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Discovery and Applications of Small Molecule Probes for Studying Biological Processes

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Abbreviations: ACD, Available Chemicals Directory; AIDS, acquired immune deficiency syndrome; ALP, alkaline phosphatase; β-AST-IV, β-arylsulfotransferase-IV; ATP, adenosine-5′-triphosphate; BCIP, bromochloroindolyl phosphate; bHLHZip, basic helix–loop–helix leucine zipper transcription factor; BH3, Bcl-2 homology 3 domain; BLT, blocker of lipid transport; BrdU, 5-bromo-2′-deoxyuridine; CAM, chick chorioallantoic membrane; cAMP, cyclic adenosine-3′,5′-monophosphate; CDK, cyclin-dependent kinase; CFP, cyan fluorescent protein; CHO, Chinese hamster ovary cell; DAMGO, [D-Ala^2, *N*-Me-Phe^4, Gly^5-ol]-enkephalin; DOR, delta opioid receptor; DPDPE, [D-penicillamine^{2,5}]enkephalin; dpf, days post-fertilization; EC_{50}, 50% effective concentration; ED_{50}, 50% effective dose; ELISA, enzyme-linked immunosorbent assay; ER, endoplasmic reticulum; FACS, fluorescence-activated cell sorting; 5-FOA, 5-fluoroorotic acid; FRET, fluorescence resonance energy transfer; FXR, farnesoid X receptor; GFP, green fluorescent protein; GlcNAc, *N*-acetylglucosamine; GSK-3β, glycogen synthase kinase-3β; GST, glutathione *S*-transferase; GTP, guanosine-5′-triphosphate; HDAC, histone deacetylase; HDL, high-density lipoprotein; Hh, Hedgehog; HIF-1, hypoxia-inducible factor-1; HIV, human immunodeficiency virus; HRP, horseradish peroxidase; HSQC, heteronuclear single quantum correlation spectroscopy; IAP, inhibitor of apoptosis protein; IC_{50}, 50% inhibitory concentration; iNOS, inducible nitric oxide synthase; ITSA, inhibitor of trichostatin A; K_d, dissociation constant; K_i, inhibition constant; KOR, kappa opioid receptor; LC–MS/MS, tandem liquid chromatography–mass spectrometry–mass spectrometry; LRET, luminescence resonance energy transfer; MEKK, mitogen-activated protein kinase kinase kinase; MMP2, matrix metalloproteinase 2; MOR, mu opioid receptor; NAD, nicotinamide adenine dinucleotide; NBT, nitroblue tetrazolium; NCI–DTP, US National Cancer Institute, Developmental Therapeutics Program; NIH, US National Institutes of Health; NMR, nuclear magnetic resonance spectroscopy; PAP, adenosine-3′,5′-bisphosphate; PAPS, adenosine-3′-phosphate-5′-phosphosulfate; PIP_2, phosphatidyl inositol-4,5-bisphosphate; Ptc, patched; PTP1B, protein tyrosine phosphatase 1B; RNAi, RNA interference; RPC, rat pituitary cell; SAG, smoothened agonist; SANT, smoothened antagonist; SAR, structure–activity relationship; SDS–PAGE, sodium dodecylsulfate polyacrylamide gel electrophoresis; Smac/DIABLO, second mitochondria-derived activator of caspase/direct IAP binding protein with low p*I*; SFK1, suppressor of FK506 1; SH3, Src homology-3 domain; siRNA, small interfering RNA; Smo, smoothened; SR-BI, scavenger receptor, Class B, type I; TGF-β, transforming growth factor β; TGF-βR, transforming growth factor β receptor; VCA, verprolin, cofilin, acidic domain-containing region of N-WASP; VSVG, vesicular stomatitis virus surface glycoprotein; WASP, Wiskott–Aldrich Syndrome protein; XIAP, X-linked inhibitor of apoptosis protein; YFP, yellow fluorescent protein.

