# Chemical genetic approaches for the elucidation of signaling pathways

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New chemical methods that use small molecules to perturb cellular function in ways analogous to genetics have recently been developed. These approaches include both synthetic methods for discovering small molecules capable of acting like genetic mutations, and techniques that combine the advantages of genetics and chemistry to optimize the potency and specificity of small-molecule inhibitors. Both approaches have been used to study protein function *in vivo* and have provided insights into complex signaling cascades.

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Current Opinion in Chemical Biology 2001, 5:360–367

1367-5931/01/$ – see front matter


Abbreviation
1-NM-PP1 1-naphthylmethylpyrazolo[3,5-*d*]pyrimidine

## Introduction

Renewed appreciation for the power of small organic molecules to address questions in cell biology has fueled an explosion of interest in chemical biology. The idea that small (MW < 700 Da) drug-like molecules can perturb the function of specific proteins is a central tenet of pharmacology. This is further bolstered by the fact that many cellular functions are carried out by small molecules (e.g. ATP, neurotransmitters, steroid hormones, prostaglandins and phosphoinositides). Using small molecules to perturb protein function is particularly useful because the effects of drugs are:

1. Rapid, potentially diffusion-limited.

2. Often reversible because of metabolism/clearing.

3. Tunable, enabling graded phenotypes by varying concentration.

4. Conditional, because they can be introduced at any point in development.

Despite these advantages, the use of small molecules to probe cellular signaling has lagged behind that of genetic or biochemical methods. Two types of genetic experiments have provided a wealth of information about cellular processes. When identification of new components of a pathway are desired, forward genetics is used. This involves generating large numbers of mutants, screening to identify those with either gain- or loss-of-function phenotypes in a process of interest, and then identifying the mutations in the specific genes that underlie the phenotype. In order to study the function of one component, reverse genetics is used. Mutations are targeted to a particular protein and the role of the protein is inferred from the phenotype of the resulting mutant.

The advantages of genetics are that it is both highly specific (a single nucleotide change in 3 billion base pairs [bps]) and highly portable (any organism can, in principle, be genetically modified). In contrast, it is often difficult to identify a small molecule that binds specifically to a single enzyme active site out of up to 30,000 proteins, some of which have highly homologous active sites. Additionally, unlike genetic systems, the synthesis of each small molecule presents unique challenges and is not sufficiently general to systematically inactivate every gene product in an organism.

Nonetheless, despite the important advantages of high specificity and portability there can be problems with traditional genetics. For example, when gene knock-outs are lethal, further study of the mutant organism is complicated. Also, most genetic mutations are not conditional; they cannot be turned on or off at will. Although conditional mutations can be introduced through the use of an inducible promoter, generation of the mutated protein occurs over hours to days. Conditional mutations can also be found that are rapidly manifested by an external stress, such as heat (TS alleles), but this stress can often have unwanted side-effects such as induction of heat stress proteins. Thirdly, knock-out phenotypes of non-essential genes can often be masked by functional compensation by related genes during development by the organism. Chemical genetic strategies using small molecules that act as mutations would complement traditional genetic studies by providing a general means to rapidly and conditionally inactivate proteins.

The key question becomes, how can we identify specific chemical inhibitors of every gene product in the yeast, worm, fly, mouse and human proteomes? The ideal drug is one that shows perfect target-specific behavior and can be given to an animal to inactivate its target almost instantaneously. In this review, we highlight two complementary approaches to generate and identify such molecules, and describe ways in which these molecules have been used to study biological processes.

## Chemistry as a genetics-like tool

To use small molecules as biological probes, both high affinity and specificity are necessary. One approach is to

find small molecules that are capable of activating or inactivating gene function by direct interaction with the gene product [1,2]. Historically, natural products have been used to accomplish this task. To expand the scope of inhibitable proteins, efforts have focused on generating libraries of molecules to be screened.

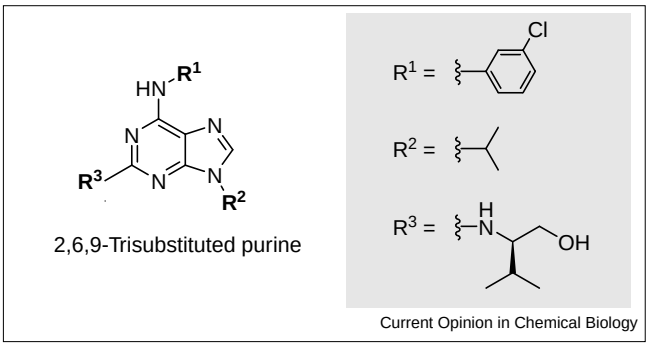
Several recent examples highlight some of the approaches that are being explored. Since the advent of small-molecule combinatorial synthesis [3–6], countless libraries of biologically interesting molecules have been synthesized [7,8]. A subset of these have been based on structural motifs (scaffolds) that are capable of binding to a variety of protein targets with high affinity [9]. These structures tend to be rigid polycyclic heteroatomic systems that are capable of orienting substituents in three-dimensional space.

