

Novel chemical genetic approaches to the discovery of signal transduction inhibitors

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Concurrent advances in both high-throughput chemistry and genomics have given rise to the field of chemical genetics as a discipline for elucidating and validating drug targets, and generating novel therapeutics. Indeed, chemical genetic approaches to drug discovery have now been applied to several important drug target classes, especially those involved in signal transduction. Chemical genetics is distinct from the broader term 'chemogenomics' which is defined as the description of all possible drugs against all possible targets (reviewed in [1]). This review covers several 'orthogonal' chemical genetic approaches and focuses on a unique analog sensitive kinase technology and its applications to kinase drug discovery.

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▼ Identification of the next generation of therapeutic targets for drug development from among the products of the ~40,000 genes in the human genome represents the paramount challenge for 'post-genomics' drug discovery. Therefore, major efforts are under way to develop technologies that can best functionally validate potential drug targets and yield pharmacologically relevant information about their mechanism of action. Such technologies are crucial to accelerating the drug discovery process.

Target validation: genetic and chemical approaches

The power of genetic approaches to the study of target function is the ability to affect the activity of a single protein in a whole organism. Thus, it is not surprising that knockout mice have become popular models for target validation. However, the use of genetics for target validation is less than ideal for at least three reasons. First, genetics-based approaches affect the entire protein target. This makes it difficult to determine the difference between an effect resulting from the loss of the entire

protein (which, for example, might serve an important scaffold function in the cell) and an effect resulting from another functional aspect of the protein (e.g. its enzymatic activity). The second disadvantage is lack of temporal control. Both gene knockouts (even sophisticated 'inducible knockouts') and RNA-based approaches (antisense and RNAi) modulate protein levels slowly. This can be problematic when studying processes such as signal transduction, which can occur on millisecond time scales. Moreover, conditional or inducible systems (e.g. temperature sensitive alleles) require secondary perturbations that can have unwanted side effects. For example, it has been recently shown that a temperature shift in yeast (from 25°C to 37°C) results in significant expression changes to >15% of the genes in the yeast genome [2]. The third disadvantage applies specifically to knockout mice; for genes that play crucial roles in embryonic development, gene knockouts often yield an embryonic lethal phenotype. This has certainly been problematic for protein kinases, as several kinase knockouts have been demonstrated to have embryonic lethal phenotypes. Thus, although genetic knockout studies have been useful for understanding the role of certain genes in mouse embryonic development, they provide little pharmacologically relevant information about the modulation of target activity in the adult organism [3].

A purely chemical approach also has its advantages and disadvantages. Whereas small molecules enable the probing of protein function in a highly controllable and pharmacologically relevant way, their major disadvantage is typically their lack of specificity. In general, small molecules found to inhibit one protein

will often inhibit multiple related proteins; thus a purely chemical approach is often unable to define the specific function of an individual target. Furthermore, it is often difficult to determine the true spectrum of action of such small molecules. These 'off-target' effects could be major contributors to toxicity, or even to positive biological and clinical effects. Thus, as tools for studying protein functions, small molecules require extensive characterization before their effects on a complex biological system can be interpreted.

Although genetics offers unprecedented target specificity, chemistry offers unprecedented temporal control over the function of target proteins. Experimental systems that can incorporate the advantages of these two approaches represent powerful tools for target validation and drug discovery in the post-genomic era.

Orthogonal chemical genetics

Recently, the benefits of combining orthogonal chemistry and genetics in drug discovery have been revealed through several studies in diverse areas of cell biology [4]. Each of the experiments uses the same fundamental approach of first modifying a small-molecule ligand (to render it 'orthogonal'), followed by changing protein structure in a complementary way to accept the orthogonal ligand. This 'lock and key' design approach takes place in stepwise fashion: first, a small molecule that binds to the protein of interest is modified (the 'key') in a manner designed to eliminate its ability to bind to its native target. This modified compound is said to be orthogonal in normal cells because it can no longer interact with its natural protein target (or, ideally, with any other target in the cell). Second, the individual protein of interest is engineered (the 'lock') to accept the orthogonal compound. Typically, a 'space creating' mutation is made in the protein because it can be readily exploited chemically. Importantly, the mutation to the protein must only affect the binding to the orthogonal compound and not otherwise modify protein function. In some situations, the engineered protein might not accept the natural ligand, thus creating a protein which itself is orthogonal.

