Chemical Genetics†

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Daniel Walsh was born in Shenandoah, Pennsylvania. He attended the University of Scranton and performed undergraduate research with Dr. Paul T. Buonora. He conducted his Ph.D. work with Dr. Young-Tae Chang at New York University. His research in Chemical Genetics focused on developing small molecule microarrays and chemical probes that target tubulin. He is currently a Hazardous Materials/WMD Specialist with the New York City Department of Environmental Protection.

Young-Tae Chang was born in Busan, Korea, in 1968. He studied chemistry in Pohang University of Science and Technology (POSTECH, Korea) and received his B.S. in 1991. After one and half years of army service in Korea, he started his graduate study at POSTECH and received a Ph.D. in 1997 under the supervision of Prof. Sung-Kee Chung, working on the divergent synthesis of all possible regioisomers of *myo*-inositol phosphates. He did his postdoctoral work with Prof. Peter Schultz at UC Berkeley and The Scripps Research Institute. In 2000, he was appointed assistant professor at New York University and promoted to associated professor in 2005. He received the NSF Career award in 2005 and his research interests include chemical genetics, molecular evolution, and artificial tongues.

1. Introduction

Chemical genetics, in the simplest terms, can be defined as a "genetics" study using "chemical" tools.[1,2] Classical genetics may study a gene function by directly removing a

† This paper is dedicated to Professor Sung-Kee Chung, my lifetime mentor, on the occasion of his 60th birthday.

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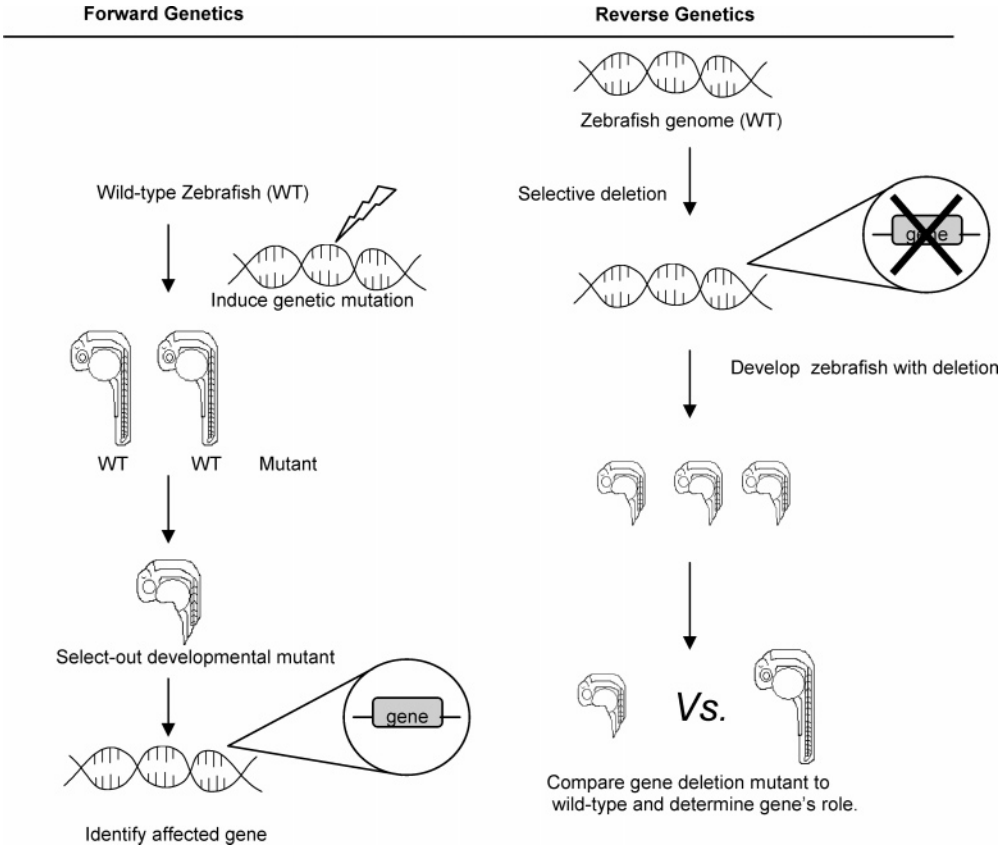


Figure 1. Forward genetics begins with a wild-type cell or organism that has its DNA mutated via chemical- or radiation-induced mutagenesis. The organisms are then screened for changes in phenotypes of interest, and that phenotype is selected out. The selected cell/organism is then analyzed to identify the mutated gene responsible by gene mapping. Reverse genetics begins with the selective targeted mutation of a gene. The phenotype induced is observed, and the mutant is compared to the wild-type to determine the gene's function.

gene product(s), proteins (genetic knockout), from the organism. In contrast, chemical genetics indirectly studies a gene's role by altering the activity of the cognate protein, using small molecule inhibitors analogously to the genetic knockout.[1,3,4]

As is classical genetics, chemical genetics is divided into two approaches, forward and reverse (Figures 1 and 2). Forward genetics (FG) operates "from effect to cause" or "phenotype (physically apparent characteristic) to genotype (genetic sequence)" and requires no specific gene target from the onset.[4−6] It studies changes in phenotype(s) such as morphology, growth, or behavior resulting from random genomic DNA mutations or deletions induced from radioactive or chemical mutagenesis and then identifies the gene responsible through mutation mapping.[3,7,8] Forward *chemical* genetics (FCG) mimics FG by substituting random mutagenesis with a collection of a library of typically unbiased (not targeted) compounds as protein function regulators in place of mutagens.[9] The first step in both FG and FCG is to screen for changes induced by either the inhibition or stimulation of a protein's function, and both go on to identify the genetic cause, but in different ways. FG goes after genetic mutation, a permanently retained marker, but FCG needs to identify the protein partner for the small molecule. This target identification is one of the greatest challenges in chemical genetics.[6,10−13]

With the help of molecular biology techniques, reverse genetics (RG) was a later development in genetics and operates "from cause to effect" (genotype to phenotype).[4,14] Reverse genetics begins with selecting a gene of interest, manipulating it to produce an organism harboring the mutated gene, and characterizing the phenotypic differences between the mutant and the wild-type organisms.[7,13] In the same context, reverse *chemical* genetics (RCG) begins with a known protein, analogous to a specific gene selection.[5] This known protein is then screened with vast pools of library compounds to identify functional ligands that either stimulate or inhibit the target protein.[15] Once a specific ligand is identified, it is introduced to a cell or organism, analogous to genetic mutation, and the resulting changes in phenotype are studied.[5]

Compared to classical genetics, chemical genetics offers a number of advantages and provides access to previously unstudied biological space.[16] Use of chemical tools offers greater ease and flexibility than classical genetic modification. Classical genetic techniques are relatively difficult to employ, especially in mammals due to their diploid genome, physical size, and slow reproduction rate, though great progress has been made in this area.[17−23] On the other hand, chemical genetics studies may be conducted on any complex cellular or animal models without any time-consuming genetic modifications that may prove lethal or in which the cell/animal can mask the phenotype through related gene functional compensation for the mutation.[4,8,14,24] Especially important is chemical genetics' promise of operating in the relevant context of human cells at physiological conditions that has strained traditional genetics techniques.[25] Therefore, chemical genetics fills a major gap in genetic studies where no, or suboptimal, model systems exist.[26] Additionally, chemical genetics also allows for the possibility of "multiple knockouts" by adding multiple specific ligands, a situation often described as a "nightmare" for a geneticist.[27]

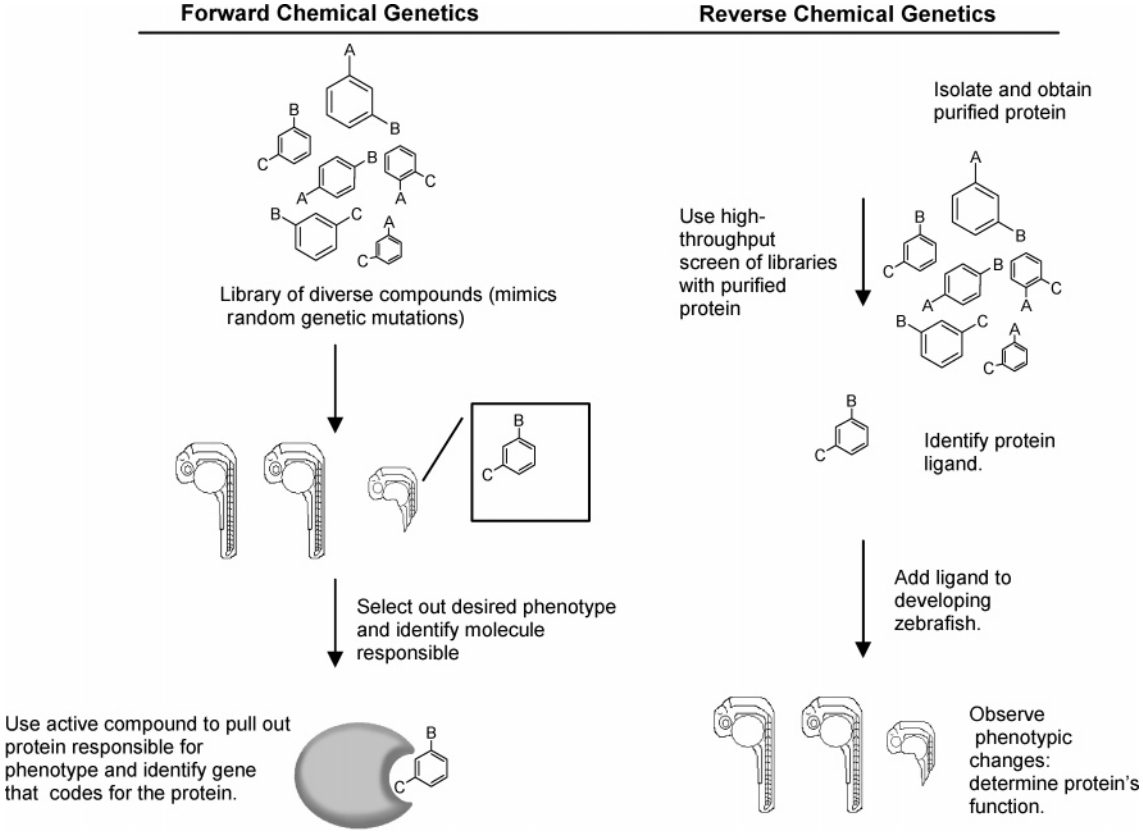


Figure 2. Forward chemical genetics: A library of compounds is designed, synthesized, or isolated from natural sources mimicking random genetic mutation. The compounds are screened in high-throughput in vivo assays. A desired phenotypic change is then sought, and the compound responsible is considered a "hit" and selected out of the library. The molecule is then typically used as a "hook" to "fish-out" the protein of interest. The protein's function and the gene responsible for it are then studied. Reverse chemical genetics: A purified protein is selected and screened in vitro against a library of compounds to identify a specific binding ligand. That selected ligand is then introduced to an in vivo system to determine the protein's role by monitoring phenotypic changes.

Additionally, classical genetic knockouts, in principle, delete the protein entirely from the organism.[3] Therefore, it is difficult to determine effects that arise from the deletion separately from those that arise from merely a particular function of the protein.[28] It is always possible that one protein may have multiple functions, and chemical genetics can potentially isolate and dissect particular functions of that protein while leaving others intact.[24] Additionally, if a gene is essential for survival or development, a total knockout, such as in classical genetics, may abolish the chance to study the later stage function of that gene since the deletion may be lethal.[29] Chemical genetics allows the use of sublethal doses of the ligand and avoids full lethality, thus providing a partial knockout phenotype.[30]

Another advantage of chemical genetics is real-time control. Chemical genetics allows for this by rapidly introducing a cell-permeable ligand at any stage that may yield the desired phenotype as quickly as diffusion-limited kinetics allows.[10,14] The chemical perturber/ligand/probe is in effect a "switch" that can turn the event under study on or off in real time and allows for kinetic in vivo analysis, something not usually possible in classical genetics.[6,17] While temporal control is available in classical genetic studies through conditional alleles, such as temperature-sensitive mutations, these alleles often have unwanted broad side effects that may interfere with the desired result.[14] Antisense oligonucleotide and RNA interference (RNAi) are other popular alternatives for conditional knockouts[12,28,29] that work by inhibiting the synthesis of the target protein from mRNA. However, because their effects are delayed until all of the already existing proteins are degraded, they are particularly ill-suited to time-sensitive studies, such as signal transduction, that occur on the milliseconds to hours time scale.[28]

Chemical genetics and classical genetics are techiniques that compliment each other well.[31] One of the greatest advantages of classical genetics is the incredible specificity of a gene knockout. While some chemical ligands can be very specific switches with specificity approaching that of a gene knockout,[1,3,32] the low specificity of other ligands often give "off-target" effects in which the probe may interact with proteins other than the protein(s) targeted. This makes defining specific protein functions very difficult since these off-target effects may lead to toxicity or false or unwanted positive/negative biological results. Not only often lacking in specificity, chemical genetics cannot yet match the generality of genetics. Geneticists can, in theory, "knockout" any gene as long as the genome sequencing is done in the given species, an ability that at this point exists as nothing more than a dream of the chemical geneticist.[27] These situations are the perfect place for the integration of chemical and classical genetics.[28]

Importantly, unlike drug development where specificity is tantamount, in chemical genetics it does not need to be completely specific, so long as it gives an identifiable phenotype that allows for the deciphering of the target protein's function and its side effect are relatively small.[12] Whereas one may desire compounds with affinity in the (sub)nanomolar range capable of producing the desired effect, in reality, compounds of low micromolar affinities are often accepted as good to reasonable candidates in chemical

genetics.[11] Despite this, and with an understanding of the necessary medicinal chemistry follow-up modifications and studies required, chemical genetics still has the advantage of immediately offering a potential drug lead, rather than simply a target gene or protein, as in classical genetics.[13,29,33] Interestingly, a lead compound developed in drug discovery that may not possess pharmacokinetic properties suitable for a therapeutic purpose may still be used as a probe in chemical genetics studies.[24] In fact, the lower pharmacokinetic property requirements for chemical genetics probes compared to drugs allows for the use of a greater variety of functional groups and a maximization of the chemical space in library constituents.[34]

This review provides a survey of contemporary research in chemical genetics with a special emphasis on the techniques and methods in use. Studies where small molecules, in concert with genomic techniques, are used to study genome-wide effects (chemical genomics) or work highly focused on pharmaceutical development through the integration of chemistry, genomics, target family design, and chemo/bio-informatics (chemogenomics) will not be thoroughly covered in this review, except where aspects of the work are relevant to or integrate chemical genetics.[3,14,27,35−52]

2. Chemical Toolboxes

Chemical genetics is dependent on the integration of techniques spanning many disciplines including combinatorial chemistry and high-throughput screening (HTS). Collections of compounds, so-called libraries, are the absolute starting point for any chemical genetics study or discussion. Here, highlights of chemical tools relevant to chemical genetics will be discussed, while leaving out the full details of library generation itself, which has already been the subject of many reviews.[53−61]

2.1. Natural Products

Nature does not lack for time, and over time many powerfully bioactive compounds have evolved. For example, colchicine has been used as a drug for over 2000 years. It is generally accepted that collections of natural products have a higher probability of delivering hits than a typical synthetic combinatorial library.[3,62]

Natural products are obtained from sources such as plants, soil, and marine sponges.[3] Most difficult in this natural product discovery is isolating any active components out of the whole mixture extract. The typical and time-consuming isolation route is known as bioassay-guided purification.[9,63,64] Purification involves iterative processes where compounds undergo multiple rounds of extraction and chromatography guided by the screening results of the successive crude extracts. These studies are hampered by overlooking potentially highly active low-abundance compounds, the cytotoxicity of one component masking the desired effect of another component, and bioactivity resulting from complicated synergistic effects.[9,63,64] In addition, determining the structure of the compound is another laborious and challenging task. Over the past decade and a half, these drawbacks led to a retreat from natural products by the majority of the major pharmaceutical companies, but the disappointment in the number of drugs arising from combinatorial chemistry and the continued benefits and development of natural products (see mevinolin and cyclosporine) are luring many back to them.[64−68] Currently, major pharmaceutical companies are re-embracing natural products and natural product libraries. Natural products need not have their use restricted to therapeutics; they can also be of great value to chemical genetics studies. Extremely exciting is the integration of traditional natural products and their scaffolds with modern combinatorial and HTS tools.[65,69,70] Perhaps success will lie

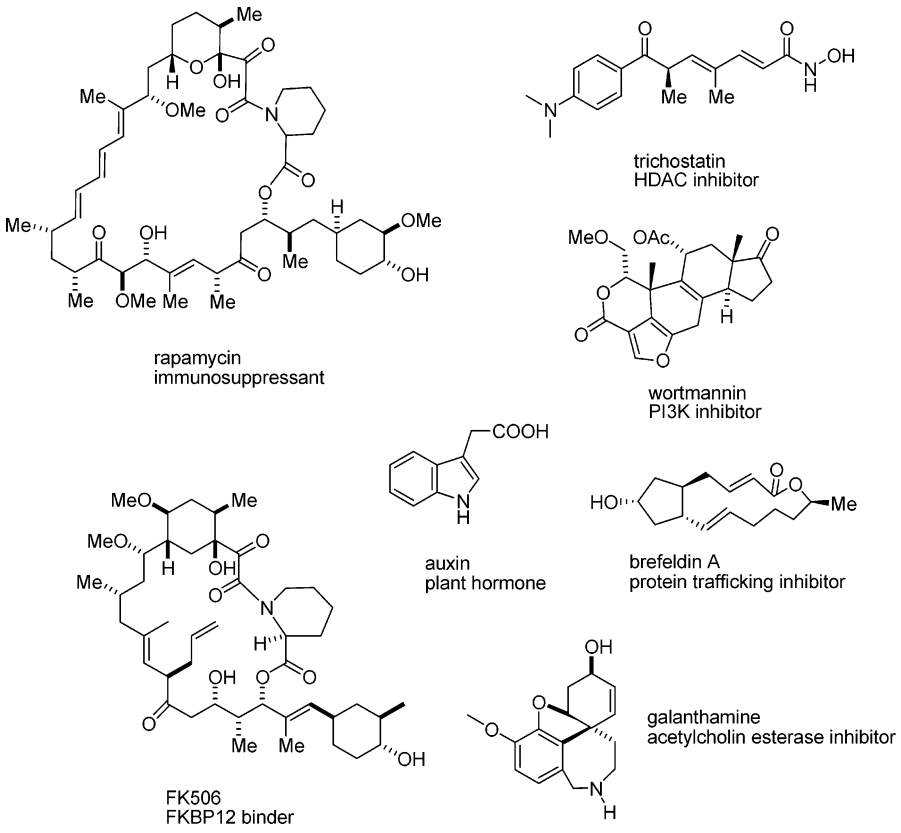


Figure 3. Representative natural products used in chemical genetics studies.

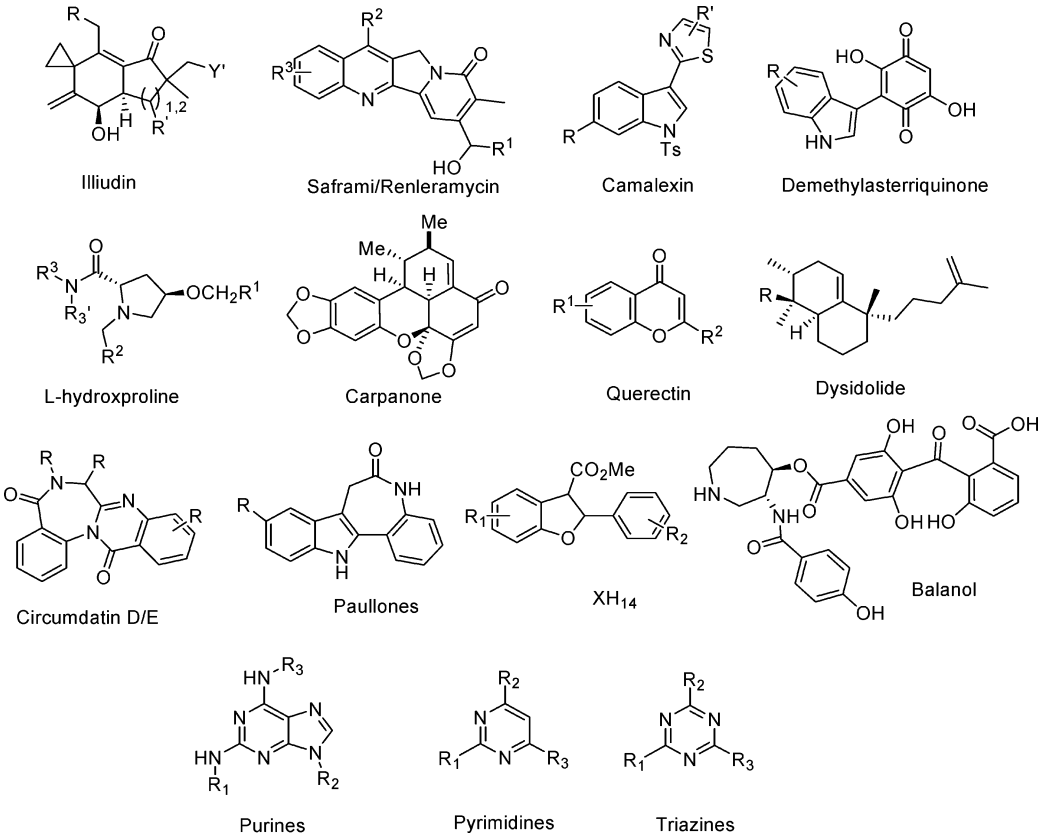


Figure 4. Representative natural-product-like compound structures.

in a balance of the old and the new. Some representative natural products used in chemical genetics studies are listed in Figure 3.

2.2. Natural-Product-Like Compounds

Natural-product-like libraries offer a highly desirable middle ground between those who seek powerfully bioactive compounds from natural libraries and those who seek the ease of synthesis found in libraries composed of small organic molecules. Natural products are typically chiral and extremely complex and contain many stereogenic centers. These structures are often highly potent and serve as attractive leads for drug development.[71] Natural products can be considered privileged structures in a biological context and an excellent starting point for library design with a high probability for biological activity.[62,72] In Waldmann's terms, natural products, based on their evolutionary selection, serve as "biologically validated" starting points for library design.[73] These compounds often contain sets of related and homologous pharmacophoric groups throughout families of natural compounds.[74] Natural-product-like libraries are those collections of compounds whose structures are based on or share high structural homology with natural products.[69] These libraries may be designed to generate derivatives of a natural product scaffold, as Nicolau et al. did in their diverse synthesis of benzopyran derivatives[75−77] or as Schreiber et al. did in their modification of a natural product scaffold, (−)-shikimic acid, to a highly diversified tetracyclic template/scaffold.[64,78] In addition, some have sought to generate natural-product-like libraries not to improve on known activity but to expand a molecule's functionality into a previously unknown areas of biological space.[79] The synthesis of natural-product-like libraries encounters many of the difficulties of synthesizing natural products, along with the challenge of synthesizing these complex structures on the solid phase.[69,71] A great deal of work has been produced, and detailed studies of scaffold design and natural-product-like libraries are well documented in the literature.[69,72,80,81] Figure 4 provides some representative structures of members of natural-product-like libraries.[69,72,82,83]

2.3. Diversity Oriented Synthesis

After the combinatorial boom of the 1990s, a well-documented disappointment in the number of quality leads has set in.[65] The compounds coming out of early combinatorial libraries simply did not perform up to the designer's expectations. Guidelines such as Lipinski's rules are helpful for generating more drug-like small molecules, but it has been argued that compounds from solid phase organic synthesis may not be chemically diverse enough to generate the desired selectivity and potency.[84,85] For example, a library of thousands or millions of compounds from a traditional combinatorial library may populate only a small area of chemical space.[62,84,86]

Diversity oriented synthesis (DOS) is a new term for a method of library construction in chemical genetics. It is also attracting attention in drug discovery for its great potential in generating valuable lead discoveries.[87] Advocates of the DOS approach point to the archetypal case of a flat aromatic or heterocyclic core dotted with various diverse appendages that has largely failed to deliver the promised drugs. In addition, the very basis of a structure−activity relationship (SAR) study is the intuitive notion that structurally similar compounds yield biologically similar profiles. Therefore, combinatorial libraries of little structural diversity will occupy a narrow window of biological space, thus limiting their

broad applicability. The compounds generated from traditional combinatorial chemistry, with their absence of multiple if any stereocenters and their limited functional group variety, do not occupy the chemical space required.[3,62,74,84]

The discrepancy between the success of natural products, especially in terms of selectivity and potency, and the as yet disappointing rate of lead generation from traditional combinatorial libraries has forced the question of whether natural products occupy regions of chemical space evolutionarily fine-tuned to be the most potent and active compounds.[88] Thus, one of the stated goals of DOS is to create extremely diverse libraries populating the maximum amount of chemical space to explore the greatest amount of biological space.

DOS is a modern development but has evolved from traditional solid-phase synthesis and particularly draws upon the split-pool/one-bead−one-compound approach.[89] Schreiber et al. have done significant work in developing DOS and optimizing methodologies to shuttle these compounds systematically through the various phases of chemical genetics studies.[90,91] It draws its name from its contrast to the traditionally used approach of target oriented synthesis (TOS). Beginning with a known target (often a natural product), TOS uses retrosynthetic analysis to work backward from a complicated product to simple and available starting materials. The goal of TOS is to get to a precise region of chemical space—a solo target or a few closely related derivatives.

Retrosynthetic analysis is not applicable to DOS since no target structure is available and thus the library cannot be targeted. Therefore, new thinking and planning strategies are required, and the idea of forward synthetic analysis has been proposed that moves in the direction of simple to complex, or reactants to products, in contrast to TOS. DOS strategies focus on maximizing diversity by utilizing branched and divergent pathways where the products of one reaction are common substrates for the next and where any follow-up chemistry must be efficient and systematic.[92,93] DOS's ability to generate nature-mimicking diversity from simple building blocks is one of its most desirable features.[92] Structural diversity is obtained by varying the building blocks and functional groups, diverse stereochemistry, and last and most interestingly, diversity generated in the compounds chemical skeletons/scaffold from a common framework. Molecules that occupy a greater degree of 3-D space or those that are more globular/spherical are a popular goal of DOS, as opposed to the relatively flat or circular molecules traditionally used in combinatorial chemistry.[88] DOS places a huge strain on synthetic planners since their proposed chemistry must be general, applicable, and compatible with all the various functional groups present in the library at any time.[94] Therefore, the reaction or process is the key to forward synthetic planning, since common reactivities are essential among library intermediates.[88]

Early DOS strategies used a one-synthesis/one-skeleton approach. This involved attaching numerous appendages to the molecular framework. The powerful effect of diverse stereochemisty on biological activity is well-known and has been a major focus in DOS. This was key to the design of some highly diverse and populated libraries (Figure 5).[95] The most promising and intriguing approach, however, is the ability to produce skeletal diversity from one common scaffold.[72,88] Within this concept, the predominant goal is to achieve structural diversity combinatorially.[93,96]

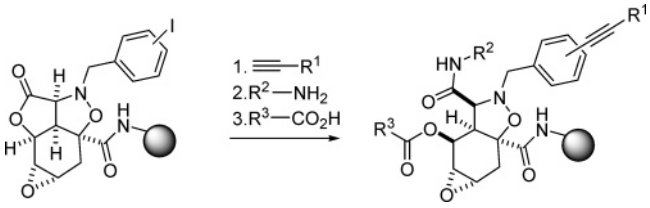


Figure 5. Two million compounds synthesized from 30 alkynes, 62 amines, and 62 carboxylic acids. The first structure includes three resin spacers, both enantiomers, and *ortho*-, *meta*-, and *para*-iodo benzyls.

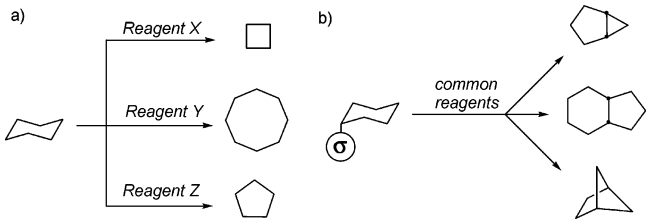


Figure 6. Skeletal diversity pathways: (a) differentiating processes, and (b) folding processes.

Schreiber et al. have sought to generalize the DOS method and describe two ways to diversify the scaffold (Figure 6). The first process, differentiation, uses different reagents to generate different scaffolds. The second process takes advantage of so-called σ elements. Schreiber describes these σ elements as pre-encoding skeletal information into a collection of products having distinct molecular skeletons using common reaction conditions. These are called "folding" processes due to their likeness to protein folding, in that abundant structural information is encoded by primary amino acid sequences that produce 3-D macromolecules in common folding buffers.[88] Figure 7 illustrates how a branching differentiation pathway in DOS give rise to skeletal diversity through consecutive Diels−Alders reactions. Figure 8 illustrates the concept of σ elements.

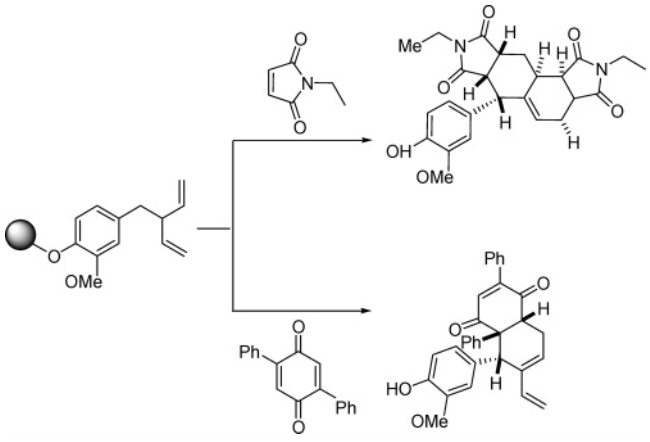


Figure 7. Representative example of differentiation DOS pathways leading to skeletal diversity.

Wong and co-workers have also exploited DOS libraries incorporating interesting scaffolds such as iminocyclitols to identify selective α-fucosidase inhibitors and *N*-acetyl-β-hexosaminidase inhibitors targeting osteoarthritis.[97,98] DOS combined with in situ microtiter plate screening has also provided sulfotransferase and HIV protease inhibitors[99−101] Numerous research reports and reviews of DOS libraries have been published.[72,80,87−90,92−96,102−113]

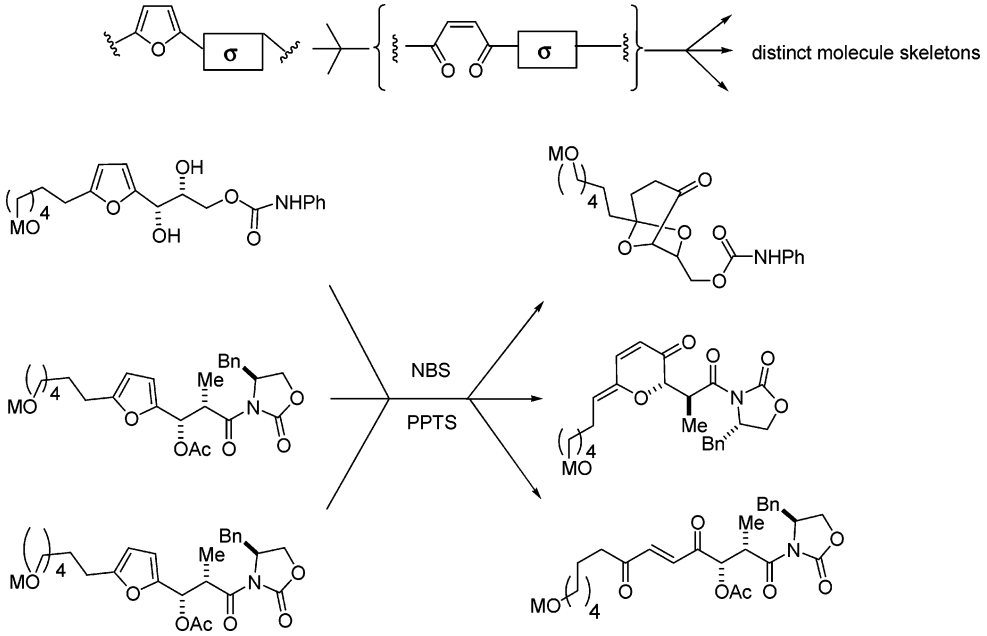


Figure 8. Skeletal diversity generating folding process—the transformation of substrates having different σ elements into products having different skeletons under a common set of reaction conditions.

2.4. Tagged Libraries

Tagged libraries represent unique opportunities in library design.[114] In this approach, libraries may be synthesized by any means and designed around any type of scaffold but must contain some functional tags integrated into the library. These tags are incorporated into the library compounds from the beginning, and they bestow some additional function into the molecules. The most common example of a tag is a fluorophore, but many more variations exist that allow the compounds to have functions extending beyond their biological activity that may aid in areas such as target identification or ligand assembly.

Peptide nucleic acids (PNA), developed by Buchardt et al., are oligonucleotide-like molecules that have their DNA backbone removed and replaced with an achiral polyamide backbone that can hybridize with DNA through strand displacement[115] PNA tags have been used in the development of tagged libraries that allow for the spatially addressable localization and identification of probes on an oligonucleotide microarray surface (Figure 9). The PNA tag encodes the synthetic history of the probe molecule and also hybridizes with a complimentary cDNA strand on the microarray surface to specifically identify the small molecule based on its location on the array. In early work, Schultz et al. combined PNA tagged libraries with microarray technology.[116,117] In one study, cathepsin inhibitors were identified by screening libraries in complex mixtures with the target protein before hybridization to the array. This spatially addressable PNA array also found use in the activity based profiling of proteases with the detection of caspase activation upon apoptosis induction. The characterization of the activated caspase and the inhibition of the caspase that executed the apoptotic phenotype were possible through this technology.