In a successful recent example, Schultz and co-workers [10,11] reported the synthesis and application of a focused library of 2,6,9-trisubstituted purines (Figure 1) in an effort to identify selective kinase inhibitors. The purine scaffold was chosen for its ubiquitous appearance in biologically important molecules. Through iterative chemical synthesis and screening *in vitro*, several inhibitors with low nanomolar affinity and some degree of specificity [12] for human CDK2–cyclin A kinase complex and *Saccharomyces cerevisiae* Cdc28p were identified. This library has also found use in other studies [13]. Similarly, syntheses of libraries that incorporate scaffolds such as benzopyran [14–16], benzodiazepine [17], biphenyl [18], and dihydropyridine [19] have appeared in the literature [7,8].

As an alternative to privileged structure library synthesis, diversity-oriented organic synthesis is being investigated. This approach exploits reactions, such as the Ugi reaction, that generate a high degree of structural complexity. By utilizing a range of different building blocks and generating intermediates that can undergo a host of reactions, a high degree of molecular diversity is introduced [20,21•].

While combinatorial approaches aimed at finding inhibitors for a particular protein target have been most effective when structural or mechanistic information about that target has been used in the design process [5,6], recently, several methods have been described that are useful even in the absence of such information. One such strategy, developed by Ellman and co-workers [22•], is to use the target itself to guide the synthesis of the library. In this method (Figure 2a), a library of monomeric low-affinity ligands is first screened at high concentration. These monomers are then crosslinked and re-screened to find more potent bivalent inhibitors. The method was validated by the discovery of a potent ($IC_{50}$ = 64 nM) subtype-specific inhibitor of c-Src (>75-fold selectivity over closely related kinases Fyn, Lyn and Lck). The advantage of this method is that it allows a high-affinity ligand to be assembled from its low-affinity components; however, weak ligand–protein interactions can be difficult to detect. To overcome this problem, Wells and co-workers [23] have developed a method for stabilizing the interaction between weakly binding molecules and their target by reversible disulfide bond formation using an endogenous (or mutagenically introduced) cysteine residue (Figure 2b).

**Figure 1**



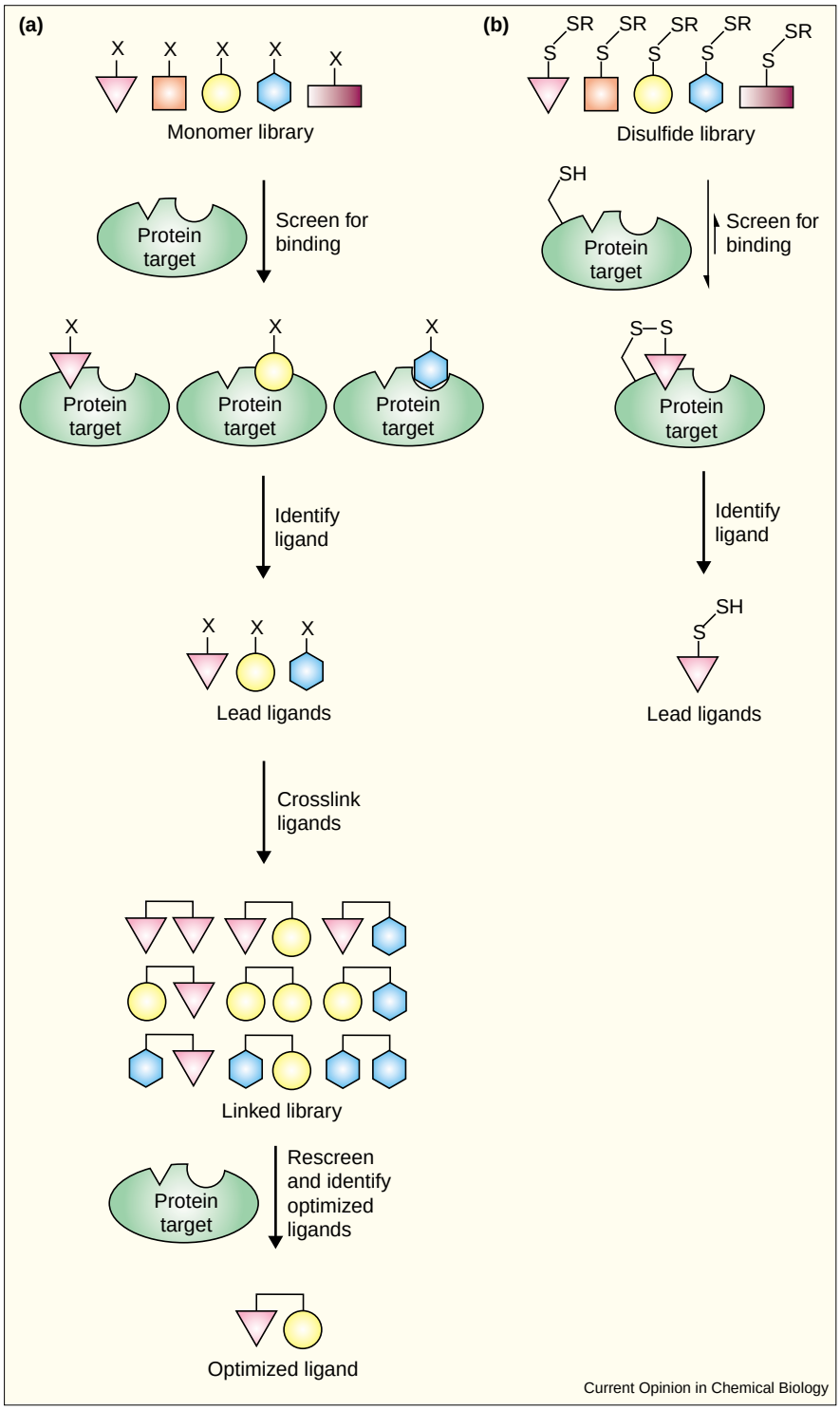

Chemical structure of the 2,6,9-trisubstituted purine scaffold. The tightest-binding inhibitor is shown, for which the $IC_{50}$ against cdc2–cyclin B is 4 nM.

