Screening for Allosteric Kinase Inhibitors in High Throughput

A Novel Fluorescence Approach for Detecting DFG-out Kinase Inhibitors

Kinases and their associated signaling pathways are largely responsible for the regulation of intracellular processes. Aberrantly regulated kinases can ultimately cause total cellular disregulation and contribute to the onset of several diseases, including cancer. Based on the fundamental understanding of kinase malfunction in cancer biology, the discovery of small organic molecules to perturb kinase function has culminated in the uprising of targeted cancer therapy [1]. As of now, a dozen kinase inhibitors are on the market and several hundreds more are in clinical trials. However, limited selectivity and the emergence of drug resistance remain fundamental challenges for current modern medicinal chemistry research for the development of kinase inhibitors that are effective in long-term treatments [2].

Personalized Cancer Therapies: Understanding Kinase Drug Resistance

Resistance mutations to classical ATP-competitive (Type I/II) inhibitors are emerging at an increasingly rapid pace and often limit the success of newly available targeted cancer therapies. In particular, point mutations at the gatekeeper position, in which a relatively small amino acid side chain (classically Thr) is mutated into a larger hydrophobic residue (Ile or Met), are common among protein kinase targets in cancer such as Bcr-Abl (T315I), c-KIT (T670I), PDGFRα (T674I) and EGFR (T790M) [3]. These mutations often result in a steric clash which obstructs inhibitor binding to the hinge region of the ATP pocket and can also increase the intrinsic enzymatic activity of some kinases (fig. 1) [4]. Therefore, the need to identify and develop reversible inhibitors which are able to overcome such mutations and bind with a high affinity is the focus of many academic and industrial research projects.

New Possibilities: Allosteric Inhibition Yields Higher Selectivity and Higher
**Potency**

Most known kinase inhibitors are Type I inhibitors - ATP-competitive compounds such as staurosporine, erlotinib (Tarceva) and dasatinib (Sprycel) - which bind in the ATP binding site and hydrogen bond with the hinge region of the kinase (fig. 2). More recently, Type II inhibitors - compounds which bind partially in the ATP binding site and extend past the gatekeeper and into an adjacent allosteric site that is present only in the inactive, or "DFG-out", kinase conformation - have been shown to possess advantageous pharmacological properties.

Many Type II inhibitors currently on the market, such as sorafenib (Nexavar), lapatanib (Tykerb), and imatinib (Gleevec), are very effective anti-cancer drugs. However, since resistance to imatinib has already been identified in Abl kinase, the answer to the problem of drug resistance does not lie simply in the binding mode of the inhibitor.

Compounds binding exclusively to less conserved allosteric sites outside the ATP pocket (Type III inhibitors) bind beyond the gatekeeper residue and, therefore, would be expected to have superior selectivity profiles and offer new opportunities for scaffold development. This allosteric site is formed when the activation loop - a crucial structural component of the substrate binding cleft participating in the recognition of substrates and influencing the arrangement of the catalytic residues - adopts the DFG-out conformation characteristic of inactive kinases [5] (fig. 3).

*In vivo*, the activation loop is believed to be in a conformational equilibrium between DFG-out (inactive) and DFG-in (active) conformations. This equilibrium can be regulated by phosphorylation of the activation loop or by a variety of protein-protein interactions. Alternatively, inhibitors binding to the allosteric site can lock the kinase in the DFG-out conformation. In recent years, the availability of structural information of inactive kinase conformations (p38α, EGFR, Abl, VEGFR and B-Raf) in complex with Type II or Type III inhibitors has intensified the search for novel inhibitor scaffolds which take advantage of this site.

**The Bottleneck in Allosteric Kinase Inhibitor Discovery: Lack of Screening Methods**

The identification of allosteric inhibitors that bind to and stabilize inactive kinase conformations is expected to provide valuable insights into the development of new chemical principles to address the most defined current challenges in kinase drug discovery, limited selectivity and drug resistance. However, the potential value of...
library screening for binders to the allosteric pocket will only be unlocked if high-throughput screening (HTS) methods are available which can discriminate between Type I and the more desirable Type II/Type III inhibitors. To address this need, we have developed a robust new method which allows for the detection and quantification of the binding of different types of kinase inhibitors in high-throughput, specifically those which utilize this less-conserved allosteric pocket and lock the kinase in an inactive state [6].