Winssinger et al. have continued work in this area by screening a 4000 member PNA library targeted to cysteine proteases against dust mite allergen extracts and identified Der p1 as a target implicated in dust mite allergies.[118] Winssinger et al. have also expanded upon the methods to produce PNA libraries through their detailed synthesis of a

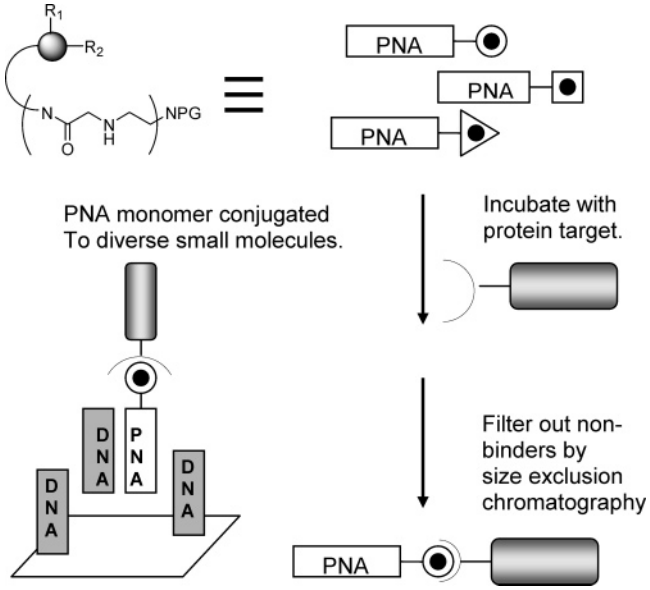


Figure 9. PNA tagged libraries.

PNA-encoded cysteine protease inhibitor library. These workers utilized an alternative solid-phase PNA synthesis based on azididopeptide nucleic acids where the azide is a mask for the amino group.[119,120] Harris et al. have significantly expanded the use of PNA-encoded microarrays in profiling proteases with rhodamine-based fluorogenic substrates through their recent demonstration of a system that allows for the screening of samples that range from single proteases to complex clinical blood samples.[121] This method utilized fluorogenic protease substrates linked to PNA. Using these latent fluorophores instead of substrates linking FRET fluorophores was advantageous since only proteolysis at the residue adjacent to the fluorophore gives a signal, and proteolysis of that bond changes the electronic properties of the fluorophore significantly enough to give rise to a large increase in fluorescence, thus aiding detection.

Click chemistry offers unique tags that can be used to generate library members through strategies such as target

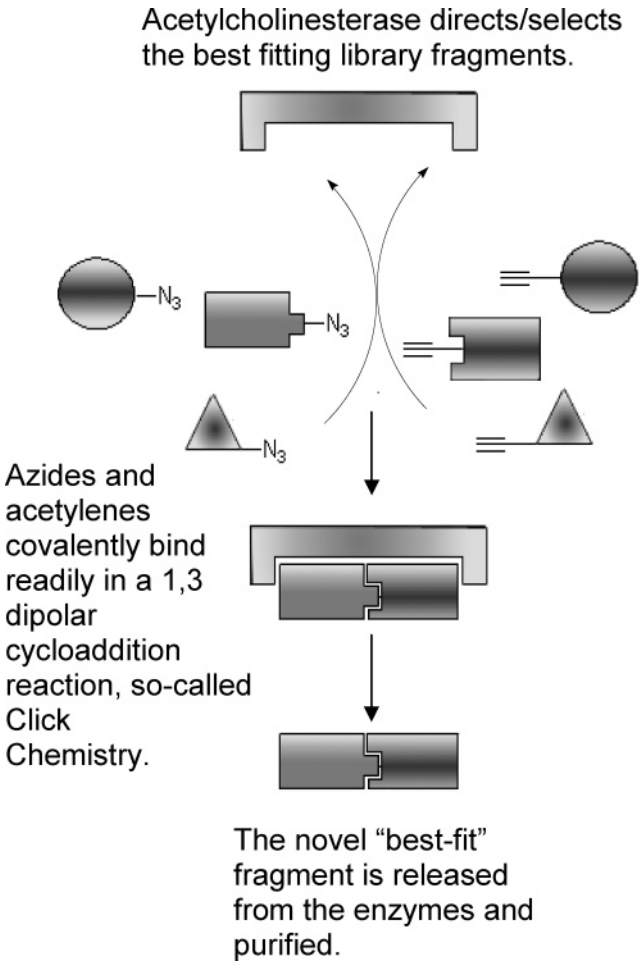


Figure 10. Target-guided ligand assembly using click chemistry.

guided ligand assembly (Figure 10). Sharpless developed click chemistry as a rapid reaction that proceeds in a short amount of time through "spring loaded" highly endothermic, irreversible reactions to form carbon−hetero bonds, one of the most common of which is a [2 + 3] Huisgen 1,3-dipolar cycloaddition between an azide and an alkyne moiety, described as an energetically "near-perfect" reaction.[122,123] Click chemistry's main advantage is the swift and clean nature of these reactions that can greatly assist library synthesis and lead discovery. Sharpless and Kolb have written an excellent review on click chemistry and its effect on drug discovery.[123]

Azide−alkyne cycloaddition has been used to generate enzyme inhibitors and has been employed as a tool in solid-phase synthesis, but one of its most exciting applications for chemical genetics is in situ screening and target guided assembly (a more detailed discussion appears in the following section).[124−127] In a classic application, Sharpless et al. used target guided selection to identify an inhibitor of acetylcholinesterase, a key enzyme implicated in Alzheimer's disease.[128] Building blocks bearing azide or acetylene functional groups were screened against acetylcholinesterase where the enzyme's active site guides the formation of the dimeric ligand. The inhibitor formed rapidly, in high yield, and stereoselectively and was shown to be of high affinity.

Kolb et al. have continued the work and optimized the conditions, in particular the mass spectroscopic analysis, expanded the throughput of screening, and identified three new highly potent acetylcholinesterase inhibitors.[129] Kolb et al. have also used this method in the screening of inhibitors for carbonic anhydrase II and identified a number nanomolar binders for this therapeutically important target.[130]

Click chemistry based tags have also been incorporated in studies of activity based protein profiling (ABPP) by Cravatt et al. (Figure 11).[131] ABPP is a chemical proteomic method that uses probes to monitor and visualize changes in protein functions or levels in the cell, especially changes that occur on a post-translational level. In a traditional ABPP study, the probes carry a tag or reporter, such as biotin or fluorescence, whose size and physical properties may adversely affect probe−target interaction, cellular uptake, and probe distribution. In the click chemistry based approach, the probe is functionalized with a tag bearing a simple azide moiety, which presents much less of a disturbance in the system. After screening and binding to the protein target, an acetylene bearing reporter is introduced where it is covalently attached to the probe−target complex for visualization. Cravatt et al. used this approach in detecting labeled proteins and, in preliminary experiments, showed that they could target glutathione *S*-transferases (GST) both in vitro and in vivo in whole proteomes.[132] The group has further expanded and optimized this approach, especially to control the high background reaction rate. This study reported the optimal conditions for click chemistry based APBB with respect to speed, bioorthogonality, and sensitivity. Screening of homogenized breast cancer cells identified several enzymes that could be labeled by activity-based probes in situ that were not previously identified in vitro.[133]

ABPP probes label the active sites of their enzyme targets, and an extension of ABPP relevant to chemical genetics would be the use of this technology to identify inhibitors.[131,134] This method is advantageous because (1) it screens

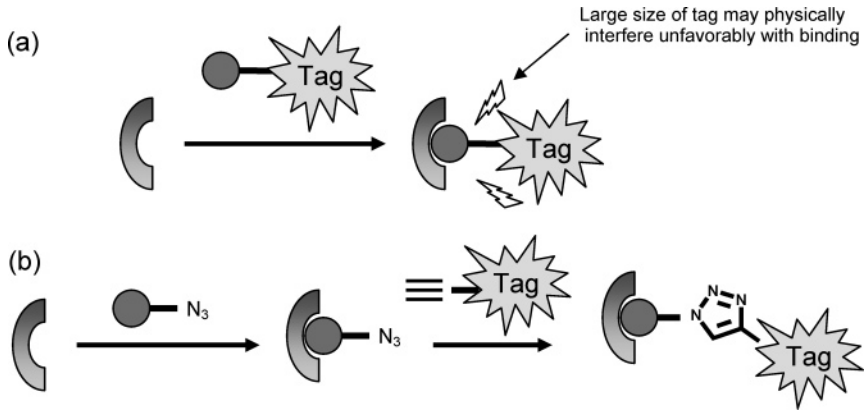


Figure 11. (a) Traditional ABPP approach and (b) click chemistry ABPP approach.

the enzymes in the native proteomes, (2) unknown enzyme−inhibitor pairs can be identified analogously to substrate screening, and (3) this technique screens many enzymes in parallel and allows for the potency and selectivety of these compounds to be concurrently evaluated.[131] For example, Bogyo and co-workers screened irreversible enzyme inhibitors that were preincubated with enzymes or proteomes and treated with ABPP probes to identify inhibitor-inactivated enzymes.[135] Here, they selected covalent bond forming inhibitors of the *Plasmodium falciparum* cysteine protease falcipan 1 and used them to demonstrate a role of this enzyme in parasite invasion of erythrocytes, thus establishing a new target for antimalarial therapeutics (Figure 12).[131,135] Additionally, Bogyo and co-workers have used chemically reactive fluorescent probes to profile and identify enzymes in complex mixtures and used a fluorescently tagged compound that was directed toward the papain family of proteases to identify selective inhibitors of individual cathepsin proteases in a competition assay with small molecule inhibitors.[136,137] These studies showed the possibility of using ABPP in lead discovery and drug development.

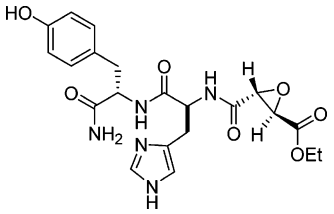


Figure 12. Specific falcipan-1 inhibitor.

Encoded self-assembling chemical libraries offer an attractive tagged library method for active probe development (Figure 13).[138] Melkko et al. designed these libraries to identify molecules of high binding affinity. Their studies originated from the belief that the probability for potent ligand identification increases with larger libraries. Their libraries are based on oligonucleotide derivatives (oligonucleotides bearing various small molecule pharmacophores) that are capable of combinatorial self-assembly in the form of dimers and trimers. These libraries can also be enriched through affinity maturation for binders on an immobilized target antigen. It is important to note that these libraries are not dynamic, in that they are stable, and the library members can exist at very low concentrations. DNA tags (six nucleotide identification code) encode each molecule. Any complexes that form can, after identification, enrichment, and decoding (by PCR, polymerase chain reaction), have their DNA tag replaced with a linker for covalent attachment of any of the identified pharmacophores. In a proof of concept experiment, these libraries were screened with bovine serum albumin and carbonic anhydrase, and nanomolar binders were identified.[138]

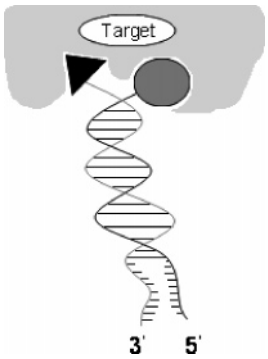


Figure 13. Encoded self-assembling chemical libraries.

Chang et al. have explored the tagged library concept by synthesizing libraries of compounds that all contain intrinsic linker tags.[114] These linker tags contain a functional group that can be easily modified to adapt the molecule to a variety of experimental requirements, but the primary purpose of this funcitonal group is target protein identification. They have developed triazine libraries containing an oligo(ethylene glycol)-based linker with a terminal primary amine. The amino end of the linker was used to immobilize the hit compounds on the solid phase, for example, agarose or microarrays surfaces, to provide affinity matrices. Their tagged strategy is applicable to any type of library scaffold and allows for rapid transfer from biological screening to target identification without the need for the time-consuming SAR study and modification of the hit compound. In their initial report, they tested these linker tagged compounds in zebrafish embryos screening and identified a hit compound, encephalazine, which suppresses eye/brain development.[139] Encephalazine was then easily immobilized on agarose beads for pull-down affinity matrix studies without the need for a cumbersome SAR study. They identified four ribosomal subunit proteins (S5, S13, S18, L28) that are implicated in brain/eye development by functional genetic knockout. These results corroborated phenotypic screens by encephalazine (Figure 14).[139]

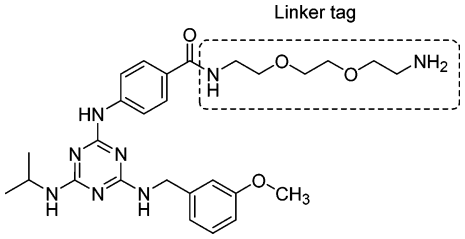


Figure 14. Encephalazine.

In a reverse chemical genetics application, a similar tagged library was immobilized on an activated glass surface by covalent bond formation. This small molecule microarray was screened with fluorescently tagged human immunoglobulin G (IgG). A number of hit compounds were identified, and their binding was confirmed and quantified by surface plasmon resonance (SPR) with the best having a K_d value of 2 μM. These compounds may have future applications in the isolation and purification of IgG (Figure 15).[140]

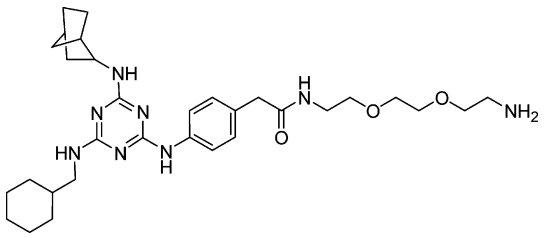


Figure 15. Example of an IgG binder.

Chang et al. have also used the tagged library strategy in the development of intrinsically fluorescent compound libraries. In many contexts, the addition of an exogeneous reporter or tag can alter the effect or cellular distribution of the original molecule. Tagging the molecule intrinsically avoids this problem. In their strategy, the probes being studied are

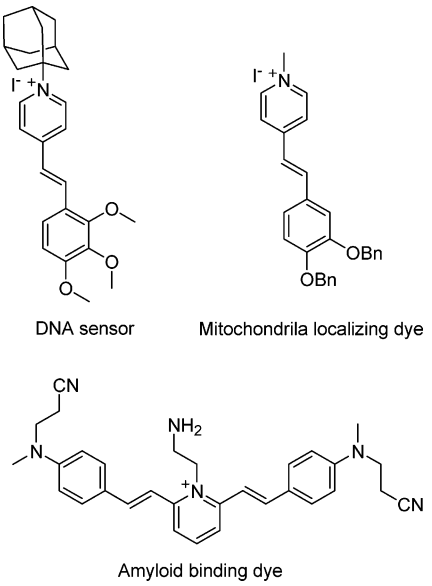


Figure 16. Example of styryl dye compounds.

all intrinsically fluorescent through their styryl scaffold, and no additional tagging is required to visualize cellular localization or for sensing applications. They have used solution-phase approaches in developing cell-permeable DNA-sensitive dyes,[141] in addition to developing a combinatorial approach that produced a number of active probes that target and localize to various cellular organelles with a wide variety of wavelengths (Figure 16).[142] They have developed a solid-phase approach to styryl dyes and used these to develop novel amyloid sensors, which are potentially valuable in Alzheimer's Disease (AD) research.[143]

2.5. Target Guided Ligand Assembly

Many successful combinatorial libraries are designed from structural or mechanistic information about the target. However, in some cases, little information is known about the target. This difficulty is only compounded when no leads exist for a target that may serve as a starting point for library design. One interesting strategy to overcome this difficulty is the use of target guided ligand assembly (TGLA). TGLA starts with no requirements of target or lead information and follows four steps: (1) a collection of aqueous soluble binding elements must be assembled that all possess a functionally reactive group capable of linking the elements together; (2) the binding elements are screened, and even weakly binding elements are identified; (3) a combinatorial library is created by linking together any identified binding elements with various length linkers; (4) this combinatorial library is then screened against the target (Figure 17).[144]

Ellman et al. used the strategy to identify subtype-selective inhibitors of the tyrosine kinase c-Src. Finding selective inhibitors among kinase families poses a formidable challenge. The potential binding elements were selected, and all contained an *O*-methyl oxime as the linkage group from which a linkage can be prepared by the condensation of *O*-methylhydroxylamine with any aldehyde. The monomers were screened with a protein tyrosine kinase enzyme-linked immunosorbent assay (ELISA), and all possible binders were identified. These were then combinatorially linked with a variety of linkers. A second round of screening identified a potent inhibitor with an IC_{50} of 64 nM and possessing 75-fold selectivity over a series of highly homologous kinases (Figure 18). Not only revealing an inhibitor, the study also provided SAR data that may aid in understanding the

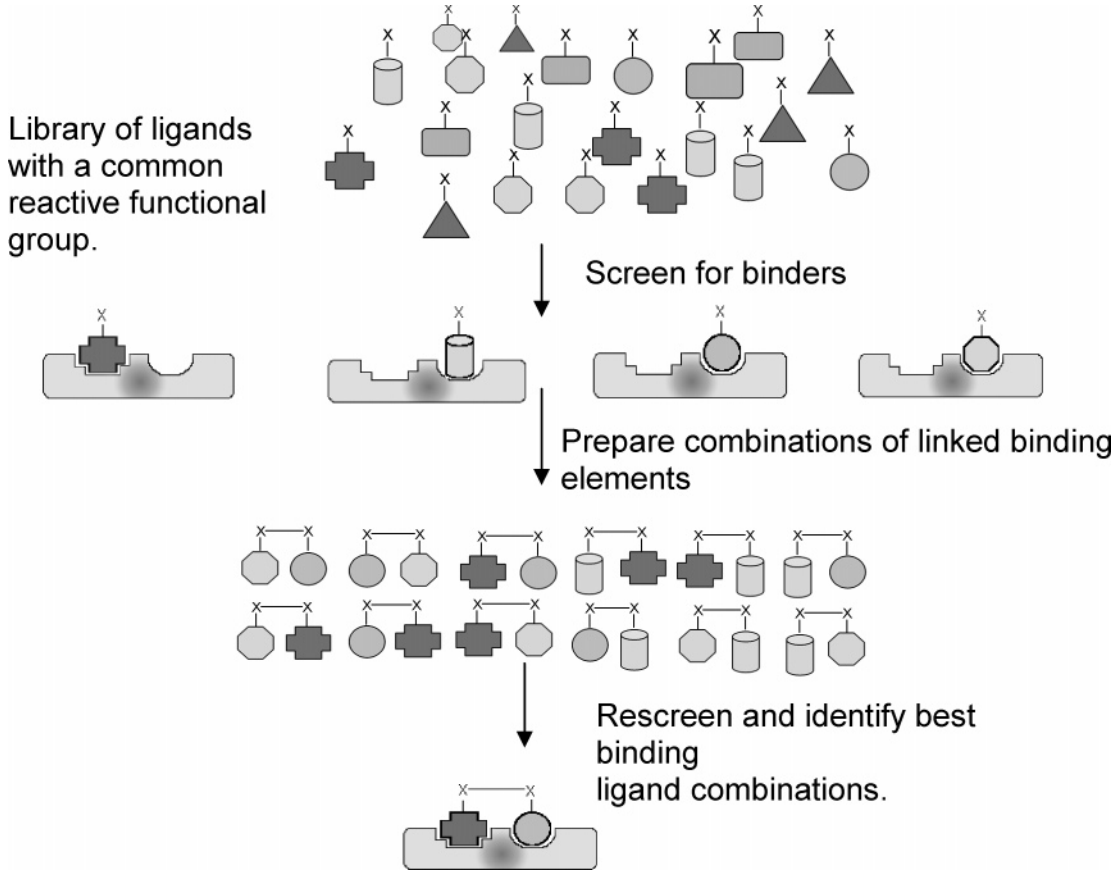


Figure 17. Target-guided ligand assembly.

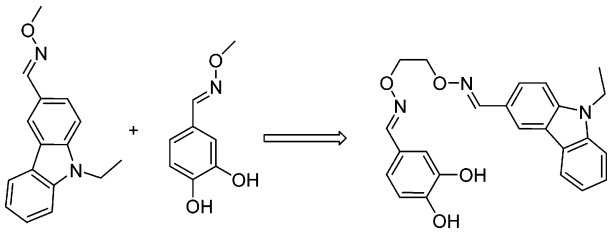


Figure 18. Assembly of a potent inhibitor from previously identified fragments, c-Src inhibitor with $IC_{50} = 64$ nM.

selectivity and affinity among kinases.[144] This strategy was similarly employed by Bertozzi et al. where they identified the first two active inhibitors ($IC_{50} = 30$ and 40 μM) for tyrosylprotein sulfotransferase 2 (TPST-2).[145]

A similar approach was presented by Wells et al. and was termed "site-directed ligand discovery".[146] Site-directed ligand discovery is closely related to what is termed "fragment-based lead discovery and assembly". Both of these approaches are HTS-based and seek to identify binding fragments for targets, and the fragments are linked or assembled at a later stage to give a potent compound. In general, a target protein contains or is engineered to contain reactive functional groups "X", and the protein is screened with a collection of fragments (or small molecules) that all possess a reactive functional group "Y", complimentarily reactive with X. Should the fragment bearing Y bind in the vicinity of the protein's X group, the two can react to form a covalent bond and be analyzed by a method such as mass spectrometry. The approach allows for the identification of low-affinity ligands, as well as providing for the ability to identify the fragment's binding location.[147] The first report of this technology by Wells et al. involved the process of "tethering". This method employed X as a free thiol and Y as a disulfide. Thiol exchange is well suited for site-directed ligand discovery since it occurs under mild conditions and is highly chemoselective. In the initial report, nanomolar inhibitors of the enzyme thymidylate synthase were assembled from primary screening of a disulfide library followed by rounds of optimization and modification.[146]

Raimundo et al. presented an excellent demonstration of the use of the tethering strategy involving the discovery of the first high-affinity ligand for a cytokine target, interleukin-2 (IL-2), and demonstrated that protein−protein interactions are potential small molecule targets.[148] As an additional example of this technology, a second generation method of tethering, termed "tethering with extenders", was presented by O'Brien and co-workers. Here the protein is modified with an extender, a small ligand that contains a reactive functionality and a (usually protected) thiol. After the reactive functionality binds to the protein, the thiol is deprotected and used for tethering to identify a ligand that binds nearby. This method expands the technology from site-directed ligand discovery to discovery plus ligand assembly.[147] This approach has been used by O'Brien and co-workers to discover potent low micromolar inhibitors of the pro-apoptotic cysteine protease caspase-3.[149] Excellent reviews of tethering and site-directed ligand discovery and fragment-based lead assembly and their application to drug discovery are available for more in-depth discussions on this topic.[147,150]

2.6. Dynamic Combinatorial Libraries

Traditional combinatorial libraries are synthesized primarily by parallel or split-pool techniques.[151] Even DOS, whose goal is to maximize diversity in chemical space, generates libraries that are static pools of discrete molecules.[152] While the use of compound mixtures fell out of favor in combinatorial chemistry, they found renewed use in supramolecular chemistry in the form of dynamic combinatorial chemistry (DCL). The dynamism of DCLs results from the reversible interchangeability possible with their components (Figure 19). In these systems, every member of the library and the

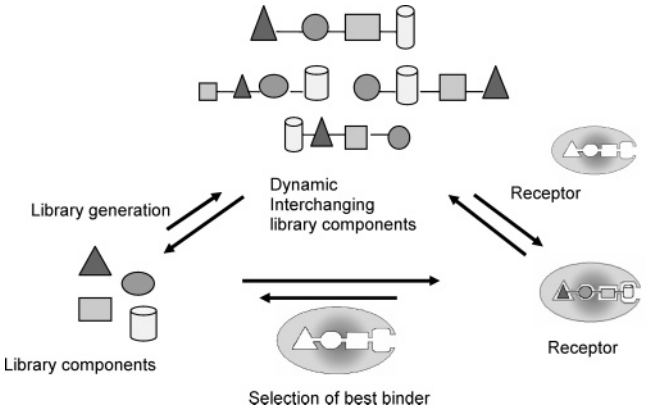


Figure 19. Dynamic combinatorial libraries.

targets themselves affect all other members of the library, particularly in terms of library composition.[152] Any stabilization of one member will result in an equilibrium shift and thermodynamic redistribution, by LeChatelier's principle, of the library mixture favoring the best binder.[153,154] Advantageously, library construction and screening can be combined in one step, since amplification of the best binder can be analytically detected.[155,156] So in reality, the use of DCLs is as much an application of HTS as library development. Whereas traditional combinatorial libraries rely on their sheer numbers, DCL libraries offer an alternative approach through self-replication and amplification.[153]

The use of dynamic combinatorial libraries requires three steps: (1) the collection or design of a library capable of undergoing reversible constituent interchange, (2) conditions whereby the library may undergo interconversion, and (3) a step that selects the "fittest" binder and possibly involves its amplification.[152] When a template or ligand is used to amplify the concentration of a member of the library, so-called "survival of the fittest", the Darwinian implications of this often lead it to being referred to as "molecular evolution". In a molecular evolution system, the fittest binder is amplified with each successive round of screening, whereas the poor binders' concentration will either be unaffected or decrease.[153,154,157] Though widely applied for many other purposes, DCLs have also been used to identify protein ligands, which makes them a promising tool for library development in chemical genetics.[158−162]

An example of a DCL in drug discovery was provided by Lehn et al. who screened an in-house patrimonial collection of 1440 compounds and identified an inhibitor of HPr kinase (HPrK/P).[163] HPrK/P is implicated in the bacterial carbon catabolite repression pathway. HPrK/P-deficient bacteria show serious growth defects and thus make HPRK/P inhibitors desirable antibiotic targets. Using the original lead as a guide, they set forth assembling headgroups that resemble the lead compound in combination with a variety of linker lengths. From 21 components, it was possible to assemble a DCL of 800 unique constituents. A radioactive phosphorylation assay was employed to identify inhibitors.

Screening revealed that the DCL contained potent inhibitors and a dynamic deconvolution protocol was used to identify the active member(s). This was done by selectively removing a particular building block and judging its effect on inhibition. A building block's importance was judged by the magnitude of the decrease in activity upon removal from the library. After identifying the important building blocks, detailed assembly of these different building blocks was undertaken, and the most active compound was identified (Figure 20). Though the compound identified from the DCL did not show a significantly improved IC_{50} when compared to the original lead (17 vs 10 μM), it did show that kinase inhibitors can be generated and screened using DCLs to provide useful SAR data.

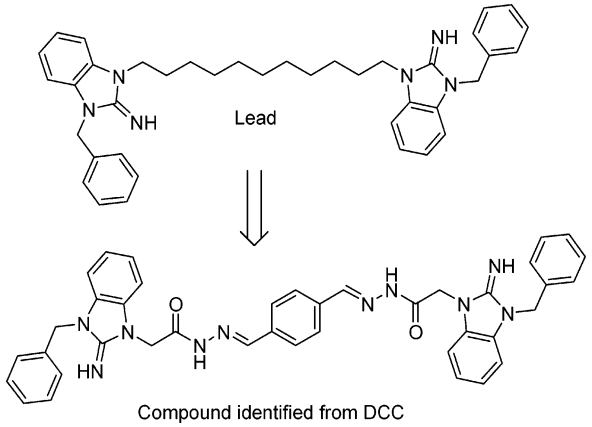


Figure 20. HPrK/P inhibitor identified by DCC.

2.7. Annotated Chemical Libraries

Generally, libraries require careful design followed by extensive screening. Truly daunting is the follow-up work to identify a hit compound's mechanistic mode of action or target protein. An alternative approach to designing and building large collections of compounds of which no known biological activity information exists is the use of an annotated chemical library (ACL). An ACL is a collection of compounds of diverse structure from various sources possessing experimentally bona fide biological activities and mechanisms. An ACL contains compounds with diverse sets of biological activity, whereas a typical library would even in a best case scenario be composed of just a fraction of a percent of active compounds. One need not synthesize even a single compound to generate an ACL; one merely needs to identify, collect, and annotate. ACLs operate by assigning previously reported biological activity to each compound without any required regard for the pathway or phenotype under study.[164] A popular example of an ACL is the National Cancer Institute's screening panel. Annotated libraries are particularly desirable in the nascent field of chemogenomics where they assist in chemoinformatics-based similarity searches.[165] Annotated reference sets have also found use in identifying ligands in in silico screening.[48]

Stockwell et al. used an ACL in a chemical genetics application in which they focused on employing their ACL in a study of biological mechanisms implicated in tumor cell viability.[164] They sought the rapid elucidation of targets and mechanisms by screening and identifying a subset of inhibitors of tumor proliferation followed by determination of the statistically enriched biological mechanisms among the identified subset relative to the whole collection. Their ACL was comprised of 2036 compounds, and these were assigned any relevant combination of 169 biological mechanism descriptors. Their ACL occupied a broader biological space than two much larger commercial libraries (20 000 Comgenex and 29 996 Chembridge) included in the study. A viability assay showed that 2.5% of the ACL inhibited cell proliferation, whereas only 0.69% of compounds from a commercial library screen showed inhibition, 4 times the number of antitumor agents for the ACL when compared to a commercial library. Additionally, a greater percentage of their ACL library compounds were more selective toward tumor cells over primary cells than the commercial library compounds. In screens with A549 lung carcinoma cells, 85 compounds were identified from a viability assay and, based on their annotation and the biological literature, they identified 28 overrepresented mechanisms among their identified compounds. Specifically from these data, they hypothesized that "ionophores" are capable of killing A549 cancer cells. Importantly, they demonstrated that ACL's can greatly accelerate the identification of the underlying biological mechanisms in chemical genetic studies.

Uesugi et al. adopted an annotated library approach in the identification of the histamine H1 receptor as a stimulator of insulin-induced adipogenesis.[166] The study of fat cell differentiation is of increasing interest due to the worldwide rise in obesity, and the preadipocyte 3T3-L1 cell line serves as a model of adipogenesis since they differentiate to mature fat cells in the presence of insulin. They screened 880 annotated compounds of known activity whose mechanisms have been well-studied in a phenotypic adipogenesis assay. They identified antihistamine compounds that inhibit differentiation followed up by siRNA analysis of the histamine H1 receptor knockdown that hindered insulin-induced adipogenic differentiation. Though the authors noted the small size and diversity of the library, this study shows the promise of the use of annotated libraries.[166]

3. Screening: An Overview

Once libraries of compounds are assembled, the next step in chemical genetics is testing them for their biological activity, that is, assays or screening. As discussed in section 1, one can conduct forward or reverse chemical genetics screens. Briefly, FCG begins with a library of compounds, screens for a response from the assay system, such as a cell morphology change, and goes on to identify the specific protein that was targeted (small molecules and their target proteins identified in forward chemical genetic screening are summarized in Table 1), whereas RCG begins with a known protein target and screens for a specific compound that alters the protein's function. Within this review, this overarching difference in approach served as a guide to the placement of a certain piece of work. Assays must be designed with maximum sensitivity, selectivity, reproducibility, and cost-effectiveness in mind.[167] Given the size of today's libraries, the option of doing anything other than HTS is something less than desirable. Screening technologies have progressed in miniaturization from high-throughput (384 well plates) to ultra-high-throughput (3456 well plates) screening (u-HTS), and their limits have not yet been reached. Marron and Jayawickreme have provided an informative review on the increased use of "well-less" lawn format screenings that have replaced well-based microtiter plates with agarose "lawns". These can potentially move beyond the level of u-HTS.[168] Abbott laboratories has developed a particularly robust screening platform called μARCS (microarrayed

Table 1. Small Molecules and Their Target Proteins Identified in Forward Chemical Genetic Screening

small molecule	activity	target protein	section and refs
encephalazine	inhibition of brain/eye development in zebrafish	ribosomal subunit proteins (S5, S13, S18, L28)	2.4[139]
SMIRs	inhibition of rapamycin activity	Tep1p (PTEN) and Ybr077cp	4.1[185]
splitomicin	yeast growth inbition	Sir2p (HDAC)	4.1[186]
SFK1	suppression of FK506 activity	Por1p (VDAC)	4.1[187]
SFK2,3,4	suppression of FK506 activity	Ald6p (aldehyde dehydrogenase)	4.1[188]
sirtinol	auxin signal increase	sirtuin	4.2[189,190]
brassinazole	BR biosynthesis inhibition in plant	DWF4 (cytochrome P450)	4.2[191]
PTK787/ZK222584	block blood vessel formation	VEGFR inhibitor	4.3[192]
tubulyzine A, B, C	slow epiboly development in zebrafish	tubulin	4.3[193]
MP576	SARS-CoV inhibition	SARS-CoV protease	4.6.1[194]
HE602	SARS-CoV inhibition	SARS-CoV helicase	4.6.1[194]
myoseverin	myotube disassembly	tubulin	4.6.1[195,196]
MP C11	pigmenting albino melanocyte	mitochondrial F1F0-ATPase	4.6.1[197]
melanogenin	pigmenting melanocyte	prohibitin	4.6.1[198]
syntab A	mitosis perturbation	tubulin	4.6.2[199]
tubacin	inhhibition of α-tubulin acetylation	HDAC6	4.6.2[200]
monastrol	block mitosis entering	mitotic kinesin Eg5	4.6.2[201]
ICG-001	down regulation of β-catenin	cAMP response element binding protein	4.6.3[202]
TWS119	neuronal differentiation	GSK-3β	4.6.3[203]
PNRI-299	AP-1 inhibition	REF-1	4.6.3[204]
Hh-Ag 1.1	inhibition of Smo	Smo	4.6.3[205]
MOL-294	NF-κB inhibition	thioredoxin	4.6.4[202]
wiskostatin	inhibition of actin assembly	N-WASP	4.8[206,207]
aminopurvalanol	cell cycle arrest at G2/M	CDK1 (cyclin-dependent kinase 1)	4.8[208]
diminutol	inhibition of mitotic spindle assembly	NQO1 (quinine oxidoreductase)	4.8[209]
ubistatin A, B	inhibition of mitotic entry	ubiquitin	4.8[210]

compound screening).[169] This gel-based technology embeds the arrays in agarose with one of the advantages being the ability to store compound libraries in a dry, inert environment in a ready to screen format.[170,171]

However, though screening methods have matched the enormity of many libraries, the screens must be reliable and reproducible, and the data must also be manageable. Not only have the number of assays increased, but assays have increased in the dimensionality of the data produced, so-called "high-content screening".[167] Therefore, a significant challenge in HTS, and especially in u-HTS, is the development of tools that allow for data management and analysis in chemical genetics.[172] For example, Stockwell et al. have made a freely available software tool termed the small laboratory information management system (SLIMS). SLIMS aids users in data collection and analysis, as well as relevant literature retrieval for aid in determining an active compound's mechanism of action.[173] They have also made freely available the VisTa program that relies on periodogram analysis to identify and correct for systematic errors in array-based screening.[174] In addition, Leonetti et al. have also made freely available a data processing tool called the ELISA data manager that aids in the treatment and archiving of small- to medium-sized libraries.[175] Another approach is to limit the number of screens and reduce the attrition rate of leads in the drug development process by using chemoinformatic tools to inform library and screening design and in deciding which leads should be brought through the drug development process.[176,177]

A key improvement, and an area of fundamental importance, is the use and expansion of model systems in chemical genetics. As a model, cellular phenotypic studies comprise a large component of chemical genetics, but new systems continue to be developed. Instead of mammalian cells, the platelet has been developed as a promising model system, particularly in phenotypic studies.[13] The platelet is amenable to small molecule based chemical genetics screens since its anucleate nature makes genetic manipulation difficult. In addition, they are readily available and show dynamic responses in biological assays.