Since the pioneering work of Hwang and Miller with GTPases in 1987 [5], there have been numerous ligand–receptor, protein–protein interaction, and enzymatic systems that have been studied using similar orthogonal chemical genetic techniques. Several of these approaches are reviewed here.

Examples of orthogonal chemical genetic approaches

RASSLs: receptors activated solely by synthetic ligands

G-protein coupled receptors (GPCRs) are the largest family of transmembrane receptors in the human genome. This

important class of therapeutic targets controls many physiological processes such as heart rate, neurotransmission, chemotaxis, cellular proliferation and hormone secretion. The repertoire of specific ligands for these receptors is extensive and should enable simple chemical control of any GPCR of interest. However, animal studies are complicated by the presence of endogenous peptide ligands, which can activate or inhibit signaling events. Furthermore, several so-called 'orphan' GPCRs have no known ligands.

To simplify the study of individual GPCRs, Conklin and co-workers [6,7] have engineered receptors that respond only to synthetic ligands and not to their endogenous native ligands. These investigators took advantage of the fact that peptide hormone binding contacts are distinct from the contacts made by small-molecule ligands: peptides usually interact with the extracellular loops of the receptor, whereas small molecules typically bind in proximity to the transmembrane domains.

An alternative approach would be to further engineer RASSLs to respond to synthetic ligands, which are orthogonal (unresponsive) to all endogenous GPCRs. There has been recent progress in this area, with the report of a 'neoreceptor–neoligand' approach applied to adenosine receptors [8]. These authors demonstrated that synthetic adenosine agonists could selectively stimulate modified A₃ receptors. With further efforts to improve the potency of these ligands, the RASSL approach could be an extremely powerful method for functionally validating the >1000 predicted GPCRs in the human genome.

An approach similar to RASSL has also been used to design and synthesize ligands for engineered nuclear hormone receptors, a unique family of transcriptional regulators. This group of receptors has both DNA binding and ligand-dependent transactivation properties. Several investigators have used the orthogonal ligand approach to selectively activate or repress gene expression by targeting specific nuclear hormone receptors such as the retinoid X receptor [9], the retinoic acid receptor [10] and the estrogen receptor [11].

These orthogonal ligand–receptor pairs are excellent tools for selectively stimulating a single member of a large family of receptors (whether GPCR or nuclear). They should be especially helpful in determining the function of orphan receptors, for which the natural ligand has not been identified.

CIDs: chemical inducers of dimerization

Protein–protein interactions are crucial to almost all cellular functions. The promiscuity and enormous complexity of these interactions, however, can make it difficult to dissect out the functional consequences of one particular

protein–protein interaction in a cellular context. To address these issues, chemical genetic methods have been developed to specifically induce or perturb protein–protein interactions in cells. Schreiber and colleagues [12] were the first to describe a method that makes use of small cell-permeable organic molecules called chemical inducers of dimerization (CIDs). CIDs have two binding surfaces that recognize one or more dimerization domains fused to target proteins; thus, upon CID treatment, the target proteins become cross-linked. The protein complex can also be rapidly dissociated with the addition of a modified CID with only one of the two binding surfaces. The dimerization domains are derived from immunophilins: cyclophilin, the FK506 binding protein 12 (FKBP12), and the FKBP12-rapamycin-binding domain of FKBP-rapamycin-associated protein (FRAP). Various CIDs have been used to perturb or initiate several cellular signaling pathways including: receptor dimerization at the cell surface [13], translocation of cytosolic proteins to the plasma membrane [14], import [15] and export [16] of proteins from the nucleus, and regulation of gene transcription [17].

Although using CIDs to control these various signal transduction events has proven to be versatile, a significant shortcoming of this approach is the promiscuity of CIDs towards endogenous immunophilins. The high expression of immunophilins in cells can lead to the formation of non-specific immunophilin–CID complexes, thereby preventing the CIDs from interacting with the desired immunophilin fusion proteins. This reduces the potency and efficacy of CIDs. To improve CID specificity for immunophilin fusion proteins over endogenous immunophilins, an orthogonal strategy was developed.

