preparations for Assay Development

In order to develop a well behaving kinase assay, the purified protein kinase needs to be produced and isolated in a way that retains its activity. We have so far produced over 300 human protein kinases and corresponding assays. We and other research groups have noticed that recombinant kinase proteins expressed in insect cells with a histidine-tag can potentially contain host cell-derived kinase activity [2]. Excluding the possibility of this contaminated activity is critical in kinase assay development to establish accurate kinase profiling. One way to eliminate this issue is to select the specific substrate for each kinase carefully. The other way is to express the kinases with a GST-tag instead. Isolating kinases with a consistent phosphorylation state is essential to avoid batch to batch differences. In addition, for kinetic analysis it is especially important that highly active kinases should be used that give a linear response. Thereat, the kinases ought to be highly activated by including their upstream kinases [3] or dephosphorylating with a phosphatase [4] if necessary.

Assay Development in MSA Platform

The gold standard method for kinase assays is to monitor the incorporation of 33P labelled adenosine triphosphate (ATP) as a measure of substrate phos-

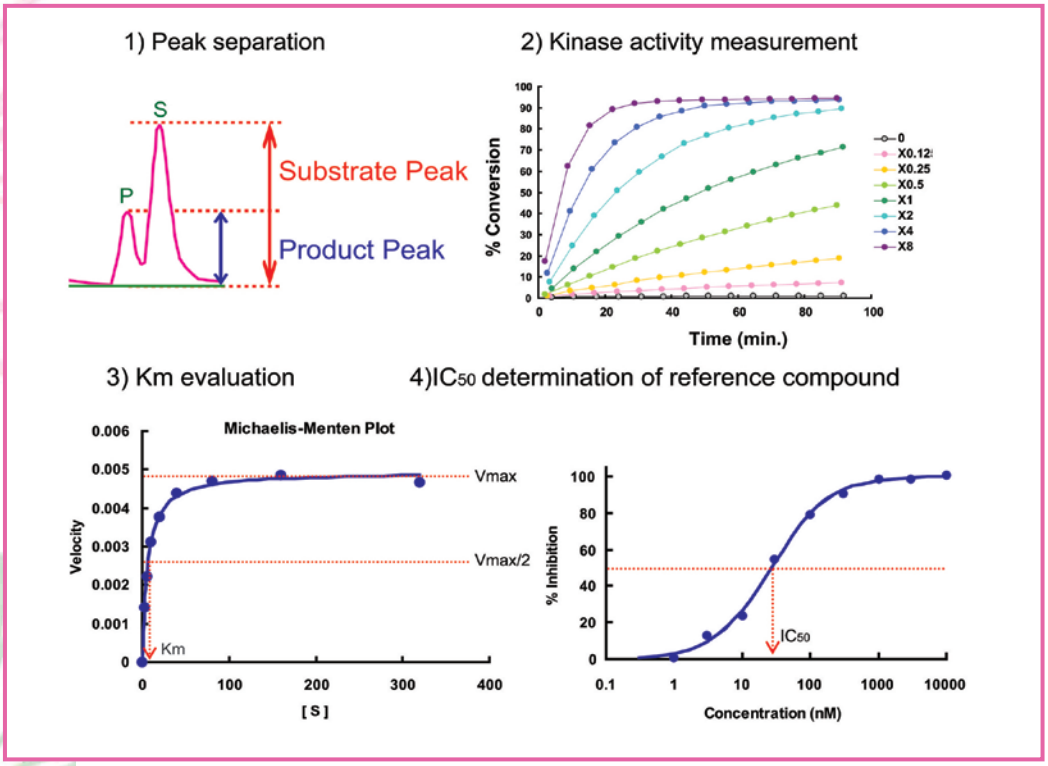

Fig. 1: Assay optimisation for the Mobility Shift Assay. 1-1) Peak represents the substrate and product. The peak height is measured and the % conversion is determined by the following equation; [product peak/(product peak + substrate peak)] x 100. 1-2) Real time monitoring for enzyme linearity 1-3) Km determination with Michaelis-Menten plot 1-4) IC50 value was determined by a known kinase inhibitor. The ATP concentration used was equivalent to the Km.