A number of reviews have been written on screening (HTS and u-HTS) in drug development and chemical genetics with some having emphasis on its role in academia.[167,178−180] Clemons has written a review that focuses on complex phenotypic screening in HTS, and Murchison et al. contributed a review on the use of high-throughput phenotypic screening using cell imaging.[181,182] However, some forms of compound screening require no physical screening at all but instead rely on virtual screening based on in silico molecular docking, as Jiang et al. described in their review, or employ virtual targeted libraries for a specific receptor.[183,184] The current review will highlight novel and interesting uses of model systems in chemical genetics within the context of describing the actual work.

4. Forward Chemical Genetic Screening

4.1. Yeast

As in genetic studies, budding yeast (*Saccharomyces cerevisiae*) is a robust and powerful tool in chemical genetics. Three significant advantages of yeast as a model organism in understanding cellular responses to chemical perturbation include its ease of growth, high genetic conservation with humans, and the size of the collection, up to 6000 genes, makes its use a very high-throughput screen.[50] In a demonstration of the state of the art of chemical genetics, that is, the integration of multiple chemical genetic, genetic/genomic, and proteomic tools, Schreiber et al. identified new components of the target of rapamycin (TOR) signaling network (Figure 21).[211−213] Rapamycin is a promising macrolide

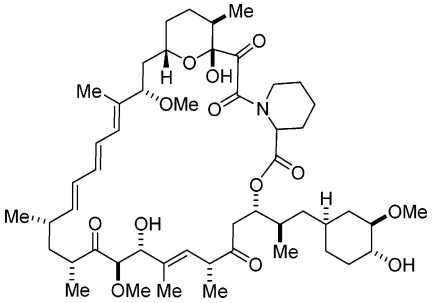


Figure 21. Structure of rapamycin.

immunosuppressant and anticancer drug. TOR is conserved from yeast to humans and regulates cell growth as a response to nutrient signals. The group's phenotypic screening in yeast sought modifiers of rapamycin-sensitive pathways. Yeast treated with rapamycin had their growth and growth-related phenotypes scored from which hit compounds were identified. This phenotypic screening identified six compounds (SMIR, small molecule inhibitors of rapamycin) that fully suppressed rapamycin's anti-proliferative effect. Full transcription profiling followed target identification using proteome chips.[211] From these SMIRs, they identified the proteins Tep1p, a PTEN homolog, and Ybr077cp (Nir1p), a component of the EGO complex, as target proteins involved in the TOR pathway in terms of phosphatidylinositide metabolism and regulation.[185]

In an interesting approach, Raikhel et al. used a chemical genetic, or as they termed it "chemical genomic", approach using yeast phenotypic screening to study vacuolar sorting in *Arabidopsis*.[214] Yeast was chosen as a simpler but more robust model for the more complex *Arabidopsis*, since the pathway under study is evolutionarily conserved. They screened a 4800 member commercial library to identify compounds that alter the delivery of carboxypeptidase Y (CPY) through the endomembrane into the vacuole. Secreted CPY was detected by antibody staining, and changes in vacuolar morphology were visualized by a selective fluorescent probe. Wild-type yeast secretes no CPY beyond the cell, but the yeast vacuolar protein sorting (*vps*) mutant secreted significantly and was used as positive control. Fourteen compounds were identified and termed the "sortins", protein sorting inhibitors; the highly potent sortin 1 was also shown to affect carboxypeptidase Y secretion in plants (Figure 22). This study showed that yeast screening, where vacuole biogenesis is not essential in itself, can still be used as tool for the study of vacuolar delivery in plants.

Schreiber et al. have used genome-wide yeast transcription profiling in studying histone deacetylase (HDAC) function

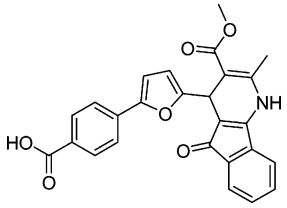


Figure 22. Structure of sortin 1.

by probing with the HDAC selective inhibitor trichostatin A (TSA) (Figure 23).[215] Their study integrated genetic transcription analysis using microarrays with chemical genetic methods to identify genes implicated in modulating HDAC activity, as well as revealing that different HDACs play distinct roles in the gene regulation of processes such as cell cycle progression, amino acid biosynthesis, and carbohydrate transport and utilization.[215]

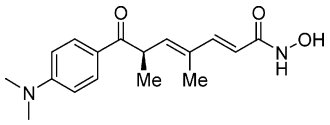


Figure 23. Trichostatin A.

In yeast, Sir2p has been an active subject of phenotypic chemical genetic studies.[186,189] Sir2p is a founding member of a family of a NAD^+-dependent HDACs, also called the sirtuins, and is involved in transcription silencing from the telomeric, rDNA, and silent mating-type loci. From a visual growth inspection screening, Simon et al. identified a compound, splitomicin, that phenocopied *sir2* loss-of-function mutant and inhibited HDAC activity in vitro (Figure 24).[186] Their results also showed that Sir2p is continuously required for silent-state maintenance in nondividing cells.

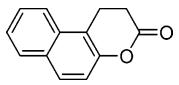


Figure 24. Splitomicin.

Schreiber et al. sought to find sirtuin inhibitors through high-throughput phenotypic screening in yeast.[189] They sought compounds that interrupt the silencing pathway at the telomeres by using a cell-based URA3 reporter screen and 1600 unbiased library compounds. In the presence of 5-fluoroorotic acid, the Sir2p inhibitor caused the expression of the URA3 gene and cell death. From this library, three compounds were identified that inhibited Sir2p transcriptional silencing in vivo in yeast. They were also shown to inhibit human SIRT2 deacetylase activity in vitro. It was further postulated that these compounds interfere with body axis formation in *Arabidopsis.*

The logical extension of the small molecule as a genetic knockout analogy holds firmly in relation to synthetic lethality screening. This traditionally genetic approach easily applies to small molecule−gene interactions.[216] In a typical chemical synthetic lethality screen, small molecules are tested in cells comprised of various genetic mutants. More lethal combinations, when compared to the wild-type, of a small molecule (and its interaction with a target) and a specific mutation, such as a gene deletion, are considered hits, and their data is interpreted. Friend et al. demonstrated an early example in their screening of compounds against 70 isogenic yeast mutants harboring defects in cell cycle repair, checkpoints, and DNA repair. Their screening of anticancer

chemotherapeutic agents against mutant strains also allowed for the generation of drug sensitivity patterns.[217] Friend et al. followed up more thoroughly on this work in an expanded yeast panel with a greater number of Food and Drug Administration (FDA) approved anticancer agents and generated comprehensive toxicity profiles for these compounds that revealed wide differences between the agents screened.[218]

In a genome-wide screen performed by Tucker and Fields using haploid yeast deletion strains, synthetic lethality was tested in the presence of four chemicals: hydrogen peroxide, menadione, ibuprofen, and mefloquine.[219] It was shown that hydrogen peroxide and menandione induced oxidative stress, whereas ibuprofen and mefloquine were toxic by unknown mechanisms. From their screening, they identified the sensitivities of 659 deletion strains, including 163 multichemical sensitive strains, 394 strains specific to hydrogen peroxide and menadione, 47 strains specific to ibuprofen, and 55 strains specific to mefloquine. Similarly, Zheng and co-workers studied the function of the target of rapamycin protein (TOR) by screening 2216 haploid yeast deletion mutants for sensitivity in the presence of rapamycin.[220] Their results identified 106 mutants with rapamycin sensitivity, and the majority of the resistant or hypersensitive genes were of known function and clustered into discrete groups. This analysis provided for a global view of TOR function and afforded valuable information for future mechanistic studies.

FK506 (Figure 25) is an immunosuppressant drug, and one of its target proteins is FK506 binding protein 12

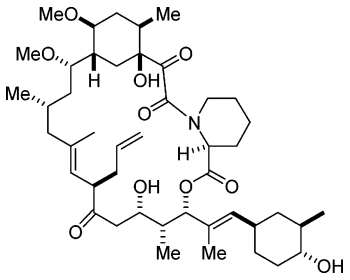


Figure 25. Structure of FK506.

(FKBP12).[221] In addition to binding FKBP12, FK506 specifically inhibits Ca^{2+}-dependent protein phosphatase calcineurin. In yeast, calcineurin is only required for survival under stress conditions such as high Na^+, Li^+, Mn^{2+}, and OH^- concentrations. To develop a tool to study calcineurin function in yeast, Schreiber and co-workers sought molecules that would suppress FK506 inhibition of calcineurin at high NaCl concentrations.[187] A total of 10000 compounds were screened and monitored for cell growth as positives, and one molecule, SFK1, a suppressor of FK506, was identified as a promising compound. While SFK1 restored growth at high salt concentrations, at low concentrations of salt, it proved lethal. An affinity pull-down experiment identified Por1p, a mitochondrial voltage-dependent anion channel, as the target of an amine-derivatized SFK1. In a series of follow-up experiments, it was also convincingly suggested that SFK1 interacts directly or indirectly with the pore protein Por1p in the outer mitochondrial membrane and that SFK1 targets the mitochondria. They then performed synthetic lethal screening of a genome-wide set of viable yeast deletion mutants in the presence of SFK1 for sensitivity, followed by transcription profiling. Yeast deletion strain screening with SFK1 yielded strains with impaired mitochondrial function and reduced sodium tolerance. In addition, mitochondrial function was linked to ionic balance, and it was suggested that calcineurin has a role in meditating that signaling network. They followed this study up by screening the additional SFKs (SFK2−4) and linked the SFKs to a specific target pathway, namely, Ald6p, aldehyde dehydrogenase.[188] In addition, they showed that this method is suitable for the dissection of networks by small molecules through their implication of other gene products in the Ald6p $NADP^+$-dependent pathway (Figure 26).

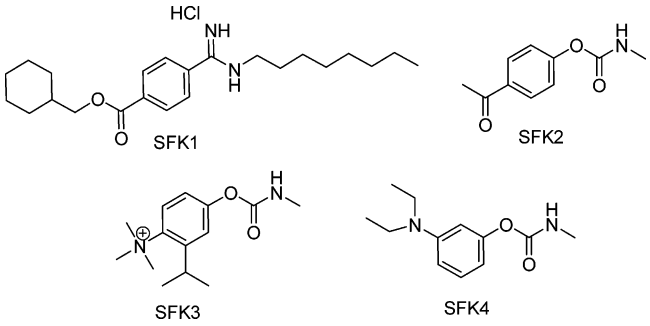


Figure 26. Structure of SFK compounds.

Boone et al. used a yeast genome deletion set in identifying targets and pathways of well-known inhibitors in a study that incorporated genetic and chemical genetic data.[222] Their proof of principle study incorporated the following steps: (1) they screened the deletion set for hypersensitivity toward 12 diverse inhibitory compounds and generated chemical−genetic interaction profiles, (2) they identified genes occurring in multiple assays and filtered out those responsible for multidrug resistance, (3) they generated genetic interaction profiles of genes that encoded the compound's targets through genetic array analysis, and (4) they clustered the chemical genetic profiles with the genetic interaction data and grouped several compounds with their known pathways or proteins.[223] In addition to demonstrating that these profiles may identify a compound's mechanism of action, they also identified a group of genes involved in multidrug resistance that may be conserved in humans.[222]

Huang et al. undertook a thorough and extremely promising study that revealed novel functions of the phosphatidylinositol (PI) metabolic pathway by screening the entire yeast deletion genome for sensitivity in the presence of wortmannin, a known small molecule inhibitor of phophatidyl inositol 3-kinase (PI3K) (Figure 27).[224,225] The screening

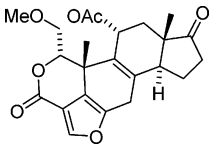


Figure 27. Structure of wortmannin.

revealed genetic interactions in the pathway and the global consequences of perturbing the pathway. From their screen, they identified 591 genes that bestow wortmannin resistance upon deletion and 476 that bestow hypersensitivity, providing a global view of the wortmannin-responsive networks within the cell. This revealed novel connections between the PI pathway and other biological processes that include the DNA replication and damage checkpoint, proteasome function, and chromatin remodeling.[224] Their results showed the ability to understand relative distances between processes affected by wortmannin and protein−protein

interactions in the proteomic subnetwork. This work shows the power of whole genome profiling to annotate the biological effects of a small molecule.[224]

Haploinsufficiency involves the lowering of a gene dosage from two copies to one in diploid cells. The result, a heterozygote, shows greater drug sensitivity if the inhibitor targets the half-deleted gene's product, rather than the wild-type. As a proof of principle, Davis et al. developed this technique and screened 233 molecularly "tagged" or "bar coded", by a 20 member oilgonucleotide, heterozygous strains that were pooled and grown in the presence of a drug, tunicamycin, a well-characterized glycosylation inhibitor.[226] They identified the known target and two hypersensitive loci by screening the oligonucleotide tag on DNA microarrays (Figure 28). Davis et al. continued and expanded their work

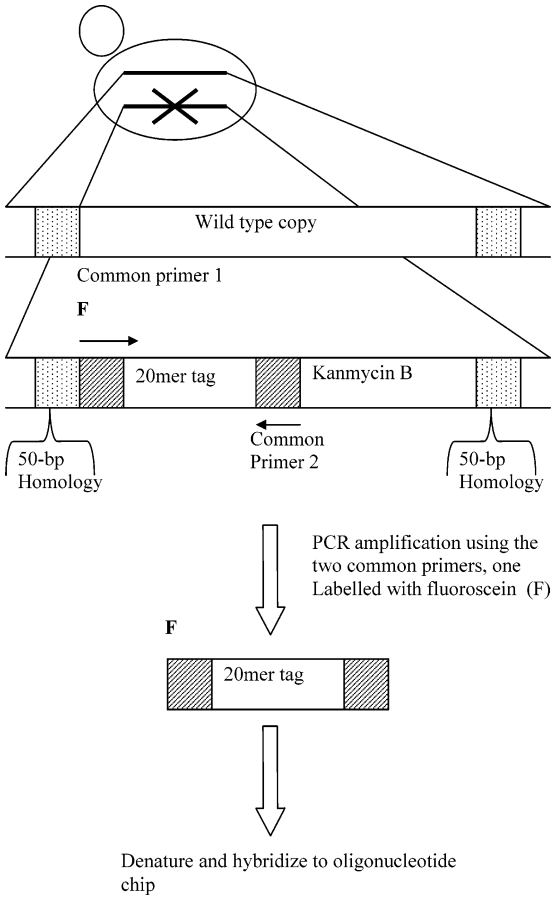


Figure 28. Heterozygote deletion construct with tag.

to include genome-wide heterozygous yeast deletion strains.[227] Again, the group followed a similar protocol of bar coding, screening the pooled strains, and following up with an oligonucleotide microarray analysis. Data analysis afforded gene-by-gene ranking of sensitivity and generated a genome-wide profile of functional interactions.[227] Eighty genome-wide experiments were performed in addition to genetic follow-up experiments to validate their results. From their screens and analysis, they were able to identify gene products that directly interact with the compounds and are dosage-limiting for growth, as well as those involved in bioavailability of small molecules to cells. Importantly, all of their studies showed previously unknown interactions that may lead to novel drug targets. This screening method may prove valuable for drug discovery by filtering and prioritizing compounds for development.[227]

Shoemaker et al. analyzed 78 mostly medically relevant compounds in a genome-wide haploinsufficiency screen that incorporated half of the yeast genome heterozygotes to determine their mode of action by DNA microarray analysis.[228] The group used "fitness profiling" to analyze the modes of action of the compounds. Advantages of this method are that it does not require any prior knowledge of a compound's mode of action and that affected biological processes are identified along with the protein target(s). In addition to validating the targets of known compounds and revealing potentially new modes of action for many of the compounds tested, they also suggested that the cholesterol-lowering drug molsidomine may function by targeting lanosterol synthase in the sterol biosynthetic pathway. In addition, a target of 5-fluorouracil was identified as the rRNA processing exosome.[228]

Roberge et al. used a yeast genome-wide drug-induced haploinsufficiency screen to identify the pathway of a compound in preclinical trials, dihydromotuporamine C (dhMotC), an inhibitor of angiogenesis and metastasis (Figure 29).[229] The target of this compound was unknown and was

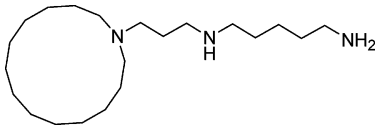


Figure 29. Structure of dihydromotuporamine C.

difficult to determine since it had no other known biological activity. Also, its structure provided no clues and did not resemble other bioactive compounds. By their method, the authors revealed that it targets sphingolipid metabolism and leads to decreased ceramide levels in yeast. Their original HTS identified 21 heterozygous deletion strains sensitive to dhMotC, and sensitivity ranking identified *lcb1*Δ/LCB1 and *tsc10*Δ/TSC10 as the most sensitive. These deletion strains were supersensitive, and the encoded gene products were members of the sphingolipid biosynthesis pathway. [229]

4.2. Plants

Plants offer an attractive platform for phenotypic screening in chemical genetics: (1) all known plant growth regulators are small molecules; the experimental protocols for analyzing plant growth regulators are well defined and can be easily adapted to unbiased chemical genetic screens; (2) the genomes of the most common systems are already sequenced and that significantly aids in target identification; (3) plant roots readily take-up small molecules, avoiding many of the permeability and transport issues in traditional chemical genetic systems.[5] In addition, due to the genetic redundancy in *Arabidopdis*, the ability to knock out all of a gene's copies and its homologues by traditional genetic means is extremely difficult, but this can be done using small molecules in a chemical genetics approach.[5]

Auxins and their signaling events are the focus of a great number of chemical genetic studies in plants. As a plant hormone, auxin regulates plant development on multiple levels, that is, cellular division, differentiation and elongation, embryogenesis, seedling growth up to flowering and senescence, etc., and its subsequent gene directing signaling events are still not clearly understood.[230−232] Theologis et al. developed a high-throughput screen in *Arabidopsis* seedlings to identify inhibitors of auxin signaling.[230] In addition to phenotypic screening that focused on root elongation, their

screening involved a 10000 member commercial library and used the BA3 line, where exogenous auxin triggers the expression of GUS (β-glucuronidase), as the reporter system.[233] Affymetrix microarray screening was also used in transcription profiling. Their screening was rapid, and active compound identification was aided by the tissue-specific expression of the GUS gene in the root elongation zone. From their library, several diverse compounds were identified, three of which interfered with the auxin-regulated proteolysis of an auxin/indole-3-acetic acid transcription factor. Two of these compounds showed phenotypes indicative of an altered auxin response that included impaired root development, and microarray transcriptional analysis showed the mechanistic similarities of the two most potent compounds. Their approach offers a rapid and robust method for the study of auxin signaling, with the targets remaining to be identified.

Hayashi et al. provided an example of forward chemical screening using natural products. Using transgenic *Arabidopsis* with a GUS auxin-inducible reporter, they identified the natural products yokonolide A and B (A82548A), isolated from *Streptomyces diastatochromogenes*, which were shown to inhibit auxin signal transduction.[232−234] Yokonolide B was shown to inhibit the degradation of AUX/IAA factors.[235]

In a straightforward example of plant phenotypic screening, Schreiber et al. screened members of the biaryl-containing DOS library in *Arabidopsis*. Through visual microscopic screening, they identified a potent compound, (*P*)-4k, that was shown to stunt development and lead to pigment loss followed by death (Figure 30).[112]

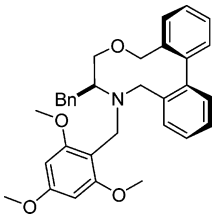


Figure 30. Structure of (*P*)-4k.

Zhao et al. investigated auxin signaling components using a chemical genetics approach and the compound sirtinol, a known inhibitor of the sirtuin family in yeast, which is known to affect root and vascular tissue development in *Arabidopsis* (Figure 31).[189,190] The group screened for compounds using

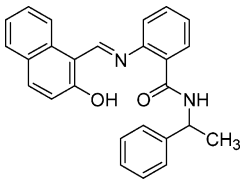


Figure 31. Structure of sirtinol.

a GUS reporter system.[233] Sirtinol was shown to upregulate ectopic expression and the auxin reporter gene. This was followed up by whole genome microarray analysis, which showed that sirtinol activated the expression of 16 000 of 23 000 auxin-inducible genes. In addition to a number of other interesting phenotypic changes, phenotypic screening with sirtinol in light- and dark-grown seedlings showed phenotypes typical of plants over-producing auxin suggesting that sirtinol activates auxin-inducible genes and increases auxin signal output.[190] Genetic screening identified *sir1* (sirtinol resistant 1) that gave auxin-related phenotypes and suggested that *sir1* regulates auxin-inducible genes. From follow-up experiments, it was suggested that *sir1* is a key regulator of many auxin-inducible genes and an upstream component in auxin signaling. This work shows the power of chemical genetics in plants in combination with genetic tools for the identification and dissection of key components of the auxin-signaling mechanisms. Additionally, Zhao et al. used genetic screens to identify mutants insensitive to sirtinol and identified *Atcand1*, a HEAT-Repeat protein that participates in auxin signaling. This is illustrative of how chemical probes identified from chemical screens are of continued interest as chemical tools well past their initial reports.[231]

The first brassinosteroids (BR) were isolated in 1979 and brassinosteroid chemistry became established in the late 1980s.[236,237] This group of approximately 60 compounds have been shown to be involved developmentally in pollen tube growth, stem elongation, the inhibition of root growth, promotion of xylem differentiation, the retardation of root abscission, and cell elongation, as well as roles in protecting plants from environmental stresses such as chilling, drought, and heat stress.[236,237] For all of these above reasons, BRs are now considered a class of plant hormones,[236] and tools to dissect their role, especially inhibitors of their biosynthesis, are particularly valuable (Figure 32).[6]

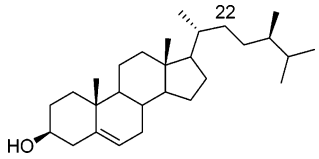


Figure 32. Campersterol, a representative brassinosteroid.

Yoshida et al. have done extensive work on BR biosynthesis inhibitors. They reported the first BR biosynthesis inhibitors that were unrelated to Gibberellic acid (GA, plant growth regulator) biosynthesis.[238] The design of these compounds were based on the molecules uniconazole and paclobutrazol, known GA biosynthesis inhibitors that also inhibit cytochrome P-450.[237] GA biosynthesis inhibitors were eliminated by rice stem elongation assays where the inhibitors are known to retard rice stem elongation and are rescued by GA treatment.[238] Their hits were then transferred to phenotypic screening in *Arabidopsis* and cress (*Lepidium sativum*) where brassinolide, a potent brassinosteroid, can reverse the effect of the BR inhibitors. BR biosynthesis inhibitors induced *Arabidopsis* dwarfs that phenocopied BR biosynthesis mutants. From cress screening, a triazole-type potent inhibitor, brassinazole, was identified, of which the target was shown to be brassinosteroid biosynthesis (Figure 33).[237,239] Therefore, brassinazole can serve as a complement to BR-deficient mutants in studies to determine the function of BRs.[239]

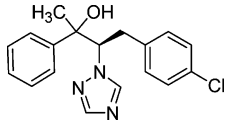


Figure 33. Structure of brassinazole.

Yoshida has continued to explore BR biosynthesis inhibitors and expanded the characterization of brassinazole. In follow up experiments with brassinazole, it was shown to inhibit the hydroxylation of the side chain C-22 position of BRs by binding to DWF4, an *Arabidopsis thaliana* cyto-

chrome P450 that catalyzes the hydroxylation.[191] In addition, brassinazole was used as an inhibitor of BR biosynthesis in light- and dark-grown *Chlorella vulgaris* where it was shown that the mevalonate pathway does not function in *C. vulgaris* and that isopentenyl biosynthesis is responsible for the synthesis of a precursor in BR synthesis.[236] As an additional example of brassinazole's use as a biological tool, Yoshida has shown that BRs function in xylem development in vivo since brassinazole inhibited secondary xylem development in cress plants.[240] Murofushi et al., in collaboration with Yoshida, developed inhibitors of BR biosynthesis based on 5-substituted pyrimidine derivatives where their activities were screened by a cress stem elongation assay.[241] Additional BR biosynthesis inhibitors, such as Brz220, have been found through *Arabidopsis* screening by Asami et al. that have shown potency and specificity greater than brassinazole (Figure 34).[242,243] In addition, triadimefon, a triazole-based fungicide, was also shown to induce brassinosteroid-deficiency-like phenotypes in *Arabidopsis*, an important implication for BR-biosynthesis inhibitors in their application to crops (Figure 35).[244]

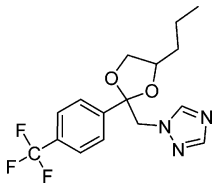


Figure 34. Structure of Brz220.

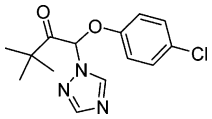


Figure 35. Structure of triadimefon.

4.3. Zebrafish

Zebrafish (*Danio rerio*) have become a promising whole organism screening method in chemical genetics for many of the same reasons that they are popularly used in developmental biology and genetics. Whole organism screening is preferred in some cases over target-based screening since it allows for a more unbiased discovery in a relevant physiological context. However, though mammals provide an excellent relevant screening context, their use is expensive, requires a great deal of space, large quantities of compounds, is strictly regulated, is laborious, and often raises ethical questions. Because of these limitations in mammals, systems such as zebrafish have been popularized due to the many advantages they present. First, zebrafish, unlike other systems such as yeast or round worms, are vertebrates with discrete organs such as the brain, sensory organs, heart, muscles, bones, etc. These organ systems are very close to their human counterparts, in terms of their high level of structure, and this aids in their suitability for chemical genetics and drug discovery. Additionally, zebrafish are small enough in their early embryonic stages to live in a well of a microtiter plate. They are also prolific reproducers, which allows for the screening of large libraries. Last, zebrafish embryos are a desirable model due to their complete transparency, which allows for the multiple observations of dynamic processes in every organ and structure without the need for dissection or sacrificing the animal.[245]

Schreiber et al. pioneered the use of phenotypic zebrafish screening and chemical genetics. Though no target identification was performed, in their important early study, they screened a 1100 random member small molecule library and identified molecules that modulated aspects of vertebrate ontogeny including the development of the central nerve system, the cardiovascular system, the neural crest, and the ear.[246] In another study, Schreiber et al. screened their biaryl-containing DOS in zebrafish embryos and identified a compound that showed interference in zebrafish development related to pigmentation, weak hearts, abnormal brains, and misshapen jaws.[112] Schreiber et al. have gone on to employ zebrafish in more sophisticated studies. Based on their previous success in identifying active compounds from the 1,3-dioxane library, they decided they would dramatically expand and diversify their previous library and began an interesting study that took advantage of a number of screening formats. Whereas their previous 1,3-dioxane library contained 1890 compounds derived from two racemic scaffolds, this library took advantage of their modular synthesis by incorporating a drastically increased number of building blocks and produced 18 000 enantio-enriched compounds where diversity was displayed on 12 stereochemically distinct classes of compounds.[111,113,247] Phenotypic screening in zebrafish identified one molecule, but not its enantiomers, that induced cardiovascular malfunction. The compound was also found to induce reversible 2:1 atrioventural block at 6 μM (Figure 36). To explain these

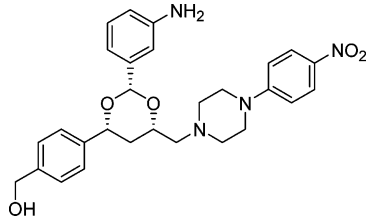


Figure 36. Cardiac malfunction inducing compound identified from zebrafish phenotypic screening.[113]

observations, they hypothesized that the target may be a cardiomyocyte-specific ion channel. Additionally, a commercial software program, QSARI (SciVision), was used to analyze the molecular descriptors, and evaluation of the diversity of the chemical space showed that the 1,3-dioxane library favorably compared to a library of 2000 known bioactive compounds, thus indicating its promise for future discoveries.

Roberts et al. have studied angiogenic signaling by using a chemical approach in zebrafish.[192] This study was an excellent example of the use of chemical compounds as a means of inducing loss-of-function in vertebrates as opposed to antisense technologies. Although the targets of the probe, PTK787/ZK222584 (a vascular endothelial growth factor receptor inhibitor), were known, this proof of concept experiment showed that this method would be a viable route for determining a novel compound's mechanisms (Figure 37).

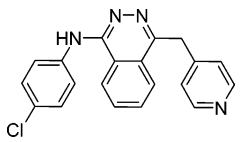


Figure 37. PTK787/ZK222584.

Treatment with PTK787/ZK222584 blocked formation of all blood vessels, a phenotype that closely matched that produced by antisense treatment. Using this method, they were

able to study angiogenic signaling and showed that overexpression of AKT/PKB, a putative effector of endothelial growth factor signaling, allowed blood vessel formation in the presence of the drug. Increased AKT/PKB activity prevented drug-induced endothelial cell apoptosis. This study demonstrated the important role of AKT/PKB in angiogenesis.

Fishman et al. have used zebrafish phenotypic screening in a couple of interesting studies. In an important early report, both chemical genetic and conventional genetic approaches were employed, and a powerful small molecule, concentramide (Figure 38), was found to phenocopy a genetic mutation

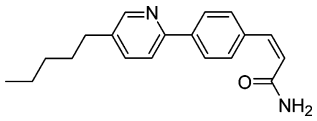


Figure 38. Concentramide.

called heart-and-soul (*has*).[248] The phenotype expressed itself by ventricle formation within the aorta. Though the phenotypic expressions were similar, *has* and concentramide were mechanistically different, with *has* disrupting epithelial cell−cell interactions and concentramide shifting the converging heart field rostrally.

Additionally, they used the suppression of a genetic mutation that served as a model of aortic coarctation.[249] The zebrafish mutation gridlock was used that has a mutated *hey2* gene in which the zebrafish have a malformed aorta preventing circulation to the trunk and tail. This resembles congenital dysplasias of the aorta in humans. The screen sought molecules that suppressed this mutation, restored circulation, and allowed zebrafish survival to adulthood. From a screen of 5000 diverse compounds, two compounds were identified that suppressed the mutation, and it was found that these compounds accomplished this through the upregulation of the expression of VEGF (Figure 39). This study

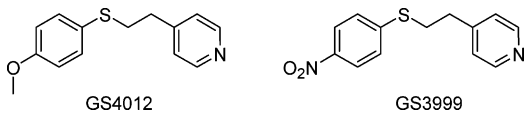


Figure 39. Two small molecules identified as gridlock suppressors.

highlights the possibility of small molecule unbiased screening overcoming genetic mutations without targeting the gene responsible and has important implications in gene therapy.

Chang et al. have also used zebrafish as a primary screen to identify a potent microtubule destabilizing entity.[193] In an effort to increase the activity of the moderately active tubulin inhibitor myoseverin, the group designed a novel triazine library utilizing computer-aided modeling and SAR analysis from myoseverin derivatives. Over 100 compounds sharing a structural motif with myoseverin were synthesized in a solid-phase orthogonal route that rapidly generated highly pure triazine compounds. Primary screening in zebrafish embryos identified compounds, the tubulyzines, that induced phenotypic changes similar to the known microtubule destabilizers myoseverin and nocodazole. These included delays in epiboly, and larger blastoderm cells in 1K cell stage embryos and somite stage embryos showed a characteristic developmental arrest. To confirm the activity, they were screened in an in vitro polymerization assay using purified bovine brain tubulin and growth inhibition was tested in U937 human leukemia cells. They identified a compound that showed a 4−10-fold improvement over myoseverin in tubulin assays and U937 cell growth inhibition (Figure 40).