In this approach, a CID was modified to contain bulky substituents that clash with the amino acid side chains in the target protein, thereby abolishing CID binding to wild-type targets. Complementary mutations were made in the target protein that restores binding to these modified CIDs. Initially, cyclophilin–cyclosporin was used as a model system; this orthogonal receptor–ligand pair provides a potent tool for inhibiting calcineurin-mediated dephosphorylation of nuclear factor of activated T cells (NFAT) in a cell or tissue-specific manner [18].

The orthogonal design strategy has also been used for FKBP. Clackson *et al.* [19] have redesigned the FKBP–ligand interface (by introducing a single amino acid mutation: F36V) to introduce a novel specificity-binding pocket. This resulted in a highly specific orthogonal ligand ($K_d = \sim 0.1$ nM), which is 1000-fold more specific for the engineered FKBP than for the wildtype protein. FKBP (F36V) has been used in a wide variety of cell systems to control diverse signaling pathways. In one example [19], a chimeric protein was

created by fusing FKBP (F36V) to the intracellular domain of the Fas receptor; this construct was expressed both in HT1080 cells and in mice. Treatment with the orthogonal ligand caused rapid apoptosis of the engineered cells (either *in vitro* or *in vivo*) that was independent of endogenous FKBP.

A potentially more subtle way of controlling dimerization is to create a small-molecule switch at the intrinsic receptor–ligand interface of the protein of interest. This method essentially combines the RASSL and CID approaches. By first introducing a hole into both the human growth hormone and its associated receptor, Schultz and colleagues [20] were able to screen a chemical library to find a small molecule that suppresses the introduced mutations, allowing much of the original binding affinity to be restored.

ASKAs: analog sensitive kinase alleles

Protein kinases represent as many as 2% of all human genes, and are involved in virtually all cellular processes and consequently many disease processes as well. Thus, kinases represent a rich source of drug targets across a wide array of large clinical indications. The success of Herceptin™ (Genentech; <http://www.gene.com/gene>), an ErbB2 specific antibody, in the treatment of breast cancer, and the potent activity of Gleevec™ (Novartis; <http://www.novartis.com>), a small-molecule abl kinase inhibitor, in the treatment of chronic myelogenous leukemia and gastrointestinal stromal tumors, have recently highlighted the importance of kinases as key oncology targets. Given the large number of kinases (at least 500 different protein kinases in humans), and the extensive homology at their most druggable site (the ATP-binding pocket), powerful methodologies will be required for efficient and meaningful kinase target validation and drug discovery.

We have been using a unique approach for studying kinases that combines the specificity of genetics with the pharmacological relevance and control of small-molecule chemistry. This chemical genetics platform provides crucial tools for the functional validation of kinases as drug targets, with multiple applications to the drug discovery process. The technology is based upon the discovery of analog-sensitive kinase alleles (ASKAs), and corresponding small-molecule analog compounds that specifically modulate ASKA activity [21]. Unlike the systems previously mentioned that require individual chemistries and structural engineering for each protein of interest, recent studies demonstrate that the ASKA approach can be applied to members of diverse protein kinase subfamilies [22]. Because the ASKA system is highly modular, it can be efficiently applied across the kinase superfamily.

Description of the ASKA technology

The key feature of the ASKA approach is the creation of a subtle but unique structural distinction between the catalytic domain of one kinase and all other kinases in the genome. This distinction is achieved by making a mutation in the ATP binding pocket of the kinase. All protein kinases have a bulky amino acid residue at a conserved position in the ATP-binding pocket. When this so-called 'gatekeeper' residue is changed to an alanine or a glycine, access to a deep hydrophobic 'specificity pocket' is created (Fig. 1). Importantly, this mutation in the ASKA is 'quiet' in terms of kinase function; numerous studies have demonstrated that protein structure [23], enzymatic activity [24], substrate specificity [23] and cellular function [22,25–27] remain intact. This method is widely applicable throughout the kinase superfamily, and does not require three-dimensional structural information; the gatekeeper residue is readily apparent from simple amino acid sequence alignments [22]. A small modular set of functional ATP analogs and potent inhibitors have been synthesized that fit only the engineered kinases. Thus, the ASKA system works efficiently across the kinase superfamily with a small number of analogs and inhibitors without the need to perform additional chemistry to analyze and validate each kinase target.