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Introduction

Modulation of protein functions is a powerful approach to dissecting and studying cellular processes. These functions can be altered by introducing perturbations at any point along the pathway from gene to RNA to protein (*Figure 2.1*).

Genetic mutations provide one method to inhibit or activate protein functions. Two strategies have been used. Forward genetics involves introducing random mutations into the genome of a cell or organism, screening the resulting mutants for a phenotype of interest, then identifying the underlying mutation. Conversely, reverse genetics involves introducing mutations into a particular gene of interest, then identifying the resulting phenotypic effects. By analysing the results of these experiments, the functions of the gene and its protein product can be deduced. The genetic approach has had a major impact upon our understanding of biology, due to its broad applicability and exquisite cellular specificity. However, there are some limitations to this approach. First, many mutations are constitutive rather than conditional. This can lead to complications, such as embryonic lethality in genetic knockout experiments involving essential genes. The effects of constitutive mutations can also be compensated by upregulation of related genes and proteins, obscuring the phenotypic effects. Some techniques for conditional activation of mutation phenotypes have been developed, such as the use of inducible promoters and temperature-sensitive mutants. However, the timescale for activation of inducible promoters remains rather long, on the order of hours or days. Furthermore, temperature changes can have broad-ranging effects on multiple proteins and biological processes, decreasing the specificity of experiments using temperature-sensitive mutants. Second, in contrast to protein activity levels, which may vary greatly in response to regulatory signals, genetic mutations are not generally tunable. Third, for mammalian systems, the slow rates of cell proliferation and reproduction and large number of genes make the forward genetic approach less efficient.

Recent advances in RNA interference (RNAi) have provided a powerful new means of modulating protein functions in mammalian cells. Small interfering RNAs (siRNA) are used to knockdown a cognate mRNA by targeting it for degradation. This approach provides improved temporal control over protein function relative to most genetic approaches; however, the timescale is still in the order of hours to days, much slower than many dynamic biological processes. Furthermore, RNAi can only lead to functional inhibition and does not provide the possibility of direct functional

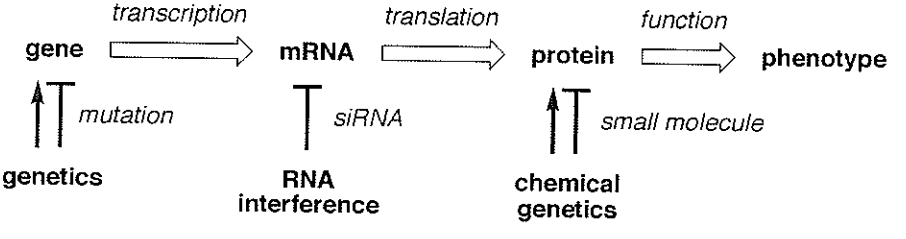


Figure 2.1. Three approaches to modulating protein function.

activation. As an additional consequence, RNAi cannot be used to dissect individual functions of multifunctional proteins, separating, for example, the roles of binding and catalysis.

Chemical genetics is a third complementary approach to modulating protein functions, through the direct interactions of small molecules with protein targets. Small molecules have many features that make them attractive tools to probe protein function. Small molecules allow conditional modulation of protein function on a fast timescale of seconds to minutes. Small molecules can either inhibit or activate protein function, and the effects are often reversible and tunable across a range of doses. Small molecules can be used to modulate individual functions of multifunctional proteins, and may also be sensitive to regulatory post-translational modifications. Small molecule probes identified using one biological model system may be readily applicable to studies in other models or organisms. Finally, small molecule probes may be used as lead structures for the subsequent development of therapeutic compounds. Despite these attractive features, there are two major challenges to using small molecules: broad applicability to any target of interest, and high cellular specificity. Notably, these are the same features that have made genetic and now RNAi approaches so valuable. Until recently, small molecule probes were identified only on an *ad hoc* basis, usually resulting from initial discovery of a cytotoxic natural product and subsequent target identification. This severely limited the applicability of the chemical genetic approach.