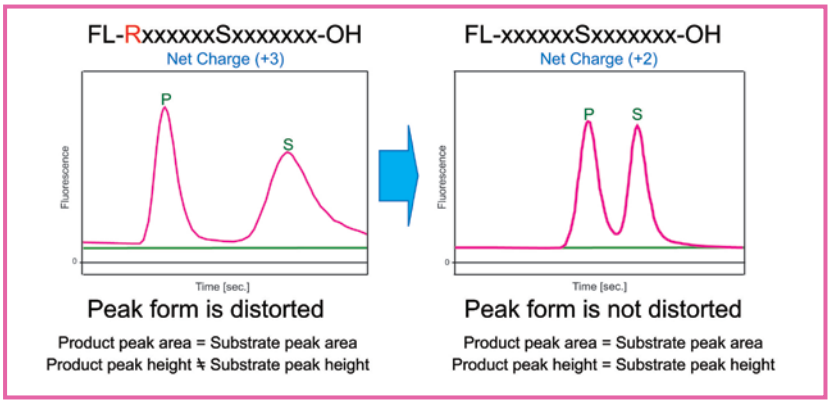

Fig. 2: Substrate optimisation. The charge of the peptide is modified to obtain a sharp peak for the MSA format.

phorylation. There are an increasing number of assay formats that have more recently been developed for measuring kinase activity, radioactive and non-radioactive, antibody-based, and non-antibody based. Many factors go into selecting an appropriate format. The trend in recent years has been shifting to more homogeneous assay formats [5]. A very promising non-radioactive and non-antibody-based technology that relies on fluorescence intensity is Caliper's mobility shift assay (MSA). This is a technology based on microfluidics on a microchip involving the electrophoretic separation of a fluorescently labelled substrate and the phosphorylated product. Among several assay platforms, MSA is one of the most accurate methods. In this system, the kinase reaction takes place in a 384-well plate, and a capillary sipper takes the sample into the microchip as the separation device for the substrate and the phosphorylated product detected via laser-induced fluorescence. Based on the separation principle of the assay, MSA is unlikely to be affected by interference from a compound's autofluorescence. The steps taken in developing an assay using MSA are illustrated in figure 1. An example of the peaks generated is shown in figure 1-1. The amount of product formed is determined by calculating the ratio of the product peak to the substrate peak. The peak separation condition should be carefully optimised to obtain the ideal peaks (fig. 2). This assay method, however, is strongly affected by the nature of the peptide, especially the charge. Therefore, the substrate design is critical to the success of the assay. One of the remarkable features of the MSA platform is the ability to measure real-time kinetics by sipping samples at various time intervals from a single well as shown in figures 1-2. Compared to other assay methods, MSA allows simplified real time kinetic analysis and accelerated assay development and optimisation. The calculation of Km values from initial velocities and the determination of enzyme linearity are shown in figures 1-3. To validate the assay, we measure the inhibition by a known kinase inhibitor (fig. 1-4). To date, we have been able to develop 209 assays with this MSA platform.

Profiling Data of Kinase Inhibitors in the Clinic

Since a protein substrate cannot be used with the MSA format, we use an ELISA format to assay kinases using a protein substrate. In order to help validate our profiling methods, we have profiled two drugs, Gleevec and Tarceva against 247

