KINASE PROFILING:
THE MYSTERIES UNRAVELED

Dr. Steven Pelech, founder, president, and CSO of Kinexus reveals the secrets and complexities behind kinases profiling.

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Future Pharmaceuticals: What does kinase profiling involve? What are its applications within the pharmaceutical industry?

About two percent of the human genome appears to encode at least 515 protein kinases, which are collectively referred to as the "kineome." Some 478 of these kinases feature highly conserved catalytic domains. More than half of these kinase domains have been now been cloned and expressed as recombinant proteins, and are commercially available to screen for inhibitors. It appears that a minimum of 100 carefully selected representative kinases from the kineome need to be tested to obtain high confidence that an inhibitory compound is really selective for a kinase of interest.

With the commercial success of the launch in 2001 of Gleevec (Imatinib) by Novartis for treatment of chronic myelogenous leukemia, there has been a frenzy of activity to develop specific protein kinase inhibitors as therapeutic drugs, primarily for oncology applications. Eight small molecule kinase inhibitors are now on the market, and at least 80 more are in human clinical trials. Upwards of 500 additional kinase inhibitors are in the discovery and preclinical stages. It has been estimated that over 30 percent of all research spending on drug development now focuses on protein kinases.

Initially, there was a lot of reluctance by the pharmaceutical industry to pursue kinases as drug targets, because it was unclear whether specific inhibitors could be developed. It took six years to bring Gleevec to clinical trials, largely due to concerns about whether the market for this drug warranted the investment. However, in 2005, Gleevec sales grossed nearly $2.2 billion.

How much potential do you believe kinase profiling has in drug development? Why?

The primary attraction of protein kinases to the pharmaceutical industry is their linkage to over 400 human diseases, and the fact that as signaling enzymes they comprise about a fifth of the potential drugable protein targets. Protein kinases play key roles in cellular regulation, and they appear to catalyze the reversible phosphorylation of more than 10,000 proteins at greater than 500,000 sites. It is no wonder that defective signaling through malfunctioning kinases can have such a profound effect on human health. It also underscores the importance of identifying specific kinase inhibitors, since there could be major side effects if too many bystander kinases are also affected by these compounds.

High throughput screening for inhibitors against a target kinase can be performed for as low a penny per assay. Counter screens with panels of protein kinases are markedly more expensive and typically cost closer to $50 per kinase. Consequently, there has been a trend to perform protein kinase profiling in counter screens later in drug development usually to verify a lead compound's specificity. However, now that vast libraries of kinase inhibitory compounds exist and a large repertoire of kinases is available at increasingly lower costs, a compelling case could be made to perform earlier and broader screening with bigger collections of protein kinases.

While only about a third of the protein kinase inhibitors in clinical trials are currently directed towards non-oncology applications, it is very likely that protein kinases inhibitors will show high utility for treatment of other diseases, particularly for neurological diseases such as Alzheimer's, Parkinson's, and schizophrenia as well as immunological disorders. A selective inhibitor for a kinase may actually have wide applications in diversified markets. With the safety and efficacy of a kinase drug established for treatment of one disease, approvals for additional indications for the same drug are likely to be forthcoming. This is what happened in the case of Gleevec, when it was discovered that it was effective for the treatment of gastrointestinal stromal tumors.

What kind of technological challenges are encountered when developing a kinase profiling service?

Several companies, including Ambit, Invitrogen, ProQinase, Upstate, and others, offer panels of up to 250 or more distinct protein kinases. The production of diverse catalytically active protein kinases for in vitro screening for inhibitors is challenging as the post-translational modifications and cofactors that are required for optimal enzyme activity for most are unknown. Commercial preparations of protein kinases almost invariably correspond to catalytic fragments produced as recombinant proteins in bacteria or insect cells, and these may be partly denatured or misfolded. Furthermore, it appears that most protein kinases actually occur in dimeric forms in their active states, and this can induce conformational changes that may affect interactions with potential drugs.

Most in vitro kinase screening today is performed with synthetic peptides that have Km's that are a thousand-fold higher than for physiological substrates. Concentrations of ATP are routinely used in these assays that are ten- to fifty-fold lower than occurs naturally in cells. Over 2000 proteins feature a conserved ATP binding site, so there is a high risk of side effects with drugs that target this pocket in protein kinases. Most of the kinase inhibitors in clinic trials are in fact competitive with respect to ATP. Despite these caveats, there still appears to be high concordance with the ability of kinase inhibitors chosen from in vitro screens to be selective in vivo.

What benefits and limitations differentiate kinase profiling from other screening methods?