In 1994, Mitchison proposed that the advent of combinatorial chemistry and high-throughput screening techniques would allow the small molecule approach to be applied to a broader range of biological targets in a systematic fashion (Mitchison, 1994; Schreiber, 1998, 2003). Furthermore, a variety of curated collections of individually synthesized compounds are now available from commercial and public sources for screening, making this approach accessible to laboratories without access to synthetic libraries. The issue of specificity remains a significant concern; however, ongoing efforts in diversity-oriented synthesis aim to generate combinatorial libraries of complex molecules that may act with high cellular specificity (Schreiber, 2000; Arya *et al.*, 2002).

Herein, we review the current progress through 2003 in identifying chemical genetic probes and applying them to the study of biological systems. We first provide a brief introduction to the methods and techniques used to identify chemical genetic probes. We then describe examples of the identification and application of chemical genetic probes, divided amongst classes of biological targets. In keeping with Mitchison's proposal that small molecule probes may be identified in a systematic, rather than *ad hoc* manner, we have focused on compounds identified by high-throughput screening of combinatorial libraries and compound collections rather than by rational design or by target elucidation of natural products originally identified on the basis of general antibiotic activity. To limit the scope of this review, we have elected to exclude studies involving natural (Turk and Cantley, 2003) and non-natural amino acid-containing peptide libraries, notably early work by Schreiber and co-workers to identify SH3 domain ligands (Combs *et al.*, 1996; Kapoor *et al.*, 1998; Morken *et al.*, 1998), and more recent work by Rana and co-workers to identify inhibitors of protein–RNA interactions in HIV (Hwang *et al.*,

2003). We have also excluded most studies involving the identification of new lead compounds for established drug targets as these have been reviewed elsewhere (Golebiowski *et al.*, 2001, 2003). Novel combined genetic–chemical genetic approaches using small molecules to induce protein dimerization (Crabtree and Schreiber, 1996; Pollock and Clackson, 2002), to inhibit specific mutated kinases (Bishop *et al.*, 2001), and to induce protein splicing (Mootz and Muir, 2002), have also been discussed elsewhere. Finally, we discuss future directions in the field aimed at identifying chemical genetic probes with improved efficiency.

Approaches to identifying small molecule probes

By analogy to genetic techniques, both forward and reverse chemical genetic screens have been used to identify small molecule probes. Forward chemical genetics involves treating a biological system with various small molecules, screening for a specific phenotype of interest, and then identifying the biological targets of the active small molecules. Conversely, reverse chemical genetics involves screening small molecules for binding or functional modulation of an individual protein target of interest, then determining the effects of the small molecule in a biological system. Again, by analysing the results of these experiments, the functions of the protein can be deduced.

A variety of techniques have been used to identify these small molecule probes and to assess their effects. A brief discussion of these techniques is presented in this section.

SOURCES OF COMPOUNDS FOR SCREENING

Both combinatorial libraries and compound collections have been used for high-throughput screening. Combinatorial libraries require an upfront investment in synthetic development. However, once active 'hit' molecules have been identified, additional quantities can be readily resynthesized for further evaluation, and a number of related analogues can usually be generated to optimize the activity of the initial hit and to develop information about structure–activity relationships (SAR). In contrast, compound collections are readily available from commercial and public sources. However, access to additional quantities of hit molecules is often limited, and access to analogues may require subsequent synthetic efforts.