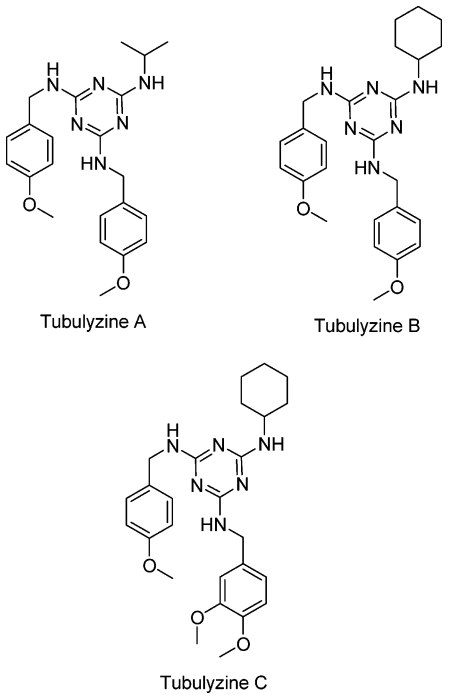


Figure 40. Tubulyzine A, B, and C.

4.4. Drosophila

For over 100 years, *Drosophila* has been used by geneticists in a great number of studies.[250,251] Their short life cycle and low cost make them a desirable animal model. The entire *Drosophila* geneome sequence is available, and many tools for target identification and validation already exist such as genome-wide genetic mutations. Though they bear fewer genes than humans, cases exist of one *Drosophila* gene representing several human genes, and this kind of feature makes it a popular model system for studying human disease pathways.[22] Additionally, *Drosophila* is an excellent compliment to *Caenorhabditis elegans* RNAi studies in which some knockouts are unavailable, such as genes expressed in the nervous system.[252] Though *Drosophila* has not been widely used in chemical genetics, its unique advantages will attract more attention from the field in the future.

Field et al. used phenotypic cell-based screening in combination with genome-wide RNAi screens to identify cytokinesis inhibitors and their targets.[253] This parallel approach allowed for the identification of inhibitors of cytokinesis, the genes involved, and information on the small molecule's targets by cross-comparing phenotypes. Normal mitotic cells that fail cytokinesis, the last step in mitosis, exhibit a two nuclei phenotype. Screening for this phenotype of a 51 000 member library comprised of commercial "drug-like" molecules, natural products, natural-product-like compounds, and 19 470 dsRNAs was performed in *Drosophila* cells and revealed 52 active small molecules, 25 of which were selected for further screening. These compounds were termed the binucleins. In addition, 214 dsRNAs, and therefore 214 proteins (25 of which were previously unpredicted), were identified from the phenotypic screening as being involved in cytokinesis and revealed the previously unknown essential role in cytokinesis of a gene, *CG4454*.

One gene, *borr,* had a profound effect on cytokinesis that was shown to be involved in the Aurora kinase pathway. Phenotypic classification revealed a small molecule inhibitor of the Aurora B pathway. This was a dramatic illustration of how this parallel approach may be useful for future studies in chemical genetics.[253]

4.5. *Caenorhabditis elegans*

For years, *Caenorhabditis elegans* has been a powerful research tool in genetics.[22] *C. elegans* has seen little use in chemical genetics thus far, but it is of growing use in drug discovery and is poised to make a large contribution. It is merely a matter of time until it becomes a wide-spread tool in chemical genetics. *C. elegans* was the first multicellular organism to have its genome completely sequenced. The knowledge and the experience that comes with it will prove invaluable in future *C. elegans* applications in chemical genetics. The worm itself is small and transparent, which allows for full visualization of its developmental processes, and contains complex structures such as a digestive tract, nervous system, and muscles. Additionally, it has a short life cycle and produces many progeny, which makes it more compatible with high-throughput screening. Advantageously, RNAi screens were first used and developed in *C. elegans*, and the large amount of experience in this field will greatly aid multipronged chemical genetic approaches, particularly target identification.[252]

Indeed, though the use and understanding of *C. elegans* has also dramatically increased, it has not been used to its full potential in phenotypic chemical genetic screens, as alluded to by Han and Hara's use of the vulval development phenotype by farnesyltransferase inhibitors as an in vivo screen for inhibitors of Ras-activated tumors (Figure 41).[254]

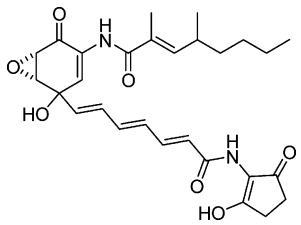


Figure 41. Ras farnesyltransferase inhibitors found in *C. elegans* screening.

An early report by Chalfie and co-workers relevant to target validation and identification and mechanism of action studies used benzimidazole anti-microtubule drugs to show that the *ben-1* gene product, β-tubulin, conveys sensitivity in *C. elegans.*[255] This was identified by screening a panel of *C. elegans* mutants with benzimidazole, and they showed that all resistant mutations mapped to a single locus, *ben-1*. The *ben-1* deletion mutants were fully resistant, and those animals remained viable and coordinated, indicating that β-tubulin is nonessential for growth and movement and that the gene's function is most likely redundant.

In a pathway dissection study, Ruvkun and investigators screened *C. elegans* mutants with a number of neurotransmitter agonists and antagonists to identify compounds that could induce or provide recovery from dauer arrest. Their results suggested that a metabotropic acetylcholine signaling pathway activates an insulin-like signal during *C. elegans* dauer arrest.[256] In an application relevant to mechanism of action studies, *C. elegans* has been used to uncover a novel gene family encoding over a dozen multipass transmembrane proteins by screening fluoxetine (Prozac).[257] The effects were independent of inhibition of serotonin and show the potential power of using probes and drugs in *C. elgans* to unveil useful biological information beyond their therapeutic targets.

C. elegans has also been used in a couple of applications relevant to toxicology. Uemura et al. developed a novel and simple method in which *C. elegans* was used as a model to determine the reproductive toxicity of chemicals including environmental pollutants.[258] van Meel and co-workers have also demonstrated the use of wild-type *C. elegans* and a transgenic strain as a rapid model for the assessment of the toxicity of pharmaceutical compounds by screening several EGFR kinases and an inactive analogue.[259]

Last, in a preliminary chemical genetics report, Geary and co-workers used *C. elegans* screening to identify presenilin inhibitors.[260] The proteins presenilin 1 and 2 and the genes that encode them are targets of Alzheimer disease (AD) therapeutics. In humans, loss of function of presenilin-1 reduces amyloid-β peptide processing from the amyloid protein precursor. However, in *C. elegans*, loss of function of presenilin-1 causes a defect in egg laying. Therefore, compounds were sought that phenocopied the egg laying defect by targeting presenilin. To make this screen of moderate throughput, the group devised an assay that measured chitinase release into the culture medium since chitinase is released by hatching eggs. Though they identified compounds from their library that phenocopied the egg laying defect, none of the compounds have made it through later rounds of assays to confirm target specificity yet. Though they failed to find specific inhibitors, they claim due to a small library size, the method developed allows for the mechanism-based discovery of AD drugs and offers a potentially useful screening method in chemical genetics.[260]

4.6. Mammalian Cells

4.6.1. Phenotypic Screening

Phenotypic screening that identifies hit compounds through morphological changes or growth inhibition in mammalian cells provides a robust platform for screening libraries in chemical genetic studies. Schweitzer et al. used this in developing a disease-specific cell-based screen to identify lead compounds for Huntington's disease.[261] They developed a model screening for Huntington's disease using cultured neural PC12 cells that are similar to neurons and exhibit characteristics of mature neurons, such as their ability to undergo growth factor withdrawal-induced apoptotic cell death. These cells are a standard model for neuronal cell biology. Their medium-throughput screening used PC12 cells incorporating a plasmid that inducibly expresses an expanded polyglutamine form of exon 1 of *Htt* with a green fluorescent protein (GFP) marker. After induction of the *Htt* transgene, the cells died within 48 h. Using the NIH Custom collection, their screening revealed 18 compounds that rescued the cells completely. These compounds represented caspase inhibitors and cannabinoids making them lead compounds in Huntington's disease treatment, in addition to their model screening system providing a robust method for researchers at large.

Yuen et al. used an interesting phenotypic assay to identify compounds that protected Vero (African green monkey kidney cell line) cells from severe acute respiratory syndrome coronavirus (SARS-CoV) associated cytopathic effects (CPE).[194] SARS-CoV was identified as the causative agent

of endemic atypical pneumonia, the infamous SARS virus, and angiotensin-converting enzyme 2 (ACE2) was identified as the functional receptor. They sought compounds that could interfere with any of the many steps involved in SARS-CoV infection by screening 50 240 compounds from a commercial library. As a model system, they used Vero cells, in which SARS-CoV effectively replicates and CPE serves as an effective phenotypic marker. Their screening identified 104 hits that prevent SARS-CoV-induced CPE. These compounds were then screened for their EC_{50}'s in a viral plaque reduction assay. To understand how these compounds may be acting, they screened the 104 compounds to determine how they affect three major pathways for viral replication, namely, viral entry, transcription, and proteolytic processing. SARS-CoV main protease (SARS-CoV M^{pro}), which plays a role in proteolytic processing, is a major target of anti-SARS-CoV drug development. Screening for SARS-CoV M^{pro} inhibitors identified a compound, MP576, with an IC_{50} of 2.5 μM. This compound also performed favorably in a docking study and was shown not to be a general protease inhibitor. Screening their hits against polynucleotide-stimulated ATPase activity of SARS-CoV helicase (SARS-CoV Hel) found a compound, HE602, that strongly inhibited its activity (IC_{50} = 6.9 μM). It was also shown to perform well in a helicase assay that indicated this compound operates similarly to inhibitors of herpes simplex virus (HSV) helicase−primase protein. Last, libraries were screened for compounds that block spike protein−ACE2-mediated cellular entry of the SARS-CoV. The compound VE607 was shown to specifically block entry with an EC_{50} of 3 μM. Their interesting results highlight chemical genetics' role in emerging disease research as well as providing useful drug targets for SARS-CoV infection (Figure 42).

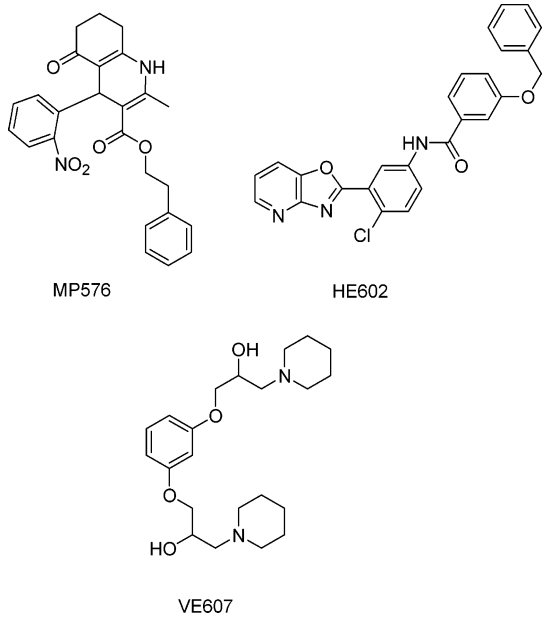


Figure 42. Structure of MP576, HE602, and VE607.

Rosania et al. presented an in-depth comprehensive report on forward chemical genetics using a cell-based phenotypic study in the discovery of myoseverin (Figure 43).[195,196] A library of 2,6,9-purines was screened in 96 well plates against murine C2C12 muscle cells induced to form myotubes. Myotube morphology was visualized using a phase contrast microscope, and myoseverin induced reversible myotube disassembly with an EC_{50} = 11 μM. In the presence

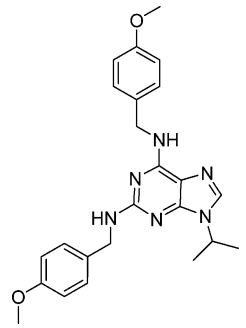


Figure 43. Structure of myoseverin.

of myoseverin, the long cylindrical myotubes were replaced with smaller chains of rounded cells producing mononucleated fragments. A biotin-modified myoseverin designed for affinity experiments revealed tubulin as the target after immunoblotting. It was shown that myoseverin did not affect actin and behaved differently than other compounds acting on the cytoskeleton, that is, colchicines and nocadazole. mRNA transcription profiling revealed that 93 genes were significantly affected by myoseverin and, interestingly, that many of these were involved in extracellular matrix remodeling and growth factors. Affected genes also included growth factor inducible transcripts as well as a number of cytoskeletal genes. These transcription profiles were consistent with the activation of pathways involved in wound healing and tissue regeneration.

Orlow et al. used a tagged triazine library in a phenotypic screening to identify compounds that correct for albinism in albino type 2 murine melanocytes.[197] Tyrosinase mistrafficking and increased retention in the endoplasmic reticulum is considered a main cause for albinism in oculocutaneous albinism type 2. From the absorbance-based melanin assay, it was shown that six compounds induced enhanced pigmentation in the form of an absolute increase in the melanin amount that was not a result of selective toxicity for melanin-deficient cells (Figure 44). Affinity

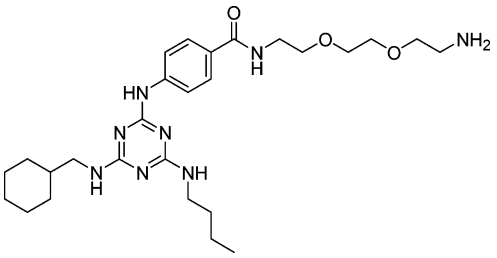


Figure 44. MPC11, a representative pigment increasing compound in type 2 albino cells.

experiments, streamlined from the use of tagged libraries, identified the mitochondrial F1F0-ATP synthase as the target protein. Competition experiments with free molecules against affinity matrices and antibody blotting against the α and β subunits of ATPase confirmed the compound's specific binding to the target. Immunohistochemical analysis showed that the triazine compounds induced pigmentation by correcting tyrosinase mistrafficking. Two known inhibitors, oligomycin and aurovertin B, of the ATPase were also shown to induce pigmentation and competed with the triazine compounds. Importantly, this report identified mitochondrial ATP synthase as a potential therapeutic target for albinism in melanocytes.[197]

Orlow et al. used a similar approach to find a pigmentation inducer in normal melanocyte.[198] After a careful SAR study,

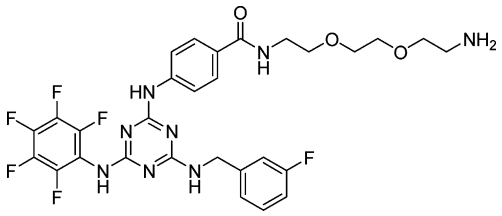


Figure 45. Structure of melanogenin.

a strong pigmentation inducer, melanogenin (Figure 45), and its target protein prohibitin were identified by an affinity matrix of melanogenin. It was shown that a treatment of melanogenin increased the expression level of tyrosinase and thus increased the enzymatic activity. Interestingly, neither RNAi of prohibitin alone nor cotreatment with melanogenin showed any significant tyrosinase upregulation. The upregulation of tyrosinase is only observed with melanogenin treatment in the presence of prohibitin, which suggests that prohibitin induces tyrosinase overexpression, but only when bound to melanogenin. This is a unique case where conventional genetics cannot identify the function of the target protein, but chemical genetics can.

Synthetic lethal screening has also found use in human cell lines. At about the same time, two groups reported chemical synthetic lethal screening in isogenic human cell lines where the lines differ by only one deletion.[262,263] In a methods development report, Canaani et al. used GFP mutants as a fluorescent reporter in a screen of lethality with inosine monophosphate dehydrogenase inhibitors.[262] In a more high-throughput application, Kinzler et al. used a similar fluorescence approach to screen 30 000 compounds and identified a novel cytidine nucleoside analogue that showed selective in vivo toxicity in *K-Ras* allene containing cells (Figure 46). This work demonstrated a broadly applicable approach for mining activator compounds targeted to the specific genetic alterations.[263]

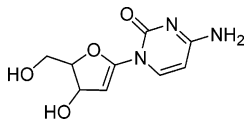


Figure 46. Cytidine nucleoside analogue sensitive for mutant *K-Ras* genotype.

Dolma et al. have performed a broad, systematic, and specific chemical synthetic lethality study that exceeded the scope of previous efforts.[264] Their screening involved an isogenic engineered tumorigenic cell line focused on six defined genetic elements and screened over 23 500 compounds. They identified a number of compounds with genotype selective activity in the presence of certain genetic elements. A novel compound called erastin was identified that was specifically active only in the presence of the genetic elements representing small T oncoproteins and the oncogenic allele of *HRAS* (*RAS^{v12}*) (Figure 47).

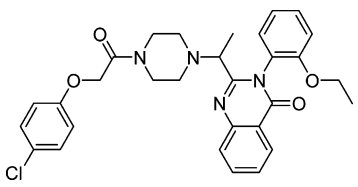


Figure 47. Structure of erastin.

Schultz et al. used a phenotypic screen that measured alkaline phosphatase (ALP) levels as a marker for compounds that induce osteogenesis in the search for selective modulators of stem cell differentiation using mouse embryonic mesoderm fibroblasts, C3H10T1/2 cells.[265] This type of control would have significant therapeutic value for several bone-related diseases. ALP's substrate, 2′-[2′-benzothiazoyl]-6′-hydroxybenzothiazole phosphate (BBTP), hydrolyzes to a highly fluorescent anionic product, which readily afforded automated screening. From a combinatorial heterocyclic library composed of various scaffolds containing 50 000 compounds obtained from a "convergent" synthesis and assayed with a primary ALP fluorescent screen, they identified a potent compound, a 2,6,9-trisubstituted purine termed purmorphamine (Figure 48). This compound had an

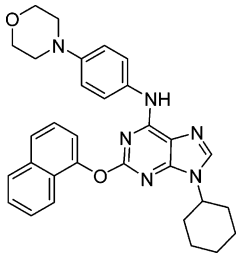


Figure 48. Structure of purmorphamine.

EC_{50} of 1 μM and morphologically changed the cells from fibroblasts to osteocytes. They followed up this screening with a bone-specific transcription factor reporter assay to confirm the induction of osteogenesis in addition to the histological staining of endogenous ALP. In studies with bone morphogenic protein 4 (BMP-4), which induces osteoblast differentiation cells, they reported that purmorphamine and BMP-4 synergistically induced trans differentiation of preadipocytes and myoblasts.[265]

4.6.2. Cytoblot

Cellular cytoblot assays were developed by Schreiber et al. and offer a robust screening platform.[266,267] Cytoblots are whole-cell immunodetection assays that resemble ELISAs and Western blotting.[266] Cytoblot detection uses a primary antibody and a secondary antibody covalently linked to horseradish peroxidase (HRP) to detect a phosphorylated protein or other molecule of interest in fixed whole cells. Following this, a chemiluminescent reaction is initiated by the addition of luminol, H_2O_2, and *p*-iodophenol. Antigen levels are visualized on film, and since these assays take place in microtiter plates, this system is high-throughput with the added benefit of being performed in human cells. In their initial report, Stockwell et al. showed the broad versatility of this system.[266] A number of biosynthetic processes were monitored by the cytoblot technology including DNA synthesis by measuring the incorporation of 5-bromodeoxyuridine in the presence or absence of transforming growth factor β, acetylation of histone H4 in response to trapoxin and trichostatin, and mitosis-associated phosphorylation of histone H3 and nucleolin in the presence of nocodazole and other anti-mitotic agents. Last, they showed the use of these assays in compound screening by identifying a marine sponge extract capable of inhibiting genotype-specific inhibition of 5-bromodeoxyuridine incorporation and by demonstrating that the small molecule suppressed the anti-proliferative effect of rapamycin among others.

Schreiber et al. used a cytoblot assay with TG-3, an antibody that recognizes phosphorylated nucleolin as a

marker of mitosis, as a primary screen to identify molecules that perturb mitosis.[199] Screening of a commercial library of 16 320 compounds revealed 139 compounds that were identified and classified as colchicine-like compounds, taxol-like compounds, or compounds with mitotic machinery targets other than tubulin. One compound, syntab A, showed a promising taxol-like effect against tubulin by stabilizing microtubules (Figure 49).[199]

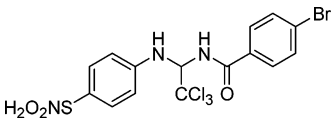


Figure 49. Structure of syntab A.

Schreiber et al. have also used this cytoblot assay to find small molecules that modulate the human chromatid decatenation checkpoint.[268] They screened 9600 compounds in a number of checkpoint assays that identified suppressors of G_2-phase arrest caused by an inhibitor of topoisomerase's enzymatic activity. It was found that these molecules, termed the suptopins, had distinct effects on cell cycle progression, microtubule stability, and the nucleocytoplasmic transport of cyclin B1. In addition, it was shown that chromatid deacetylation functions independently of the chromatin deacetylation checkpoint (Figure 50).[268]

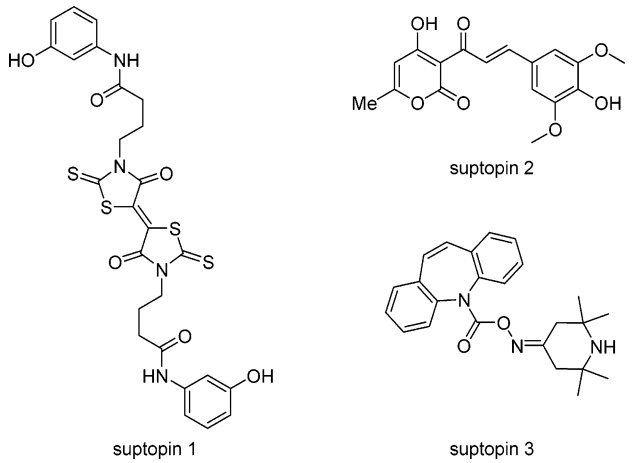


Figure 50. Structures of suptopins.

Schreiber and co-workers have continued to expand the use of cytoblot assays.[200] They sought to find domain-selective small molecule inhibitors of HDAC6-mediated tubulin deacetylation. HDACs are zinc-dependent hydrolases that mediate chromatin remodeling and gene expression and possess HDAC activity that is inhibited by the small molecule TSA (Figure 23).[200,268] Determining a role for protein acetylation other than histones is a major goal toward understanding the targets and role of HDACs. TSA increases the acetylation of α-tubulin, but it lacks selectivity for nuclear HDACs. This complicates the interpretation of studies using this molecule. Therefore, they sought a selective inhibitor of tubulin deacetylase activity to uncouple α-tubulin acetylation from the nuclear consequences of HDAC inhibitors.[200] They screened a 7392 member deacetylase biased 1,3-dioxane library in acetylated α-tubulin and acetylated lysine cytoblot assays in A549 cells. From the screen, they identified an α-tubulin-selective inhibitor, tubacin, and demonstrated that its target was class II HDAC6. Inhibition of HDAC6 had no effect on cell cycle progression but did affect cell motility. In addition, the fact that α-tubulin acetylation is involved in the localization of microtubule-associated proteins suggests that these molecules may have a therapeutic application in antimetastatic and antiangiogenic agents.[268] Tubacin and a related molecule were later structurally dissected to identify the key elements of selective HDAC inhibitors (Figure 51).[269]

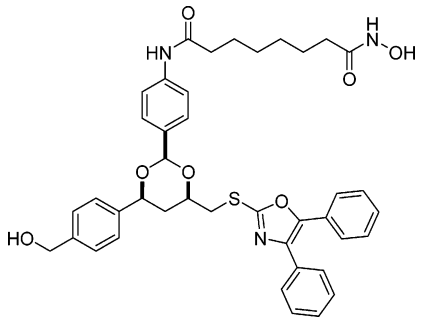


Figure 51. Structure of tubacin.

To further probe cellular activities of TSA, Schreiber and co-workers identified additional small molecule TSA suppressors (ITSA).[270] Small molecules that overcame the cell cycle blockade of cells arrested at the G1 and G2 phases by TSA were considered hits. From 9600 small molecules, they identified 23 ITSAs, benotriazole-based compounds. Their primary screening used a TG-3 cytoblot assay where the TG-3 antibody measured increases in phosphonuclein levels, indicative of a protein modification state of mitotic arrest. Follow up assays, including a BrdU cytoblot, confirmed the ITSAs ability to suppress TSA's anti-proliferative activity, but the direct target of the compounds remains elusive (Figure 52).[270]

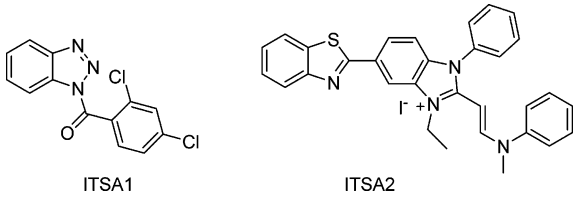


Figure 52. Example of ITSA compounds.

Olson et al. have used modified cytoblot assays as part of a study to understand the role of HDACs in cardiac hypertrophy through the use of HDAC inhibitors. Their results demonstrated that these inhibitors showed a dose-dependent blockade to hypertrophy. HDAC's involvement in controlling cardiomyocyte hypertrophy suggests a role for these compounds as therapeutics for cardiac hypertrophy and heart failure, in addition to their role as potential anticancer agents.[271]

Mitchison et al. employed a cytoblot assay that detected the increased phosphorylation of nucleolin as a marker for cells entering mitosis in the search for compounds that affect mitotic machinery but do not directly target tubulin.[201] Screening 16 320 commercial small molecules identified 139 hits. After exclusion of direct tubulin-targeting compounds, 86 compounds were screened in a phenotypic assay to examine the distribution of actin, chromatin, and microtubules. One compound exhibited an interesting phenotype in which the bipolar mitotic spindle was replaced by a monastral microtubule array surrounded by a ring of chromosomes—this compound was called monastrol (Figure 53).[201] Monastrol was shown to target the mitotic kinesin Eg5, thus

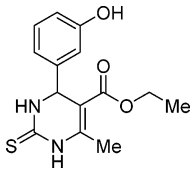


Figure 53. Structure of monastrol.

preventing spindle pole separation during mitosis and producing a spindle checkpoint-dependent mitotic arrest.[272,273] In addition, it was shown that monastrol competes with ATP binding to Eg5; it inhibits microtubule-stimulated ADP release from Eg5, but it does not compete with microtubule binding. This indicated that monastrol binds to a novel allosteric site in the motor domain.[274]

Sebti et al. have developed a phosphotyrosine signal transducer and activator of transcription (STAT) 3 cytoblot.[275] STAT3 plays a critical role in tumor malignancy and is of therapeutic importance. Their screen identified JSI-124 (cucurbitacin I) from the National Cancer Institute (NCI) Diversity Set that reduced the levels of phosphotyrosine STAT in v-Src-transformed NIH 3T3 cells and human cancer cells potently and rapidly (Figure 54). It was shown that this

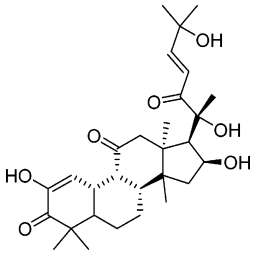


Figure 54. Structure of cucurbitacin I.

molecule affected the level of tyrosine phosphorylated Janus kinase (JAK), but not Src. The compound inhibited tumor growth and increased viability in mice with melanoma constitutively activated by STAT3, and these results support the use of targeting the JAK/STAT3 pathway for anticancer therapeutic discovery.

In an example of the utility of chemical synthetic lethal phenotypic screening used in combination with cytoblot techniques, Leder et al. identified a mitochondriotoxic compound, F16, that selectively inhibits tumor cell growth. Specifically, it inhibited the proliferation of mammary epithelial *neu*^{T}-overexpressing cells in addition to other cells.[181,266,276] This example is notable due to its parallel screening approach where cells lacking and expressing the *neu*^{T} oncogene were screened. This approach provided HTS data that was equivalent to comparative chemical genomic profiles of the two states (Figure 55).[181]

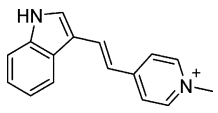


Figure 55. Structure of F16.

4.6.3. Gene Reporter

Beyond cell-based phenotypic screening, cell-based reporter methods that typically rely on transcription-based reporters are another attractive option. Reporter gene assays are now a standard tool in chemical genetics, and the sophistication and scope of these studies continues to increase. Rosen et al. presented an early example of high-throughput screening using a luciferase reporter system in cultured cells.[277] Their system sought to identify compounds that activate the transmembrane granulocyte-colony stimulating factor (G-CSF) receptor and, therefore, activate the G-CSF signal transduction pathway. They screened a murine myeloid cell line, NFS60, that contained a G-CSF responsive reporter construct with a synthetic STAT binding element linked to a minimal promoter and the luciferase gene. STATs, receptor associated proteins, are activated downstream of G-CSF binding and are involved in transcription regulation. Their luciferase assay identified a compound, SB 2478464, that induced G-CSF-like tyrosine phosphorylation and formation of granulocytic colonies in vitro (Figure 56). Their

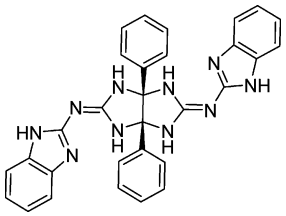


Figure 56. Structure of SB 2478464.

results showed that a small, nonpeptidyl molecule has activities associated with a protein hormone, and that a small molecule can activate a large receptor protein requiring dimerization for activation.[277]

Schreiber et al. undertook integration of chemical genetics, by way of cellular screening, and genomic tools in the form of transcription profiling with cDNA microarrays.[267] They used a cell-based screen for small molecules that activate the p3TPLux reporter gene in a transfected mink lung epithelial cell line. The reporter was composed of a luciferase coding gene and a DNA sequence sensitive to TGF-β. Follow up cytoblot screening was used to determine whether TGF-β-like compounds block DNA synthesis. A total of 16 000 compounds were screened, and four active compounds were identified, two of which were selected for follow up microarray screening. From microarray screening, one compound was shown to activate genes implicated in metal ion homeostasis. They analyzed the effect of various metal ions, but interestingly, they found that copper alone could activate the reporter and that copper, but not other metals tested, cooperated synergistically with one of the compounds, indicating that this compound acts as a copper transporter.[267]

Gudkov et al. used a *lacZ* reporter gene activated in response to a p53-sensitive promoter in a mouse ConA cell line.[278] p53 is a target of cancer research due to its role in inducing apoptosis in dangerous or damaged cells and because the p53 gene is functionally absent in human tumors. In addition, p53 is indicated in the toxic effects of anticancer treatments. They screened a 10 000 member commercial library in the presence of a p53 inducer, doxorubicin, and identified an active compound, pifithrin-α (Figure 57), which

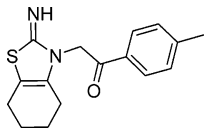


Figure 57. Structure of pifithrin-α.

blocked activation of p53-responsive *lacZ* in the presence of Dox, UV light, and γ radiation. From cellular follow-up experiments and in vivo screening, it was shown that

pifithrin-α protected mice from the lethal genotoxic stress associated with anticancer treatment without leading to tumor formation.[278]

Reporter-based screening has also been used in a number of other studies to identify active compounds for in depth analysis. Kim et al. used GFP and chloramphenicol acetyltransferase (CAT) reporter gene assays in HeLa cells to identify a compound from a 400 member commercial library that induced the assembly of the interferon-β (IFN-β) enhanceosome through stimulations of all the enhancer-binding activator proteins.[279] It was shown that this compound, CG18 (Figure 58), stimulated the mitogen-activated

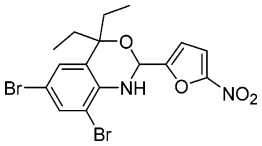


Figure 58. Structure of CG18.

protein kinase kinase kinase 1 (MEKK1), which is a member of a family of proteins involved in stress-mediated signaling pathways. They used CG18 to thoroughly examine these pathways and found that the MEKK family may integrate signaling transduction pathways that lead to the activation of IFN-β.[279]

In addition, Pasco et al. employed a luciferase gene reporter system with a cyclooxygenase-2 (COX-2) promoter to screen for compounds that inhibited the stimulation of COX-2 activity.[280] Their library consisted of 968 extracts from 266 plants, and 12 active extracts were identified. They further identified a known anti-inflammatory compound, shikonin (Figure 59), from *Arnebua euchroma* as a specific

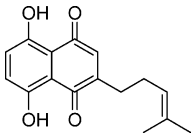


Figure 59. Structure of shikonin.

active compound. Follow-up screening showed that the family of shikonins were active and demonstrated the utility of HTS reporter assays to find specific inhibitors of drug targets.

