Small-Molecule Kinase-Inhibitor Target Assessment

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The identification of the cellular targets of small-molecule protein kinase inhibitors is a significant hurdle to assessing their therapeutic potential for many diseases. Here we review several biochemical and genetics-based approaches to identifying inhibitor targets. We also describe a chemical-genomics approach to kinase-inhibitor target identification and validation that matches transcriptional signatures elicited by a drug of unknown specificity and those elicited by highly specific pharmacological inhibition of engineered candidate kinase targets.

Protein kinase inhibitors have emerged as promising therapeutic molecules for the treatment of a number of diseases including cancer and asthma.[1] Over the past decade, significant efforts have been devoted to identifying potent and highly selective inhibitors of kinases associated with disease. However, due to the large size of the protein kinase superfamily and the fact that most kinase inhibitors bind in the highly conserved ATP-binding pocket, such inhibitors have proven to be difficult to identify even with technologies such as combinatorial synthesis, structure-based design, and high-throughput screening.[2–4] Thus, it is widely accepted that even the most specific kinase inhibitors suppress other kinases besides the intended target ("multiplex" inhibition).

The central question that remains to be answered is whether kinase inhibitors achieve their therapeutic effects because of, or in spite of, their lack of specificity. The traditional model of targeted therapeutics ascribes to pharmacological agents that are as close to monospecific as possible to avoid detrimental side effects. However, the prominence of kinases as key nodes in convergent signal transduction pathways in processes such as angiogenesis[5] and ErbB-driven cancers[6] suggests that inhibition of multiple pathways might be preferable, or even necessary, to block aberrant signaling. Because the answer to this question is likely to be different when considering different inhibitor and disease combinations, a key challenge in the development of molecules as molecular therapeutics is to assess their true spectrum of cellular targets.

The standard biochemical approach to addressing this problem relies on specificity screens with in vitro inhibition assays against panels of purified kinases.[7] It is not yet possible to biochemically assay every kinase in the genome, although this challenge is being undertaken by a number of groups using technologies such as protein arrays.[8] In a complementary approach, bead-immobilized kinase inhibitors have been used as reagents to affinity-purify putative inhibitor targets from cell lysates. Using this strategy, Gray and co-workers have identified targets of the cyclin-dependent kinase (CDK) inhibitor purvalanol.[9,10] A group at Axxima Pharmaceuticals has further refined the technology to identify kinases that bind to the p38 MAP kinase inhibitor SB203580.[11] In both cases, the authors identify additional targets besides the known targets of these inhibitors and verify that inhibition of these kinases occurs in intact cells. Specifically, purvalanol was found to inhibit p42 and p44 MAP kinases and SB203580 was found to inhibit the kinase RICK. Both groups also identify non-kinase proteins that might be relevant targets of the inhibitor, thus highlighting a potentially important advantage of this approach over kinase-inhibition assays.

A number of groups have addressed the problem of drug target identification using genetics-based approaches, with the design of elegant genome-wide screening strategies in the model organism *S. cerevisiae*. In a haplo-insufficiency profiling (HIP) approach, a library of diploid yeast strains with heterozygous deletions of each gene is screened for drug sensitivity either in a single culture with a competitive growth assay[12,13] or by screening the 6000+ strains in parallel.[14] The utility of a HIP screen is predicated on a gene-dosage effect; the drug is able to preferentially inhibit the growth of the strain that contains only one copy of the gene for its target. This approach has now been used to identify candidate targets for a large number of chemical agents, such as methotrexate, 5-fluorouracil, molsidomine, and dihydromotuporamine C. In a genome-wide synthetic lethal screen,[15] a drug is screened at a concentration that is normally sublethal against a library of haploid yeast strains with individual gene deletions. Deleted genes that result in increased drug sensitivity might be potential direct drug targets or genes that are involved in the same cellular pathways as the drug target. Synthetic lethal screens on molecules such as rapamycin and fluconazole, besides representing a technical tour de force, have identified targets consistent with the known mechanisms of action.

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In another approach, and one that has recently been applied to kinase inhibitors, the yeast 3-hybrid screening system was adapted to identify putative targets of several CDK inhibitors.[16] Unlike the genetic screens described above, 3-hybrid screening in principle detects only direct interactions between an inhibitor and protein-binding partners. By using this system, a number of targets of the CDK inhibitor purvalanol were identified. Interestingly, this screen resulted in the identification of both overlapping and divergent targets compared to the affinity-purification study by Gray and colleagues described above.