**Assay Concept**

The abundance of available structural information of active and inactive kinase conformations, as well as protein sequence alignments guided us to the identification of amino acid positions suitable for fluorophore labeling in order to detect conformational changes in the kinase domain triggered by allosteric ligand binding. The principle of the fluorescent-labeled kinase assay for allosteric binders is highlighted in figure 4.

We employed fluorophores that are commonly used in the formation of protein-fluorophore conjugates, are relatively small in size and highly sensitive to polarity and/or conformational changes of acrylodan-labeled Kinases which occur as the kinase is stabilized in the inactive DFG-out conformation by these types of inhibitors. The observed spectral changes afforded the possibility for ratiometric fluorescence read-outs, which serve as an internal correction for dilution errors which may exist in binding assays where several samples are used to examine inhibitor binding in a titration series.

**Development and Validation of the Assay System Using the Serine-threonine Kinase p38α**

Once the protein was labeled in the appropriate position on the activation loop, a small library of known allosteric inhibitors for p38α was characterized using endpoint fluorescence measurements following pre-incubation with a range of concentrations for each compound. These experiments yielded binding curves from which the Kd for each ligand could be determined directly. The affinities of the different compounds were comparable to published values obtained with an alternative p38α-specific assay system. Relationships between affinity and inhibitor structure also matched published trends. In a second step, measurements were made in real-time to obtain the kinetics of binding (k_{on}) and dissociation (k_{off}) for a few select compounds. The expected slow binding rate of Type II or Type III allosteric compounds was observed and the experimentally determined rate constants for binding and dissociation also closely agreed with reported values.
determined with another p38α-specific assay system. Thirdly, we verified that the assay responds similarly to multiple drug scaffolds by testing several reference compounds known to either bind or not bind to p38α. For higher throughput screening of compound libraries, the assay was successfully adapted to a 96 or 384-well format, the latter of which is the standard screening format used by most large pharmaceutical companies. The average Z-factor for the Kd determinations using the 96-well and 384-well plate formats was found to be 0.88 +/- 0.3 and 0.85 +/- 0.6, respectively, which is more than suitable for reliable results in large screening campaigns.

Application to a Tyrosine Kinase cSrc: Identification and Development of Potent cSrc Inhibitors Which Overcome Drug Resistance Mutations and Stop Cancer Cell Growth

By applying this powerful new assay system for identifying ligands which bind to the DFG-out conformation of the tyrosine kinase cSrc, [6] we were able to address a clinically relevant issue - how to design kinase inhibitors to treat patients with cancers that are driven by a subset of drug resistance mutations and are no longer responsive to available standard therapies. Using this system, we identified the first known ligands shown to bind exclusively within the allosteric pocket of inactive cSrc and employed protein X-ray crystallography to understand the chemical principles of how one of these compounds is able to retain activity on drug resistant cSrc. Iterative structure-based design cycles resulted in the synthesis of potent Type II inhibitors which show low nM activities on both wild type and drug resistant cSrc variants and helped to uncover a unique feature of a subset of these compounds, a surprising flexibility in the vicinity of the gatekeeper residue which allows these inhibitors to overcome a dasatinib-resistant gatekeeper mutation [9].

Conclusions

Since HTS projects are expensive in both time and resources, much work is done prior to starting a large screening campaign to assess the quality of an assay on a smaller scale and to predict if the assay would be useful in a high-throughput setting. The work presented here with the serine-threonine kinase p38α and its successful adaptation to the tyrosine kinase cSrc suggests that application of this assay system to other Kinase targets such as EGFR, Abl, PDGFR and Kit may lead to the development of more specific drugs which may be able to overcome kinase drug resistance, an issue that has received significant attention recently. Its application will be particularly fostered by first answering the question of which kinases can adopt targetable inactive conformations.

References

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