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Drug Discovery

Harnessing the Power of Kinase Inhibitors

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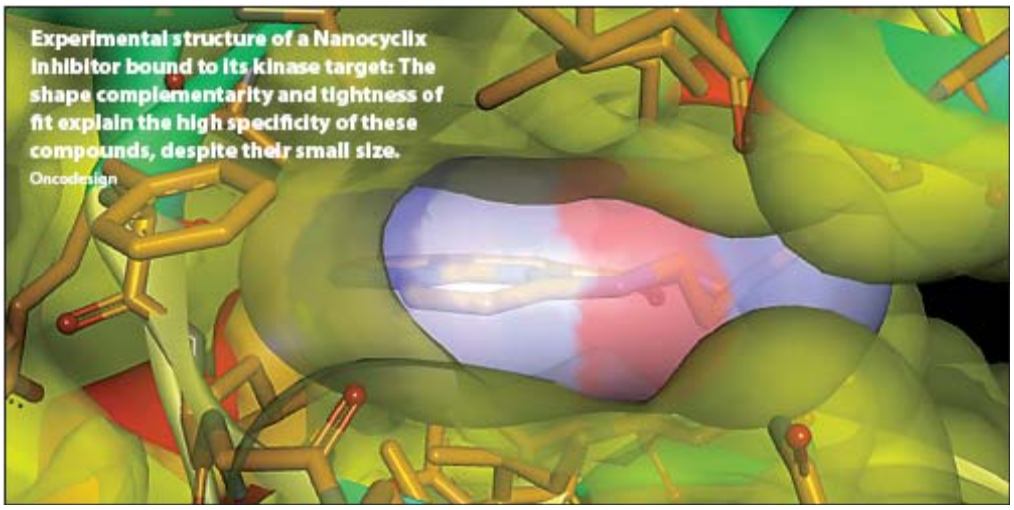
The ~600 kinases that make up the human kinome comprise a group of exciting druggable targets that can be modulated using small molecule or monoclonal antibody approaches.

There are currently 15 FDA-approved kinase inhibitor drugs, yet there remains much uncapped potential, as the biological function of ~90% of the kinome is unknown.



Methods have been designed for improving small molecule type I kinase inhibitors, which target the ATP binding pocket common to all kinases in their active form, as well as type II inhibitors, which bind to the same area as type I molecules, but also to an additional allosteric binding pocket in the inactive DFG motif conformation of the kinase. Novel assays have identified kinase targets, improved the function of certain kinase inhibitors, and led to the discovery of new inhibitor classes to accelerate drug discovery.

Sheraz Gul, Ph.D., vp and head of biology at **European ScreeningPort**, indicates that he has had a long-standing interest in kinases, particularly NF-κB inducing kinase (NIK). NIK is known to phosphorylate IKK-α at Ser-176, the same site where IKK-α autophosphorylates.

Dr. Gul has noticed a few discrepancies over the years with regard to reported NIK biochemical assays: various suppliers who sell recombinantly expressed and purified NIK have shown that it is catalytically active in biochemical assays when using a number of substrates (peptide and the generic kinase substrate myelin basic protein), however not when using IKK-α protein or a peptide derived from it as substrates.

Using such non-IKK-α derived substrates is somewhat of a concern, as myelin basic protein will undergo phosphorylation by most kinases. Additionally, it is possible that a highly active kinase partner bound to NIK may be responsible for the observed phosphorylation of non-IKK-α-derived substrates in biochemical assays.

In light of the intriguing aspects of how physiologically relevant NIK activity arises, Dr. Gul concludes that, rather than developing a biochemical assay, a cell-based assay to search for NIK inhibitors that prevent IKK-α phosphorylation would more effective. However, cell-based assays do have their disadvantages, primarily that most hits often exhibit cytotoxicity.

His research team began by performing a dual transfection assay of catalytically inactive full-length IKK-α and full-length NIK in insect cells, and have translated this to a HEK293 cell-based system and performed proof-of-concept screens against compound libraries with both assays. The screen that utilized the former assay yielded a sizeable number of hits, some of which were shown to prevent p52 translocation into the nucleus from the cytoplasm, as this would be the downstream effect of inhibiting NIK.

As an alternative to the dual transfection assay, Dr. Gul's research team made the surprising finding that batches of cells that were separately transfected with NIK and catalytically inactive IKK-α, which were mixed together upon cell lysis, did not result in the catalytically inactive IKK-α undergoing phosphorylation, indicating that its NIK- mediated phosphorylation can only occur in a native cellular environment. This was all the more reason to prioritize cell-based assays over biochemical assays when searching for NIK inhibitors that prevent IKK-α phosphorylation.