One important limitation common to all these strategies is not so much in target identification but in target validation. Biochemical strategies are not carried out in intact cells, while genetic screens rely on indirect readouts such as cell growth to identify binding interactions. Thus, detection of the binding interaction between an inhibitor and its target(s) is by necessity decoupled from the process of evaluating specific cellular phenotypes elicited by treatment with the inhibitor. Without time-consuming validation, it is unclear whether or not a detected interaction is relevant in a cellular context. The cellular environment is a complex milieu with factors such as drug bioavailability, subcellular localization, and target abundance able to affect an inhibitor's in vivo spectrum of targets. For instance, recent evidence suggests that the inhibitor BAY 43-9006, originally developed against Raf kinase, might exert many of its cellular effects through inhibition of VEGFR in diseases such as renal-cell carcinoma.[17,18] The dual EGFR/ErbB-2 inhibitor GW572016, while sharing the same 4-anilinoquinazoline core as other tyrosine kinase inhibitors with similar in vitro specificity, appears to have unusually strong potency in cells due to a unique binding mode that results in a comparatively slow off-rate.[19] These examples suggest that identification of candidate kinase inhibitor targets alone is unlikely to reveal unambiguously the in vivo mechanism of action. In fact, in vivo target validation often becomes apparent only after a drug has entered clinical trials (BAY 43-9006). The ultimate confirmation of a drug's primary in vivo target comes from the observation of drug-resistant mutants, as is well documented in the case of Gleevec,[20] a potent inhibitor of the oncogenic fusion kinase BCR-Abl used to treat chronic myelogenous leukemia.

One of the best ways to ascertain an inhibitor's in vivo efficacy is through analysis of biomarkers that are known to be regulated by the catalytic activity of the kinase of interest. For instance, the inactivation of the ribosomal protein S6 kinase was used as a reliable marker of clinical activity of rapamycin derivatives through inhibition of mTOR.[21] Confounding the forward genetics analysis of cellular effects of kinase inhibitors is that the roles of the putatively targeted kinases are often not completely understood. This makes target validation through a biomarker-based strategy problematic.

A powerful method to abrogate the activity of specific proteins is through the use of short interfering RNA (siRNA) to silence the expression of those genes. The generality and ease of deployment of this technology make it an indispensable tool for studying kinase signaling. Gewirtz and co-workers have recently demonstrated that siRNA targeting the Lyn kinase induces apoptosis in BCR-Abl-expressing leukemic cells that are resistant to the BCR-Abl inhibitor Gleevec, while normal hematopoietic cells remained viable.[22] This study illustrates the potential of using siRNA for kinase-target validation, as it revealed a unique dependency of a diseased cell on Lyn kinase signaling. The caveats associated with siRNA for target validation are the same as for approaches that use genetic perturbations to abolish target activity. Pharmacological inhibition of a kinase is mechanistically different from blocking expression of the kinase altogether. If the kinase must ultimately be inhibited by a small molecule (in disease treatment, for instance), the effects of siRNA against the kinase may or may not recapitulate the cellular activities of the eventual drug candidate.

One promising alternative to address this issue utilizes a chemical genetic strategy developed by our laboratory to generate "prevalidated" monospecific inhibitors of engineered kinases. A space-creating mutation at a conserved bulky amino acid residue (the "gatekeeper")[23] in the ATP-binding pocket renders kinases susceptible to ATP-competitive inhibitors, such as 1-NA-PP1 and 1-NM-PP1,[24] that have been designed to be poor inhibitors of nonengineered kinases. Importantly, it appears that most protein kinases are amenable to inhibitor sensitization at this residue while still allowing for sufficient kinase catalytic activity to retain normal cellular function.[25] By replacing the endogenous kinase with its analogue-sensitive counterpart in cells, we have used these chemical genetic tools to elucidate the cell biology of kinases involved in a number of different signal-transduction pathways.[26–28]

We have adapted these chemical-genetic tools into a screen that can be used to assess the molecular etiology of specific cellular effects exerted by a given kinase inhibitor. We envisioned using analogue-sensitive alleles coupled with the appropriate cellular assays to generate a molecular *reference pro-*