With a small-molecule library in hand, it becomes possible to perform forward- or reverse-style chemical genetic experiments, complementary techniques directed toward different goals. In reverse chemical genetics, the goal is to determine the biological function of a protein using a small molecule to inhibit its activity *in vivo*. To accomplish this, first the target is chosen, then the chemical library is screened for potent and selective inhibitors, then the inhibitors are used to elicit a phenotype. Alternatively, in forward genetics the goal is to find proteins that are sensitive to small molecule inhibition. This is accomplished by screening diverse libraries of molecules, identifying those that elicit an interesting phenotypic response, and identifying the protein target.

To date, small-molecule libraries have been used primarily in the drug-discovery process, not for dissecting complex cell signaling pathways. The lead compounds that have been discovered could be powerful tools for performing reverse chemical genetics, thus complementing the available natural products that have proved indispensable for studying cell biology. However, it remains an open question as to whether molecules generated by these methods will have the required specificity to be useful as biological probes. A study of the specificity of 28 commercially available, supposedly specific, protein kinase inhibitors against approximately 30 kinases revealed that all but two drugs had more than one protein target *in vitro* [24•]. Admittedly, kinases have a high degree of homology in their active sites, and other protein targets could be less problematic.

An important example of the use of chemical libraries to perform forward chemical genetics comes from the Schreiber and Mitchison labs [25•]. To find novel

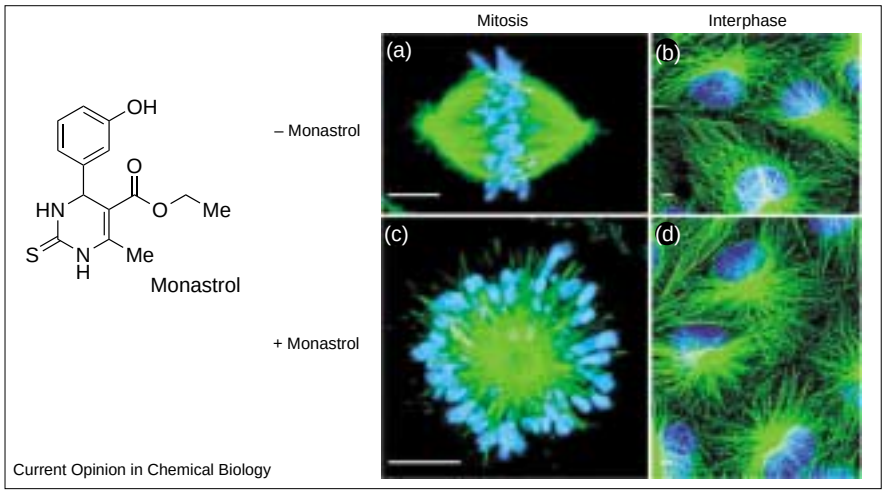
**Figure 2**




Methods for identifying small-molecule ligands based on target-guided self-assembly. (a) Monomers showing low affinity for their protein targets can be identified by performing screens at high concentrations. Optimization of monomeric lead compounds has been achieved by crosslinking the ligands and re-screening to identify highly specific dimeric inhibitors. (b) Identification of weakly binding leads has also been achieved using reversible tethering methods.

inhibitable proteins in mitosis they devised a screen to distinguish changes in mitotic spindle formation from changes in general tubulin polymerization. Small-molecule inhibitors of mitosis are potential anti-cancer drugs and to date all known inhibitors of the mitotic machinery, including Taxol and epothilone, target the same protein, tubulin. Their screen identified monastrol, a molecule that inhibits normal mitotic spindle formation (Figure 3a,c), but does not affect normal tubulin function (Figure 3b,d). By correlating this with a known mutant phenotype, and testing inhibition *in vitro*, they were able to show that the molecular motor protein Eg5 is a target of monastrol. Hence, this

**Figure 3**




Monastrol causes monoastral spindles in mitotic cells. Immunofluorescence staining (α-tubulin, green; chromatin, blue) of BS-C-1 cells treated for 4 hours with 0.4% DMSO (control, a,b) or 68 μM monastrol (c,d). No difference in distribution of microtubules and chromatin in interphase cells was observed (b,d). Monastrol treatment of mitotic cells replaces the normal bipolar spindle (a) with a rosette-like microtubule array surrounded by chromosomes (c). Scale Bars, 5 μm.

study has provided an important new clinical drug lead, a new therapeutic target, and a potential tool for probing cell biology.

The forward chemical genetic approach has now been extended to the study of whole organisms. Using the zebrafish *Danio rerio*, Schreiber and co-workers [26•] have shown that small molecules from diverse libraries can elicit interesting phenotypes during development. This example highlights a key challenge for forward chemical genetics, that is to identify the protein targets of small-molecule inhibition. For phenotypes that cannot be easily correlated with a unique mutation, some methods are available including affinity purification [27], yeast three-hybrid systems [28,29], display cloning [30,31], and protein microarrays [32,33]. A potentially useful genetic solution is to generate and isolate mutations that abrogate drug-sensitivity. Recently, this idea has been used to identify the target of a small-molecule plant hormone that had long eluded identification [34•].

