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Review

Functional interrogation of kinases and other nucleotide-binding proteins

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The largest mammalian enzyme family is the kinases. Kinases and other nucleotide-binding proteins are key regulators of signal transduction pathways and the mutation or overexpression of these proteins is often the difference between health and disease. As a result, a massive research effort has focused on understanding how these proteins function and how to inhibit them for therapeutic benefit. Recent advances in chemical biological tools have enabled functional interrogation of these enzymes to provide a deeper understanding of their physiological roles. In addition, these innovative platforms have paved the way for a new generation of drugs whose properties have been guided by functional profiling.

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1. The beginnings of activity-based proteomics

The origins of activity-based proteomic profiling (ABPP) can be found in the growth of bio-organic chemistry and mechanistic enzymology that emerged post-World War II [1–3]. Intense interest blossomed in understanding the nature of enzymatic catalysis in the context of the physical organic principles that govern the conversion of substrate to product and the structural elements of the enzyme/substrate interactions that lead to the remarkable rate accelerations that enzymes can achieve. Major advances in this understanding of enzyme action came from the design and analysis of small molecules, such as affinity labels and mechanism-based inhibitors, which could modify the enzyme in specific ways to reveal the mechanistic and structural aspects of catalysis in an era that largely pre-dated the stunning advances in and accessibility to structural biology instrumentation (for early successes, see [4,5]).

This early work was critical to the understanding of catalysis and also the appreciation that common mechanistic motifs and structural elements united enzymes into families that could potentially be interrogated with common chemical probes. The work over 60 years ago on the stoichiometric inhibition of serine hydrolases, such as chymotrypsin, trypsin, and cholinesterase, by diisopropyl fluorophosphate [6–8] is the lineal ancestor of serine hydrolase ABPP that has become the signature work of the field [9,10,2].

The advances in genomics, separations technologies and mass spectrometry were instrumental in the transition from enzyme chemistry to proteomic chemistry that ABPP represents. The ability to move protein purification from an upfront, often long and tedious, procedure to a back-end deconvolution of native proteomic mixtures has greatly expanded and accelerated the data analysis as will be discussed. In addition, the proteomic mixture provides a richer substrate to explore the effect of protein–protein and other cellular interactions that can affect ABPP and gives clues to the cellular behavior of enzymes, their substrates and inhibitors.

In this review, we will focus on the development of ABPP for the protein kinases (PKs) that comprise the kinome. The challenge that interrogation of the kinome by ABPP presents is daunting and multifaceted. Several approaches have been explored with varying degrees of success and will be discussed.

2. The kinome challenge

The human kinome consists of a nonredundant set of 518 PK genes, divided into 9 groups, 134 families and 201 subfamilies [11]. It is the largest enzyme complement in mammals constituting 1.7% of the human genome. Despite the broad range of percent protein sequence identities among kinome members, sequence comparisons have shown that virtually all PKs have at least one conserved lysine residue within their active sites. The ATP binding loop region contains one lysine residue in all "typical" PKs with

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few exceptions [12,13]. A second lysine is found two residues to the C-terminus of the catalytic aspartic acid of the HRDLK motif in the majority of serine/threonine kinases. These two motifs, along with other areas of the protein, play a major role in the binding of ATP, the common substrate for all PKs, and the catalysis of the phosphoryl group transfer of the γ-phosphate of the ATP to the protein substrate.

The key role of the PKs in a wide range of complex cellular signaling functions and pathway cascades makes them prime candidates as targets for drug design [14–17]. This is reinforced by the fact that nearly half of the 518 human PKs have been mapped to disease loci or cancer amplicons. In addition, the design of inhibitors of the common ATP binding site has been most tractable for the medicinal chemists. Thus, a major challenge has been designing potent competitive inhibitors of ATP for the target kinase that also demonstrates acceptable selectively against the rest of the kinome – a considerable challenge (for notable recent successes, see [18–20]).

A potentially greater challenge of kinome drug discovery is the issue of species differences. Not surprisingly, specific kinases show species variability in their primary protein sequences that can lead to differences in potency that must be considered in preclinical animal and toxicology models. This is a common problem in most discovery projects regardless of the target under investigation. However, other kinomes of other species suggests that this problem goes deeper.

Consider the mouse kinome [21]. The mouse is arguably the preeminent species for early efficacy and toxicity analysis of preclinical drugs. The mouse kinome is the probably the best understood behind the human. There are 540 predicted PKs in the mouse kinome with orthologues for 510 of the 518 human PKs. Eight human PKs have no mouse orthologues; the mouse has 40 unique orthologues. Some of these unique orthologues can be traced to gene loss, retrotransposition, and incomplete genome sequence. Orthologous kinase pairs vary in sequence conservation along their length, with many species-specific sequence inserts exist and alternative splicing. There are 97 mouse PK pseudogenes that are all distinct from the 107 human PK pseudogenes.