***Editorial Board Member**.[*] Professor Kevan Shokat is one of the leading investigators in the new field of chemical genetics. His research focuses on the development of novel chemically based tools to decipher signal-transduction pathways on a genome-wide scale. He and his colleagues have developed a method for producing small molecules that are specific for a particular target in a signaling cascade by combining protein design and chemical synthesis. Professor Shokat received his Ph.D. in 1991 from UC Berkeley and completed post-doctoral work at Stanford in 1994, after which moved to Princeton University as an Assistant Professor of Chemistry and Molecular Biology. In 1999, he relocated to the Bay Area, where he is currently Professor in the Department of Cellular and Molecular Pharmacology at UC San Francisco and Professor of Chemistry at UC Berkeley. He is a Pew Scholar, a Cottrell Scholar, a Searle Scholar, a Glaxo–Wellcome Scholar, and an Alfred P. Sloan Fellow.*

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file of cellular effects caused by inhibition of candidate kinases. Target identification would be achieved by matching cellular effects elicited by treatment with the kinase inhibitor to these reference profiles. This methodology could also address the important and often elusive question of whether any kinase targets remain unidentified after conducting a screen. Assuming that the cellular assays measure a broad spectrum of inhibitor effects and that reference profiles for all relevant kinases are available, iterative matching of reference profiles would allow targets of the kinase inhibitor that span all of its cellular activities to be identified.

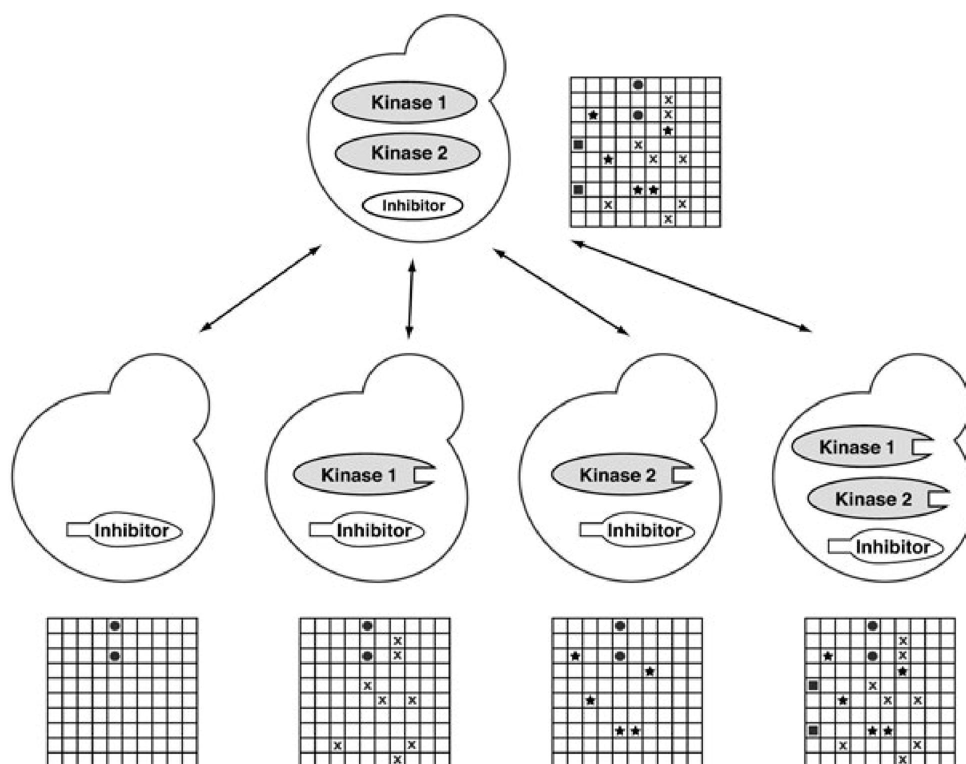

Figure 1. *The targets of a kinase inhibitor of interest can be identified by deconvoluting its global transcriptional expression profile as measured by DNA microarrays. Gene expression changes arising from nonspecific xenobiotic effects (●) or specific inhibition of kinase 1 (×), kinase 2 (*), or kinases 1 and 2 (■) are identified by profiling wild-type or yeast strains carrying the appropriate analogue-sensitive allele(s). In this case, the kinase inhibitor is identified as being a multiplex inhibitor of kinases 1 and 2.*