Another complication in performing forward chemical genetics is that small molecules can have multiple modes of action and inhibit multiple targets. The mechanism of vancomycin inhibition is a case that demonstrates this. Although one mode of action of vancomycin is binding to the D-Ala–D-Ala motif in the peptidoglycan, recent work by Kahne and co-workers [35•] demonstrates that a mechanism independent of peptide-binding can also operate.

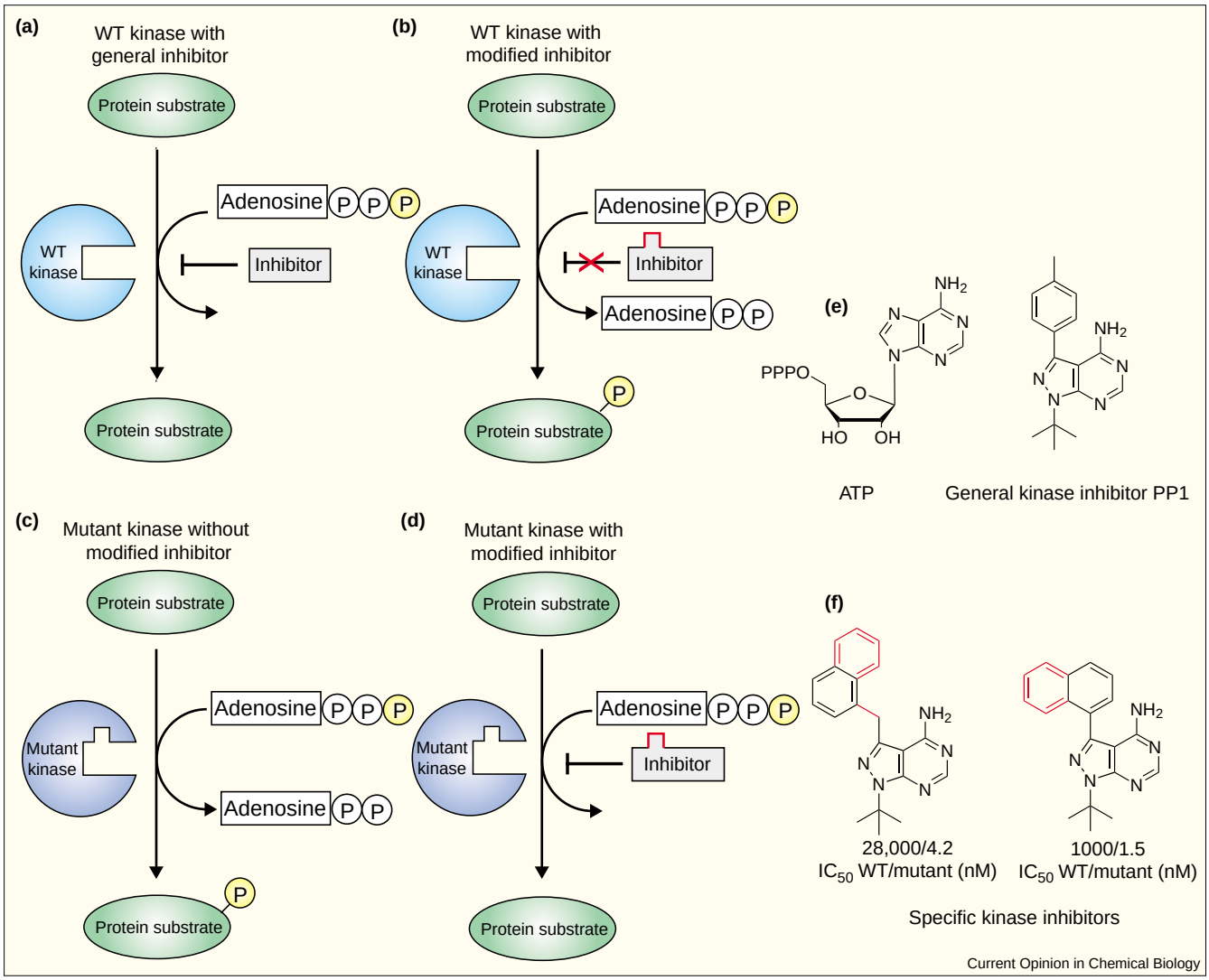
Overall, the various methods for generating and using libraries in both forward and reverse chemical genetics offer the potential to discover new small molecules that will be useful for probing biological processes and providing new drugs. We expect that the examples discussed herein will serve as a foundation for continued discovery in cell biology.

## Chemistry coupled to genetics

A fundamentally different way of using chemistry to study biology is to combine chemistry with genetics in one experimental regime [36]. When trying to perturb biological function using small molecules, the central problem is to find small molecules that interact specifically with a desired protein target. Generating specific drugs is especially challenging when the protein target shares a high degree of homology with other proteins in the cell. When using drugs as biological probes, however, one is not limited to modifying the small molecule. Recombinant DNA technology enables modulation of protein structure by introducing point mutations or even entire protein domains. Thus, it is possible to start with a high affinity but non-specific inhibitor and confer specificity by site-directed mutagenesis. Alternatively, one can genetically introduce protein domains that already possess high affinity and specificity. Pioneering work by Hwang and Miller [37] demonstrated that by interchanging the hydrogen-bonding donor–acceptor groups between substrate and enzyme, they could convergently engineer a GTPase to uniquely accept XTP, allowing them to probe a number of GTPase-dependent processes [38–40]. Elegant realization of the latter idea has been demonstrated by Schreiber and co-workers [41], in which grafting of protein domains that dimerize in a small-molecule-dependent fashion provides a chemically inducible means of associating target proteins.