In vitro screening for protein kinase inhibitors clearly benefits a target-driven drug discovery approach. However, our knowledge of the normal and pathophysiological functions of the vast majority of the protein kinases and their regulation is extremely deficient. Remarkably, all the kinase inhibitors in clinical trials and those that have already been approved collectively target only about two dozen kinases, and these are non-proprietary. For some targets such as Neu or p38 MAP kinase, more than a dozen different companies have competing drugs in clinical trials. Including those in pre-clinical studies, perhaps four dozen kinases are being actively pursued for discovery of inhibitors by the pharmaceutical industry at present. This means that as much as 90 percent of the protein kinases are largely neglected. The serendipitous discovery of selective inhibitors for these other protein kinases in broad screens should facilitate their eventual characterization and evaluation of their suitability as therapeutic targets.

An alternative strategy to in vitro protein kinase screening is to track the expression and phosphorylation of protein kinases and their substrates in lysates from cell lines exposed to compounds in their culture media. This can also be performed with tissue

extracts from animals treated with drug leads in vivo. This is much more revealing than in vitro kinase profiling, since the drugs are tested in a physiological setting in living cells. Furthermore, this can yield a lot of information about the more obscure protein kinases that could advance them as attractive drug targets.

Gene microarrays have become extremely popular for profiling changes in mRNA levels of thousands of proteins, including protein kinases. The dogma is that changes in mRNA expression will be reflected by similar alterations in protein levels. At Kinexus, we recently performed an extensive survey of protein kinase expression data from gene microarray analyses of 30 human tissues that has been deposited in the Gene Expression Omnibus available from the U.S. National Center for Biotechnology Information. Surprisingly, we found that more than 90 percent of the human protein kinases genes are ubiquitously expressed at appreciable levels in all of the human tissues tested.

We have developed two methodologies at Kinexus to track protein kinase expression and phosphorylation in cell and tissue extracts using highly validated antibodies. One of these is called Kinetworks™, which is based on multi-immunoblotting with up to 40 antibodies simultaneously on a mini-SDS-PAGE gel. Over the last seven years, we have used the Kinetworks™ technique to analyze over 10,000 diverse cell and tissue lysates. Many of these results are available to the scientific community through our online databank KiNET. Our findings have revealed that there are profound differences in kinase protein levels based on species, tissue, gender, age, and disease status. It is clear that the correlation between mRNA and protein levels for kinases in human tissues is extremely poor and the mRNA data can be misleading.

Most protein kinases are regulated by direct phosphorylation, so assessment of the specific phosphorylation states of these kinases can provide an indirect measure of their activity status. Again, the thousands of Kinetworks™ analyses we have performed have shown there is often an inverse correlation between the amount of active and total species of a given protein. In retrospect, this is not surprising, since cells probably possess a reservoir of inactive proteins that are poised for rapid activation by phosphorylation. Once phosphorylated and activated, these proteins are also tagged for speedy degradation. In view of this, we believe that the phosphorylation status of proteins is likely to show the tightest correlation with the phenotypic changes in cells in response to extracellular stimuli. Since there appears to be over 500,000 human phosphorylation sites, the phosphoproteome would appear to be a rich source of biomarkers for disease diagnosis.To track the expression of phosphorylation of protein kinases and their substrates in higher throughput and lower cost, we recently developed the Kinex™ antibody microarrays. These microarrays are printed with over 600 pan- and phospho-site-specific antibodies. While these protein microarrays are extremely sensitive and reproducible, they do generate a high degree of false positives and negatives. This is largely due to the cross-reactivity of antibodies and the occurrence of proteins in complexes. Nevertheless, these antibody microarrays have tremendous potential for discovery of biomarkers and utility for both patient and drug profiling.

Are there any other techniques with growing demand? For example, how is small interfering RNA (siRNA) revolutionizing bioscience research?

Apart from the use of microarrays, there is growing adoption of so-called liquid chips based on Luminex™ bead technology for tracking protein kinase expression and phosphorylation. So far, only about two dozen protein kinases can be quantified with commercial kits from companies like Becton Dickinson, Bio-Rad, Invitrogen and Upstate. A major limitation of this technology is that it can only be used to track a maximum of maybe 20 to 30 signaling proteins at a time. There appears to be problems in identifying suitable capture and reporter antibodies pairs for most protein kinases. At Kinexus, we have tested over 2800 commercial antibodies in-house by Western blotting with about a 75 percent failure rate. It is even more difficult to identify capture antibodies that perform well for immunoprecipitation.