The ASKA technology has multiple applications to the drug discovery process. These include:

- Pathway-based target identification, including the identification of direct kinase substrates, facilitating the understanding of target function in the context of disease-relevant cellular pathways.
- Cell-based and *in vivo* model systems for pharmacologically relevant target validation.
- Chemical genomic profiling: using microarrays to determine the effects of highly specific drug-mediated kinase inhibition on gene expression.
- Direct coupling of chemical genomic profiling to high-throughput and high-content drug screens.
- Preclinical *in vivo* models with reference compounds to establish therapeutic index and provide a source of biomarkers.

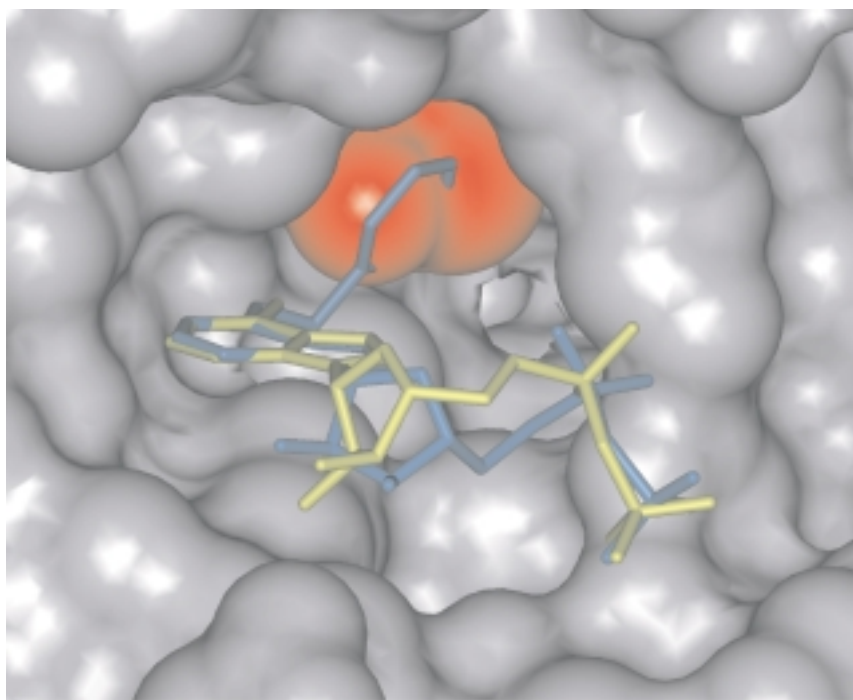
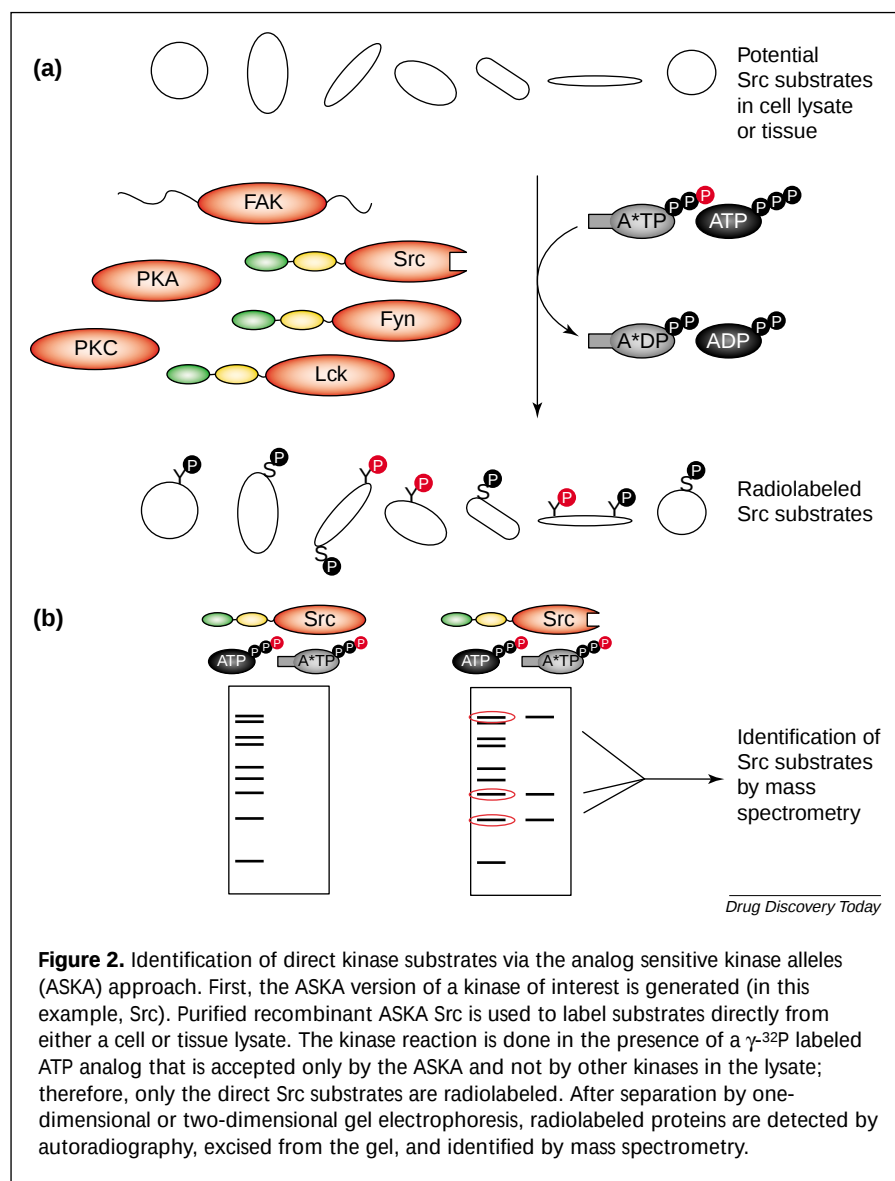