Kahn et al. used a luciferase-based transcription assay, TOPFLASH, to measure β-catenin/TCF (T cell factor) transcription in transformed colorectal cells.[202] β-catenin responsive genes are activated in most colon cancers, and antagonists of this pathway are actively sought. From their screening, they identified an active compound, ICG-001 (Figure 60), that antagonized β-catenin/TCF transcription and

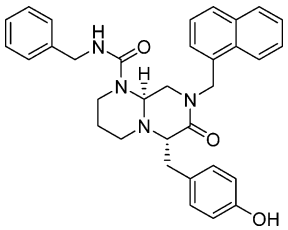


Figure 60. Structure of ICG-001.

down-regulated β-catenin/T cell signaling by binding to the cyclic AMP response element-binding protein. ICG-001 was also shown to selectively induce apoptosis in carcinoma cells and was efficacious in mouse xenograft cancer models, thus verifying this compound's potential therapeutic benefit.[202]

Stockwell et al. reported the identification of an upregulator of the survival motor neuron (SMN) protein through a *SMN*-minigene luciferase gene reporter study in CC44a cells that only produced luciferase during splicing and transcription.[281] Low levels of the SMN protein is a cause of spinal muscular atrophy, a leading genetic cause of infant mortality; upregulation of the genes responsible for the SMN protein is a desirable activity for a drug. Their library contained approximately 47 000 compounds from various sources that included their ACL, commercial sources, and others. They identified indoprofen (Figure 61), a known COX inhibitor,

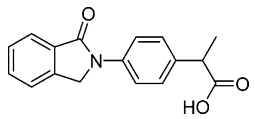


Figure 61. Strucutre of indoprofen.

which increased SMN-related luciferase production, the number of nuclear germ fibroblasts (an indicator of increased SMN protein production), and viability in a spinal muscular atrophy mouse model. In addition, no other COX-targeting NSAID (nonsteroidal antiinflammatory drugs) showed similar activity, indicating that it operated in a COX-independent manner.

Schultz et al. have used high-throughout reporter-based screening to identify small molecules that control stem cell fate.[203] They screened a large combinatorial kinase-directed heterocyclic library in mouse P19 EC (embryonal carcinoma) cells because, like embryonic stem cells (ESC), these cells are pluripotent, have broad embryonic differentiation potentials, and do not exhibit frequent spontaneous neuronal differentiation.[108] A luciferase assay using the pTα1-Luc reporter was inserted into the specific neuronal marker, neuronal Tα1 tubulin. A number of compounds were identified as inducing neuronal differentiation. They selected a compound that, after SAR analysis of the primary screen, was a model for follow-up pyrrolopyrimidines that were synthesized and screened to identify a potent compound called TWS119 (Figure 62). Follow up phenotypic screening

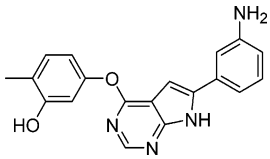


Figure 62. Structure of TWS119.

showed specific neuronal differentiation with the correct neuronal morphology. Affinity analogues utilized in the pull-down experiments suggested the target as GSK-3β. Biochemical studies and analysis of the mechanism of action suggested a role for GSK-3β in the induction of mammalian neurogenesis in ESCs.[203]

Orlow et al. adopted a chemical genetic approach to understand the "cross talk" between signaling pathways.[282] They sought to understand how components of the yeast pheromone response pathway are involved in other signal transduction networks. They did this by screening a library of 100 compounds of various modes of action to identify those that activate the pheromone responsive genes *FUS1* and *RLM1* by a *lacZ* reporter system. They found that the catecholamines (L-DOPA, dopamine, adrenaline, and nor-

adrenaline) activated these genes, but their effect could be reversed by the antioxidant *N*-acetyl-cysteine, suggesting that the gene activation response was a result of oxidative stress. Further genetic analysis showed that the cellular response to oxidative stress differs depending on the nature of the oxidant.

Kahn et al. used *luc* reporter gene constructs in human lung epithelial A549 cells to identify specific small molecule inhibitors of activator protein (AP-1).[283] They sought these since an oxidant/antioxidant imbalance in the lungs leads to the activation of AP-1 and nuclear factor κB (NF-κB), a characteristic of asthma. Using a small library of β-strand mimetics, they identified a compound, PNRI-299 (Figure 63),

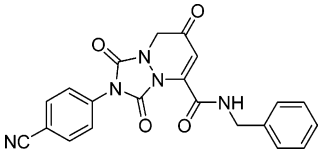


Figure 63. Structure of PNRI-299.

that specifically inhibited AP-1 but did not affect NF-κB; they had previously found a small molecule inhibitor of NF-κB.[284] Affinity pull-down experiments identified the target of PNRI-299 as the oxidoreductase, redox effector factor-1 (REF-1). This compound showed positive results in mouse asthma models and validated AP-1 as a therapeutic target for asthma. They followed up with a detailed report of this compound's synthesis and its effect on leukotriene C4 synthase.[204]

Porter and investigators used a chemical genetic reporter-based screen to interrogate the Hedgehog signaling pathway.[205,285] Hedgehog (Hh) signaling mediates the differentiation of multiple cell types during embryogenesis, facilitates tissue repair, and is a target in Parkinson's disease and diabetic neuropathy therapeutics. The Hh response is controlled by two transmembrane proteins, Patched (Ptc), a negative regulator of Hh signaling, and Smoothened (Smo), an activator. Their screen sought compounds that could interfere with the inhibition of Smo by Ptc, could activate Smo independent of Ptc, or might act downstream of Smo.[205] Using a cell line responsive to Hh signaling, they screened a 140 000 member small molecule library with a luciferase reporter assay. A number of agonsists were identified, but one, Hh-Ag 1.1 (Figure 64), was selected for further study.

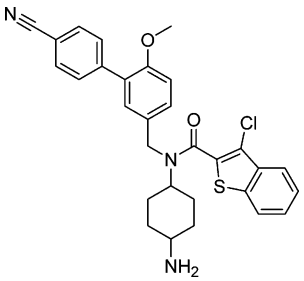


Figure 64. Structure of Hh-Ag 1.1.

This compound promoted cell-type-specific proliferation and differentiation. Through genetic deletion and biochemical studies, it was shown that Hh-Ag 1.1 acts directly by binding to Smo, which can have its activity modulated by small molecules. This was an excellent study that demonstrated identificaton of an active small molecule while providing control of important signaling pathways.[205,285]

4.6.4. Fluorescent Imaging

Fluorescent imaging based approaches, such as high-throughput automated fluorescence microscopy techniques and cell imaging, are a promising method for the identification of active compounds.[286]

Yarrow et al. developed a high-throughput image-based cell migration screening method that phenotypically analyzed scratch wound healing using automated microscopy. This method is readily adaptable for use with a number of perturbations including small molecules.[182] Kau et al. also employed this technique to identify inhibitors of the export of FOXO or Forkhead family of transcription factors.[287] This study was interesting because they isolated small molecules that restored a function of phosphatase and tensin homologue (PTEN), in cells that lack PTEN.[287,288] PTEN is a tumor suppressor protein and the counter partner of PI3K, which dephosphorylates 3-phosphate from phosphoinositides. FOXO restricts cell growth, but normally not when PTEN is absent. In cells lacking PTEN, FOXO1 is improperly localized in the cytoplasm and does not inhibit cell cycle progression. Therefore, it is reasoned that forcible relocation of FOXO1 to the nucleus, as a result of the action of small molecules, would overcome this aspect of PTEN deletion and inhibit the tumorgenicity of PTEN null cells. The automated image-based screen visualized the subcellular localization of FOXO1 by immunostaining a tagged FOXO1. A screen of an 18 000 member commercial library revealed two classes of small drug-like molecules: (1) those that target the general nuclear transport machinery and (2) compounds specific to the PI3K/Akt/FOXO1 signaling pathway.[287] Extensive follow up screening revealed that the first class of compounds targeted the nuclear export receptor CRM1, while the other implicated a novel role for calmodulin as a mediator of FOXO1 nucleocytoplasmic localization and regulation.[287]

Phenotypic cellular high-throughput imaging has also been used in studies involving membrane traffic.[286] Kirchausen et al. used this screening to identify 26 inhibitors of exocytosis. Secretion is a dynamic and important pathway whose mechanisms and components are poorly understood. Few modulators exist due to a lack of pharmaceutical interest because of the high toxicity of the known modulators. Brefeldin A (BFA, Figure 65) is a useful reagent for studying

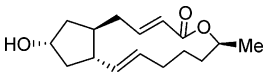


Figure 65. Structure of brefeldin A.

Golgi function, but it produces phenotypes of unknown origin. Because of this, small molecule compliments were sought to further investigate the secretory pathway.[289,290]

Over 10 000 compounds from a commercial library were screened by automated microscopy, and the behavior (exocytosis from the endoplasmic reticulum (ER) to the plasma membrane) of the temperature-sensitive mutant of the surface glycoprotein (VSVG, vesicular stomatitis virus G protein) VSVG^{ts}-GFP was visualized. A temperature shift induced VSVG^{ts}-GFP to migrate from the Golgi apparatus to the plasma membrane. Screening of molecules that disrupted this process revealed compounds that fell into the following phenotypic categories: (1) ER exit block, (2) Golgi exit block, (3) Golgi fragmentation, and (4) vacuole formation. A focused study of a selected ER blocking compound, Exo1 (Figure 66), revealed that it, like BFA, caused a redistribution

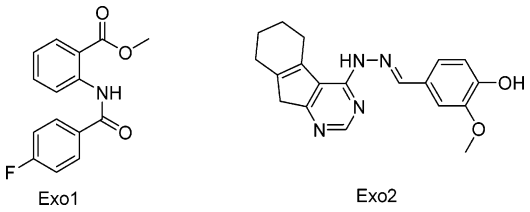


Figure 66. Structure of Exo1 and Exo2.

of Golgi contents but, unlike BFA, did not have a great effect on the trans-Golgi network. Though they did not identify the precise target of Exo1, follow up experiments revealed it to be different from BFA's target.[286,291] This type of screening also identified a related molecule, Exo2 (Figure 66), that was used to study the function of the Golgi in cholera toxin trafficking by disrupting the Golgi apparatus without disrupting the trans-Golgi network.[292] Last, Exo1 and Exo 2, along with other compounds identified from the previous screening, were used to study their effects on endocytotic membrane traffic.[293] Two compounds were found to disrupt Golgi-to-cell surface traffic without disrupting ER-to-Golgi transport. These are a novel class of compounds that elevate endosomal and lysosomal pH, down-regulate cell surface receptors, and impair recycling of internalized transferrin receptors to the plasma membrane. In addition, they inhibited ATP hydrolysis through ATPase and were found to be potent ionophores.[293]

Shair et al. tested their synthetic library in a fluorescent phenotypic assay monitoring the ability of the compounds to block the trafficking of a VSVG-GFP fusion protein.[79] While they based their DOS library on galanthamine, a potent acetylcholinesterase inhibitor, they hoped to extend the derivative's activity into different areas of biological space. From their library of 2527 compounds, they identified a potent compound termed secramine that blocked VSVG-GFP movement from the Golgi apparatus to the plasma membrane as visualized by fluorescent microscopy. Interestingly, galanthamine did not have a similar effect. This paper was noteworthy because it showed the possibilities of exploring new biological space based on a natural product scaffold whose derivatives have significantly different activities compared to the parent compound (Figure 67).[79]

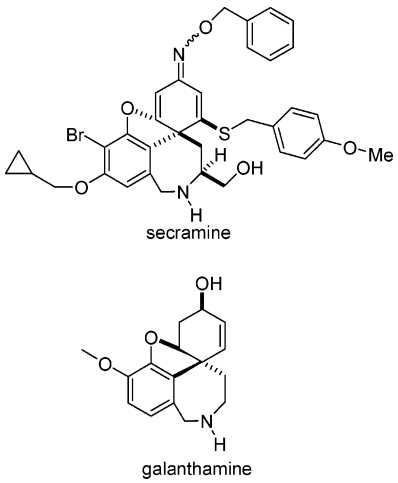


Figure 67. Structure of secramine and galanthamine.

Wessling-Resnick et al. also used a mammalian cell-based fluorescent technique to monitor non-transferrin-bound iron uptake and transferrin-mediated iron transport.[294] This screen used a metal-sensitive fluorophore, calcein, that is quenched when bound by iron. They used a cell-permeable calcein derivative that gets hydrolyzed and trapped in the cell. Decreased fluorescence indicated iron uptake, as well as allowed for the monitoring of cell viability. From screening the NCI Diversity set, they identified 10 compounds that inhibited iron transport. Two compounds were shown to block the uptake of iron mediated by transferrin, but the others did not. Therefore, this represented the first small molecule inhibitors of iron transport and a powerful forward chemical genetic screening method for monitoring iron uptake.[294]

Mitchison et al. developed a new cell-based high-throughput screen to identify small molecules that block calcium-triggered nuclear import of the nuclear factor of activated T cells (NFATs).[295] These compounds were sought to improve upon the currenly prescribed immunosuppressant drugs FK506 and cyclosporin A, which often have debilitating side effects when used in the treatment of organ transplant rejection. Briefly, these drugs operate by targeting the calcium-dependent calcineurin that itself dephosphorylates and promotes NFAT transcription activation. These NFATs then transactivate the cytokine genes responsible for the regulation of the proliferative responses of T cells. Since NFATs are critical for T cell activation, they are desirable drug targets. Screening of a commercial library employed HeLa cells expressing a GFP-tagged NFAT. They monitored for compounds that inhibited the nuclear import of the tagged GFP−NFAT and identified 14 compounds. It was shown that these compounds operated upstream of calcineurin and NFAT by targeting calcium mobilization instead of calcium-activated phoshatase calcineurin. In fact, it was shown that they interfered with calcium mobilization involving store-operated calcium (SOC) channels, which raises the possibility for therapeutic modulation along these lines. Last, these compounds showed cytokine gene suppression in T cells, and significantly, these compounds lowered the IC_{50} of cyclosporin A when used together, which may have implications for multicomponent therapeutics.

Kapoor and co-workers have searched for probes that affect the dynamics of cell division.[296] They identified a novel, cell-permeable small molecule, HR22C16, through a high-throughput forward chemical genetics phenotypic cell-based screen. As a primary screen, they used BS-C-1 cells (African green monkey kidney cells) in a fluorescent microscopy based morphological assay and stained the actin cytoskeleton using rhodamine phalloidin. Further screening revealed a monoaster phenotype in the microtubules and chromosomes hinting at Eg5 inhibition. In vitro screens revealed an $IC_{50} = 800$ nM for HR22C16, compared to $IC_{50} = 14\ \mu M$ for monastrol. Next, they developed a tracerless, solid-phase DOS pathway to HR22C16 analogues and identified a compound "**6**" that showed increased potency ($IC_{50} = 90$ nM) (Figure 68). In an interesting application, they devised inactive analogues that bear a photolabile "cage" that upon irradiation with a He−Cd laser releases the active compound. This caging of a molecule provides excellent temporal control of an active compound's effect and is especially advantageous in studies on time-sensitive processes such as cell division.

Bertozzi et al. employed immunofluorescence detection as the primary screen to visualize poly(α-2,8-sialic acid) (PSA) in NT2 neuronal cells with a monoclonal antibody to PSA 12F8.[297] PSA is a linear homopolymer found mainly on the neural cell adhesion molecule. It has been implicated

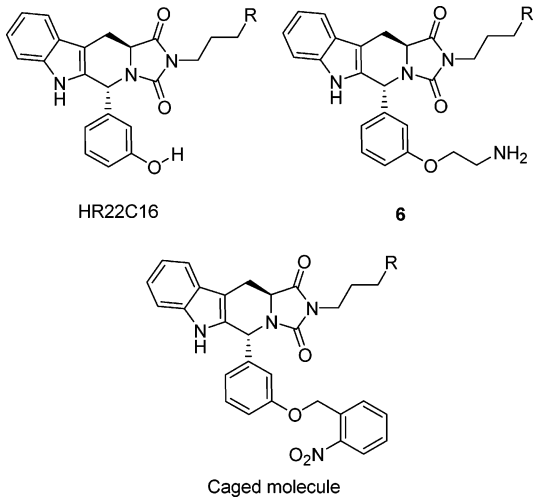


Figure 68. Structure of HR22C16, the more potent analogue "**6**", and the caged molecule.

in many pathological processes, but importantly, it is a marker of several tumors. From their screening, Bertozzi and co-workers reported a specific and reversible modulator of PSA expression, *N*-butanoylmannosamine (ManBut). Administration of ManBut leads to the biosynthesis of unnatural chain-terminating derivatives in the biosynthetic pathway and of the appearance of unnatural sialic acid residues on the cell surface. ManBut provides temporal and tunable control in a dose-dependent fashion by altering the endogenous sialic precursor pools. The amount of truncated PSAs formed was directly correlated to the dosing of ManBut. PSA modulation was only available through genetic means or enzyme degradation, but this approach provides exquisite control of PSA biosynthesis and presents ManBut as a useful tool for studying the effects of PSA biosynthesis (Figure 69).[297]

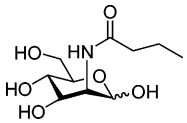


Figure 69. Structure of ManBut.

Kahn and co-workers used a similar immunofluorescence assay in the identification of an inhibitor of the NF-κB pathway.[284] NF-κB is a target of interest due to its role in regulating a wide array of genes and especially its important role in immunological regulation, in particular its role in inflammatory disease states. The group sought inhibitors of the NF-κB pathway by screening a library of compounds built around a constrained β-strand template.[298] This assay measured NF-κB driven transcription by detecting the vascular cell adhesion molecule (V-CAM), an adhesion marker expressed on the surface of endothelial cells. V-CAM transcription activation results from NF-κB binding to two sites on the V-CAM promoter gene. V-CAM was visualized through a secondary antibody screen, and inhibitors of V-CAM expression were sought. They identified an inhibitor, MOL-294, that does not inhibit the proteosome complex, IκB phosphorylation, or the nuclear translocation of NF-κB. Biochemical follow-up assays suggested that one of the targets of MOL-294 is the cellular redox protein thioredoxin (Figure 70).[202]

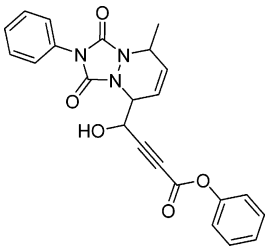


Figure 70. Structure of MOL-294.

4.7. Chemical Genetic Networks and Multidimensionality

Chemical genetics can be expanded to studies of chemical genetic networks where compounds interact analogously to genetic networks and also to studies where the data expands dimensionally away from one compound−one response phenotypic studies. An example by Schreiber et al. demonstrates both of these concepts by creating "chemical genetic maps" of chemical and biological multidimensional space.[299] This study incorporated DOS, parallel and hierarchical HTS screening and analysis, and multidimensional data analysis. They assembled a collection of 1,3-dioxane-based compounds biased toward metal chelation, more specifically the zinc-dependent HDACs. This study identified 617 small molecule inhibitors of intracellular deacetylation from a 7392 member library.[181,216] The data were analyzed by looking at the connectivity of a compound's response in multiple assays and its relations to other compounds. This study highlighted the possibility of using small molecules to dissect complex biological networks and illustrated practical examples of the use of computational methods. The development of chemical genetic networks by graph theory and the mapping of chemical space will allow for the development of more selective inhibitors.

Schreiber et al. expanded upon this work to incorporate chemical genomic profiling of a biological network. [300] This study used graph theory and perturbation screening with wild-type and nine isogenic yeast deletion strains, each missing a component of the spindle assembly/cell polarity network. All possible pairwise combinations of 24 small molecules of known biological activities were used to generate a chemical genetic profile; in total this gave 5760 perturbations. A chemical genomic profile was defined as "the ability of combinations of small molecules to interact antagonistically or synergistically" to provide "a chemical tool to resolve differences between biological networks."[300] This study showed that varying the architecture of a genetic network is a means for analyzing the diversity of small molecules. It was also shown that small changes in the genome, one deletion, can have a significant impact on the effect of small molecules. This tool aids in the characterization of molecular diversity, chemical space annotation, and the characterization of perturbation in biological networks.[300]

Schreiber et al. used DOS and chemical genetic multidimensional analysis to scrutinize the effect of macrocyclization. They screened bicyclic products and their monocyclic precursors that incorporated changes in stereochemistry for differences in cellular response over 40 parallel cell-based assays. Their analysis used hierarchical clustering to classify the compounds, all of which were closely related structural derivatives. This study was able to quantify the various diversity elements and group the compounds based on their response to the assay panel. This type of analysis

will be important in understanding biological responses to small molecules as well as in compound design.[301]

Last, Bussey et al. in an interesting integration of genetic and chemical genetic approaches screened a set of yeast deletion mutants to score synthetic interactions in a study of the interconnectivity of genes involved in β-1,3-glucan assembly. They also screened this mutant collection for sensitivity and resistance to the β-1,3-glucan synthase inhibitor caspofungin.[302] Integration of all the data showed overlapping sets of genes implicated in compensatory chitin synthesis *FKS2* regulation, protein mannosylation, and the *PKC1*-dependent cell integrity pathway.[302]

Multicomponent screening and data analysis was revealed to be a powerful tool in a report by Stockwell and co-workers.[303] In this paper, they devised a high-throughput screen to identify therapeutically useful combinations of small molecules. They screened pairwise combinations of approximately 120 000 different two component combinations of reference listed drugs. This study incorporated a wide variety of assays including a *Candida albicans* proliferation assay, a colony-forming unit assay, a dye efflux assay, tumor necrosis factor α (TNFα) and IFN-γ ELISAs, an A549 tumor cell in vitro BrdUrd incorporation assay, and an A549 tumor cell in vivo mouse xenograft assay. The data from these assays showed unexpected activities from combinations of compounds that the authors proposed arose from interactions between the compounds' pathways of action. The use of compounds of known biological activity dramatically aids in the understanding of the pathway(s) on which a compound may be acting. These types of studies will also be useful in understanding the interconnectivity of various networks. Of particular note, they identified a number of interesting combinations of active compounds, such as antipsychotic and antiprotozoal agents that showed no antitumor activity individually but were active when combined. This type of result is consistent with previous reports of treatment-specific gene expression changes where very little overlap in genetic response was seen between patterns of two single agents versus the pattern of their combined administration.[216,304]

4.8. Cell-Free Systems

Cell-free *Xenopus* egg extracts provide a versatile environment in the phenotypic screening of compounds for the study of a variety of processes.[195,203,208,305,306] One of the most studied areas using this method is cell division and the cytoskeleton. Kirschner and Rosen have used this system in the study of N-WASP (neural Wiskott−Aldrich syndrome protein) inhibitors.[206,207] Actin cytoskeleton regulation involves numerous signaling steps. In one model of this mechanism, membrane-proximal signals recruit and activate members of the WASP family of proteins, which in turn activate the ARP2/3 complex and promote the generation of new actin filaments.[206] The cell-free extract system provides a model to study this signaling pathway. When phosphoinositide-containing synthetic lyposomes are added to *Xenopus* egg extracts, actin polymerizes on the vesicle surface.[307] This process relies on the presence of three proteins, GTPase cdc42, N-WASP, and ARP2/3.[307−309] The use of pyrene-labeled actin monomers allowed for fluorescence measurements due to their 20−30-fold fluorescent increase upon polymerization.[307] In the first report, a 384 member cyclic peptide library was screened and a cyclic peptide, 187-1, was identified: cyclo($_L$Lys-$_D$Phe-$_D$Pro-$_D$Phe-$_L$Phe-$_D$Pro-$_L$Gln)$_2$. Purified protein assays and photo-cross-linking using a biotin/benzophenylalanine 187-1 derivative were used to identify the target of the compound as N-WASP. It was shown that this compound prevented activation of the ARP2/3 complex by allosterically stabilizing the auto-inhibited conformation of N-WASP.[206,207] Later work using the same screening system identified a small molecule inhibitor, wiskostatin (Figure 71), from a commercial library that chemically inhibited N-WASP. NMR analysis revealed that wiskostatin inhibited N-WASP activation by binding to the GTPase domain in an auto-inhibited conformation.[305]

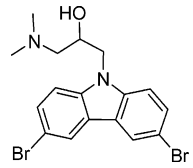


Figure 71. Structure of wiskostatin.

Heald and co-workers have used the *Xenopus* egg cell-free extract system in phenotypic screening that identified inhibitors of the mitotic spindle assembly. They screened a 2,6,9-trisubstituted purine library from which several hit compounds and their unique targets were discovered, aminopurvalanol/Cdk1,[208] myoseverin (Figure 43)/microtubule,[310] and more recently diminutol (Figure 72)/NADP-dependent quinine oxidoreductase (NQO1).[209] A clear understanding of NQD1's role remains to be elucidated, but this novel regulatory protein is involved in microtubule binding and is a necessary component in microtubule morphogenesis and cell division.

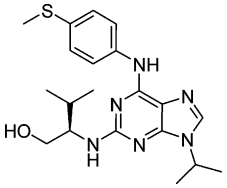


Figure 72. Structure of diminutol.

Ubiquitins and the ubiquitin−proteasome pathway regulate a host of processes in eukaryotic cells and are an area of intense research.[311,312] A chemical genetics approach by King et al. used unbiased screening to identify compounds or unknown targets relative to cell division and discovered inhibitors of proteasome-dependent degradation.[210] Their approach sought compounds that would stabilize cylcin B and prevent its ubiquitin-dependent degradation in *Xenopus* extracts. Cyclin B degradation regulates exit from mitosis and requires activation of an E3 ubiquitin ligase, anaphase-promoting complex/cyclosome (APC/C). A luciferase-modified cyclin B was used to monitor its degradation. The screen sought compounds that inhibit mitotic entry and the cyclin degradation machinery. Their miniaturized assay system screened 109 123 compounds and identified 22 inhibitors. Compounds were classified and rescreened to determine the nature of their activity, for example, compounds that block mitotic entry vs direct proteolysis inhibitors. Two selected compounds that bound to ubiquitin chains and blocked interactions with proteasome-associated receptors without affecting 26S assembly or peptidase activity were named the ubistatins (Figure 73). These compounds, especially ubistatin A, were shown to specifically bind to 48-lysine-linked ubiquitin chains.[210] This study is interesting not only for the

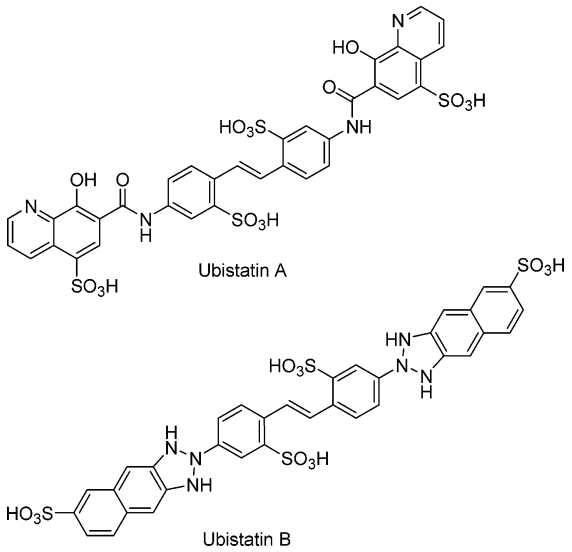


Figure 73. Structure of ubistatins.

insight into the ubiquitin−proteasome pathway and the promise these compounds have in studying this pathway, but also for the value of unbiased chemical screens combined with the biochemical *Xenopus* extract system to identify compounds with unique and unexpected activities and mechanisms.[210,273]

Cell lysates provide another avenue for forward chemical genetic screening. Wang et al. have used the HTS of 184 000 compounds from an in-house library to identify a compound, α-(trichloromethyl)-4-pyridine-ethanol (PETCM, Figure 74),

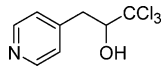


Figure 74. Structure of PETCM.

that provided insight into the regulation of programmed cell death (apoptosis).[313] This compound, along with 28 others, was identified as being an activator of caspase-3 in extracts of a panel of cancer cells. This was a cell lysate assay where caspase-3 was monitored by cleavage of a colorimetric substrate. Using biochemical fractionation and PETCM, they studied the pathway that regulates mitochondria-initiated caspase activation. They identified the oncoprotein prothymosin-α (ProT) and the tumor suppressor putative HLA-DR associated proteins (PHAPs) as important regulators, but with opposing effects; ProT negatively regulated caspase-9 activation by inhibiting apoptosome formation, whereas PHAP promoted caspase-9 activation after apoptosome formation. It was later shown that PETCM inhibits the anti-apoptotic activity of ProT and promotes the formation of active caspase-3. ProT knockout by RNAi also sensitized cultured cells to apoptosis. Using this approach, they revealed the regulatory roles of the oncoprotein ProT and the tumor suppressor PHAP in apoptosis.[15,313]

5. Reverse Chemical Genetic Screening

5.1. In Vitro Screening

Purified enzyme assays provide a robust method for the identification of active compounds as the first step of reverse chemical genetics. Schultz et al. exploited this initially by identifying potent kinase inhibitors.[314,315] In particular, they sought inhibitors of cyclin-dependent kinase (CDK), which is a key component of cell division and a promising target for anticancer therapeutics. Structural analysis of known inhibitors and the ATP binding site of CDK indicated that they should focus on producing 2,6,9-trisubstituted purines, as opposed to the traditional approach of diversifying the ribose ring. They synthesized a diverse purine combinatorial library[316,317] and identified two potent and selective compounds for CDK2, purvalanol A and B; the more potent purvalanol B showed an IC_{50} of 6 nM against a CDK2/cyclinA complex (Figure 75). Cellular screening to determine the effects of these compounds was undertaken with purvalanol A and was evaluated for tumor growth and inhibitory activity in 60 human cancer cell lines. They also measured a genome-wide response to the compounds by measuring mRNA levels in yeast using high-density oligonucleotide arrays. Transcription arrays with purvalanol A identified five down-regulated genes related to the cell cycle. This work clearly demonstrated the promise of these purine libraries combined with reverse chemical genetics for generating powerful biological probes.[314,315]

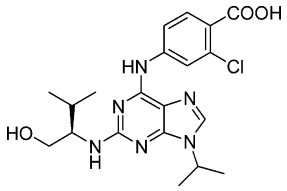


Figure 75. Structure of purvalanol B.

Willson and co-workers used a reverse chemical genetics approach in the functional analysis of orphan nuclear receptors in the regulation of bile acid metabolism.[49] Their approach focused on understanding the roles of two orphan nuclear receptors, FXR (NR1HR) and PXR (NR112), in the regulation of bile acid metabolism. To screen a 9000 member combinatorial library, they employed a cell-free ligand-sensing assay (LiSA) in which upon binding of an agonist to an allophycocyanin-labeled FXR, recruitment of europium-labeled coactivator protein SRC1 was detected by FRET (fluorescence resonance energy transfer). From this screening, a smaller follow-up library was made, and a cell reporter screen revealed a potent compound, GW4064, with an EC_{50} of 80 nM in cells (Figure 76).[4] This compound was used to identify genes regulated by FXR in the liver, including some involved in bile acid synthesis and transport. It was also shown that PXR (NR112) is a lithocholic acid receptor that controls biosynthesis and metabolism of bile acids and that FXR and PXR cooperate to control biliary and urinary bile acid secretion. This was a thorough use of reverse chemical genetics in the understanding of receptor function.[49]

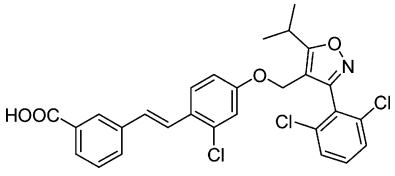


Figure 76. Structure of GW4064.

Saltiel and co-workers used a small molecule inhibitor of the mitogen-activated protein kinase kinase 1 (MEK1) to define a role for MEK in colon cancer signaling in mice.[318] This full study included in vitro and mouse tumor growth studies that demonstrated MEK as a relevant colon cancer

target. Navre et al. used a fluorescence-based quenching assay with purified enzymes to identify potent and selective inhibitors of collagenase-1 from a diketopiperazine combinatorial library.[319] Schaeffer et al. used a combinatorial library along with membrane-based screening of five subtypes of somatostatin receptors to identify subtype selective agonists of the somatostatin receptor, which were used to study downstream signaling events of the activated receptor.[320] This study demonstrated the use of small molecules to activate, rather than inactivate, specific members of a protein family.[17]

Small molecules that target proteins involved in the cytoskeleton, other than tubulin and actin for which potent inhibitors exist, are extremely valuable. A focus of this search was for small molecule inhibitors of myosin II, a protein involved in muscle contraction, cytokinesis, and cell migration, for which previous inhibitors showed poor cell permeability and lacked specificity.[321] Straight et al. screened a commercial 16 300 member library against purified rabbit muscle myosin subfragment (s1) and measured actin-activated ATPase activity by a luminescence assay. From this screen, aryl sulfonamides were selected as a framework for the generation of a minilibrary from which screening identified the commercially available *N*-benzyl-*p*-toluenesulfonamide (BTS, Figure 77) with an IC_{50} of approximately

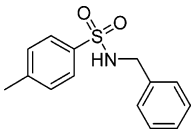


Figure 77. Structure of BTS.

5 μM. They sought to understand the mechanism of BTS's action and through a competition experiment showed that BTS does not compete for the nucleotide binding site of myosin, but it does weaken F-actin's interaction with myosin.