Our laboratory has developed a combined chemical genetic method to specifically inhibit protein kinases [42–44]. Protein kinases form a large enzyme family and play a significant role in nearly all signaling pathways [45]. The active site of protein kinases is well conserved and makes specific inhibition of a desired kinase challenging [46]. To generate protein-inhibitor specificity, we use protein design to engineer a functionally silent yet structurally significant mutation into the active site of a kinase of interest. In our case, this mutation is the replacement of a conserved bulky

**Figure 4**




Kinase-specific inhibition can be achieved by coupling chemistry and genetics. **(a)** Wild-type kinases are inhibited by a non-specific inhibitor. **(b)** Wild-type kinases are not inhibited by non-specific inhibitor analogs containing a sterically bulky functional group. **(c)** Engineered kinases show normal kinase activity. **(d)** Engineered kinases are inhibited by inhibitor analogs that contain a sterically bulky functional group. **(e)** Chemical structures of ATP and the general kinase inhibitor PP1. **(f)** Chemical structures of specific kinase inhibitors (structure in red indicates modified group), $IC_{50}$ WT/mutant (nM). WT, wild type.

residue with glycine or alanine, thus creating a new pocket in the active site. Importantly, these mutations do not usually affect kinase activity in a significant way (Figure 4c). Separately, a non-specific inhibitor of the wild-type enzyme (Figure 4a,e) is chemically modified with substituents that specifically complement the mutation introduced into the active site (Figure 4d,f). Importantly, the new inhibitor analogs are designed to be unable to inhibit any wild-type kinases via steric clashes (Figure 4b). In a cellular context, it is difficult to prove perfect specificity. Experiments have shown that our inhibitors have no off-target effects *in vitro*; furthermore, addition of our inhibitors to wild-type *S. cerevisiae* has resulted in few transcriptional changes [47•]. Using this approach, several analogs of a pyrazolo[3,5-*d*]pyrimidine-based kinase inhibitor (PP1) have been found that inhibit engineered kinases with nanomolar $IC_{50}$ values and without significant inhibition of wild-type kinases [44,48].

This combined chemical genetic strategy is potentially general for many kinases, but several requirements must be met. First, it must be possible to introduce the desired mutant allele into the organism of interest. Second, the stability and activity of the engineered enzyme must be unaltered. Third, the inhibitor must be bioavailable. Work in our labs and in collaboration with others has demonstrated that these requirements can be met in most circumstances. This technique has been used to probe the functional role of CaMKIIα in learning and memory in mice (JZ Tsien, personal communication), v-Src in

transformation of 3T3 cells [44], and Cla4 and Cdc28 in cell-cycle regulation in *S. cerevisiae* [47•,49•].

An example that highlights the ability of chemical genetics to complement traditional genetics is the use of this technique to clarify the role of Cdc28 in the cell cycle of *S. cerevisiae*. Cdc28 is the primary cyclin-dependent kinase involved in cell-cycle control. Use of mutants containing a temperature-sensitive Cdc28 allele generated by traditional genetics suggested that the most critical role for Cdc28 was to control the transition from G1 to S phase. Surprisingly, experiments done in collaboration with David Morgan (UCSF) showed that inhibitor-sensitive Cdc28 mutants arrested at the G2/M transition when treated with 1-naphthylmethylpyrazolo[3,5-*d*]pyrimidine (1-NM-PP1). This discrepancy was not due to off-target effects of the drug, because this compound induced no toxicity or cell-cycle arrest in wild-type yeast. Thus, the difference in cellular phenotype was the result of a fundamental difference in how cdc28 function was altered by the two approaches.

The discrepancy between the chemical and temperature-induced inhibition of cdc28 can be explained from a structural perspective. Cdc28 functions in two ways, as a catalyst and as a scaffold for other components of the cell-cycle machinery. Typically, temperature-sensitive mutants unfold at elevated temperature thus resulting in a loss of both of these functions. ATP-competitive inhibitors, such as 1-NM-PP1, block only kinase catalytic activity. Thus, in this case, the chemical method is a more specific probe of protein function.

Moreover, the observed discrepancy is consistent with what is known about cdc28 catalytic activity. The kinase activity of Cdc28 is maximal at the G2/M transition and is therefore expected to be most sensitive to inhibition at this stage, consistent with observed G2/M arrest at low doses of 1-NM-PP1. This model predicts that higher inhibitor concentration should result in earlier cell-cycle arrest. This was also demonstrated. Thus, the ability of this chemical genetic approach to probe protein function more specifically highlights the advantages of using chemical genetics as a complement to traditional genetics.