There has been a lot of excitement about the application of RNAsi to selectively abrogate proteins. This is clearly an important technique to dissect out the roles of protein kinases in physiological processes. One study from the Max Planck Institute of Molecular Cell Biology and Genomics showed RNAsi depletion of only about 30 out of over 600 kinases in HeLa cells actually inhibit cell proliferation by more than 67 percent. This can be interpreted to show that the vast majority of protein kinases are not individually required for cell survival. This is probably good news for kinases as drug targets in view of the high evolutionary conservation and ubiquitous tissue distribution of most kinases. These results support the notion that protein kinases signaling networks are highly redundant and loaded with extensive feedback mechanisms. In one Kinetworks™ study that we undertook with staurosporin-treated human Jurkat T cells, we observed as much increased phosphorylation of proteins with this non-specific kinase inhibitor as we saw reduced

phosphorylation in other proteins. This dynamic compensatory capacity of cell signaling systems probably accounts for why many protein kinase inhibitors are well tolerated. It is likely that these compensatory mechanisms also kick in when a kinase is malfunctioning from spontaneously acquired or somatic gene mutations.

In the industry, there's still debate about what constitutes an effective drug that inhibits kinases. Where do you stand on this? Are there any new arguments in the discussion?

Compelling arguments have been made that semi-selective kinase inhibitors that affect a range of receptor-tyrosine kinases may be more desirable drugs than those that exhibit high specificity. Such semi-specific inhibitors may service larger markets. It may be more difficult for cancer cells to develop drug resistance by mutation of the drug binding sites in multiple targeted kinases. It is certainly easier to develop less specific drugs. My own feeling is that if I wanted non-specific kinases inhibitors, I would drink a lot more green tea and eat more tofu, but only for a short period, since there is a link to increased occurrence of Alzheimer's disease associated with high consumption of these items later in life.

The real fruit of the last 20 years of cancer research has been the identification of nearly 100 oncogenes, most of which specify protein-tyrosine kinases. Oncogenic mutations appear most frequently in protein kinases. Full blown cancer can arise from the loss of function of just a couple of tumor suppressor proteins and the gain of function of just a few oncoproteins through mutation of the genes that encode these regulatory proteins. There may be millions and even billions of various combinations of tumor suppressor protein inactivations and oncoprotein activations that underlie all the cancers in our population. Every cancer patient may possess a unique set of molecular lesions that give rise to their cancer. Another study that we completed at Kinexus was the analysis of the phosphorylation status of about 80 key regulatory proteins in 40 different human breast tumor cell lines. Each of these cell lines showed profound differences in the phosphorylation status of the proteins that were tracked. Not one breast tumor cell line resembled another. We have examined several hundred tumors cell lines by Kinetworks™ multi-immunoblotting, and the message is the same. All of these tumor cell lines are extremely different with respect to their phosphoproteomes.

Cancer cells appear to acquire high dependency on these oncogenes, so defining which oncoproteins are activated in each patient and specifically targeting them is likely to produce the most dramatic recoveries with the fewest side-effects. This is really the Holy Grail of personalized medicine. But this is completely contingent on the development of highly specific kinase inhibitors and the ability to track large numbers of protein kinases accurately and cost effectively in patient biopsies.

How can organizations utilize advanced profiling techniques most advantageously? What is the business case for using services like yours rather than doing it in-house?

Setting up the infrastructure to perform extensive in vitro or in vivo kinase profiling within most companies really does not make financial sense, never mind the lost opportunity that arises from the inordinate time it takes to establish such operations. The development of the reagents and technology to perform such analyses is costly, time consuming, and technically challenging. While the need for "systems biology" approaches is frequently expounded, in reality large scale proteomics analyses are not supported by government and charitable granting agencies, so it is almost impossible for academic institutions to carry out this kind of research. The availability of cost effective commercial proteomics services makes this feasible. The affordability of our services is reflected by the fact that Kinexus has provided its Kinetworks™ and Kinex™ services to over 650 academic laboratories in addition to over 150 companies.

What do you see as key drivers for kinase profiling in the future?

In vitro protein kinase screening would greatly benefit from the development of ultra-specific peptide substrates for enzyme activity assays. This could be facilitated by the elucidation of the consensus phosphorylation site sequences for all of the protein kinases and incorporation of the optimum sequences for secondary binding sites for substrate recognition. Better preparations of full length human protein kinases with the required post-translational modifications and cofactors are also need.

Better antibodies and ultimately affinity peptides for improved capture of kinases and their substrates on microarrays are urgently required. These reagents will be invaluable for the development of disease diagnostic protein microarrays for use with tissue biopsies and biofluid specimens to support personalized medicine delivery. As we more fully understand the architecture of kinase signaling pathways in normal and diseased tissues, we will finally be able to fully exploit the growing pharmaceutical arsenal of specific protein kinases inhibitors and deploy them in a rational manner to radically improve how we treat human disease.

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