Combinatorial chemistry involves the rapid, efficient synthesis of small molecule libraries in which various building blocks and reactions are used to introduce structural diversity at multiple sites in each molecule. Major efforts have been directed toward the development of flexible, efficient synthetic routes to combinatorial libraries (Dolle, 2003). In addition, a number of specific techniques have been developed for combinatorial chemistry. Both solid phase (Guillier *et al.*, 2000; Yu, and Bradley 2002) and solution phase synthetic techniques have been used. Related fluorous phase (Zhang, 2003) and soluble polymer phase (Boyle and Janda, 2002) techniques are also available. Solid phase synthesis offers the advantage of rapid purification of support-bound intermediates; however, translation of solution phase chemistry can be challenging. Libraries can be generated using parallel, split–pool, or mixture synthesis. Parallel synthesis provides each library member in a spatially

separated format, but library size is limited to approximately 10 000 compounds due to practical considerations. Split–pool synthesis allows larger libraries to be synthesized easily in a one-bead–one-compound format (Furka *et al.*, 1991; Lam *et al.*, 1991). However, because positional information is lost during this process, recursive deconvolution or encoding techniques (Affleck, 2001; Braeckmans *et al.*, 2002) must be used to determine the structures of active compounds. One approach has been to couple chemical tags directly to the solid support during library synthesis to generate a binary code that can be read by tag cleavage and gas chromatographic analysis (Ohlmeyer *et al.*, 1993; Nestler *et al.*, 1994). Radiofrequency tags have also been used to direct the split–pool process and to track individual compounds (Moran *et al.*, 1995; Nicolaou *et al.*, 1995).

Among the commercial sources of compound collections, the ChemBridge Corporation (http://chembridge.com) is certainly the most widely cited in the academic literature. While the original collections were curated from academic laboratories in the former Soviet Union, ChemBridge has expanded its inventory to include over 330 000 compounds from a variety of sources. These compounds have been selected on the basis of computed structural diversity and drug-like properties. A number of other commercial suppliers have emerged in recent years. Notably, many of these are now expanding their offerings to include combinatorial libraries. The National Cancer Institute Developmental Therapeutics Program (NCI–DTP, http://dtp.nci.nih.gov) also has several collections of compounds available to the public, comprised of individually synthesized compounds, natural products, and crude marine, plant, and fungal extracts. Recently, a Training Set of 230 compounds with well-defined biological activities and a larger Diversity Set of 2000 structurally diverse compounds have been assembled from these collections to facilitate early screening efforts. Stockwell and co-workers have recently assembled an 'annotated' collection of 2036 commercially available compounds with known biological activities (Root *et al.*, 2003). The authors propose that this mechanistic information may facilitate investigations of new activities identified by screening this collection. The MDL (http://www.mdli.com) Available Chemicals Directory (ACD) of commercially available compounds has also been used as a database for *in silico* screening using molecular modelling. The NCI maintains similar databases of its compound collections.

Of the 56 studies reviewed herein, 25 (45%) describe active molecules from combinatorial libraries, and 16 (29%) describe active molecules from the ChemBridge collection. The remainder were derived from other compound collections or undisclosed sources.

HIGH-THROUGHPUT SCREENING

A variety of techniques has been developed to allow high-throughput screening of combinatorial libraries and compound collections (Croston, 2002; Walters and Namchuk, 2003). Broadly, these can be divided into cell-free and cell-based assays. Cell-free assays may screen for binding to an individual protein target or for activity in a functional biochemical system. These formats allow the assay components to be carefully controlled; however, the applicability of the resulting small molecule probes to studies in cellular systems must be assessed separately. Cell-based screens

inherently test for cellular activity, but may be complicated by the presence of multiple potential targets in each system.

Binding assays can be performed with compounds that are still immobilized on a water-compatible solid support, such as TentaGel, or with compounds that have been cleaved from the solid support, then immobilized onto the wells of a microtitre plate or printed onto an appropriately functionalized glass microscope slide to form a high-density small molecule microarray (MacBeath *et al.*, 1999; Hergenrother *et al.*, 2000; Barnes-Seeman *et al.*, 2003). Binding is detected using a protein target that is labelled with a fluorescent tag, or with biotin to allow conjugation to streptavidin–alkaline phosphatase and subsequent staining with nitroblue tetrazolium and bromochloroindolyl phosphate (NBT/BCIP). Binding of unlabelled protein targets can also be detected using an enzyme-linked immunosorbent assay (ELISA). Alternatively, soluble small molecules can be assessed by competitive binding assays with a known ligand and the protein target of interest. The ligand is either fluorescently labelled for analysis by fluorescence polarization, or radiolabelled for analysis by scintillation counting. Modulation of binding interactions between two appropiately labelled partners can also be monitored by fluorescence resonance energy transfer (FRET). Notably, FRET can also be used in cell-based assays to detect binding interactions between labelled fusion proteins. Finally, biochemical assays often involve enzymatic turnover of a designed substrate that yields, directly or indirectly, a fluorescent signal amenable to automated analysis.