In addition to using combined chemical genetics to inhibit proteins, it is also possible to probe the activity of enzymes and receptors using modified agonists and substrates. Hence, we have applied our strategy toward identifying kinase substrates. By synthesizing $\gamma[^{32}P]$-labeled analogs of ATP that contain a sterically bulky functional group and using the engineered kinases previously described, we can selectively label the direct phosphorylation substrates of a kinase of interest [50,51]. Conklin and co-workers [52,53•] have applied a chemical genetic method to the study of seven-transmembrane G-protein-coupled receptors. By introducing changes in the agonist-binding domain they have generated receptors that are activated by a synthetic agonist, but not the endogenous peptide hormone ligand. By providing the researchers with exclusive control over the activation of these receptors, influence over a number of physiological processes including heart rate and cell proliferation has been demonstrated.

Approaches that combine chemistry and genetics offer a means of generating small molecules that act rapidly and reversibly, yet offer the portability and specificity of genetics.

## Conclusions and future directions

The intersection between genomics, proteomics, combinatorial organic synthesis, natural-product screening, and protein design has created an exciting environment for the development of powerful new tools that have been termed chemical genetics. To date, our view of biological phenomena has been shaped largely through the use of genetics. Yet, we know that genetics has limitations. The use of chemical agents to probe pathways provides information that, when superimposed with genetic and biochemical data, gives a higher resolution understanding of biological processes. An ongoing challenge is to identify biological questions that benefit from the application of small-molecule approaches. Another is to continue to develop tools that are sufficiently specific and portable to address these questions. In meeting these challenges, chemical genetics offers the potential to augment and even profoundly expand our understanding of biology.

## Acknowledgements

We are grateful to V Denic, HF Luecke, JW Taunton and members of the Shokat laboratory for helpful discussions and critical comments on this manuscript. PJA thanks the Susan G Komen Breast Cancer Foundation (PDF 2000 78) and the American Cancer Society (PF-01-095-01-TBE), and MSK thanks the National Institutes of Health (AI10611-01) for postdoctoral fellowships. KMS thanks the National Institutes of Health (CA70331-05 and AI44009-03), GlaxoSmithKline and the Sandler Family Supporting Foundation for generous financial support.

## References and recommended reading

Papers of particular interest, published within the annual period of review, have been highlighted as:

- • of special interest
- •• of outstanding interest

1. Stockwell BR: Chemical genetics: ligand-based discovery of gene function. *Nat Rev Genet* 2000, 1:116-125.
2. Stockwell BR: Frontiers in chemical genetics. *Trends Biotechnol* 2000, 18:449-455.
3. Bunin BA, Ellman JA: A general and expedient method for the solid-phase synthesis of 1,4-benzodiazepine derivatives. *J Am Chem Soc* 1992, 114:10997-10998.
4. Dewitt SH, Kiely JS, Stankovic CJ, Schroeder MC, Cody DMR, Pavia MR: "Diversomers" – an approach to nonpeptide, nonoligomeric chemical diversity. *Proc Natl Acad Sci USA* 1993, 90:6909-6913.
5. Ellman JA: Design, synthesis, and evaluation of small-molecule libraries. *Acc Chem Res* 1996, 29:132-143.
6. Thompson LA, Ellman JA: Synthesis and applications of small molecule libraries. *Chem Rev* 1996, 96:555-600.
7. Patchett AA, Nargund RP: Topics in drug design and discovery: privileged structures – an update. In *Annual Reports in Medicinal Chemistry*, Vol 35. Edited by Trainor GL. San Diego: Academic Press; 2000:289-298.

8. Arya P, Chou DTH, Baek MG: Diversity-based organic synthesis in the era of genomics and proteomics. *Angew Chem Int Ed Engl* 2001, 40:339-346.

9. Evans BE, Rittle KE, Bock MG, DiPardo RM, Freidinger RM, Whitter WL, Lundell GF, Veber DF, Anderson PS, Chang RSL *et al.*: Methods for drug discovery: development of potent, selective, orally effective cholecystokinin antagonists. *J Med Chem* 1988, 31:2235-2246.

10. Gray NS, Wodicka L, Thunnissen AM, Norman TC, Kwon S, Espinoza FH, Morgan DO, Barnes G, LeClerc S, Meijer L *et al.*: Exploiting chemical libraries, structure, and genomics in the search for kinase inhibitors. *Science* 1998, 281:533-538.

11. Chang YT, Gray NS, Rosania GR, Sutherlin DP, Kwon S, Norman TC, Sarohia R, Leost M, Meijer L, Schultz PG: Synthesis and application of functionally diverse 2,6,9-trisubstituted purine libraries as CDK inhibitors. *Chem Biol* 1999, 6:361-375.

12. Knockaert M, Gray N, Damiens E, Chang YT, Grellier P, Grant K, Fergusson D, Mottram J, Soete M, Dubremetz JF *et al.*: Intracellular targets of cyclin-dependent kinase inhibitors: identification by affinity chromatography using immobilised inhibitors. *Chem Biol* 2000, 7:411-422.

13. Armstrong JI, Portley AR, Chang YT, Nierengarten DM, Cook BN, Bowman KG, Bishop A, Gray NS, Shokat KM, Schultz PG, Bertozzi CR: Discovery of carbohydrate sulfotransferase inhibitors from a kinase-directed library. *Angew Chem Int Ed Engl* 2000, 39:1303-1306.

14. Nicolaou KC, Pfefferkorn JA, Roecker AJ, Cao GQ, Barluenga S, Mitchell HJ: Natural product-like combinatorial libraries based on privileged structures. 1. General principles and solid-phase synthesis of benzopyrans. *J Am Chem Soc* 2000, 122:9939-9953.