Straight et al. used the same assay to identify a specific inhibitor of nonmuscle myosin II.[322] They identified a compound, blebbistatin (Figure 78), that blocks myosin II-

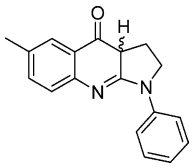


Figure 78. Structure of blebbistatin.

dependent cell processes. Blebbistatin inhibited the ATPase and gliding motility activities of human platelet nonmuscle myosin II but did not inhibit myosin light chain kinases. It was shown in a later study by a different group that blebbistatin's inhibition stems from its high-affinity binding to the myosin−ADP−P_i complex and its interference with the phosphate release process.[323,324] While blebbistatin inhibited contraction of the cleavage furrow, it did not disrupt mitosis or contractile ring assembly.[322] With the use of blebbistatin, MG132 (a proteasome inhibitor), and other drugs, it was shown that ubiquitin-dependent proteolysis has a role in cytokinetic phase exit in mammalian cells. Follow-up experiments showed that continuous signals from microtubules are required to maintain the cleavage furrow position, and these signals control myosin II localization independently of other furrow components.[322] Blebbistatin has been further used in studies to probe its specificity within the superfamily of myosins, its role in inhibiting pancreatic adenocarcinoma cellular invasiveness, and its specificity for myosin II in *Dicytostelium*.[325−327]

A fluorescent polarization assay, namely, a high-throughput fluorescent donor displacement assay, was employed by Walker and co-workers in identifying selective inhibitors of the glycosyltransferase MurG.[328,329] This in vitro assay involved the HTS of *Escherichia coli* MurG, a nucleoside diphospho-glycosyltransferase (NDP-Gtf) involved in murein biosynthesis, and measured the displacement of a fluorescently labeled UDP-GlcNAc derivative from a glucosyl donor site by monitoring the fluorescent polarization change upon addition of an inhibitor.[328] Screening of a large commercial library revealed a number of selective MurG inhibitors, and it is possible that this screen may be generalized to identify inhibitors of other Gtf's.[329]

Lastly, Nash et al. have described a potentially useful screening method for ranking protein ligand binding.[330] Their technique, termed affinity-selection mass spectrometry (AS-MS), makes possible the rapid assessment of binding affinities of mixtures of compounds generated from DOS. The multidimensional chromatographic technique proceeds by the following steps: (1) ligands are selected from a library screening in the presence of a known competitive binder, (2) unbound compounds are chromatographically separated from the protein−ligand complexes, and (3) further chromatography separates ligands from the protein−ligand complex for quantification and identification by MS. This technique is rapid and unique because it requires no compound modification, tags, or immobilization—the compounds are "tagged" by their molecular weight and decoded by MS. This technique is general across protein classes, can determine the nature of cooperation of ligands (allosteric or competitive), and allows for the multiple hits from HTS to be rapidly triaged in terms of binding affinity.

5.2. Chemical Inducers of Dimerization

Small molecule dimerizers, or chemical inducers of dimerization (CIDs), were one of the first systemized tools in chemical genetics to reveal the potential of small molecules in dissecting biology.[1] These dimerization systems allow for the modulation and regulation of proteins even where ligands for a particular protein do not exist.[331] This early technology grew into the chemical genetic mainstays of the three-hybrid system and the orthogonal analogue sensitive allele approach pioneered by Shokat and his colleagues. On the chemical genetics time scale, the groundwork for this technology was laid out some time ago, and most of the current studies rely on the same basic systems. For that reason, an in-depth review of all the applications of this technology is outside the scope of this review. However, excellent reviews on this topic are available,[1,331−337] and here we wish to give a flavor of the basic premises and wide-utility of this technique.

In nature, the ligand-mediated dimerization of proteins is employed to regulate almost all signaling events, particularly gene expression and signal transduction.[331] For example, transcription factors dimerize to promote receptor recognition of specific DNA sites, recruitment of components of the transcription machinery, and activation or repression of transcription.[338] With nature as a guide, the CIDs are cell-permeable synthetic molecules that regulate protein−protein interactions and have been instrumental in understanding the roles of proximity and orientation effects in biology.[331,339−341]

Studying protein−protein interactions is difficult solely with small molecules due to their poor efficiency and selectivity in disrupting these interactions.[336] Among other implementations, CID systems have been used in controlling signal transduction,[339,342−347] gene expression/transcription[338,348−351] and in the study of Fas receptor mediated signaling pathways and cell death,[352−355] insulin/PDGF receptor signaling,[356] erythropoietin,[357] TGF-β,[358,359] cytosolic signaling components,[360] the regulation of protein secretion,[361] a tyrosine kinase of unknown function,[362] manipulating protein splicing,[363] the regulation of growth factors in human cells and large animal models,[364−370] and enzyme activity assays.[371,372] They have also been used to recruit intracellular signaling proteins to the plasma membrane, such as Sos[373] and Lck.[1,374] A CID offshoot, the three-hybrid system, has been used to identify the protein targets of small molecules, as will be discussed later.[375−377]

In general, the procedure involves the cellular expression of two ligand binding proteins that are separate transcription factor elements. One ligand binding protein is fused to a DNA binding domain, while the second is fused to a transcriptional activation domain. When the ligand (CID) is added, it induces the dimerization of the two proteins and recruits the transcriptional machinery to transcriptional enhancer elements occupied by the DNA binding protein. The two proteins bridged and brought into proximity by the CID are now able to activate gene expression as a transcription factor.[338] The ground-laying work by Schreiber and Crabtree developed two types of dimerizers that have informed many studies, namely, "dumbbell-shaped"[339,342,353,354,378] and "rapamycin shaped".[1] In the majority of CIDs, the dimerization domain is derived from immunophilins, such as the FK506 binding protein 12 (FKBP12).[221,379] CIDs from FK506 generally have two binding surfaces that interact with the dimerization domain fused to the protein, thus effecting dimerization, but modified CIDs exist that possess only one binding surface, and these are used for disrupting dimerization.[336]

First-generation CIDs retained their affinity for endogeneous immunophilins, which seriously reduced their efficacy, but modified CIDs soon appeared. As shown by the above examples, many have adopted CIDs as a now standard tool in exploring biology, such as the study of receptor dimerization at the cell surface.[28] However, one of the most critical advances in CID was the introduction of the "bump−hole" concept. This was demonstrated in an original report on inhibitors of phosphatase and, as greatly expanded upon by Shokat and co-workers, became one of the most productive chemical genetic tools.[380−382] The idea is to create "bumps" (normally, bulky side chains) on the ligand and "holes" on the receptor (a site-specific mutation to produce a deeper binding pocket) that act as an orthogonal ligand/receptor pair where the wild-type receptor has little affinity for the modified inhibitor and likewise the mutant receptor has lowered affinity for the endogenous ligand. This "bump−hole" strategy has been used to generate calcineurin-resistant derivatives of FK506 that operate orthogonally to existing dimerizer systems and avoid problems arising from endogenous calcium.[383] As a practical example, AP1903 (Figure 79) was produced with bulky side chains that prevented binding to wild-type targets but bound well to a mutant FKBP12 designed with a deep binding pocket, thus making it orthogonal, more specific, and more suitable for in vivo studies.[384] Gilgenkrantz et al. used this approach by expressing a modified caspase-3 in the livers of transgenic mice in which, upon injection of the CID, hepatocyte apoptosis was induced within 48 h, thus providing tight control of this process applicable to a number of model systems.[385]

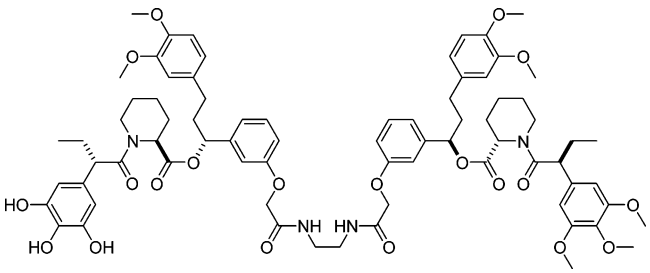


Figure 79. Structure of AP1903.

Crabtree et al. have introduced a system that overcame a previous limit of CID technology, namely, it allows dimerization to regulate the loss of function of a protein.[386] In their system, proteins encoded by the glycogen synthase kinase-3 (*GSK*-3β) gene were tagged with an 89 amino acid FRB* that destabilizes GSK-3β, in effect, a loss-of function allele. Full function and activity could be restored to this protein upon addition of a rapamycin derivative that induced the binding of GSK-3β FRB* and FKBP12 and consequently stabilized GSK-3β FRB*. Addition of FKBP12 as a competitor rapidly reverses the stabilization. This inducible stabilization can be applied to wide range of genes and provides for tight control of protein levels in developmental and physiological studies.

Peterson et al. have described a CID-based system they termed a "yeast tribrid system".[338] Contrary to most systems involving noncovalent dimerization, this system uses the covalent biotinylation of a specific lysine residue of a target protein. In this system, biotin protein ligase BirA was coexpressed in yeast with a streptavidin−LexA fusion protein and Avitag, a 15 amino acid peptide substrate of BirA, or BCCP (biotin carboxy carrier protein) biotin acceptor peptides fused to the B42 activation domain. Biotin addition resulted in BirA-mediated biotinylation of the biotin acceptor protein, recruitment to LexA DNA sites, and activation of reporter gene expression.[338] The expression of BirA, Avitag, and streptavidin allowed for the construction of a yeast tribrid system that allowed for protein dimerization to be controlled by the addition of exogeneous biotin. This system provides a potent, low toxic alternative CID-based approach for controlling cellular processes.

Last, Bertozzi et al. have developed small molecule switches for Golgi-resident sulfotransferases, GlcNAc6ST-1 and GlcNAc6ST-2, responsible for L-selectin ligand biosynthesis.[387] The modulation of glycan function through the regulation of sulfotransferases is a desirable target for studying these processes. The inducible enzymes possess activities and substrate specificities comparable to the wild-type. Two advantages of this system were exploited: (1) the need for Golgi localization for cellular substrate activity and (2) the modularity of their catalytic (Cat) and localization (Loc) domains. They fused the rapamycin binding proteins FRB and FKBP12 to the Cat and Loc domain of fucosyltransferase 7 (FucT7), respectively, and their dimerization was induced upon treatment with rapamycin. These Golgi sulfotransferases possess the same domain organization across the superfamily, implying that this system may find broad use. This tunable system offers a means to assess the impact of Golgi distribution on the activity of catalytic

domains and may offer the possibility of studying temporally controlled induction/suppression of enzyme activities in living systems.

5.3. Orthogonal Chemical Genetics

One of the most significant areas of chemical genetics takes a reverse and more importantly an "orthogonal" approach. Within orthogonal chemical genetics, a protein is engineered such that it interacts specifically and only with a specially designed probe partner. The method is orthogonal since these probes only interact and affect the function of the engineered protein, without affecting any of the native protein. This orthogonality of the engineered protein and probe to the native protein allows one to study and dissect the function of one member a closely related and highly homologous protein family. As discussed earlier, a purely genetic approach suffers in that it knocks-out the entire gene product and makes dissecting particular aspects of the protein's function impossible. In addition, no real temporal control is possible. In a complete deletion, the "knock-out" exists from the beginning and is permanent, and conditional alleles and antisense techniques operate quite slowly or require secondary perturbations. Also, in systems such as mice, many knock-outs result in lethal phenotypes. Of course, the chemical genetics approach overcomes many of these problems, particularly in terms of temporal control, but small molecules typically suffer from a lack of specificity, especially specificity among a family of related proteins. While target specificity can be demonstrated, the global off-target effects are difficult if not impossible to characterize.[28]

The orthogonal approach is a powerful blend of the advantages of both genetics and chemical genetics. The temporal control of chemical genetics is combined with the specificity of genetics, but with the improved ability to target one specific member of a protein family.[388] A general approach is applied in these studies in which a ligand is made orthogonal by modification and, in some cases, the protein is engineered in such a way as to accept solely the orthogonal ligand, thus making an orthogonal protein. The orthogonal ligands are simply "analogues" of the native, original activator or inhibitor.[336] The ligand is altered so as not to be able to interact with its natural ligand, and likewise, the native proteins cannot accept the orthogonal modified ligand. Typically, the protein is altered by introducing a "space creating" mutation that does not affect the protein's function and is exploited in the design of the orthogonal ligand.[28]

The first example of this was reported by Hwang and Miller.[389] In their work, a single mutation was made in the *E. coli* elongation factor Tu (EF-Tu) by oligonucleotide site-directed mutagenesis. This mutant protein had a reduced affinity for GDP and a drastically increased affinity for xanthosine 5′-triphosphate (XTP) but retained all of the functional properties of the wild-type protein. In addition, the mutated site is conserved in the homologous sequences of all GTP regulatory proteins making it a potentially general approach.[389] However, these enzymes relied entirely on XTP and could not be used in in vivo studies.

The use of analogue-sensitive kinase alleles (ASKA) represents one of the dominant implementations of the orthogonal reverse chemical genetics approach. Kinases play a role in almost every cellular process and are a major target of drug research, as evidenced by Gleevec, the Bcr-abl kinase inhibitor used in the treatment of chronic myelogeneous leukemia.[86,390,391] However, the number of kinases, at least 500 in humans, and their high level of homology in their most druggable site makes drug discovery and the functional analysis of specific kinases extremely difficult.[28] The discovery of ASKA and the small molecule analogue compounds that modulate the ASKA activity present an opportunity to study diverse members of the kinase subfamilies and can be generalized across the kinase superfamily without the need for individual chemistries and protein structural engineering.[28,392,393]

ASKA was developed by Shokat in the 1990s as an orthogonal chemical approach to study the complex roles of kinases in signaling pathways and cellular processes.[336,392,393] In the ASKA platform, the central feature is the creation of a unique structural distinction between the catalytic domain of one kinase and all the other kinases in the genome. This unique pocket is created on a selected kinase by mutating a large, bulky residue conserved in the ATP binding pocket across almost all protein kinases to a glycine or alanine. This residue is referred to as a "gatekeeper" (Figure 80).[28] No

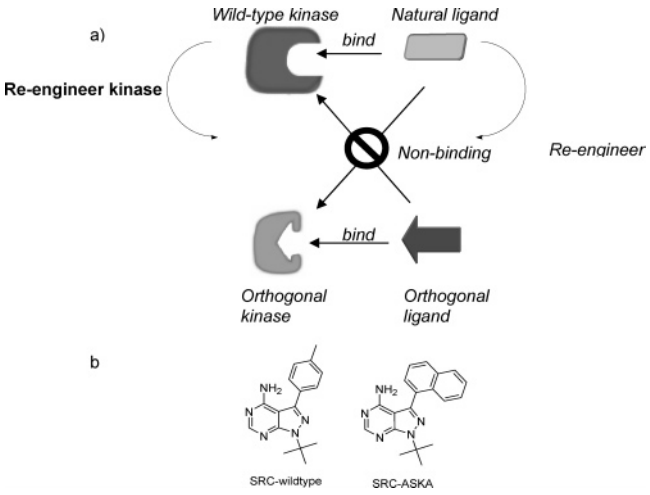


Figure 80. Outline of ASKA technology.

3-D structural information from crystallography is required because the gatekeeper is recognizable from amino acid sequence alignments.[393] Importantly, it has been demonstrated that the mutant kinases maintain their structure and this mutation does not alter the phospho-acceptor site,[394] enzymatic activity,[395] substrate specificity,[394] and cellular function.[28,393,396−398] In addition, this modular approach can be applied across the kinase superfamily since the ASKA inhibitor can be accepted by diverse kinases.[28,396] These ASKA inhibitors only weakly bind to the wild-type kinases, their activity is reversible, they are cell permeable, and they have good pharmacokinetic properties including low toxicity.[336,399]

The mutant kinase and the inhibitor represent a "bump" and "hole" approach. There are certain requirements for both components. (1) The sensitized kinase must possess a large enough residue that its removal creates a suitable pocket, (2) that residue must not be occupied by an alanine or glycine in other protein kinases, (3) the mutation should be functionally silent, (4) the mutation should use the A*TP with high catalytic efficiency, and (5) the identified residue should be generalizable to other protein kinases, though this is not a strict, but ideal, requirement. On the other hand, the allele-specific inhibitor also requires (1) that the binding orientation of the parent compound must be known or easily predictable, (2) that the ASKA analogues must be synthetically acces-

sible, and (3) that the inhibitors should be cell permeable and have good pharmacokinetic properties.[392,400]

Shokat and co-workers initially focused on developing ASKAs designed around the oncogenic tyrosine kinase v-Src. They sought ways to selectively study the role of this kinase, particularly its direct cellular substrates. However, this issue was complicated due to the huge number of kinases in the cell. They identified a functionally conserved ATP binding pocket in v-SRC (Ile338) that could be mutated to glycine (v-Src#1′, v-Src-as1) or alanine (v-Src-as2) but did not alter the function of the kinase.[392,395,396,401] Residue 338 is conserved throughout the kinase family with bulky side chains (threonine, isoleucine, leucine, methionine, or phenylalanine), the removal of which creates a path for access to the binding pocket.[392,402] The gatekeeper has been exploited in the design of serine/threonine and tyrosine kinases.[400,403] The engineered kinase was designed to accept a nonnatural phosphate donor substrate (A*TP) that is poorly accepted by the wild-type kinase. As a lock and key compliment to the removal of the bulky residue on the kinase, the A*TP analogue is designed to bear a bulky substituent at the N-6 position of ATP. Examples include N^6-(benzyl) ATP, N^6-(phenethyl) ATP, and N^6-(cyclopentyl) ATP.[395,400,402,404] This acceptance of the modified γ-^{32}P-labeled nucleotide allows for the selected kinase to be traced in the presence of other protein kinases. This strategy has been applied to identify an analogue of a pyrazo-lo[3,5-*d*]pyrimidine (PP1, Figure 79) based inhibitor that showed potent inhibition with an IC_{50}= 1.5 μM, without significant inhibition of the wild-type kinase.[24,405] Importantly, Shokat and co-workers have shown their inhibitors have no in vitro off-target effects, and the addition of their inhibitors to wild-type *S. cerevisiae* resulted in few transcriptional changes.[24,393,406] Recent work has used PP1 to identify an even more potent kinase inhibitor and this inhibitor, 3-benzylpyrazolopyrimidine triphosphate (3-benzyl-PPTP), was accepted by three divergent kinases suggesting its possible general use across the kinase superfamily (Figure 81).[407]

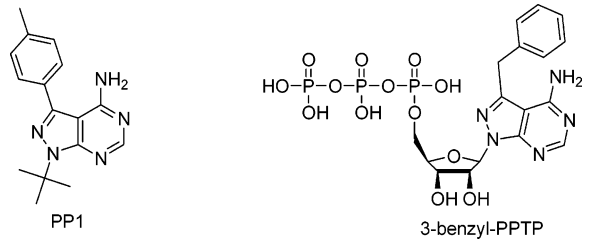


Figure 81. Structure of PP1 and 3-benzyl-PPTS.

This approach has been used in a large number of reports, often in collaboration with Shokat's lab, and several reviews are available.[24,28,388,392,400,406] Here, we wish to provide a general overview and highlights. This technique has been used to explore the role of v-Src in the transformation of 3T3 fibroblast cells,[405] and the role of the cyclin-dependent kinase Cdc28 (CDK1) in a budding yeast strain whose inhibition caused pre-mitotic cell cycle arrest distinct from the arrest seen in temperature-sensitive *cdc28* mutants. This validated the approach as a generalizable system for inducing conditional alleles,[393] and it was this approach that provided for a more specific probe of protein function than the use of temperature-sensitive alleles. It did this by only inhibiting one functional aspect of the sensitized kinase, as opposed to the total loss of function from protein unfolding in the temperature-sensitive mutant.[24,393]

Drubin and co-workers undertook a thorough examination of the role of the function of Cla4p, a p21-activated kinase from budding yeast.[397] Drubin et al. have also used the ASKA approach to study the cortical actin cytoskeleton in yeast where 1NAPP1 treatment of an ASKA mutant form of actin-regulating kinase (Prk1) provided rapid and reversible control of cortical actin dynamics that was not available through traditional approaches.[391,408] An important example by O'Shea et al. was the study of the inhibition of the cyclin-dependent kinase Pho85 in budding yeast that revealed its role in the regulation of genes involved in the cellular response to environmental stress and the crucial role of the kinase in a metabolic pathway.[398] This is important because these findings were missed by deletion/knockout analysis alone.

This strategy has also been used to identify direct targets of protein kinases. In one case, Ronai et al. used a c-Jun amino-terminal kinase mutated to accept a [γ-^{32}P]N^6-(phenethyl) ATP analogue that resulted in the specific phosphoryaltion at amino acids 216 and 353 of its target heterogeneous nuclear ribonucleoprotein K, hnRNP-K.[400] This same strategy was used by Ganem and co-workers to identify Kaposi's sarcoma-associated herpesvirus K-bZIP protein as a direct substrate of baculovirus-expressed mutant CDK2/cyclins (CDK2-as1) in BCBL-1 cell extracts.[409]

Shokat and co-workers have also found surprising features of v-Src substrates by their ASKA method.[410] They identified several novel cytoskeletal substrates involved in actin assembly and identified the protein Dok-1 as a direct substrate of v-Src. From studying the direct substrates of v-Src, they were able to develop a model for the assembly of a retrograde signaling pathway in v-Src transformed cells. Interestingly, they showed data that contradicted the commonly accepted models of kinase specificity by demonstrating a progressive phosphoryaltion model in which the v-Src phosphoryaltion of substrates can be regulated at the level of protein complex formation. In addition, they showed only a limited number of sites on heavily phosphorylated proteins are directly phosphorylated by v-Src. This suggests that a prediction of direct phosphorylation sites based solely on the optimal sequence specificity of a particular kinase may be misleading.[400,410]

Two recent examples of the ASKA system have been used in vivo in mice. The first example focused on the study of the v-ErbB oncogene in a nude mouse tumorigenesis model.[411] The v-ErbB ASKA was shown to be fully functional in NIH 3T3 fibroblasts as well as in mice. The designed inhibitor, NaPP1, selectively inhibited the v-ErbB ASKA in vitro and inhibited ASKA-induced tumors upon treatment in mice, but the wild-type did not. The group also targeted PI3K using an epidermal growth factor receptor (EGFR) inhibitor in a demonstration of combinatorial efficacy in cancer therapies through the targeting of two targets in a complex signaling cascade.[336,411]

Another interesting approach was the use of in vivo conditional protein knockout technology in the study of α-Ca^{2+}/calmodulin-dependent protein kinase II (αCamKII) in ASKA transgenic mice.[412] This system allowed for inhibition of the kinase on the minute time scale. When this kinase was overexpressed in mice, it caused learning and memory deficits that were corrected upon continuous treatment with their designed analogue inhibitor. Discontinuing the administration of the analogue inhibitor restored the pretreatment phenotypes. This ability to monitor the role of the protein with tight temporal control revealed that CaMKII

reactivation during the first post-learning week is essential for the consolidation of long-term memories in the brain.[336,412]

A number of additional studies using this technology have been reported. Hayday et al. demonstrated the use of ASKAs in complex immunological systems by developing a novel $p56^{Lck}$ ASKA and used this in combination with aggregate fetal thymic organ culture to analyze a crucial role for the kinase in mammalian cell development, particularly lymphoid development.[413] It was shown that $p56^{Lck}$ showed a dose-dependent response in thymocyte development, and the ASKA approach was superior in many regards to previously available methods for the study of developmental immunology.[413] Klionsky et al. have also used this approach in identifying a novel role for Apg1 distinct from its catalytic kinase activity in the induction of autophagy.[414]

Hahn et al. used AKSAs of Kin28 and Srb10, homologues of CDK7 and CDK8, to determine their role in RNA polymerase II (Pol II) transcription, something previously unclear.[415] Inhibition of these kinases both in vitro and in vivo dramatically decreased transcription, indicating their role in the promotion of transcription, and both were shown to have overlapping roles in promoting the ATP-dependent dissociation of the preinitiation complex (PIC) into the scaffold complex. In addition, these engineered kinases were used to identify previously unknown substrates within the PIC.[415]

Hollingsworth et al. used the ASKA method to understand the role of Mek1 kinase in double-strand breaks (DSB).[416] They showed, contradictory to other reports, that Red1, a meiosis-specific chromosomal component in budding yeast, was not a substrate of Mek1 and that Mek1's activity functions downstream of *RED1*. This again illustrated that precise pathway dissection is possible by the ASKA method.

Last, the ability to study a kinase's role in a precise high-resolution execution point experiment was demonstrated by Winey et al. in their exploration of the role of Mps kinase in kinetochore attachment during mitosis.[417] Mps1 was known to have roles in the mitotic checkpoint and in spindle pole body (SPB) duplication, but they used an ASKA Mps1 to identify a novel role for the kinase after SPB. They showed that cells with inhibited Mps1 function exhibited defects in mitotic spindle formation, sister kinetochore positioning at the metaphase, and chromosome segregation during anaphase.[417]

This technology may be applied more broadly to other enzymes and in this case may be more appropriately called analogue sensitive enzyme alleles (ASEA). The ATP motor proteins myosin and kinesin have been studied in this manner. These proteins are involved in controlling muscle contraction, vesicle trafficking, and cell motility. Mutants of these proteins have been engineered to respond to analogue inhibitors, thus providing tight control over specific motors to determine their function.[28,336] An initial report sought to design an isozyme-selective inhibitor by creating a mutation of a Tyr61 to glycine in rat myosin-1β.[418] The mutated myosin functioned near wild-type levels, and it actually increased its ATPase activity and actin-translocation rate. The analogue inhibitors inhibited ATP hydrolysis by more than 40-fold over the wild-type and thus locked the Y61G myosin-Iβ tightly to the actin. An inhibitor was found, N^6-(2-methylbutyl) ADP (Figure 82), that abolished Y61G-mediated actin filament motility but not that of the wild-type. An in vivo study in transgenic mice used the mutant myosin-Iβ to explore its role in hair cells, the sensory cells of the ear.[419] The mutant myosin was expressed in utricular

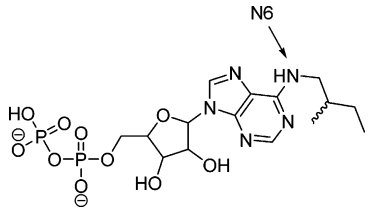


Figure 82. N^6-(2-Methylbutyl) ADP.

hairs of the transgenic mice, and the inhibitor was shown to rapidly block adaptation to positive and negative deflections in the transgenic but not wild-type mice. This clearly demonstrated the role of myosin-Iβ in the hearing process.[419]

Mercer et al. have used the ASEA method in understanding the role of a sensitized myosin Vb mutant.[420] The myosin V family, composed of three members, has been implicated in human and murine disease, synaptic transmission, secretion, and plasma membrane recycling. In particular, myosin Vb has been implicated in membrane transport, specifically transferrin recycling. It was shown in this study that inhibition of the mutant myosin Vb in HeLa cells inhibited transferrin uptake and led to increased levels of plasma-membrane transferrin receptor. This suggested that myosin Vb functions in traffic between peripheral and pericentrosomal compartments. This chemical genetic method complimented other genetic approaches in the understanding of myosin Vb function and shed new light on this process.

Kinesins are force-generating ATPases that influence cytoskeleton organization in cells and are responsible for cellular movement along microtubules, spindle formation, and chromosome segregation during cell division.[28,421] Their role in cell division makes them a major target in oncology. As in the other systems discussed, the kinesin superfamily bears a conserved bulky group that upon mutation to an alanine or glycine opens up a binding pocket that accepts an N^6-substituted ATP molecule.[28] Kapoor and Mitchison targeted kinesins in the presence of other motor proteins. They developed a cyclopentyl-adenylimidodiphosphate analogue that inhibited the mutant in microtubule-gliding assays but did not inhibit the wild-type kinesin or Eg5, another superfamily member.[421] In this study, removal of the Arg14 residue disrupted the kinesin ability to react with endogenous ATP, thus making the mutant dependent on the analogue. Hydrolysis, not release of ADP, is the rate-dependent step in kinesis, so the group used a non-hydrolyzable form of the analogue to specifically inhibit the mutant kinesin. The allele-specific inhibition and activation of mutant kinesins may be valuable in clarifying their role in cellular processes.[28,421]

Conklin and co-workers first reported a derivative application of this concept to study another large protein family, G protein coupled receptors (GPCRs). This approach was termed receptor activated solely by synthetic ligands (RASSL).[422,423] GPCRs encompass over 1000 distinct receptors and are involved in a myriad of physiological processes from hormone secretion to alterations in heart rate.[424,425] Because of this, they are a major target in drug discovery.[425,426] In addition, GPCRs modulate whole-animal physiological responses that cannot be monitored in purely cellular studies.[424] The diversity of receptors, ligands, and responses has complicated the study of their in vivo function. Though ligands exist for GPCRs, it is difficult to restrict receptor activation to one cellular subpopulation since related receptor subtypes may be activated by the same ligand. Therefore, it

was critical to design methods that would allow for the activation of one specific receptor.[423]

RASSLs overcame many of these obstacles since these genetically engineered receptors are insensitive to endogeneous ligands and can be activated by synthetic small molecules. Typically, mutations are made in a receptor for which a known high-affinity synthetic ligand is available. The mutation reduces the affinity for the endogeneous ligand, but the affinity for the synthetic ligand is retained. In addition, one can control where and when the RASSLs are expressed in vivo through tetracycline-regulated gene-expression technology.[423] The RASSL remains silent in the animal until the synthetic ligand is administered, upon rapid and reversible activation of the receptor, the activated phenotypes and signaling pathways may be studied.[336,424] Additionally, RASSLs may be used to identify orphan GPCRs with unknown ligands.[336]

A number of reviews cover this technology in depth;[336,388,423,424,427] here we simply summarize some of the successes in the area. Conklin et al. first introduced RASSLs based on the human κ opioid receptor, a G_i-coupled receptor.[422] Their prototype receptors showed 200−2000-fold reduction in affinity for the endogeneous receptors but also showed a signaling response to the small molecule spiradoline, and proliferation was induced by RASSL activation in rat-1a tissue.[422] Conklin et al. used these receptors in transgenic mice where activation of the Ro1 G_i-coupled opioid receptor in the heart was expressed by a decreased heart rate.[428] They also showed that these RASSLs can be inducibly expressed in several other mouse tissues and could be used to study and activate G protein signaling in a controllable fashion.[428] Another study using the same technology allowed the group to induce and reverse cardiomyopathy in adult mice and showed that prolonged high levels of Ro1 expression led to physiological responses consistent with cardiac myopathy.[429] This study provided significant insight and control into the dissection and role of G_1 signaling in cardiac pathology.

Colpaert et al. have demonstrated a RASSL approach by mutating the transmembrane domain V in the Ser200Ala and Ser204Ala α_{2A}-adrenoceptors in a study that presented evidence for multiple ligand activation sites.[427,430] Liggett et al. designed a modified β_2-adrenergic receptor for use in a therapeutic receptor−effector complex (TREC) suitable for gene therapy.[431] Conklin and co-workers have gone on to engineer a melanocortin-4 receptor to control G_s signaling in vivo that can be used to control G_s signaling responses such as heart rate and cell proliferation.[432] Likewise, Dumuis et al. described the first RASSL related to serotonin receptors (D100(3.32)A G_s-coupled 5-HT_4 receptor or 5-HT_4-RASSL).[433] This receptor was generated by a single mutation and was insensitive to serotonin but responsive to the synthetic ligand.

In a very interesting study, RASSLs were used to understand the role of receptors in mammalian sweet and umami taste.[434] An opioid RASSL was used to show that dedicated pathways establish attraction or averseness to tastes. This was done by expressing a RASSL for a substance that in the wild-type is normally tasteless and unattractive, but when this complimentary RASSL was expressed in sweet cells, the mice became attracted to it.[434]

Finally, Liang et al. introduced a designer ligand/receptor pair around an adenosine A_3 receptor.[435] Modified adenosines were synthesized, and the receptor was engineered to accept these designer ligands with greater affinity than the wild-type. This group termed these concept "neoceptors", since the ligand recognition profile does not need to correspond to the parent native receptor.[435,436] Extensive modeling of the adensosine receptor has also proven fruitful in providing new ligand/receptor pairs.[437] Additional neoceptor−neoligand pairs have been reported for the antiinflammatory A_{2A} adenosine receptor that have significantly expanded the range of positions for ligand and receptor mutation that would not have been predicted by modeling.[438]

5.4. Disruption of Protein–Protein Interactions

Small molecule inhibitors and modulators of protein−protein interactions are targets of interest in current drug research and are important in signal transduction studies.[439−444] Methods for analyzing protein−protein interactions continue to be developed that may be used as tools in drug discovery and chemical genetics. For example, Piwnica-Worms and co-workers have used luciferase complementation imaging (LCI) in the development of a method that allows for the real-time detection and characterization of regulated and small molecule induced protein−protein interaction in cells and living animals.[445] In terms of our chemical "knockout" analogy, the interruption of protein−protein interaction may be employed as a temporally controlled partial knockdown of one aspect (interaction) of a protein's function.

The small molecule reverse two-hybrid approach is related to the CID method and can be used to detect small molecules that disrupt protein−protein interactions. In this method, one seeks compounds that disrupt dimerization, rather than utilizing small molecules to induce it. Schreiber and co-workers developed a small molecule reverse two-hybrid system (RTHS) that sought compounds that disrupted protein−protein interactions identified by a survival phenotype in cells.[446] In their system, the cytoplasmic domain of the R1 subunit of transforming growth factor-β type I receptor was fused to the LexA DNA-binding domain, and FKBP12 was fused to the B42 transcriptional activation domain (AD). With no inhibitor present, LexA binds to the DNA binding site and B42 is brought into close proximity by the interaction between FKBP12 and R1, which resulted in the transcription of a *Ura3* reporter. When the ura3 protein was expressed, cells were sensitive to the protoxin 5-fluoroorotic acid (5-FOA); however, when the FKBP12 and R1 dimerization was disrupted by FK506, the dimerization and transcription of *URA3* was diminished, and the cells survived.[446] This provided a general method for studying protein−protein interactions that evolved from systems employed in chemical genetics.