Figure 1. Structural basis for analog sensitive kinase alleles (ASKA) selectivity. X-ray crystal structure of c-Src ASKA with N⁶-benzyl-ADP bound. Gray: rendered surface of the active site; red: threonine found in wildtype c-Src, added back in this rendering; blue: N⁶-benzyl-ADP; yellow: 5'-adenylyl-imidodiphosphonate (AMP-PNP) added as reference. Several amino acids in the rendered image have been removed to better visualize the crucial interaction residues. This image reveals the steric hindrance between the benzyl ring of N⁶-benzyl ADP and the threonine residue at the 'gatekeeper position'. A glycine at this position flattens the pocket and allows N⁶-benzyl ADP to efficiently bind. Partially visible is the deep hydrophobic pocket adjacent to the gatekeeper that can be accessed in the ASKA by the PP1-based analog inhibitors. Image courtesy of Mike Eck, Harvard University (adapted from [23]).

ASKA-based pathway mapping

By using functional ATP analogs that are uniquely accepted by the ASKA, direct substrates of the mutated kinase become uniquely tagged, thereby facilitating their rapid identification by mass spectrometry (Fig. 2). Although there are other methods for identifying kinase substrates (e.g. expression cloning, yeast two-hybrid, and peptide library screening), this unique chemical genetic approach is a rapid path to identifying direct native substrates from relevant cell or tissue lysates. This approach has recently been used to determine the direct substrates of Jnk-2 [28] and v-Src [29]. Importantly, although several known substrates were identified in this second study, several novel v-Src substrates were also revealed. Elucidation of kinase pathways by direct substrate identification is important for 'pathway placement' and functional annotation of orphan kinases; it also provides crucial additional functional information about known kinase drug targets. Knowledge about relevant substrates could predict potential adverse or



known [22], they are cell permeable [25], and have excellent bioavailability and low toxicity in mice (K. Shokat and M. Velleca, unpublished). Thus, a specific kinase can be validated as a drug target by treating an ASKA in cells or whole animals with the orthogonal inhibitor and studying the genomic, proteomic, cellular, physiologic and/or phenotypic consequences of such inhibition.