15. Nicolaou KC, Pfefferkorn JA, Mitchell HJ, Roecker AJ, Barluenga S, Cao GQ, Affleck RL, Lillig JE: Natural product-like combinatorial libraries based on privileged structures. 2. Construction of a 10,000-membered benzopyran library by directed split-and-pool chemistry using NanoKans and optical encoding. *J Am Chem Soc* 2000, 122:9954-9967.

16. Nicolaou KC, Pfefferkorn JA, Barluenga S, Mitchell HJ, Roecker AJ, Cao GQ: Natural product-like combinatorial libraries based on privileged structures. 3. The 'libraries from libraries' principle for diversity enhancement of benzopyran libraries. *J Am Chem Soc* 2000, 122:9968-9976.

17. Herpin TF, Van Kirk KG, Salvino JM, Yu ST, Labaudiniere RF: Synthesis of a 10,000 member 1,5-benzodiazepine-2-one library by the directed sorting method. *J Comb Chem* 2000, 2:513-521.

18. Neustadt BR, Smith EM, Lindo N, Nechuta T, Bronnenkant A, Wu A, Armstrong L, Kumar C: Construction of a family of biphenyl combinatorial libraries: structure-activity studies utilizing libraries of mixtures. *Bioorg Med Chem Lett* 1998, 8:2395-2398.

19. Gordeev MF, Patel DV, England BP, Jonnalagadda S, Combs JD, Gordon EM: Combinatorial synthesis and screening of a chemical library of 1,4-dihydropyridine calcium channel blockers. *Bioorg Med Chem* 1998, 6:883-889.

20. Lee D, Sello JK, Schreiber SL: Pairwise use of complexity-generating reactions in diversity-oriented organic synthesis. *Org Lett* 2000, 2:709-712.

21. • Schreiber SL: Target-oriented and diversity-oriented organic synthesis in drug discovery. *Science* 2000, 287:1964-1969.

This review gives a very clear description of Schreiber's strategy for diversity-oriented organic synthesis, provides a complete report of the progress made to date, and identifies several useful concepts for planning diversity-oriented syntheses.

22. • Maly DJ, Choong IC, Ellman JA: Combinatorial target-guided ligand assembly: identification of potent subtype-selective c-Src inhibitors. *Proc Natl Acad Sci USA* 2000, 97:2419-2424.

The identification of highly subtype-selective inhibitors of c-Src has been a longstanding challenge in chemistry and biology. The method presented in this paper for using a protein target to guide in the construction of selective inhibitors is significant in its simplicity and success.

23. Erlanson DA, Braisted AC, Raphael DR, Randal M, Stroud RM, Gordon EM, Wells JA: Site-directed ligand discovery. *Proc Natl Acad Sci USA* 2000, 97:9367-9372.

24. • Davies SP, Reddy H, Caivano M, Cohen P: Specificity and mechanism of action of some commonly used protein kinase inhibitors. *Biochem J* 2000, 351:95-105.

This *in vitro* study of the specificity of several commonly used protein kinase inhibitors illustrates the difficulty of finding inhibitors that are truly specific for their protein target.

25. • Mayer TU, Kapoor TM, Haggarty SJ, King RW, Schreiber SL, Mitchison TJ: Small molecule inhibitor of mitotic spindle bipolarity identified in a phenotype-based screen. *Science* 1999, 286:971-974.

This paper describes the discovery of monastrol, the first small-molecule modulator of the mitosis machinery that does not target tubulin. Monastrol targets the molecular motor protein, Eg5, and should prove to be a useful tool for studying mitotic mechanisms.

26. • Peterson RT, Link BA, Dowling JE, Schreiber SL: Small molecule developmental screens reveal the logic and timing of vertebrate development. *Proc Natl Acad Sci USA* 2000, 97:12965-12969.

This paper demonstrates initial progress toward the use of forward chemical genetics in a vertebrate. Several small molecules were found that affected zebrafish development.

27. Taunton J, Hassig CA, Schreiber SL: A mammalian histone deacetylase related to the yeast transcriptional regulator Rpd3p. *Science* 1996, 272:408-411.

28. Drees BL: Progress and variations in two-hybrid and three-hybrid technologies. *Curr Opin Chem Biol* 1999, 3:64-70.

29. Lin H, Cornish VW: ***In vivo*** protein–protein interaction assays: beyond proteins. *Angew Chem Int Ed Engl* 2001, 40:871-875.

30. Sche PP, McKenzie KM, White JD, Austin DJ: Display cloning: functional identification of natural product receptors using cDNA-phage display. *Chem Biol* 1999, 6:707-716.

31. Sche PP, McKenzie KM, White JD, Austin DJ: Corrigendum to: "Display cloning: functional identification of natural product receptors using cDNA-phage display". *Chem Biol* 2001, 8:399-400.

32. MacBeath G, Schreiber SL: Printing proteins as microarrays for high-throughput function determination. *Science* 2000, 289:1760-1763.

33. Kodadek T: Protein microarrays: prospects and problems. *Chem Biol* 2001, 8:105-115.

34. • Inoue T, Higuchi M, Hashimoto Y, Seki M, Kobayashi M, Kato T, Tabata S, Shinozaki K, Kakimoto T: Identification of CRE1 as a cytokinin receptor from ***Arabidopsis***. *Nature* 2001, 409:1060-1063.

