Chemistry & Biology, Vol. 12, 1057–1062, October, 2005, ©2005 Elsevier Ltd All rights reserved. DOI 10.1016/j.chembiol.2005.09.011

Previews

The Kinome Is Not Enough

Mechanism of action studies are essential to link observable effects on cells with molecular targets of small molecules. Caligiuri and coworkers [1] describe how yeast three-hybrid screening identified kinases that might mediate an intriguing tumor cell-specific antiproliferative effect.

The search for selective small-molecule inhibitors of protein kinases has an interesting history. Despite initial concerns that ATP-competitive inhibitors would lack selectivity, remarkable results have been achieved with small molecules that are able to differentiate between kinases possessing a high degree of sequence similarity (for a review, see [2]). Selectivity assessments have been based largely on data from in vitro kinase assays, although frequently they are supported by functional and phenotypic data obtained using whole cells. The number of kinases available for assay, however, has limited the conclusions that can be drawn about individual inhibitors. The discovery that the human kinome consists of ~518 kinases [3] has underscored the challenge of profiling kinase inhibitors, and it has become increasingly evident that primary sequence similarity between proteins is not the most important determinant of inhibitor sensitivity. Recently, several approaches have been applied to the evaluation of kinase inhibitor specificity, and the results have been enlightening. These approaches include (1) expansion of the panel of kinases available for in vitro assay of functional activity [4, 5], (2) display cloning [6, 7], (3) affinity chromatography [8, 9], and (4) the yeast three-hybrid approach (Y3H) [10, 11]. The consensus from these studies is that small-molecule kinase inhibitors are less selective than generally has been claimed, typically inhibiting a cross-section of kinases that in some cases are phylogenetically quite distinct. These methods are beginning to reveal important properties of clinical kinase inhibitors, including marketed drugs and drug candidates, as well as important constituents of the cell biology toolbox. In this issue of *Chemistry & Biology*, Caligiuri and coworkers [1] describe the application of Y3H to mechanism of action (MOA) studies of a putative Cdk inhibitor, RGB-286147.

Y3H is an extension of Fields and Song's two-hybrid method [12, 13], which was developed to detect proteins that associate in cells by linking their association to transcriptional activation of a specific reporter gene, e.g., one required for growth on histidine-deficient media. In Y3H (Figure 1), the DNA binding (DB) and activation domains (AD) of a transcription factor are expressed separately as fusion proteins. Linking the DB domain to a well-characterized protein (e.g., DHFR) and the AD domain to a library of potential target proteins then requires that assembly of the requisite transcription factor, and therefore reporter gene activation, be mediated by a hybrid molecule (dimerizer), consisting of an anchor compound (e.g., methotrexate) linked to a compound whose protein target may be unknown, such as a kinase inhibitor. Kley and coworkers have exploited this technique to develop tools for specifically probing whole proteome cDNA libraries for targets of small-molecule kinase inhibitors. In previous work, they demonstrated the success of Y3H in identifying and confirming targets of the purine-derived kinase inhibitor purvalanol B [14]. Remarkably in both that report and in the latest study [1], the only targets identified for these small-molecule kinase inhibitors were protein kinases.

The pyrazolopyrimidinone RGB-286147 was selected for MOA studies based on the properties it exhibits in cell-based assays [1]. Despite being prepared initially as an inhibitor of Cdk1 and Cdk2, its effects on cells are inconsistent with exclusive inhibition of these target kinases. Of particular note is the observation that RGB-286147 displays differential antiproliferative activity against noncycling HCT116 human carcinoma cells (IC_{50} = 51 nM) versus noncycling human fibroblasts (IC_{50} > 1 μM), which are not transformed and therefore more akin to nontumor ("normal") cells. In contrast, RGB-286147 is similarly antiproliferative toward HCT116 cells and human fibroblast cells when they are actively cycling.

A Y3H screen using a dimerizer of methotrexate connected via a PEG linker to RGB-286147 identified several targets for this small molecule [1]. All of the proteins identified and confirmed as targets, save one, are CDK or CDK-related kinases, illustrating a remarkably narrow spectrum of inhibitory activity. The important confirmatory studies using yeast clones specifically expressing kinase or kinase domain proteins, or employing a related PEG-tethered pyrazolopyrimidinone as an affinity probe, expanded the set of potential target kinases to 14. Inhibitory potencies for eight of these kinases were measured to validate the selection and demonstrate functional binding.

This study identifies a relatively small number of potential protein targets for RGB-286147, one or more of which may mediate its antiproliferative effect on noncycling HCT116 cells [1]. Once a complete set of inhibition data is available, it would be interesting to know whether any of the potential targets of RGB-286147 are differentially expressed in the sensitive HCT116 cells. Further sleuthing has the potential to pinpoint a new molecular target for therapeutic intervention with small molecules that could be effective against noncycling cells in a tumor mass. It may be possible to exploit this target to increase the chances of tumor eradication using chemotherapy.