In other kinomes, greater differences appear. Remarkably, the chimpanzee kinome has 587 predicted PKs. No human orthologue with greater than 95% sequence identity could be identified for 160 PKs. Variations in chimpanzee kinases compared to human kinases are brought about also by differences in functions of domains tethered to the catalytic kinase domain [22]. Little published work on other kinomes is available so they must be considered works-in-progress. Based on a library of hidden Markov models (see http://www.compbio.dundee.ac.uk/kinomer [23]), the rat kinome has 521 predicted PKs and the dog kinome, 656 PKs. Even allowing for revisions and modifications of these data, it is clear that significant interspecies differences will exist among higher mammalian kinomes. From a drug development perspective, these differences can be highly problematic. Since drug candidates are evaluated in a number of accepted preclinical species (such as mouse, rat, dog and non-human primate) for toxicity and, in some cases, efficacy, the potential for a kinase inhibitor to have different selectivity and off-target profiles in the kinomes of different species is potentially significant. Thus, if a toxicity signal is observed in the dog for example, the development team is faced with the problem of determining if the signal suggests toxicity relevant to the human use of the drug or if it is idiosyncratic to the dog kinome.

Substrate-based, recombinant kinase profiling platforms are largely focused on a large library recombinant human PKs; the kinomes of other species have not been as extensively cloned to provide broad profiling capabilities. Developing a general kinase assay that could function across the kinomes of virtually all species would be a breakthrough in the field and greatly beneficial in understanding comparative cross-species responses.

3. Non-activity based kinome profiling platforms

As interest in PKs as mediators of key biological processes has grown, numerous elegant solutions have been proposed for the study of these proteins en masse (see Fig. 1). Kinases as a class have several features in common, perhaps most significant is the common use of ATP as a co-substrate. While having a class of over 500 proteins with common active site features has made drug discovery difficult, it has in many ways simplified the task of establishing class-wide analysis platforms. All of the profiling platforms have in common the ability to determine competition between a test compound and a known probe compound, which is typically either ATP itself or a broadly non-selective inhibitor. Differences, described below, among the platforms include: whether the probe is bound to a resin or free in solution; whether the probe binds covalently to target proteins or not; for covalent probes, whether they bind via mechanism-based interaction or via photoactivated labeling; and finally the kinases are identified either by mass spectrometry of naturally occurring protein or by genetic methods by making use of kinase/nucleic acid fusions. The availability of these very broad kinase profiling methods combined with the demonstration that limited subsets of kinases are unable to accurately indicate compound selectivity have thrust these methods into the mainstream of drug discovery. Additionally, the ability to identify kinases based on an intact active site from natural extracts has provided a very useful addition to broad shotgun-style proteomic and genomic analyses.

One of the original large-scale kinase profiling platforms made use of a library of recombinant kinases expressed on the surface of T7 bacteriophage [24]. By determining number of phage particles bound to immobilized kinase inhibitors in the presence of a titration of free test compound, the assay could determine potency of test compound for the displayed kinase. From 113 kinases in 2005, this platform has expanded to cover at least 440 assays, including protein and lipid kinases, oncogenic or drug resistant mutants, and several kinases from pathogens [25]. Originally

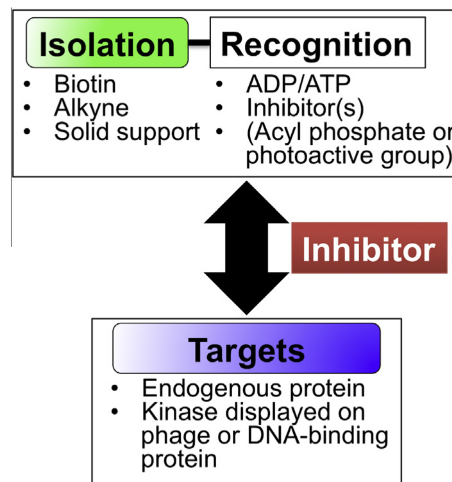

Fig. 1. Components involved in functional interrogation of kinases and other nucleotide-binding proteins. These systems determine binding of a probe (upper box), which includes isolation and recognition elements, to target proteins (lower box), which are either endogenous proteins identified after isolation by mass spectrometry, or fusion proteins wherein the protein is identified by analysis of fused nucleotides. Optionally, binding between probe and targets can be competed by soluble test inhibitors to quantitatively characterize inhibitor/target binding properties.

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