Using homologous recombination in embryonic stem cells, as well as traditional transgenic approaches (on kinase knockout backgrounds), ASKA 'knockin' cell lines and mice can be created (Fig. 3). In these cell and whole animal systems, the wildtype kinase is replaced in the genome with its functional ASKA homologue. By using the orthogonal inhibitors, the ASKA cells and mice can then be evaluated in multiple disease models to enable functional validation of kinases in both physiologically and pharmacologically relevant manners. Using this highly specific 'chemical switch' approach, a kinase can be thoroughly validated as a drug target for one or more diseases either before, or in parallel, with lead identification and optimization, but well before investing significant resources in taking inhibitors through costly clinical development.

synergistic side effects that can occur when a kinase is inhibited. Substrates can also represent additional potential drug target opportunities downstream of kinases; this could be particularly important in the case of kinases that are ubiquitously expressed, where tissue-specific (and therefore more disease-specific) substrates might be better targets. Finally, the genes identified through gene expression analysis as transcriptionally regulated by specific kinase inhibition (see chemical genomic profiling later) also represent potential downstream drug targets (or protein therapeutics) within an individual signal transduction cascade.

Target validation using ASKAs

Highly potent small-molecule inhibitors have been developed that are uniquely accepted by the ASKAs. These inhibitors are among the most specific and potent kinase inhibitors

ASKA-based drug screens

The unique sensitivity of ASKAs to the cell permeable inhibitors forms the basis of high-throughput and high-content drug screens that offer high specificity and selectivity. An important aspect of the validation approach outlined previously is the 'chemical genomic profiling' step. This refers to the determination of the effect of specific drug-mediated kinase inhibition on gene expression. Direct gene expression read-outs in cells, generated by highly specific ASKA inhibition, form the basis of high-throughput, primary, cell-based drug screens. Likewise, the complete set of genes up- or down-regulated in an inhibitor study (and measured by microarray analysis) represents a comprehensive genomic 'blueprint' of specific kinase inhibition (Fig. 4). This blueprint forms the basis of an array-based, high-content assay for optimizing potency and specificity in a

kinase inhibitor lead series. Importantly, as two studies have recently shown [22,27], the blueprints generated by the ASKA approach are fundamentally different from those generated by knockdown or knockout technologies. In one example [27], the role of a kinase in a crucial metabolic pathway was revealed only upon acute chemical inhibition of the ASKA, and was missed by pure knockout analysis.

Finally, the kinase substrates identified through the ATP labeling studies can also be used for both biochemical and cell-based drug screens. Secondary and tertiary drug screens using natural kinase substrates might have advantages over pseudo-substrate-based screens.

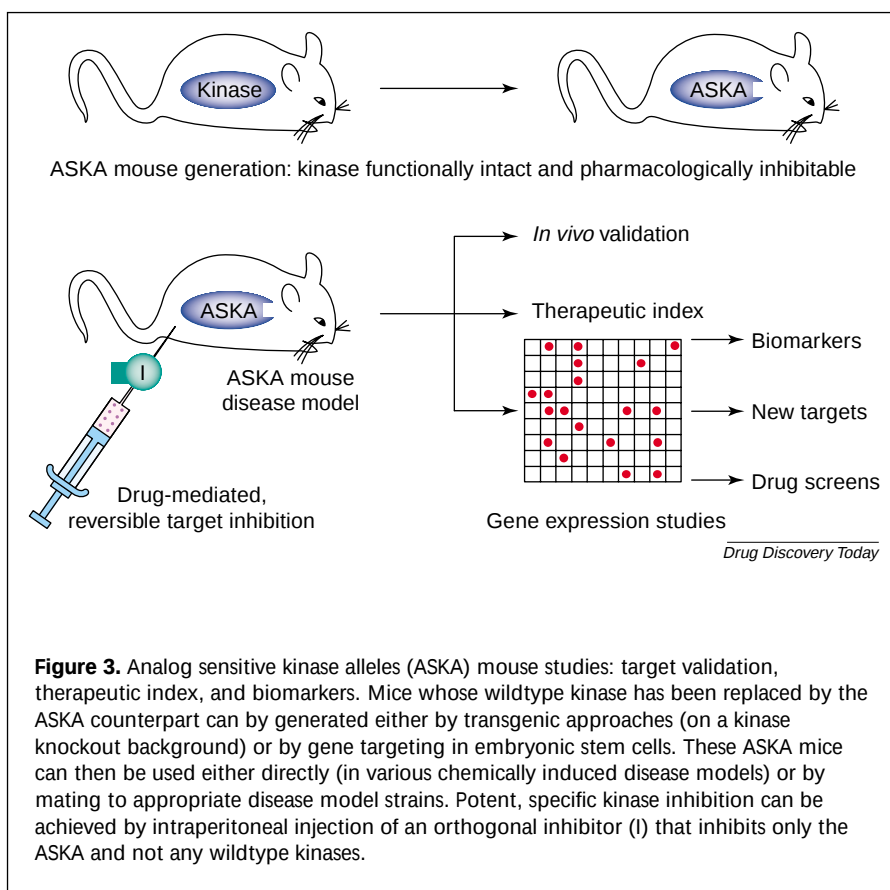
Orthogonal kinase inhibitors as reference compounds