A similar RTHS method has also been applied with peptide inhibitors of the dimerization of HIV-1 protease.[447] As an example of the continued use of RTHS, Benkovic et al. exploited the RTHS approach and integrated it with their split intein-mediated circular ligation of peptides and proteins (SICLOPPS) technology, which produces cyclic peptides intracellularly.[448] This system allowed for the co-compartmentalization in the host cells of RTHS with genetically encoded small molecule libraries that provided for the coupling of all the system components to DNA encoding. This provided a systematic method for the identification of small molecules that modulate protein−protein interactions.[448]

As an additional example, Vogt et al. identified small molecule agonists of Myc/Max dimerization.[449] Interfering with Myc/Max dimerization is of interest due to its ability

to regulate Myc activity. This is a target of cancer therapy for its role in tumorigenesis linked to its activating effect on transcription and cell growth, and its repression of differentiation. This is an interesting study since the compounds screened were identified from a combinatorial library, FRET was used to monitor the disruption of dimerization, and two of the inhibitors (Figure 83) identified were shown to

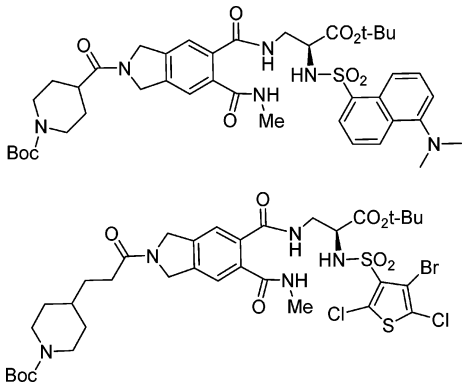


Figure 83. Structures of Myc/Max antagonists.

interfere with Myc-induced oncogenic transformation in chicken embryo fibroblast cultures. In addition, it was an excellent integration of protein−protein interaction screening and cellular phenotypic screening.[449]

Yuan et al. used a fluorescent polarization technique to identify inhibitors of the interaction between the BH3 domain and Bcl-x_L (Figure 84).[450] The Bcl-2 family proteins are

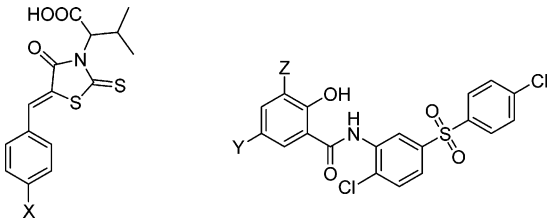
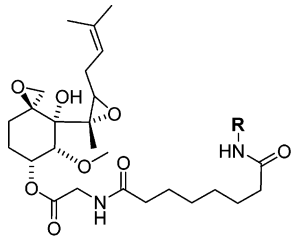


Figure 84. Representative scaffolds of inhibitors of interaction between BH3 and Bcl-x_L.

implicated in apoptosis regulation, though their precise mechanism is not clearly understood. However, it has been shown that BH3 domain mediated homodimerization and heterodimerization are key factors in regulating the apoptotic functions of the Bcl-2 family. In particular, they focused on interfering with the formation of the Bcl-x_L/Bak BH3 complex. They screened 16 320 commercial compounds in a fluorescent polarization assay that monitored the displacement of a fluorescently labeled BH3 domain of BAK from a GST−Bcl-x_L.[451] They identified three compounds that inhibited the BH3 interaction both in vivo and in vitro and induced apoptosis, underscoring the important role of the Bcl-2 family in anti-apoptosis and the maintenance of cellular homeostasis. These compounds were termed BH3 inhibitors and operated in the micromolar range.[450]

5.5. Targeted Protein Degradation

Crews et al. reported an interesting reverse chemical genetics approach to study protein function by manipulating protein levels through selective in vivo targeted protein degradation via the ubiquitin−proteasome pathway induced by a small molecule.[452] Chemical genetic approaches typically attempt to inhibit a protein's function, and few approaches are available that regulate protein levels or target posttranslational processes. As was discussed previously in this report, the ubiquitin−proteasome pathway has a central role in protein degradation and is a desirable target in the control of protein levels in the cells.[311,312] Selectively targeting a protein for its degradation provides an intriguing method for inducing phenotypes and studying protein function, as well as a potential therapeutically relevant approach to remove disease-related proteins.[453] They have developed specific ubiquitination-inducing small molecules called proteolysis targeting chimeric molecules (PROTACS) that are composed of (1) a target-specific ligand, (2) a linker, and (3) a ligand for an E3 ubiquitin ligase (Figure 85).[454,455] E3



R= GGGGGGRAEDS*GNES*EGE-COOH
or GGGGGGDRIIDS*GLDS*M-COOH
*phosphorylated serine

Figure 85. Structure of PROTAC-1.

ligase is an integral part of the ubiquitin−proteasome pathway and is the means by which substrate specificity is conferred. They initially reported three PROTAC molecules, PROTAC-1, -2, and -3, that selectively targeted the degradation of methionine aminopeptidase 2 (MetAP-2), the human estrogen receptor (hERα), and the androgen receptor, respectively.[454,455] However, these were in vitro models requiring cellular microinjection. Next, their goal turned toward in vivo protein degradation induced by the addition of the PROTAC to the cells and that involves a minimum of molecular biological manipulations.

In the subsequent study, Crews et al. turned to two different systems.[452] PROTAC-4 used an orthogonal bump−hole approach with an FK506 derivative, AP21998, as the bump and a mutant $FKBP12^{F36V}$ as the hole (Figure 86). A seven amino acid sequence, ALAPYIP, was chosen as the E3 recognition domain. Additionally, a polyarginine tag was included to improve cell permeability. Cellular levels of FKBP12 were monitored with a EGFP (enhanced green fluorescent protein)−FKBP12 fusion protein construct. The screening, microscopy and Western blotting, revealed that cells treated with PROTAC-4 lost EGFP−FKBP12 and retained their viability.

PROTAC-5 was constructed using a testosterone−androgen receptor (AR) ligand−receptor pair and the same ALAPYIP recognition domain and polyarginine tag seen in PROTAC-4. Similar screening showed the efficacy of PROTAC-5 in degrading a GFP-AR and maintaining cell viability, and the need of the ligand and recognition domain to be linked to affect degradation. They have successfully demonstrated this as a promising system for chemical genetics. While the proteins were fluorescently modified for visualization in these examples, this need not be the case. In addition, this system is modular and may be generalized to a wide variety of proteins and small molecule ligands. This system may be used as a new form of chemical knockout for phenotypic studies or in the identification of

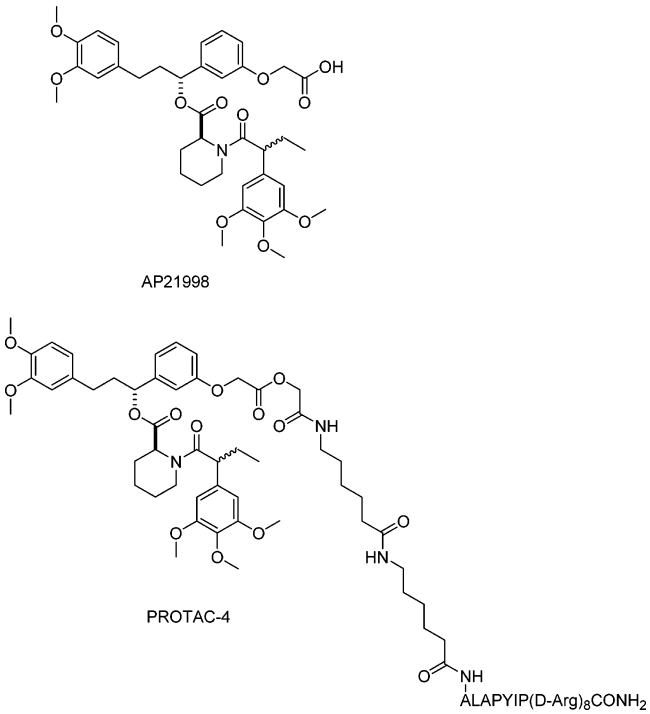


Figure 86. Structure of AP21998 and PROTAC-4.

new ligands for proteins by monitoring a protein's degradation.[452]

5.6. Screening on Beads

Reverse chemical genetic screens using purified proteins may be directly carried out on the synthesis beads themselves; so-called bead staining.[4] Strop et al. used this approach to identify factor Xa inhibitors from a peptide library composed of L-amino acids.[456] Critical to the hemostasis pathway is the proteolytic cleavage of factor X to factor Xa as part of the coagulation cascade. Factor Xa goes on to convert prothrombin to thrombin again by proteolytic cleavage. Thrombin plays a central role in platelet formation and therefore hemostasis. Inhibition of factor Xa is a desirable target for anticoagulants in that it overcomes many of the problems associated with thrombin inhibitors, such as a very narrow therapeutic window.[456] In their report, they synthesized an L-amino acid octamer library, and the primary screening was performed on-bead. The library was screened in the presence of factor Xa conjugated with streptavidin alkaline phosphatase. A colorimetric assay using BCIP (5-bromo-4-chloro-3-indolyl phosphate) was used to identify binders. It was shown that several stained reproducibly but did not stain when the factor Xa was inactivated by preincubation with active site inhibitors. Follow-up analysis revealed that the minimum inhibitory sequence required was a tripeptide, L-tyrosinyl-L-isoleucyl-L-arginyl. From the original hits, peptide mimetics were designed that showed improved potency and high selectivity for factor Xa over other serine proteases. One compound identified showed a K_i of 0.003 μM compared to 40 μM for thrombin. In vitro and ex vivo coagulation assays confirmed the activity of this compound making it a desirable target for anticoagulant therapy.[456]

Kodadek et al. have used on-bead peptoid (N-substituted oligoglycines) library screening in the identification of protein ligands for use in proteomics.[457] The development of protein-detecting arrays is hampered by a lack of specific protein-binding agents. Protein capture agents exist such as antibodies or nucleic acid aptamers, but low weight synthetic protein ligands are desirable due to there economical ease of production and stability.[457] Peptides offer the synthetic versatility but suffer, as do most synthetic molecule−protein complexes, from low binding affinities when compared to antibodies. Additionally, peptides are sensitive to proteases. Kodadek and co-workers have devised a system to overcome these two issues. They showed that two noncompeting modest-affinity ligands immobilized at a high density on a surface can cooperate and achieve high affinity and specificity without the need for linker optimization.[458,459] To overcome the protease sensitivity, they chose to synthesize a library of peptoids that provide for highly diverse libraries since they incorporate primary amines as a "submonomer" diversity element of which hundreds are available. The synthesized 78 125 member compounds were screened on-bead with a fluorescent microscope after incubation with a Texas Red-conjugated Mdm-2 protein fused to a maltose binding protein. Mdm-2 is a regulator of p53 and, therefore, an anticancer target. In addition, they also sought binders for Texas Red-labeled GST. This immobilized on-bead peptoid screening was advantageous because it simulated the conditions of their future use, namely, protein-detecting arrays. They identified binders of Mdm2 and GST with K_D's of about 37 and 62 μM. These compounds will be used in the future in the development of higher affinity co-immobilized ligands (Figure 87).[457−459]

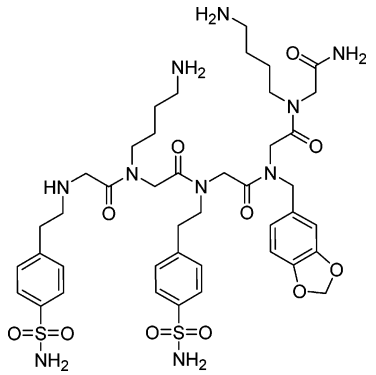


Figure 87. Structure of GST binding peptoid.

5.7. Wide-Angle X-ray Solution Scattering

Fischetti et al. have developed a new reverse chemical genetics method and reported a proof of principle experiment in which they used wide-angle X-ray scattering (WAXS) to detect changes in protein structure upon ligand binding as a moderate-throughput screen in the discovery of new probes.[460] Chemical genetic studies are often hindered by the need to design custom assays to explore certain functions, but in some cases this is impossible. Therefore, generic approaches are desirable that may be applied in any case. Fischetti et al. proposed that their general biophysical method using WAXS may overcome this difficulty. WAXS is able to generate data sensitive to secondary, tertiary, and quartenary structural elements.[461] Short-angle X-ray scattering (SAXS) has been used to generate data about the size and shape of a protein, but it does not give data concerned with conformational changes. WAXS extends upon SAXS by collecting data at wider angles and providing more sensitive structural data at low concentrations. To determine whether WAXS is suitable for chemical genetic studies, they analyzed the

WAXS data response to structural changes of proteins in the presence of known ligand binders. Analysis of the data showed significant structural changes that correlated with known crystallographic data in the presence and absence of the ligand. In addition, it was shown that the magnitude of change in WAXS data was significantly greater in the presence of ligand-induced structural changes than in a case where ligand binding does not result in structural changes. Protein structural changes included domain rotation in transferrin in the presence of iron, the hinge bending motion of the maltose binding protein in the presence of maltose, change in the shape of the binding cleft in alcohol dehydrogenase in the presence of NAD^+, calmodulin refolding in the presence of calcium, and side chain reorientations in adipocyte lipid binding protein and ricin in the presence of arachidonic acid and neopterin, respectively. It was shown that WAXS is sensitive enough to detect large ligand-induced changes such as domain movements, but also smaller changes such as side chain rearrangements. Therefore, this highly accurate method may become a valuable tool to identify induced changes in native proteins and overcome a serious bottleneck in chemical genetic studies.[460]

5.8. Gene Expression Regulation

Alternative "knockout" methodologies exist that function by operating on target genes other than the proteins themselves. For example, engineered zinc finger peptides targeting unique genome sequences through gene transfection have the ability to modulate gene expression.[462−465] In addition, nucleic-acid-based technologies exist that are known to inhibit DNA/RNA function. These systems or molecules include antisense triplex-forming oligonucleotides, ribozymes, aptamers, and small interfering RNAs.[466−468] Of course by regulating gene expression one therefore affects protein levels or function, and while these above techniques are powerful, they comprise their own dynamic field that lies outside the scope of this chemical genetics review.

Golub et al. have described a new method termed gene expression-based high-throughput screening (GE-HTS) for identifying compounds that modulate biological processes. Unlike techniques such as cellular phenotypes or reporter constructs, this is done by performing RT-PCR and quantifying the PCR amplicons by mass spectrometry.[469] This screening system requires no specialization or customization of the assay or the reagents and, as opposed to other gene expression based techniques, it requires no previous target validation. These signatures are collections of genes implicated and involved in the process under study. As a validation of this method, they sought to identify compounds that can induce differentiation of acute myeloid leukemia cells by monitoring the gene expression of a five gene signature. From a library of 1739 compounds, they identified eight that gave a signature representative of differentiation and further validated these hits in follow-up screening. This is a general method in which gene expression signatures are "surrogates" for cellular states of interest.

However, one particularly interesting area deserves mention through its use of small molecules for gene regulation. The work of Dervan and his colleagues occupies a significant wing of chemical biology. Although often overlooked in protein-biased surveys of chemical genetics, the regulation of gene expression by small molecules recognizing DNA and the inevitable effects on protein expression levels and their resulting phenotypes corresponds well to the chemical genetic knockout analogy.[470−477] In the regulation of endogenous gene expressions, DNA-binding small molecules have affinities and specificities for DNA sequences sufficient to disrupt key regulatory proteins bound to genomic DNA.[475,478,479] Their pyrrole-imidazole compounds can be designed to target any predetermined DNA sequence.[475] In a classic early example, Dervan and co-workers reported an eight-ring polyamide that targeted a specific region of the transcription factor TFIIIA binding site and interfered with 5S RNA gene expression in *Xenopus* kidney cells.[475] By monitoring transcription both in vitro and in vivo, it was shown that their polyamide was able to enter cells, transit to the nucleus, and disrupt transcription complexes on the chromosomal 5S RNA genes (Figure 88).

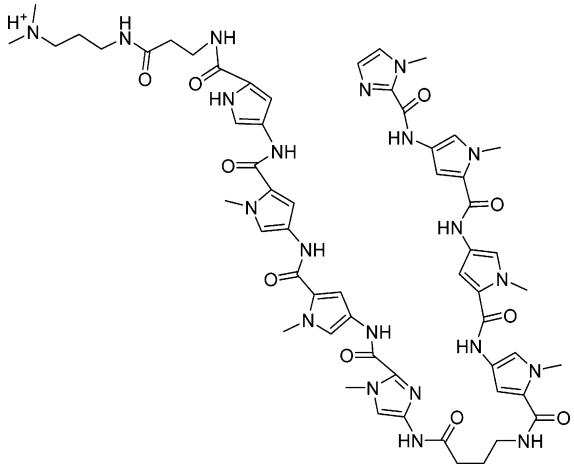


Figure 88. Structure of 5S RNA gene regulator.

Two recent reports by Dervan and his colleagues illustrate unique examples of this technology in in vivo chemical genetic studies. One approach used a DNA-binding small molecule conjugated to an alkylating agent.[474] It was shown that alkylating agents, such as nitrogen mustards, conjugated to DNA-specific polyamides can inhibit transcription elongation at the polyamide binding site in vitro and in cell culture, but previous reports did not study the effects on genomic transcription. In this report, they screened five polyamide−chlorambucil (ChI) conjugates in tumorigenic human carcinoma cells, SW620, and identified one compound that blocked cancer cell proliferation by the down-regulation of the transcription of a specific gene encoding a key component of chromatin. Polyamides with differing DNA sequence specificity were screened microscopically for morphology changes in the cells, and one compound induced an enlarged, flattened, and irregularly shaped morphology. These cells remained viable but failed to divide. Fluorescent labeling of the compound showed its cell permeability, and microarray and siRNA analysis identified the down-regulated gene target as *H4C*, a gene encoding member G of the nucleosomal histone H4 family. Additionally, it was shown that this compound arrested the cell cycle at the G2/M stage without cytotoxicity. Ligation-mediated PCR showed that this conjugate ligand occupies its chromatin target in live cells. A mice study demonstrated that these cells were no longer tumorigenic when injected. It also demonstrated that this compound arrested tumorigenic metastatic colon carcinoma cells upon injection in live mice. This approach revealed a potential new target for cancer research and demonstrated a potentially powerful method for identifying additional new gene targets.[474]

In another report, Dervan et al. inhibited a vascular endothelial growth factor (VEGF) with their DNA-specific small molecules.[473] Angiogenesis is critical for the growth and the metastatic spread of tumors, and VEGF is a key regulator of the process. High levels of VEGF pervade many cancers and part of the responsibility of its elevated levels is due to chronic hypoxia. Hypoxia triggers a multifaceted response, and the inhibition of VEGF, which lies downstream of the heterodimeric hypoxia-inducible factor 1 (HIF-1), is capable of suppressing tumors in model systems.[473] In this report, a sequence-specific DNA-binding molecule inhibited the binding of the HIF-1α/ARNT (aryl hydrocarbon nuclear translocator) heterodimer to its cognate DNA sequence, and this in turn down regulated the *VEGF*. They designed their molecule to target a DNA sequence that encompasses a hypoxia response element (HRE) in the *VEGF* promoter. It was shown that a specific synthetic pyrrole-imidazole bound the HRE and disrupted its binding to HIF. In HeLa cells, real-time quantitative PCR showed lower levels of VEGF mRNA and of secreted protein by ELISA. This pathway-specific approach showed lowered levels of a number of hypoxia-inducible genes and transcriptional analysis provided insight into the activity of multiple hypoxia-inducible genes. This approach of targeting transcription factors by interfering with protein−DNA interactions offers a potentially powerful way of specifically targeting certain phenotypes, as was shown in this case with a cancerous phenotype.[473]

5.9. Small-Molecule Microarrays

Conceptually, small molecule microarrays (SMM) promise to be ultra-high-throughput reverse chemical genetic approaches (Figure 89). However, they have not yet matured

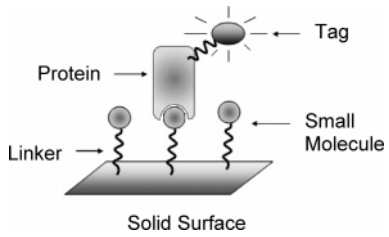


Figure 89. Small molecule microarray.

to their full utility as other microarray approaches have, for example, DNA microarrays. Since being introduced in the late 1990s by Schreiber and co-workers,[480] a large portion of the advances in this field have been devoted to optimizing the technology and not to fully developed chemical genetic/chemical biology studies.[481,482] Here, we wish to highlight some of the practical implementations of this technology relevant to chemical genetic studies in addition to our later discussion of their use in target identification.[483]

In a landmark paper, Schreiber used DOS and SMM to identify inhibitors of Ure2p in dissecting glucose signaling.[247] Ure2p is a central repressor of genes involved in nitrogen metabolism, can switch to a prion form, and is part of a signaling cascade downstream of the Tor proteins. In an effort to find the first small molecule inhibitors, they screened an unbiased 3780 member 1,3-dioxane DOS library that was arrayed on functionalized glass slides. The slides were screened with a fluorescently tagged Ure2p, and eight specific binders were identified and resynthesized for cellular screening. The compounds were screened in a *PUT1*−*lacZ* reporter system, since *PUT1* expression is known to be repressed by *URE2*. One compound activated this reporter, similar to the activity induced by rapamycin, and was termed uretupamine A. SAR studies of synthesized analogues led to the identification of a more potent derivative called uretupamine B, and SPR was used to determine the dissociation constants of 18.1 and 7.5 μM for uretupamine A and B, respectively (Figure 90). Follow up screening used transcription profiling with *ure2*Δ yeast deletion strains and showed that the compounds were acting specifically in the cell and only affected genes directly under control of Ure2p. Additional yeast deletion screening showed that the uretupamine pathway of action was involved in *NIL1*, Ure2p, and glucose signaling pathways. This study demonstrated the systematic use of diverse small molecule libraries and SMMs to identify highly specific compounds that can be used to study and dissect cellular systems with precise control.

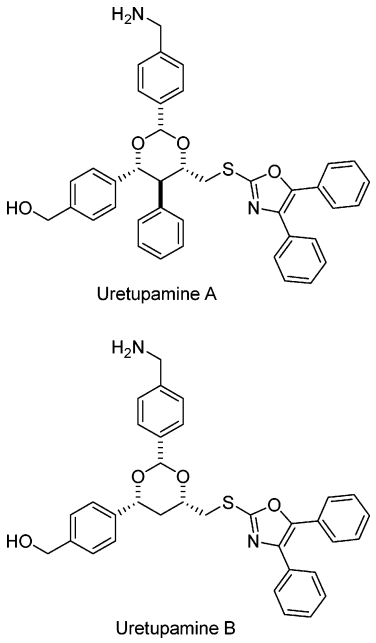


Figure 90. Structure of uretupamine A and B.

Schreiber and co-workers continued their work with SMMs in the identification of an inhibitor of a transcription factor from a 12 396 member DOS library, itself an assembly of three libraries obtained from various DOS synthetic routes.[484] The libraries were printed onto chlorinated slides and screened against a Hap3p−GST fusion protein. Hap3p is a subunit of the yeast transcription factor complex involved in the nutrient-response signaling network in aerobic respiration. After screening, a Cy5 (cyanine 5) tagged fluorescent anti-GST antibody was used to visualize any specific hit. Two reproducible hits from a dihydropyrancarboxamide library were identified from the screening. One compound was shown to bind to GST, while the other, haptamide A, was validated as a Hap3p binder and was shown to bind with a disassociation constant of 5.03 μM by SPR. A thorough SAR study was conducted, and a more potent analogue, haptamide B, was synthesized and shown to have a K_D of 0.33 μM (Figure 91). Cellular whole-genome and deletion transcriptional profiling assays with haptamide B provided evidence that haptamide B selectively inhibited Hap2/3/4/5p-mediated transcription.

The Schreiber group has expanded upon their SMM technology by developing slides derivatized with a diazobenzylidene moiety that allowed for the attachment of

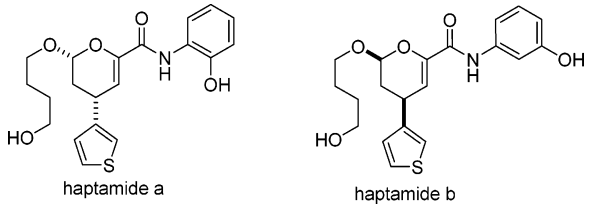


Figure 91. Structure of haptamide A and B.

compounds bearing acidic protons, such as phenols, carboxylic acids, and sulfonamides.[485] Though itself interesting, they further showed the utility of this new platform by screening a DOS-derived phenol-containing fused bicycle and tetracycle library against a fluorescently tagged calmodulin. From this screening, 16 hits were identified, 13 of which were validated by SPR with the strongest binder having a $K_D = 0.12\ \mu M$.

In another study, Schreiber et al. used SMMs as a preliminary screening of a modularly synthesized 18 000 stereochemically diverse and skeletally distinct 1,3-dioxane library (Figure 92).[113] Structural characterization relied on a

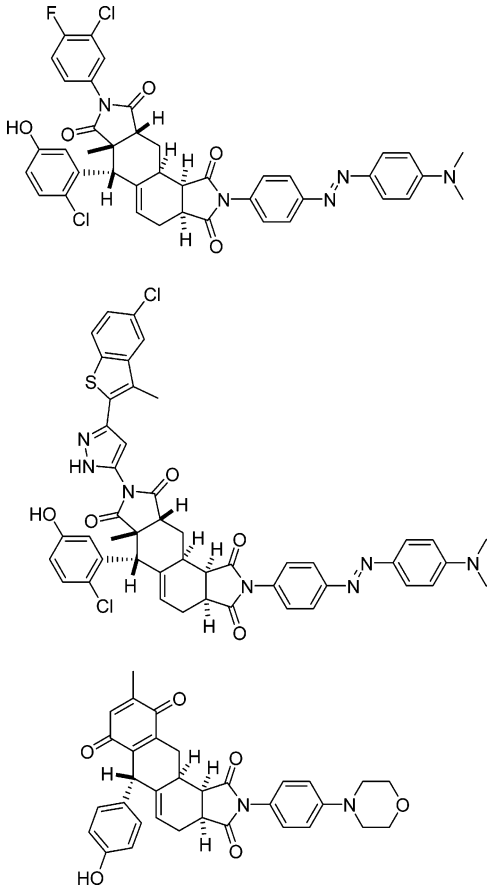


Figure 92. Calmodulin ligands.

combination of binary encoding with mass confirmation and spatial segregation.[90] Screening of a SMM, created by printing library subsets using diisocyanate capture chemistry, with a fluorescently labeled calmodulin showed a patterned incorporation of heptamethyleneamine elements in the molecular scaffold that was identified as a calmodulin-specific binding element. This screening was only part of a study that included phenotypic zebrafish screening in which an inhibitor of heart function was identified whose enantiomer was shown to be inactive. This study showed the ability of small molecule microarrays to screen stereochemically diverse sets of molecules in preliminary screens.[113]

SMMs have also found use in profiling enzyme activities,[486,487] and in an interesting application by Diamond et al., caspase inhibitors were identified by screening compounds that were not covalently bound to the slide.[488] In that study, the compounds were printed in glycerol, the enzyme was sprayed onto the array as an aerosol, and inhibitors were identified by a fluorogenic assay (Figure 93).

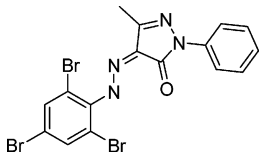


Figure 93. Caspase inhibitor identified by SMM.

Stockwell et al. developed a novel and sophisticated platform for small molecule microarrays that was applied to chemical synthetic lethal screening.[483] In their platform, small molecules are printed onto a 200 μm diameter polymeric disk on a standard microscope slide. The polymer chosen was the biodegradable lactide/glycolide copolymer (PLGA) that was used for its nontoxic and slow-release properties. Over the printed areas was seeded a monolayer of cells; no more than a hundred cells per disk are required. This method places no functional group "handle" demands on the printed compounds, and the compounds were shown to be released over a period of several days. Though they showed that phenotypes other than cell death could be monitored, their most interesting result came from synthetic lethal screening. They printed known compounds, screened against a number of cancer-related genes with siRNA technology, and primarily looked not for lethal combinations but for compounds that increased or deceased activity. From their screening, they identified a compound, macbecin II, that has reduced activity in cells with RNAi-mediated decrease in the expression of tuberous sclerosis 2. Though this is a promising method, the authors do state that it is limited by small printing densities, cross-contamination, and the lack of any ability to control the timing of release of the small molecule.[483]

5.10. Phenotypic Response

Once small molecule regulators for specific proteins are elucidated as the first step of reverse chemical genetics study, a phenotypic response test is followed up with to confirm the protein's function in vivo. As an example of the utility of a chemical tool being useful beyond its initial report, monastrol has been used to answer a long-standing question in biology, namely, whether bipolar spindle formation is essential for the initiation of cytokinesis in mammalian cells.[273,489] To do this, monastrol was used to induce mitotic arrest with monopolar spindle formation. Cells were induced to exit mitosis, and despite their lack of a bipolar spindle, the cells initiated furrowing and cytokinesis in the region of microtubules distal to chromosomes. Therefore, it was shown that overlapping microtubules in the bipolar spindle are not essential for the initiation of cytokinesis.

The wide use of monastrol illustrates an important development in chemical genetics. It has been integrated in a number of studies well beyond its original report and has clearly demonstrated how the ligands and probes discovered from chemical genetic screening can become powerful tools in the broader field of chemical biology. In addition, the

monastrol story demonstrates the true power of chemical genetics, namely, how multiprobe approaches used in in-depth studies can reveal tremendous insights into biology. This idea is illustrated by the integrated use of monastrol and Aurora kinase inhibitors to dissect the role of Aurora kinase in cell division.[490−493]

The Aurora family of serine/threonine kinases is essential for mitotic progression. For example, Aurora kinase A has a crucial role in mitotic spindle formation and centrosome maturation, while Aurora kinase B is a "chromosomal passenger" protein essential for chromosomal progression and cytokinesis.[493] A number of chemically unrelated Aurora kinase inhibitors have been described, and additional members of the Aurora kinase pathway have been identified.[253,490−492] For example, Peters and co-workers identified a compound, hesperadin (Figure 94), from their work in the

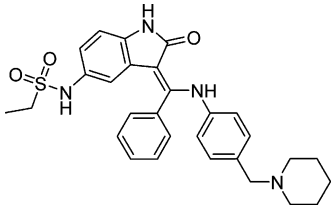


Figure 94. Structure of hesperadin.

synthesis of novel indolinones that inhibited HeLa cell proliferation but did not stop growth.[490] It was shown that hesperadin is an inhibitor of chromosome alignment and segregation and induces this phenotype by inhibiting the function of Aurora kinase B. Through their studies, they showed that Aurora B is required to generate unattached kinetochores on mono-oriented chromosomes, which could promote bipolar attachment and maintain checkpoint signaling.[490]

Likewise, Miller and co-workers identified a piperazine-based compound, VX-680, that was also shown to be a potent and selective inhibitor of Aurora kinases that blocked cell-cycle progression and induced apoptosis in a number of human tumor types (Figure 95).[493] This suggested the validity of the Aurora kinases as a target for multiple human malignancy therapy.

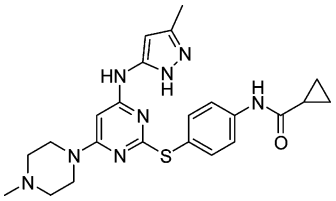


Figure 95. Structure of VX-680.

Another group, Taylor et al., identified a quinazoline compound, ZM44739, as a novel, selective Aurora kinase inhibitor, specifically Aurora kinase B, from a large small molecule library (Figure 96).[492] They used this molecule to

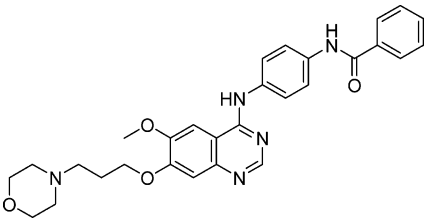


Figure 96. Structure of ZM447439.

probe Aurora kinase function and showed that by targeting checkpoint proteins (BubR1, Mad2, and Cenp-E) to kinetochores, Aurora B couples chromosome alignment with anaphase onset.[492] Taken together, all these results suggest that Aurora B may be required for the proper bipolar attachment of chromosomes to the spindle during mitosis.[490,492] Also, it was suggested in previous work that Aurora kinase B promoted proper bipolar attachment of chromosomes to the spindle by suppressing or reversing the incorrect syntelic (kinetocore−microtubule) attachments.[494] Though studies with hesperadin supported this idea, it was not clear how.[276,490]

Kapoor and co-workers sought to answer this question by employing a multitool approach with monastrol and Aurora kinase inhibitors.[491] This involved the staged reversal of Eg5 and Aurora kinase inhibition. Monastrol arrested cells in mitosis, and after its removal, an Aurora kinase inhibitor was added. Cells were prevented from exiting mitosis by the inclusion of a proteasome inhibitor, and while in mitosis the spindle poles separated due to the restoration of Eg5 activity. However, many of the chromosomes were improperly attached, which indicated that Aurora kinase activity is required to correct these attachments. Interestingly, when the Aurora kinase inhibitor was removed, it was shown that controlled activation of Aurora kinase can correct chromosome attachment errors by selective disassembly of kinetochore−microtubule fibers that do not come under appropriate tension as a result of proper bipolar attachment. This study showed the dramatic power of the products of chemical genetics and also showed the unique opportunity provided by the use of multiple reversible inhibitors in the study of complex biological processes.[273,491]

As an another example, caged molecules were used in a study by Yaffe and co-workers in a study of the role of 14-3-3 protein in G1 arrest and S-phase checkpoint function.[495] This study began with known phosphopeptide inhibitors that bind to a binding motif recognized by all 14-3-3 family members.[496] Classical genetics suffers when studying the modular phophopeptide-binding domain of the 14-3-3 family of proteins due to their high redundancy/homology, which leads to compensation of the knocked-out gene. A chemical genetics approach using UV-inducible inhibitors that target all the 14-3-3s through a conserved binding motif allows for the study of this family of proteins with unique temporal control and rapidity. The group developed an efficient solid-phase route to the phosphoserine compounds "caged" with a 1-(2-nitrophenyl)ethyl cage. An in depth study followed that showed that the caged molecules displaced endogenous ligands and caused premature cell cycle entry, release of G1 cells from interphase arrest, and loss of the S-phase checkpoint after DNA damage, along with high levels of cell death. The authors demonstrated that this caged approach is a useful means for studying protein function and may be generalized to the study of a number of other phosphopeptide-binding domain systems.[495]

6. Target Identification

Target identification is an integral part of chemical genetics studies and is its most rate-limiting and challenging part. Much of the difficulty arises from the weakly binding compounds often identified in chemical genetic screening. The possibility of success in target identification greatly increases with increasing binding affinity. Whereas nanomolar or picomolar binding constants will make target

identification much easier, mid-micromolar results are much more common. However, methods exist and are being developed to overcome these problems if compound/library development cannot match the task and produce stronger binders.[11] Some simple approaches exist such as "the guess and test", where one hypothesizes a target and performs in vivo tests to validate it, or "guilt by association" identification, where targets are implied or hinted at through studies such as mRNA transcription profiling.[29,497]

The most commonly used tool in target identification is the pull down experiment—the classic "fishing experiment" using an affinity matrix. This typically involves the attachment of the "hit" molecule to a solid-phase resin such as agarose gels. The solid linked material is then exposed to a cell extract, commonly by passing the extract over a column of the immobilized material (Figure 97).