This paper describes a genetic screen used to identify the unknown receptor of a plant hormone.

35. • Ge M, Chen Z, Onishi HR, Kohler J, Silver LL, Kerns R, Fukuzawa S, Thompson C, Kahne D: Vancomycin derivatives that inhibit peptidoglycan biosynthesis without binding D-Ala-D-Ala. *Science* 1999, 284:507-511.

This study demonstrates that vancomycin derivatives containing modified carbohydrates operate by a different mechanism than does vancomycin. The newly discovered mechanism does not depend on peptide binding, and these results highlight the difficulty in demonstrating the mode of action of small-molecule modulators of protein function.

36. Bishop A, Buzko O, Heyeck-Dumas S, Jung I, Kraybill B, Liu Y, Shah K, Ulrich S, Witucki L, Yang F *et al.*: Unnatural ligands for engineered proteins: new tools for chemical genetics. *Annu Rev Biophys Biomol Struct* 2000, 29:577-606.

37. Hwang YW, Miller DL: A mutation that alters the nucleotide specificity of elongation factor Tu, a GTP regulatory protein. *J Biol Chem* 1987, 262:13081-13085.

38. Weijland A, Parlato G, Parmeggiani A: Elongation factor Tu D138N, a mutant with modified substrate specificity, as a tool to study energy consumption in protein biosynthesis. *Biochemistry* 1994, 33:10711-10717.

39. Powers T, Walter P: Reciprocal stimulation of GTP hydrolysis by two directly interacting GTPases. *Science* 1995, 269:1422-1424.

40. Jones S, Litt RJ, Richardson CJ, Segev N: Requirement of nucleotide exchange factor for Ypt1 GTPase mediated protein transport. *J Cell Biol* 1995, 130:1051-1061.

41. Spencer DM, Wandless TJ, Schreiber SL, Crabtree GR: Controlling signal transduction with synthetic ligands. *Science* 1993, 262:1019-1024.

42. Bishop AC, Shah K, Liu Y, Witucki L, Kung C, Shokat KM: Design of allele-specific inhibitors to probe protein kinase signaling. *Curr Biol* 1998, 8:257-266.

43. Bishop AC, Shokat KM: Acquisition of inhibitor-sensitive protein kinases through protein design. *Pharmacol Ther* 1999, 82:337-346.

44. Bishop AC, Kung CY, Shah K, Witucki L, Shokat KM, Liu Y: Generation of monospecific nanomolar tyrosine kinase inhibitors via a chemical genetic approach. *J Am Chem Soc* 1999, 121:627-631.

45. Hunter T: Signaling – 2000 and beyond. *Cell* 2000, 100:113-127.

46. Sicheri F, Moarefi I, Kuriyan J: Crystal structure of the Src family tyrosine kinase Hck. *Nature* 1997, 385:602-609.

47. • Bishop AC, Ubersax JA, Petsch DT, Matheos DP, Gray NS, Blethrow J, Shimizu E, Tsien JZ, Schultz PG, Rose MD *et al.*: A chemical switch for inhibitor-sensitive alleles of any protein kinase. *Nature* 2000, 407:395-401.
This paper describes a general chemical genetic method for generating inhibitor-sensitive alleles for both protein tyrosine and serine/threonine kinases. This approach was applied to the study of the central cyclin-dependant kinase, cdc28, where a comparison between the temperature- and inhibitor-sensitive alleles was performed.

48. Hanke JH, Gardner JP, Dow RL, Changelian PS, Brissette WH, Weringer EJ, Pollok K, Connelly PA: Discovery of a novel, potent, and Src family-selective tyrosine kinase inhibitor: study of Lck- and FynT-dependent T cell activation. *J Biol Chem* 1996, 271:695-701.

49. • Weiss EL, Bishop AC, Shokat KM, Drubin DG: Chemical genetic analysis of the budding-yeast p21-activated kinase Cla4p. *Nat Cell Biol* 2000, 2:677-685.
This paper provides an example of the use of a inhibitor-sensitive kinase to dissect the role of Cla4 in budding yeast.

50. Shah K, Liu Y, Deirmengian C, Shokat KM: Engineering unnatural nucleotide specificity for Rous sarcoma virus tyrosine kinase to uniquely label its direct substrates. *Proc Natl Acad Sci USA* 1997, 94:3565-3570.

51. Habelhah H, Shah K, Huang L, Ostareck-Lederer A, Burlingame AL, Shokat KM, Hentze MW, Ronai Z: ERK phosphorylation drives cytoplasmic accumulation of hnRNP-K and inhibition of mRNA translation. *Nat Cell Biol* 2001, 3:325-330.

52. Coward P, Wada HG, Falk MS, Chan SDH, Meng F, Akil H, Conklin BR: Controlling signaling with a specifically designed G(i)-coupled receptor. *Proc Natl Acad Sci USA* 1998, 95:352-357.

53. • Redfern CH, Coward P, Degtyarev MY, Lee EK, Kwa AT, Hennighausen L, Bujard H, Fishman GI, Conklin BR: Conditional expression and signaling of a specifically designed G(i)-coupled receptor in transgenic mice. *Nat Biotechnol* 1999, 17:165-169.
This paper describes the use of convergent protein engineering and chemical synthesis to study G-protein-coupled receptors, allowing control over physiological events in mice, including cell proliferation and heart rate.