In addition to rigorously establishing the role that a particular kinase plays in a disease process, ASKA mice should provide crucial information regarding therapeutic index. By using the orthogonal inhibitors as well-characterized reference compounds, ASKA-based *in vivo* studies will be able to establish mechanism and target-based efficacy and toxicity for most protein kinases. Such information should prove useful in the preclinical testing of development candidates, because it will allow distinction between mechanism (target)-based and compound (off-target)-based toxicities.

With the FDA demanding more rigorous proof of mechanism of action, there is a crucial need to have physiologically relevant biomarkers or surrogate markers for kinase inhibitors that are entering clinical development. The ASKA approach provides at least three significant sources of these biomarkers: (1) proteins identified through kinase substrate identification studies; (2) secreted proteins revealed by inhibitor treatment of ASKA mice; and (3) chemical genomic profiles (microarray analysis). These markers can be used to correlate the degree of kinase target inhibition with therapeutic efficacy in either preclinical studies or clinical trials.

ASEAs: analog sensitive enzyme alleles

More broadly, the ASKA technology can be applied to others enzymes. ASEA technology will be useful for gene products belonging to an enzyme family with a conserved and well

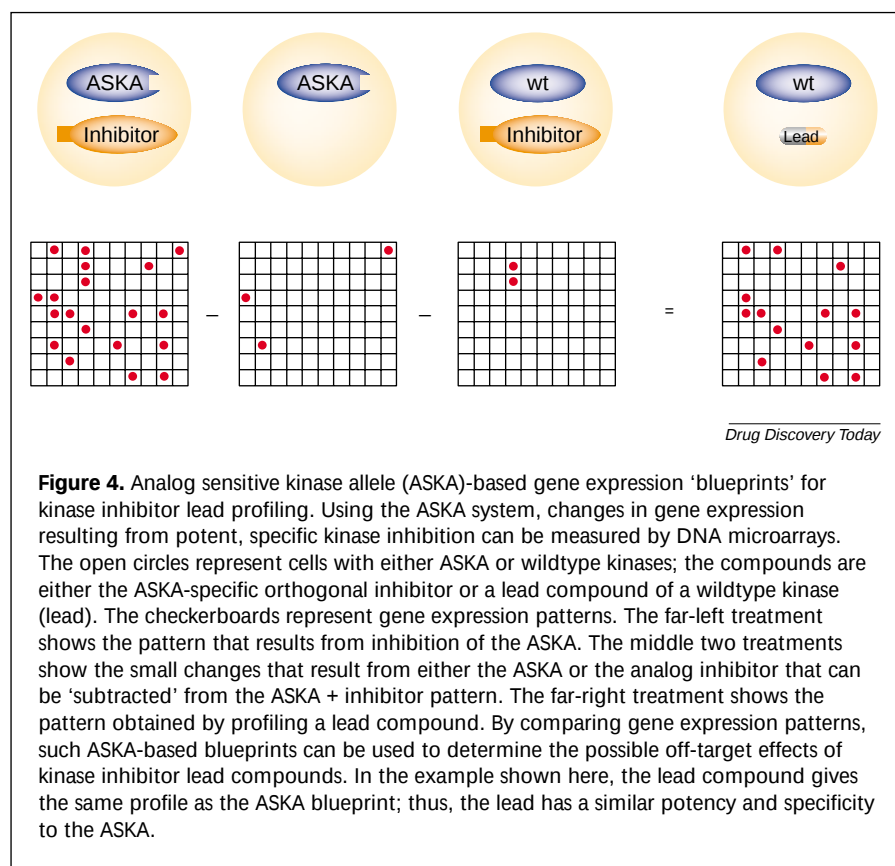


studied active site. Indeed, the ASEA approach has already proven useful for the study of enzymes other than protein kinases.