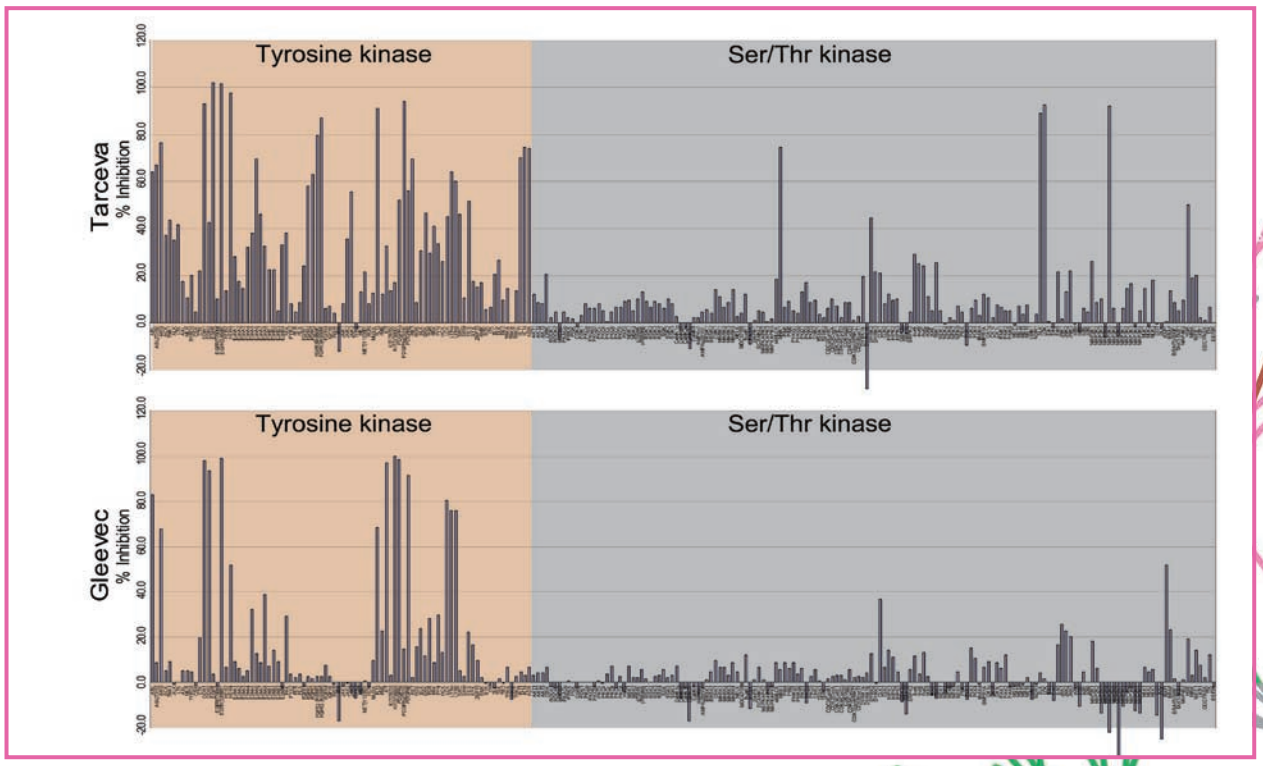

Fig. 3: Kinase profiling results of Gleevec and Tarceva. Each compound was tested at 1 μM with the ATP concentration at the Km.

kinases as shown in figure 3. At 1 μM concentration, Gleevec inhibited by more than 80% the following kinases: ABL, PDGFRα PDGFRβ, KIT, KIT[V650G], EGFR[L858R], DDR1, DDR2 and LCK. Whereas, Tarceva at 1 μM concentration inhibited by more than 80% the following kinases: EGFR, EGFR[L858R], FGFR3[K650M], FLT3, HER4, LOK, MAP2K5, PDGFRα [T674I] and SLK. These results obtained at Carna Biosciences are consistent to what has already been reported [6, 7].

Conclusions

The MSA platform is a powerful tool for the evaluation of kinase activity. Any drug discovery kinase program requires the determination of a compound's selectivity across a broad panel of kinases. With this information, the prediction of unwanted side effects is possible. Furthermore, any small molecule regardless of its target, kinase or otherwise, should be profiled against a variety of targets including kinases to learn its potential liabilities.

Acknowledgement

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