"NIK is clearly an unusual kinase, as there have been no reports of a biochemical assay being developed that specifically makes use of its well-known substrate, IKK-α (or relevant kinase inactive mutant)," Dr. Gul says.

"It is not necessary that every kinase assay be cell-based, but for those that have characteristics like NIK, you will get many hits in a biochemical assay whose activities will not translate to a cell-based assay. Therefore, in order to mitigate the risk of this all-too-common scenario, it should be best practice to use a panel of both biochemical and cell-based assays as early as possible."

Phenotypic Screening

"Over the past 15 years, **Array BioPharma** has synthesized over 10,000 kinase inhibitors, which together inhibit about 95% of the entire kinome," says David Chantry, Ph.D., senior director of translational and cellular biology. "By applying this state-of-the-art collection of kinase inhibitors, we have invented a cell-based phenotypic screening platform that enables discovery of novel drug targets."

Dr. Chantry also notes that the alternative global approaches of molecular-driven target discovery, such as using an shRNA knockdown approach or systematic knockouts in animal models, have not been very fruitful when it comes to identifying targets for small molecule drug discovery.

Array BioPharma's phenotypic screening platform has multiple differentiating features from other platforms, most notably that Array researchers are able to reverse engineer from the screen back to the molecular targets in a chemoinformatic approach. All of the inhibitors in the curated collection have demonstrated cell activity at 1 μM or less, and data has been collected regarding in vitro properties of microsomal stability, permeability, and solubility.

In total, Array BioPharma has collected ~1 million data points outlining the in vivo physical properties of their kinase inhibitor collection. Its library of diverse kinase inhibitors covers the vast majority of kinases, says Dr. Chantry, most of which have completely unknown functions.

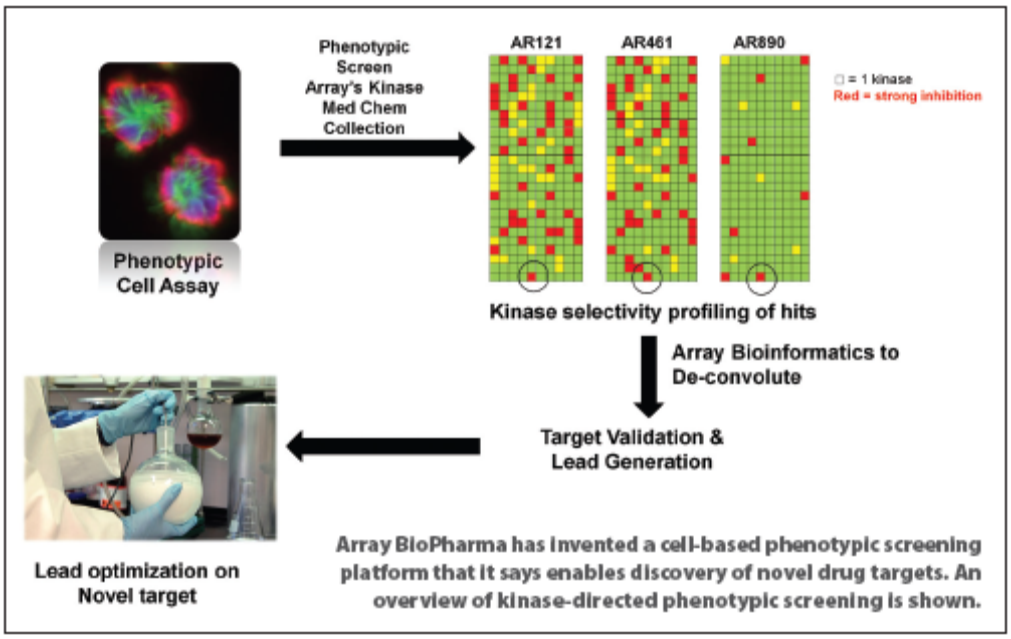
"Our approach ends up working like a Venn diagram: we obtain an overlapping set of information for each group of inhibitors that exhibit the same cellular phenotype in our assays," adds Jim Winkler, Ph.D., vp of discovery biology and translational medicine.

"There might be one inhibitor that inhibits 20 kinases across the kinome, and another that inhibits 15, most of them different from the first inhibitor; but if you do this for enough inhibitors, they will ideally only have one kinase as a common target. Using all of the selectivity data we have collected for each inhibitor, we end up combining target validation and target identification in the same process."

Depending on the therapeutic application, each phenotypic screen will be unique in terms of the number of hits and the nature of the intersection of shared

kinases that are inhibited, but the end result is a manageable list of potential targets, which can then be further analyzed by traditional approaches like RNAi knockdown.

A subset of the inhibitor library was recently used to demonstrate the strength of the platform. The researchers were interested in identifying a single kinase target that would affect cytokine production by both innate and adaptive immunity, as there are currently targets that affect each type of immunity separately but no single approach for both.