One convenient and well-established technology that could be used as the probe for such a screen is the DNA microarray. Without the need for explicit biochemical assays of each protein, a gene-array signature represents a genome-wide scan of the cellular effects of an external stimulus, such as a drug treatment.[29] Given the importance of protein kinases as fundamental components of the cell's signaling apparatus, it seems likely that perturbation of most kinases is likely to impact downstream transcription; this makes the DNA microarray particularly suitable for use in profiling the effects of kinase inhibitors. This cell-based assay also reads out only inhibitor–target interactions that are physiologically relevant, independent of possibly misleading in vitro binding affinities.

Initially, such a screen might be carried out in a model organism, such as *S. cerevisiae*, in which it is possible to conduct genetic manipulations rapidly with great precision. This would allow the construction of a set of yeast strains in which the genes for individual kinases have been replaced with their analogue-sensitive counterparts. Profiling the transcriptional effects arising from inhibition of each kinase would identify specific sets of transcripts that could serve as a diagnostic transcriptional "signature" (Figure 1). Observation that the drug of interest also elicited the same transcriptional signature would be powerful evidence that it targeted that particular kinase in cells. It is also possible to generate yeast strains containing multiply sensitized kinases that might be used to identify effects of kinase inhibitors that require simultaneous inhibition of two or more kinases. For instance, it has recently been shown that inhibition of the two yeast CDKs Kin28 and Srb10 is required to fully suppress transcriptional activation.[30] Thus, an exhaustive application of this technology would result in the identification of phenotypic changes elicited both by inhibition of individual or multiple kinases.[31]

Of course, for the comparative profiling approach outlined in Figure 1 to have the optimal opportunity for success, a number of technical conditions must be met. Ideally, the strength of inhibition of the target kinase by the inhibitor should be equivalent to the inhibition of the analogue-sensitive allele of that kinase by the PP1 analogue. Cell signaling is also sensitive to factors such as cell-cycle status and time following stimulus. Thus, depending on the kinases involved, a comprehensive analysis would in principle require reference profiles spanning a range of inhibitor doses and time points. In practice, we have found that using microarrays as a readout is suitable to address both of these issues, as acute inhibition of kinases for as little as ten minutes results in gene-expression changes sufficient to use as a reference profile, while the magnitude of the response gives a semiquantitative measure of the strength of inhibition.[25,32] Another issue concerns nonspecific gene-expression changes resulting from treatment of cells with small molecules (Figure 1, bottom left), presumably arising from interactions between these molecules and non-kinase targets (such as drug efflux pumps). The cell's transcriptional response to the stress caused by the presence of foreign molecules may differ between different chemical species such as

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PP1 analogues and the kinase inhibitor that is being profiled. These gene-expression changes will most probably have to be weeded out through the comprehensive identification of stress-response genes through transcriptional profiling of the effects of a wide range of cellular stimuli and environmental changes, as has been successfully done in yeast.[33]

More widespread deployment of this technology in validating mammalian kinases as drug targets requires the continued advancement of techniques to do gene knock-ins to generate the necessary mice or cell lines.[34] Although the genomics and genetic tools in mammalian cells have not yet reached the level of sophistication of their yeast counterparts, analogue-sensitive alleles can still be used to identify specific biomarkers, such as cell proliferation, arising from kinase inhibition. A recent study by Witte and co-workers of the drug Gleevec has shed more molecular detail on its exact mechanism of action.[35] They used an analogue-sensitive version of BCR-Abl to show that while inhibition of BCR-Abl alone by Gleevec is able to suppress cell proliferation in cells lacking the kinase KIT, inhibition of BCR-Abl and KIT together is necessary for drug efficacy in cells expressing KIT. These results suggest that dual inhibition of multiple kinases may be a general mechanism for therapeutic action by protein kinase inhibitors.

Analogue-sensitive alleles are powerful chemical genetic tools that might facilitate the dissection of the mechanism of action of kinase inhibitors. These tools have also yielded insights into the mechanisms by which cellular transformation becomes dependent on aberrant kinase signaling[36] and the possibility of exploiting synergistic contributions from inhibition of both protein kinases and lipid kinases in treating cancer.[37] Information garnered from these studies and others should prove useful in designing and testing the next generation of kinase-targeted therapeutics.

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