More generally, the work by Caligiuri and coworkers illustrates the value of proteome-wide target screening to mechanistic studies of compounds that may not perform as originally intended but that still display an interesting profile in cells [15, 16]. Thus, the identification of molecules that affect cells in a specific and desirable way followed by the application of chemical genetics tools such as Y3H to identify a cross-section of

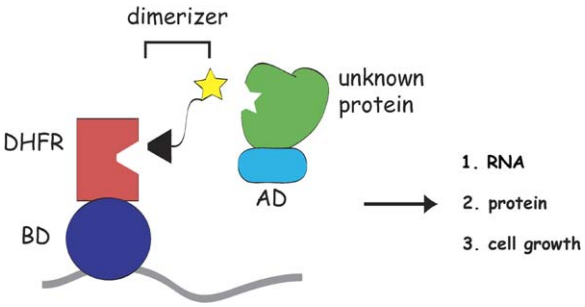

Figure 1. Schematic of a Yeast Three-Hybrid System

Transcription factor DNA binding (BD, dark blue) and activation (AD, light blue) domains are fused to DHFR (red) and an expressed library of proteins (green), respectively. A library of chemical dimerizers comprised of methotrexate (black) and a candidate small molecule (yellow) joined by a linker has the potential to bind to both DHFR and the unknown, target protein. Binding reconstitutes the transcription factor activity leading to expression from a reporter gene promoter (gray) and an observable phenotype. Figure provided by L. Szewczak.

activities (binding affinities and target proteins) will create a fingerprint against which future molecules may be compared. One can envisage the optimization of kinase inhibitors for activity against a specific subset of kinase targets or a particular signaling pathway that has been associated with the biological effect of interest. Importantly, these affinity-based techniques are not limited to compounds that bind to an enzyme active site, but in principle could be extended to allosteric inhibitors and inhibitors of proteins that lack a catalytic function.

Affinity chromatography and Y3H are complementary, since each is subject to different limitations including the use of cell lysate versus intact live cells, the use of mammalian versus yeast cells, and very likely different influences attributable to protein expression levels. Both techniques are limited by the requirement to modify chemically or to immobilize the biologically active probe molecule. Y3H shares an advantage with display cloning, namely that identification of the target protein is linked to identification of the corresponding gene, facilitating its identification and subsequent protein overexpression. The visual read-out from Y3H (cell growth), and its potential for automation at many steps, offers considerable potential for parallel screens against different proteomes, multiple protein classes, or mutants of the same protein. The selection of compounds matching a specific binding profile from a library of potential inhibitors also is conceivable. The future extension of three-hybrid technology to mammalian cells will expand the scope of compounds that can be used, increase the potential for competition assays, as well as provide a more direct relationship to phenotypic data from mammalian cells. As such powerful tools become increasingly available, it no longer will be sufficient to focus on a small number of proteins in evaluating inhibitor selectivity. Chemical tools will have to meet a higher standard of characterization, and we all may have to be more circumspect in our use of the word "selective."

Peter L. Toogood
Pfizer Global Research and Development
Michigan Laboratories
2800 Plymouth Road
Ann Arbor, Michigan 48105

Selected Reading

1. Caligiuri, M., Becker, F., Murthei, K., Kaplan, F., Dedier, S., Kaufmann, C., Machl, A., Zybarth, G., Ricahrd, J., Bockovich, N., et al. (2005). Chem. Biol. *12*, this issue, 1103–1115.
2. Cohen, P. (1999). Curr. Opin. Chem. Biol. *3*, 459–465.
3. Manning, G., Whyte, D.B., Martinez, R., Hunter, T., and Sudarsanam, S. (2002). Science *298*, 1912–1934.
4. Davies, S.P., Reddy, H., Caivano, M., and Cohen, P. (2000). Biochem. J. *351*, 95–105.
5. Bain, J., McLauchlan, H., Elliott, M., and Cohen, P. (2003). Biochem. J. *371*, 199–204.
6. Sche, P.P., McKenzie, K.M., White, J.D., and Austin, D.J. (1999). Chem. Biol. *6*, 707–716.
7. Fabian, M.A., Biggs, W.H., Treiber, D.K., Atteridge, C.E., Azimioara, M.D., Benedetti, M.G., Carter, T.A., Ciceri, P., Edeen, P.T., Floyd, M., et al. (2005). Nat. Biotechnol. *23*, 329–336.
8. Knockaert, M., and Meijer, L. (2002). Biochem. Pharmacol. *64*, 819–825.
9. Daub, H., Godl, K., Brehmer, D., Klebl, B., and Muller, G. (2004). Assay Drug Dev. Technol. *2*, 215–224.
10. Licitra, E.J., and Liu, J.O. (1996). Proc. Natl. Acad. Sci. USA *93*, 12817–12821.
11. Kley, N. (2005). Chem. Biol. *11*, 599–608.
12. Fields, S., and Song, O. (1989). Nature *340*, 245–246.
13. Chien, C.T., Bartel, P., Sternglanz, R., and Fields, S. (1991). Proc. Natl. Acad. Sci. USA *88*, 9578–9582.
14. Becker, F., Murthi, K., Smith, C., Come, J., Costa-Roldán, N., Kaufmann, C., Hanke, U., Degenhart, C., Baumann, S., Wallner, A., et al. (2004). Chem. Biol. *11*, 211–223.
15. Shim, J.S., and Kwon, H.J. (2004). Expert Opin. Ther. Targets *8*, 653–661.
16. Hart, C.P. (2005). Drug Discov. Today *10*, 513–519.

Chemistry & Biology, Vol. 12, October, 2005, ©2005 Elsevier Ltd All rights reserved. DOI 10.1016/j.chembiol.2005.10.001

Small Molecules Driving Myotube Fission

In this issue of *Chemistry & Biology*, Duckmanton et al. [1] have rigorously studied myotube fragmentation, or "cellularization," triggered by microtubule-disrupting agents. They convincingly demonstrate that cellularization remains integral to myogenic dedifferentiation, but is insufficient for reentry of the mononucleate progeny into the cell cycle.

For decades, researchers have examined in great detail the mechanisms underlying the spontaneous