In addition to the clinical relevance of this phenotype, Array BioPharma already had inhibitors of the p38 and MEK pathway in its collection to provide useful internal controls. Using a reverse-engineering approach, Dr. Chantry and colleagues discovered and validated a novel kinase target that regulates cytokine production by cells of both the innate and adaptive immune system.

“We are interested in using this phenotypic screening platform to drive collaborations with other companies, to discover novel targets, and develop clinical candidates,” Dr. Winkler adds. “We would like to work with collaborators that have in-depth biology expertise in their particular therapeutic area, and an understanding of which are the most relevant phenotypic screens that can lead to novel therapies for patients with serious unmet medical needs.”

Cyclic Kinase Inhibitor Discovery

Oncodesign has utilized a novel approach for identifying highly selective kinase inhibitors by synthesizing macrocyclic molecules (containing large cycles) with a low molecular weight, says Jan Hoflack, Ph.D., CSO.

Dr. Hoflack explains that this Nanocyclix® platform has generated high selectivity across the kinome because the cyclical nature of each inhibitor provides for the utmost degree of three-dimensional shape complementarity with their kinase target. Similar to a type 1 inhibitor, the macrocyclic molecule binds to the kinase's ATP binding site, but with three major determinants: a strong hinge region interaction, an interaction close to the gatekeeper residue, and an interaction in the ribose pocket.

"These are truly differentiated compounds, as they allow us to block kinases for which currently no inhibitors exist. They take us into novel chemical space, and as such create first-in-class opportunities against unexplored kinases. No other noncyclic molecule can do this type of binding: the cycle forces our macrocyclic inhibitors into a particular conformation, and there is high selectivity because the binding is based purely on shape," Dr. Hoflack comments.

The assay development and high-throughput screening system at the European ScreeningPort, Hamburg, Germany.

The macrocylic nature of Oncodesign's molecules creates a three-dimensional shape that perfectly fits with the kinase, according to Dr. Hoflack. This leads to a selectivity among kinases that would normally be obtained in a linear molecule by adding longer chains, thus increasing the size of the molecule in order to reach the required selectivity. Because of this, these macrocylic inhibitors are about half the size of conventional kinase inhibitors: they typically have a molecular weight around 300.

"Any molecule with a molecular weight more than 400 will have difficulty in getting through the blood-brain barrier if targeting the CNS, and for any application, a molecular weight above 500 is expected to lead to problems related to optimizing the ADME and PK properties," according to Dr. Hoflack. "Oncodesign offers a new technology, where we obtain high selectivity and potency but still use small molecules."

Dr. Hoflack indicated that Oncodesign's diverse collection of over 50 kinase scaffolds and 300 linkers comprising a total of 5,000 compounds in the

macrocyclic platform is very different from a high-throughput screening collection. These 5,000 inhibitor compounds have already displayed strong potencies in the low nanomolar range against the kinome, including both previously characterized kinases and kinases that have been unexplored by the pharmaceutical industry.

Once an initial ATP scaffold/linker combination with a good fit has been identified, the compounds are further optimized by modifying the length and functionality of the cyclic linker. Preferred inhibitors typically bind to 1–4 kinases on average, but Dr. Hoflack and colleagues have been driving the specificity further, as well as improving solubility of the compounds.

The Nanocyclix platform has been utilized in various therapeutic areas, especially for unexplored kinases. Cell-based phosphorylation assays have shown promise for macrocyclic inhibitors of RIP2, which is a target for autoinflammatory diseases like asthma, IBD, and rheumatoid arthritis, and LRRK2, a key kinase active in the brain in Parkinson's disease.

These and other inhibitors are currently being examined in vivo. Oncodesign has also been examining how to improve the safety of conventional kinase inhibitors in pancreatic and ovarian cancer cell lines, specifically by preventing resistance to targeted treatments by applying the Nanocyclix platform.

Leyi Gong, Ph.D., program director of medicinal chemistry at **SRI Biosciences** (a division of SRI International), investigated a class of bicyclic molecules called quinolones, while previously working at **Roche**. Dr. Gong and her colleagues at Roche were interested in JNK inhibition for a number of disease indications.

They implemented three different approaches in their study to identify inhibitors of JNK: a high-throughput screening approach, using a few million of Roche's compounds, a focused screening approach, where they collected in-house kinase inhibitors and inhibitors from the literature into a focused library for screening, and an approach just using literature-published leads. This resulted in the discovery of a novel series of kinase inhibitors, 4-quinolones. These compounds are highly selective because they were first identified as solely targeting JNK-1,-2, and -3.