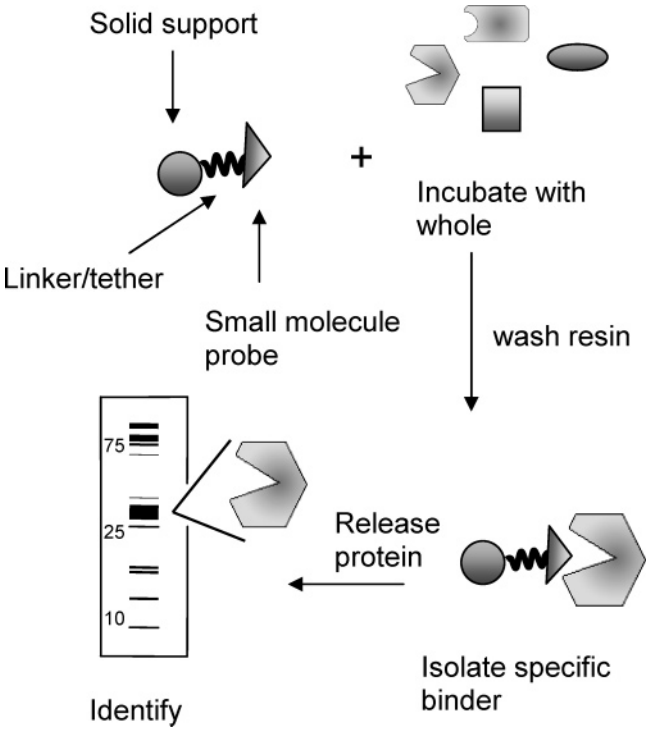


Figure 97. Outline of pull-down assay.

An early example was the synthesis of trapoxin from which a number of trapoxin analogues and the identification of its target played a key role in HDAC research.[1,498,499] Trapoxin is a powerful natural product used in anti-cancer research that has profound effects on mammalian cell growth and morphology. Interestingly, it restores oncogenic cells back to a normal morphology.[498] An analogue, K-trap (Figure 98), was synthesized and used to immobilize an affinity matrix from which, after passage of a mammalian cell extract, electrophoresis, and staining, two proteins were identified. One of these was identified as Rpd3, which was shown to be a HDAC. Along with the later identification of a number of other HDACs, this provided a major step forward in this area of research.[11] In addition, K-trap and trapoxin have been integral in studies identifying additional HDAC inhibitors, such as depudecin, another natural product that like trapoxin is known to revert oncogenic cells.[500]

Geldanamycin (Figure 99) is one of a class of unique compounds that can revert tyrosine kinase-induced oncogenic transformation. Using morphological screening for the reversion of v-Src-transformed 3T3 cells, Whitesell et al. screened

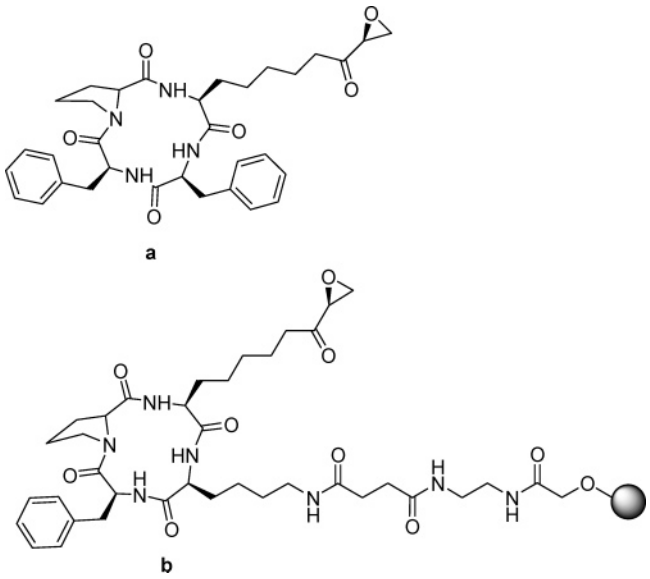


Figure 98. Structure of (a) trapoxin and (b) immobilized affinity analogue K-trap.

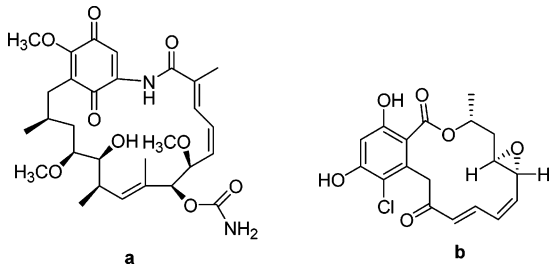


Figure 99. Structure of (a) geldanamycin and (b) radicicol.

a number of benzoquinone ansamycins and identified a position on geldanamycin amenable for derivatization. They prepared a tethered geldanamycin, immobilized it on beads, and incubated these in cell extract. Silver staining revealed a 90 kD protein that was identified as heat shock protein 90 (HSP90). The identification of this class of compound provided for the first molecules capable of studying HSP90 function.[501,502] HSP90 is an anti-cancer related target due to its role as a molecular chaperone that is a key in the conformational maturation of oncogenic signaling proteins. Additionally, HSP90 inhibitors are known to selectively kill cancer cells, even though HSP90 is expressed at high levels in cancer and normal cells. One study has shown that the selectivity of one geldanamycin derivative, 17-allylamino-geldanamycin (17-AAG), is based on an activated high-affinity conformation of HSP90 in malignant cells.[503] This immobilized geldanamycin derivative was also used in a competition assay to identify radicicol, a macrocyclic antifungal antibiotic, as a competitive HSP90 inhibitor (Figure 99).[504] Nakano et al. showed that radicicol binds HSP90 through an immobilized radicicol derivative and Western blotting.[505] HSP90 research is a broad far-reaching field and new compounds, such as the antibiotic novabiocin, which allows for the dissection of interplay between the different domains of HSP90,[506] continue to be developed. Detailed reviews on the role of chemical genetics in HSP90 research are available.[51,502]

Another important early example that has had a major impact in chemical genetics was the identification of the receptor for the immunosuppresant FK506.[507−509] Using a FK506 derivative, affinity matrices were prepared, and the

FK506 binding protein (FKBP12) was identified and determined to be a peptidyl-prolyl-*cis*−*trans*-isomerase. Of course this study was aided by the extremely high affinity between the target and the ligand, but it serves as the archetypical example of affinity pull-down experiments, especially in light of the broad use of the FK506−FKBP12 ligand pair in chemical genetics.

Another classic example by Crews et al. reported the target of a leading anti-angiogenic/angiostatic compound TNP-470, a derivative of fumagillin (Figure 100).[510−512] They

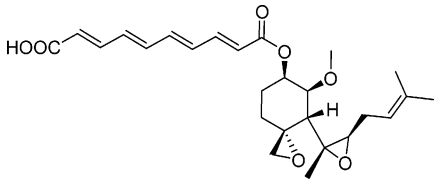


Figure 100. Structure of fumagillin.

made a TNP-470 derivative possessing a tethered biotin. Fumagillol−biotin detected the presence of binding proteins in human umbilical vein endothelial cells (HUVEC) after lysing and SDS/PAGE. After purification and analysis, a 67-kD protein band was identified as the metalloprotease methionine aminopeptidase (Met-AP-2). In addition, fumagillin was shown to be highly specific to this protein. This study was aided by the epoxide functionality on fumagillin, which presumably forms a covalent bond with the target, thus aiding the affinity purification process.

Rosania et al. used 2,6,9-substituted purines in a phenotypic screening to identify compounds that promote cellular differentiation in a myeloid leukemic cell line.[208] One compound, aminopurvalanol, was identified as promoting the expression of differentiated characteristics in human leukemic cells. Though this is interesting in itself, the group went on to identify the target of this molecule through affinity matrices prepared from a carboxy derivatized form of their hit compound. Importantly, the compounds were screened in cell-free *Xenopus* egg extracts that can progress through the entire cell cycle ex vivo. SDS/PAGE and Western blotting analysis identified the target as CDK1. In a human leukemic cell extract immunoprecipitation study, it was shown that aminopurvalanol inhibits both CDK1 and CDK2, and these results indicated that aminopurvalanol preferentially targets the G_2/M-phase transition in vivo.[208]

Affinity matrices were employed by Gray and co-workers in a search for the target of hymenialdisine (Figure 101).[513]

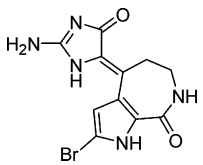


Figure 101. Structure of hymenialdisine.

Hymenialdisine (HMD 1) was isolated from natural sponges in the 1980s based on its antiproliferative effects on cultured lymphocytic leukemia cells. It was shown to be a kinase inhibitor with nanomolar activities against CDKs, Mek1, GSK3β, and CK1 and with micromolar activity against Chk1. They sought to expand the utility of the pyrrolo[2,3-*c*]azepine skeleton by synthesizing diverse HMD analogues and screened these analogues for additional targets by affinity chromatography using mouse brain extracts. Detailed studies revealed nanomolar binding against 11 new targets including p90RSK, KDR, c-Kit, Fes, MAPK1, PAK2, PDK1, PKCθ, PKD2, Rsk1, and SGK. Their analogues showed higher potencies and selectivities when compared to the parent HMD compound, which makes a strong case for the development of this scaffold as a useful therapeutic.[513]

Though commonly used, affinity experiments are beset with drawbacks. First, the compounds need to be derivatized to include a handle for attachment to the resin, unless they intrinsically bear some functional tag that allows for it.[139] The so-called tether effect can alter the activity of the compound, and tedious SAR studies are required to optimize the attachment point. As a solution, this SAR work could be avoided by introducing the intrinsic linker tag strategy.[114] Two other requirements are needed for any hope of success in affinity matrix experiments: (1) high-affinity ligands and (2) a high abundance of target. On a column, immobilized compounds display an area of highly localized hydrophobic stickiness that attracts proteins in aqueous buffer and leads to nonspecific binding.

Kodadek et al. have overcome this problem by mixing mammalian cell extracts bearing the target with large amounts of *E. coli* extract that saturates nonspecific binding sites and competes away nonspecific eukaryotic proteins.[457] Regardless of specific and nonspecific binding, the kinetic half-life of binding must be sufficient to survive stringent washing; this is only possible with extremely strong binding ligands. In addition, unless there is some target enrichment, many targets are low abundance proteins that will be lost among nonspecific binding bands in a gel.[11] Natural product scientists have had greater success in this area, since natural products typically provide much stronger binding. In addition, many contain reactive functional groups, such as an epoxide in epoxomicin, that form covalent bonds and aid in target isolation. [514]

Another interesting alternative is photoaffintity labeling, which involves attaching a photoaffintity moiety and a reporter tag. While this method does require significant SAR knowledge, it does not require immobilization on solid support.[11] Photoaffinity simply uses a photoactivated covalent cross-linking group that forms a bond upon irradiation and a reporter, such as a radioactive isotope or biotin, that allows for isolation or ease of identification. Libraries have been designed bearing photoaffinity groups to specifically exploit this approach.[515]

Labeling proteins with "heavy" and "light" tags and screening the "hit" compound versus an inactive control, followed by mass spectrometric comparison of the two samples, is another approach that avoids many of the common pitfalls in affinity methods.[11] Techniques such as stable isotope labeling with amino acids in cell culture (SILAC) and isotope-coded affinity tagging (ICAT) exemplify these techniques.[516,517] Oda et al. have incorporated the ICAT strategy into a detailed multipart quantitative platform for identifying drug targets.[518] Their systematic strategy sought to overcome many of the problems associated with low-affinity ligand and nonspecific interactions. Their strategy involved the following steps: (1) positive and negative hits were immobilized on affinity matrices and the total amount of binding proteins were enriched in this manner; (2) the proteins were then labeled using ICAT agents; (3) the tagged proteins were isolated, identified, and relatively quantified by LC-MS; (4) candidate proteins were selected by DNA array-based transcription; (5) SPR was employed

to confirm the direct interaction between the active compounds and the selected proteins. A proof of principle experiment was performed with the anticancer agent E7070 (Figure 102), currently in clinical trials, and they identified at least one of its targets to be cytosolic malate dehydrogenase (MDH).[518]

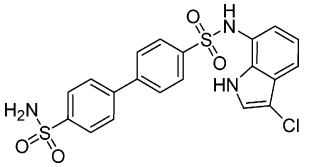


Figure 102. Structure of E7070.

Genetic approaches also exist to tackle this problem. A promising approach is the yeast three-hybrid system (Y3H) (Figure 103).[343,376,383,519,520] This work evolved from yeast two-hybrid screens and has grown in use since its first report by Licitra and Liu in detecting small molecule−protein interactions.[375] Three components are required for these studies: (1) a protein containing a DNA binding domain fused to a small molecule with a ligand binding domain, (2) a protein with a transcriptional activation domain fused to another ligand binding domain, and (3) a bivalent small molecule. The small molecule is composed of a known ligand with an affinity for the protein containing the DNA binding domain, a probe portion of the molecule that is being tested for novel protein binding, and a linker connecting the two faces of the bivalent small molecule. Should the probe portion of the small molecule bind to the protein bearing the transcriptional activation domain, that protein is brought into a proximal relationship with the DNA that allows for the activation of the downstream reporter gene indicating successful target identification.[337] In the case of Licitra and Liu, they used a cross-linked dexamethasone−FK506 heterodimer that induced dimerization of two chimeric proteins, the hormone binding domain of the rat glucocorticoid receptor fused to a LexA DNA-binding domain and FKBP12 fused to a transcription activation domain of the bacterial protein B42. Upon formation of this three hybrid complex, a reporter gene was activated. From their proof of principle cDNA library screen in Jurkat cells, they identified clones of FKBP12 and showed that the Y3H could be used to identify and screen for targets of small molecules.[375]

Advantages of the Y3H system are that the identification of ligand binding proteins is linked to the selection of the cDNAs that encode the proteins, that phenotype and genotype are closely linked, and that these systems are explored in vivo.[521] However, one drawback of this approach is that it is limited to simple, unicellular organisms, but work is underway to overcome this hurdle.[11] Though Licitra and Liu's report was promising, it suffered in that it required nanomolar or subnanomolar affinities for one ligand−receptor pair to operate properly.[375,522] Therefore, important improvements have focused on increasing the sensitivity of the assays through modification of their components.[522]

Cornish and co-workers have done significant work optimizing and improving the three-hybrid system. Initially, they reported an improved anchor system derived from heterodimeric dexamethasone (Dex)−methotrexate (Mtx) where the low picomolar binding between the Mtx and its target, dihydrofolate reductase (DHFR), and Dex and its target, rat glucocorticoid receptor (rGR) significantly improved sensitivity and was adaptable to other fusion proteins bearing GR and DHFR fusion proteins (Figure 104).[519] Interestingly, they have shown that although *E. coli* DHFR−Mtx and murine DHFR−Mtx bind in the low picomolar range, only the *E. coli* DHFR−Mtx system significantly activated transcription, and hypotheses to explain this were presented.[348] They have also optimized the system by integrating the genes encoding the DNA binding domain and the DHFR fusion protein into the chromosome, which resulted in a stabilized transcription readout.[523] Studies to quantify and characterize the relationship between ligand−receptor affinity and transcription readout showed that the Y3H system's sensitivity was suitable for drug discovery but was limited by a small dynamic range.[524]

Similar to Cornish's approach, Meldrum and co-workers used a Dex−Mtx system employing the GAL4 transcription factor.[376] Large numbers of GAL4 activation libraries are available, thus making this system attractive for screening. As a proof of principle, this system was able to isolate four

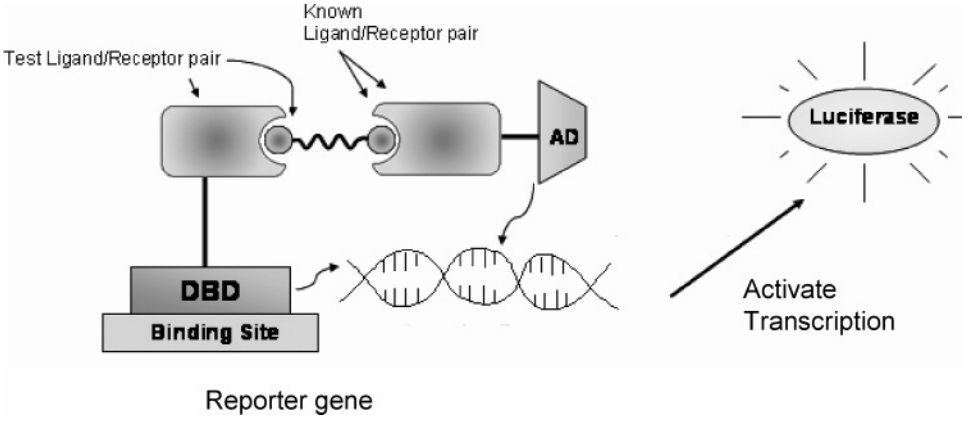


Figure 103. Yeast three-hybrid system.

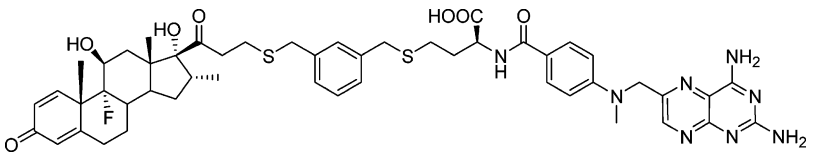


Figure 104. Structure of Dex−Mtx.

library clones of mouse dihydrofolate reductase DHFR using the Mtx-based heterodimer from a library of 5×10^6 clones. Johnsson et al. have used a new anchor system based on a DNA repair enzyme, O^6-alkylguanine-DNA alkyltransferase (hAGT), and the nucleotide analogues O^6-benzylguanine derivatives (BG). These BG derivatives have been shown to be suitable as dimerizers for the control of transcription in yeast in vivo through the covalent labeling of the fusion proteins with small molecules.[525,526]

Peterson et al. used an anchor based on an estradiol−estrogen receptor and a mutant biotin−streptavidin that has been modified to use an estrone (*O*-carboxymethyl) linked to biotin that resembled their previous efforts, but required dramatically fewer synthetic steps, 2 compared to 14. These systems have been shown to be able to efficiently activate transcription in yeast through the ER (ERα or ERα fusion proteins) mediated display of small molecules to target proteins.[377,527]

Kley et al. have used a Y3H system in the first report of the identification of novel drug targets and, of particular interest, identified CDK inhibitors.[521,522] They used roscovitine, purvalanol B, and indenopyrazole linked to a methotrexate and identified known and previously unknown targets at low micromolar binding strengths, and these new targets were validated by in vitro studies. This study is a major step forward in generalizing the three-hybrid approach for drug discovery.[521,522] For more detailed information, Kley has written an excellent review on the use of chemical dimerizers and the three-hybrid approach.[337]

Brown and co-workers have reported a novel cell-based forward chemical genetic approach that used multicopy suppression to determine the cellular targets and potential resistance mechanisms for novel antibacterial leads identified from primary high-throughput screening.[528] Previous assays to identify antibiotics suffered from acellular assays that could identify active compounds readily but provided no information on the mode of action or target of the novel agents. Additionally, they suffered from target-based screening approaches that gave compounds of known mode of action that were often inactive in bacteria due to poor cell permeability.[529] This method focused on molecules that suppressed growth cessation to identify the inhibitor's targets and resistance mechanisms. A commercial small molecule library was initially screened for growth inhibitors in the *E. coli* strain MC1061, a hyperpermeable rough lipopolysaccharide mutant strain. Forty-nine compounds from the growth inhibition screen were selected as leads for a suppressor strain assay that operates on the principle that compounds with antibacterial activity will be inactive or possess reduced activity in organisms that overproduce the cellular targets of those compounds. Three known antibiotics with known targets were used as a proof of concept to demonstrate the validity of their system. Analyses with their selected compounds showed that a majority of the suppressors identified coded for the multidrug efflux pump AcrB, indicating that these compounds were efflux substrates. Additionally, two compounds gave clones coding for the gene *folA*, which encodes dihydrofolate reductase (DHFR), were proven to target DHFR in vivo, and were competitive binders in vitro.[528,529]

cDNA microarrays, the now common and prolific genomic tool, have been used in target identification. However, the results provide clues and indirect evidence of the target, rather than the direct validation seen in many other approaches.[30] A representative use of this approach was presented by Marton et al. in the identification of the transcription factor Gcn4 as a secondary target of FK506.[530] PNA microarrays, closely related to DNA microarrays, have also been used in target identification.

Cellular microarrays that express specific cDNAs at defined locations can also be used in target identification (Figure 105).[531,532] An additional genomic approach is the

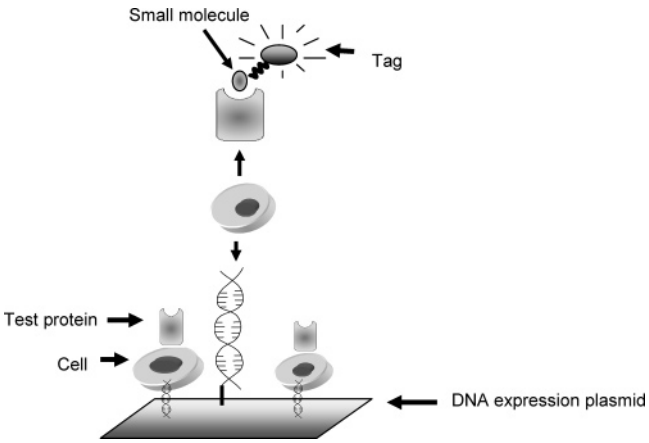


Figure 105. cDNA expression plasmids.

use of synthetic lethality or yeast deletion screens. This approach assembles collections of heterozygous mutants and screens for their hypersensitivity to small molecules.[17,226,533] Lum et al. screened 78 compounds in tagged *S. cerevisiae* heterozygotes where they identified lanosterol synthase as a target of molsidomine and the rRNA processing ribosome as a potential target of 5-fluorouracil (Figure 106). They also reconfirmed known targets for a number of other compounds tested in this proof of principle study.[228]

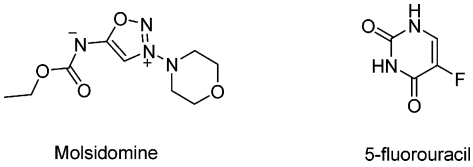


Figure 106. Structures of molsidomine and 5-fluorouracil.

Tanaka et al. introduced another interesting strategy called "drug Western".[534] In this approach, tagged small molecules are used to probe electrophoretically resolved cell extracts or cDNA expression libraries. Drug Westerns involve bacteriophages infecting bacteria grown in a Petri dish with a cDNA library. Lysis caused by the viral infection leads to a clearing, called a plaque, containing a single member of the library. The proteins in the plaque are transferred to nitrocellulose where they are screened with tagged small molecules. Any hit plaques are isolated, a single virus is purified, and the target protein is identified by DNA sequencing.[497] An example is the screening of two million plaques in which a sulfonamide drug was identified as inhibiting the transcription factor NF-YB.[534]

Phage display provides an additional target identification technique (Figure 107). In phage display, the cDNAs are expressed with the proteins of the viral coat. Large numbers of phage can be produced, targets can be selected by an affinity matrix, and the selected phage can be easily amplified for identification.[497] cDNA phage display has been used in the identification of the FK506 binding partner FKBP12,[535] in antibacterial drug discovery,[536] and in the identification

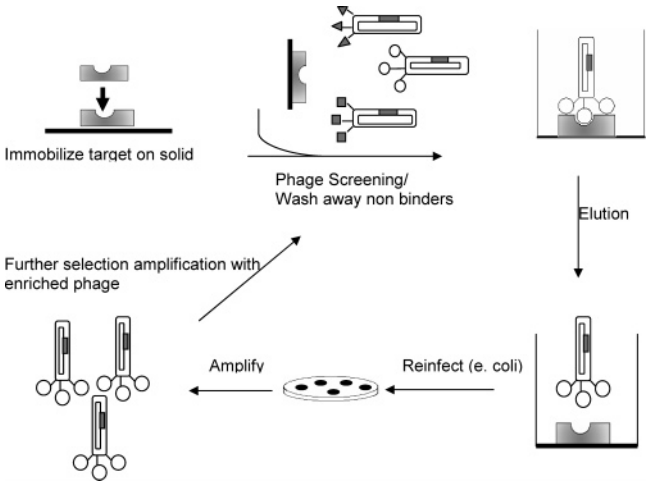


Figure 107. Phage display.

of a doxorubicin target, hNopp140.[537] In a related mRNA display approach, McPherson used a library of mRNA−protein fusion molecules with FK506 to pull down full-length FKBP12, holding great potential for future studies.[538]

Kahne et al. used a mixed chemical genetic and genetic approach to identify the gene target responsible for the activity difference between vancomycin and glycolipid derivatives of vancomycin.[539] Vancomycin is a drug used in the treatment of resistant Gram-positive bacterial infections. Vancomycin binds to D-Ala-D-Ala in peptidoglycan precursors and inhibits the transglycosylation step in peptidoglycan synthesis. Vancomycin-resistant strains express a D-Ala-D-Lac motif to which vancomycin cannot bind. However, hydrophobic substitution to the carbohydrate moiety on vancomycin overcomes this resistance through a different pathway and does so more rapidly. Based on this, the group sought to determine the genetic basis. This was accomplished by employing a standard genetic screening method in which they screened for genetic mutations that confer resistance. Of the resistant strains identified, they all contained the mutation *yfgL* with no known gene function. Through follow up experiments, they implicated *yfgL* as conferring resistance to vancomycin derivatives, but not to vancomycin itself. Interestingly, a genetic approach would have failed, since the *yfgL* deletion is lethal. Since it was shown that *ygfL* gives rise to a discernible phenotype only in the presence of small molecules that perturb transglycosylation in peptidoglycan synthesis, they proposed that *yfgL* is involved in regulating that process.[539]

Proteomic-based approaches have also been employed in target identification. Phillips et al. used a proteomics approach to identify the targets of LAF389, an analogue of bengamides, which are natural products isolated from marine sponges.[540] Bengamides are of interest due to their ability to inhibit tumor growth in a number of human cell lines, and unique activity in NCI 60 screens suggested a novel target. This proteomics approach was chosen since other methods failed to illuminate the target. One of the key advantages of a proteomics-based approach is that it operates at the level of posttranslational modification. This was the motivating factor in choosing 2-D gel analysis for their studies to identify changes in protein levels or posttranslational modifications in bengamide-treated cells. The gel analysis revealed nine proteins that underwent an isoelectric point change when treated with bengamides, and notable changes occurred in the 14-3-3 protein family. Detailed follow-up studies showed that the change to the isoelectric point was due to the retention of the initiator methionine. This suggested that methionine amino peptidases (MetAp) were being inhibited. In vitro studies confirmed that two isozymes, MetAp-1 and MetAp-2, were inhibited.[533,540]

An additional proteomic based approach is protein microarrays (Figure 108).[30,211,541] Protein microarrays derive from

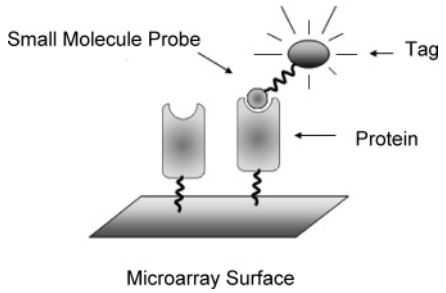


Figure 108. Protein microarray.

and are complimentary to DNA microarrays. Here, collections of proteins are immobilized on a microarray surface and probed for specific binding with tagged small molecules or probed by comparing the profiles of healthy versus diseased tissues.[541,542] These have been used in identifying protein kinase substrates and antigens.[543,544] While a very direct approach, protein microarrays suffer from the lack of large numbers of purified and stable proteins available for immobilization on the microarray surface. Though they have found great utility in proteomics, their development has been slow due to a number of technical challenges.[211,541−543,545,546] One of the most potentially valuable uses for protein microarrays may be in target identification,[547] and a recent report by Schreiber and co-workers used proteome chips as part of their study. [185]

Last, biotech and pharmaceutical companies have developed small molecule based high-throughput screening systems. Obviously, most of these are focused on therapeutics and pharmacological development. However, many of these strategies are easily transferable to more academic studies and have already produced useful information for the community at large. For example, ACADIA's R-SAT (receptor selection and amplification technology) offers a useful lead identification and target validation strategy.[43] Briefly, their system uses cells expressing a drug target gene that in the presence of a small molecule agonist induces partial oncogenic transformation. Release from contact inhibition results in rapid cellular growth in cells expressing the target and offers a readily detectable phenotype-based target identification strategy.[43] This strategy has been used in discovery of the first ectopically binding subtype selective muscarinic m1 agonist[548]among other implementations.[43,549]

7. Conclusion

It is clearly evident that the field of chemical genetics is dynamic and rapidly growing. Chemical genetics has now grown into a major wing of chemical biology and a standard tool of many researchers. The proof of principle stage of chemical genetics has long passed and many are seeking to optimize and expand upon the existing tools. The increasingly diverse libraries being generated will provide a rich source of probes for future studies. While technologies such as small molecule and protein microarrays will surely find greater and more systemized use, it will be interesting to see what

technologies will be further integrated into chemical genetic studies. This concept of integration, something chemical genetics was founded upon, appears to be one of the field's future trends. More and more in the future, simple reports of an inhibitor being identified from one type of screening may well be overshadowed by "full-story" studies that incorporate inhibitor identification with in depth biochemical and genetic analysis. As is already happening, these studies will incorporate much more sophisticated methods for managing and interpreting the data to further integrate and map biological and chemical space in a multidimensional fashion. This type of integration may soon blur the lines between terms such as chemical genetics, chemical genomics, and chemogenomics because studies will incorporate various aspects of all these techniques. As the field matures and moves closer to its lofty goal of having a full chemical toolbox to study each gene product, it will be fascinating to see how these probes will be employed as common tools in a greater variety of diverse biological studies and what kind of unique insights they will provide.

8. Abbreviations

ABPP	activity-based protein profiling
ACL	annotated chemical libraries
ALP	alkaline phosphatase
AP	activator protein
ASKA	analogue-sensitive kinase alleles
ASEA	analogue-sensitive enzyme alleles
BFA	brefeldin A
BR	brassinosteroids
CDK	cyclin-dependent kinase
CID	chemical inducers of dimerization
COX	cyclooxygenase
CPE	cytopathic effects
DCL	dynamic combinatorial chemistry
DHFR	dihydrofolate reductase
DOS	diversity-oriented synthesis
ELISA	enzyme-linked immunosorbent assays
ER	endoplasmic reticulum
FCG	forward chemical genetics
FG	forward genetics
GA	gibberellic acid
G-CSF	granulocyte-colony stimulating factor
GFP	green fluorescence protein
GPCR	G protein coupled receptor
GSK	glycogen synthase kinase
GUS	β-glucuronidase
has	heart-and-soul
HDAC	histone deacetylase
Hh	hedgehog
HRE	hypoxia response element
HTS	high-throughput screening
ICAT	isotope-coded affinity tagging
IFN	interferon
ITSA	small molecule TSA suppressors
JAK	Janus kinase
NFAT	nuclear factor of activated T cells
NSAID	nonsteroidal antiinflammatory drugs
N-WASP	neural Wiskott−Aldrich syndrome protein
PCR	polymerase chain reaction
PI	phosphatidyl inositol
PI3K	phophatidyl inositol 3-kinase
PNA	polyamide nucleic acid
PSA	poly(α-2,8-sialic acid)
PTEN	phosphatase and tensin homolog
RASSL	receptor activated solely by synthetic ligands
RCG	reverse chemical genetics
RG	reverse genetics
RNAi	RNA interference
RTHS	reverse two-hybrid system
SAR	structure−activity relationships
SARS-CoV	severe acute respiratory syndrome corona virus
SLIMS	small laboratory information management system
SMM	small molecule microarrays
SMN	survival motor neuron
SPR	surface plasmon resonance
TGF	transforming growth factor
TGLA	target-guided ligand assembly
TNFα	tumor necrosis factor α
TOR	target of rapamycin
TOS	target-oriented synthesis
TSA	trichostatin A
u-HTS	ultra-high-throughput screening
V-CAM	vascular cell adhesion molecule
VEGF	vascular endothelial growth factor
VSVG	vesicular stomatitis virus G protein

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