Turning GTPases into XTPases

The first engineered protein in a signal transduction pathway to accept a non-natural substrate was a member of the GTPase family of enzymes [5]. GTPases control many biological processes including translation, protein translocation, vesicle transport, nuclear import, and signal transduction. GTPases typically exist in both GTP- and GDP-bound states. The enzymes interchange between these two states by hydrolyzing bound GTP to GDP + P_i followed by the binding of GTP.

Through protein engineering and organic chemistry, Hwang and Miller [5] first demonstrated that the substrate specificity of a GTPase could be completely switched from GTP to XTP (xanthosine 5'-triphosphate). Although this strategy has been generalized to the study of many GTPases, it is distinctly different from other ASEA approaches (see later), because the mutant enzymes lose their ability to utilize GTP and are dependent on the orthogonal XTP for activity. Thus, they lack practical use for study in mammalian *in vivo* systems.



Conditional alleles of kinesin and myosin motors

The myosins and kinesins are part of a large family of ATP-hydrolyzing motor proteins. Many different cellular processes are controlled by these force-generating enzymes, including muscle contraction, vesicle trafficking and cell motility. To dissect the specific role of individual members of these families, mutant motors capable of interacting with unnatural ATP analogs have been developed to gain chemical control over individual motors in cells.

Gillespie *et al.* designed a system to study the involvement of myosin 1- β in the hearing process [30]. By making a space-creating mutation within the ATP-binding pocket of myosin 1- β , these investigators produced a mutant that is potently inhibited by bulky N⁶-substituted ADP analogs. Specifically, mutation of rat myosin 1- β tyr61 to glycine creates an enlarged binding pocket capable of uniquely accepting N⁶-substituted adenosine analogs. *In vitro* motility assays show that the mutant myosin is capable of functioning at or near wildtype levels in the presence of ATP, whereas its activity is potently inhibited by the N⁶-substituted ADP analogs. A transgenic mouse bearing a mutant myosin 1- β has recently been generated [31]. Studies of this mouse have shown that myosin 1- β mediates the slow component of adaptation by hair cells, the sensory cells of the inner ear.

The large kinesin family of ATP-driven motors is also amenable to a similar strategy of generating allele-specific inhibitors. Kinesin and kinesin-like proteins are responsible for the intracellular movement of organelles along microtubules, spindle formation, and the segregation of chromosomes at cell division. Indeed, their crucial role in cell division has made them popular drug targets for oncology. Kinesin superfamily members have a conserved ATPase domain with structural similarities to myosin. Examination of the X-ray crystal structure of human kinesin reveals conserved charged groups (Arg14 and Arg16). Mutation of one of these bulky groups to either alanine or glycine provides an enlarged ATP-binding pocket specifically capable of accepting an N⁶-substituted ATP molecule.

Kapoor and Mitchison have reported the use of this chemical genetic approach to produce kinesin allele-specific activators and inhibitors [32].

However, the removal of the charged group in the binding pocket disrupts the ability of kinesin to function with endogenous ATP, thus making the mutant reliant on the N⁶-substituted analog for function. Because hydrolysis of ATP, not release of ADP as in the myosin system, is the rate-limiting step in the kinesin system, they made use of a non-hydrolyzable form of the analog to specifically inhibit the mutant kinesin. Such allele-specific activators and inhibitors should enable clarification of the role of individual kinesin family members in various cellular processes.

Conclusions

For many important drug target classes, it is now possible to modify individual proteins in cells or whole organisms so that they can be specifically modulated with small molecules. This approach combines the specificity of genetics with the pharmacological relevance of small-molecule chemistry. As the pharmaceutical industry copes with a glut of potential targets, rigorous chemical genetic approaches should not only improve our understanding of target function, but also provide tangible drug discovery assets, such as drug screens, biomarkers, and valuable therapeutic index information, to truly enable genome-based drug discovery.

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