Dr. Gong and colleagues modeled the predicted binding mode of this inhibitor series and later obtained the inhibitor-bound JNK x-ray crystallography, and they noticed that this unique binding mode was shared with another series published by **Takeda Pharmaceutical**. By combining their hits from high-throughput screening with Takeda's hits, this led to the researchers engineering a new series of quinolones.

The cyclic structure of this quinolone series accounts for this intrinsic JNK selectivity by accessing a unique binding pocket, which prevents the inhibitor

from binding to any other kinases. These type I inhibitors (with a representative molecular weight of 450) bind to the ATP pocket on JNK, but also bind to another unique binding pocket that does not exist on other kinases aside from JNK-1,-2, and -3.

The researchers used structure-based drug design to improve potency and various physical and chemical properties. At first, the series appeared to highly lipophilic, but structure-based drug design was implemented to generate lower lipophilicity while maintaining potency and increasing solubility. The potency was also improved by introducing a series of small lipophilic groups to allow the inhibitor to have better contact with JNK.

"In in vitro assays, there was really a clear distinction between binding to JNK or any other kinase," Dr. Gong explains. "We profiled the quinolone compound against 317 different kinases and obtained a quantitative measurement for JNK inhibition: the compound had a K_d of 50–200 nm for JNK-1,-2, and -3 but greater than 10 uM for any other kinase in the test. Because of this selectivity, when we began in vivo work, we were confident that what we saw could be attributed only to the activity of our quinolone inhibitor."

The in vivo model induced inflammation in the mouse lung to mimic an asthmatic response. To examine how one of the lead quinolone compounds reduced inflammation, Dr. Gong and her collaborators measured neutrophil levels in BAL (Brochoalveolar lavage) and AP-1 (transcription factor family phosphorylated by JNK) activity in the lung, and saw a reduction in both four hours after the inhibitor was administered at 10 mg/kg.

Dr. Gong pointed out that there are many opportunities for this work to progress, as a number of different JNK inhibitors in the literature besides quinolones present this unique binding mode that she and her colleagues have investigated.

"The quinolone structure has shown to be very important for JNK selectivity, and we have also identified other compounds that could have a similar effect due to their specific binding mode. Other cyclical chemical templates from **AstraZeneca**, **Abbott Laboratories**, and Takeda Pharmaceutical mimic the quinolone binding motif, in which they also occupy the unique JNK binding pocket not present in other kinases."

In Silico Chemistry Modeling

Amedeo Caflisch, Ph.D., who runs a computational structural biology lab at the University of Zurich, along with his colleagues developed an in silico method, termed anchor-based library tailoring approach, or ALTA, to probe compounds for high-throughput screening. The innovative element of this fragment-based in silico method is the use of molecular dynamics simulations to

find and validate the best binding modes obtained by docking molecular fragments.

The researchers usually begin with a large ZINC library, which is an online public database containing more than 20 million commercially available compounds. Using a novel algorithm developed by Hongtao Zhao, Ph.D., a scientist at the university, the compounds are then decomposed into 100,000–500,000 different fragments, as many of the fragments may overlap between compounds. These fragments are docked onto the binding site of the target of interest and are ranked by favorable binding free energy.

The high-ranking anchor fragments (usually around 1,000) that exhibit the best binding to the protein target are then identified in the original library, and the researchers extract which parent compounds contain these anchor fragments. This fully automated procedure filters down the original list of 20 million compounds to 20–100,000, and only a small percentage of these will be active, which are finally filtered down to 20–50 compounds with molecular dynamics simulations.

“This has become a very useful method in the lab because we always have a good hit rate with few false positives,” according to Prof. Caflisch. “While most docking programs are usually designed for rigid protein docking, our method takes into account the full flexibility of the protein target. We also use an accurate treatment of the effects of the solvent.”

In collaboration with the research group of Prof. Cristina Nevado in the department of organic chemistry, Prof. Caflisch’s lab uses the in silico description of the binding mode obtained by docking and molecular dynamics simulations, as well as x-ray crystallography, to guide the synthesis of derivatives to improve potency, selectivity, and solubility. This has proven to be a very efficient procedure, he explains, as the synthesis of only 5–10 derivative compounds for a particular protein target can improve potency by 10,000-fold.

The ALTA methodology has been applied toward various studies by Dr. Zhao and colleagues, including the identification of selective type I and II EphB4 tyrosine kinase inhibitors. These inhibitor classes have been tested against the NCI-60 panel of human cancer cell lines and appear to significantly reduce the proliferation of a selected group of CNS, breast, and colon cancer cell lines.

Pharmacokinetics of a selection of compounds has further been evaluated by oral and intravenous administration in mice, and these tyrosine kinase inhibitors demonstrate high plasma concentration for up to several hours and good bioavailability. GEN