For cell-based screens, reporter gene assays are often used to screen for molecules that modulate signalling pathways leading to a specific transcription factor. Reporter genes, such as firefly luciferase, green fluorescent protein (GFP), horseradish peroxidase (HRP), and β–galactosidase (*lacZ*/β–gal), are incorporated downstream of the transcription factor-responsive promoter. Modulation of the signalling pathway is detected by observation of the reporter gene signal. Because there are multiple potential targets, we have categorized reporter gene assays as forward chemical genetic screens herein. In another approach, whole cell immunoassays, also called cytoblot assays, can be used to probe for up- or downregulation of a particular epitope (Stockwell *et al.*, 1999a). Finally, ongoing advances in pattern recognition software are allowing automated fluorescence microscopy to be pursued as a method to screen for molecules that induce a particular phenotype of interest (Dove, 2003; Yarrow *et al.*, 2003).

Of the 56 studies described herein, 30 (54%) involved forward chemical genetic screens, and 26 (46%) used reverse chemical genetic screens. Furthermore, 31 (55%) cell-based assays were used, in comparison to 25 (45%) cell-free assays. Binding assays were used in 14 (25%) cases, biochemical assays in 13 (23%) cases, reporter gene assays in 12 (21%) cases, and microscopy-based assays in 9 (16%) cases.

TARGET IDENTIFICATION AND VERIFICATION

Identification of the biological target of a small molecule arising from a forward chemical genetic screen can be a major challenge. Moreover, even in the case of small molecules identified using reverse chemical genetic screens, verification of cellular specificity must still be addressed. Broadly, two approaches have been used

for target identification and verification, based upon physical interaction or biological function.

Affinity chromatography is one approach that has been used to identify proteins from crude cell lysates that bind to an immobilized small molecule. However, this requires chemical modification of the small molecule in a manner that does not affect its activity. Furthermore, low abundance targets may not be detected in the presence of non-specific binding. cDNA expression-based methods provide potential alternatives, involving phage display, protein microarrays, and yeast three-hybrid systems. Phage display involves cloning a cDNA library into a filamentous phage, such as M13 (Tanaka *et al.*, 1999). The gene products are displayed on the surface of the phage, and can be selected by binding to an affinity matrix. This method normalizes protein concentrations and allows identification of low abundance receptors. Protein microarrays of normalized concentrations can also be generated by printing proteins onto chemically derivatized glass slides at high spatial densities (Templin *et al.*, 2002; Wilson and Nock, 2003). The slide is then probed with a labelled small molecule and binding is detected by fluorescence or staining. Finally, in yeast three-hybrid systems, a dimeric molecule is synthesized by coupling the small molecule of interest with a known partner, such as dexamethasone (Licitra and Liu, 1996). The known partner binds a cognate receptor protein, such as the glucocorticoid receptor, that is fused to the LexA DNA-binding domain. The cDNA library is expressed as fusion proteins with the LexA activation domain. Thus, binding of the small molecule of interest to a protein target recruits the LexA activation domain and drives transcription of a downstream reporter gene.

Functional approaches to target identification and verification can be carried out using existing genetic and chemical genetic tools in cellular systems, or by analysis of bioinformatic data. If a putative protein target can be proposed, then the effects of the active small molecule can be tested in systems in which the target is knocked out, overexpressed, or mutated. For example, in a chemical epistasis experiment, a small molecule with high specificity should not perturb mRNA transcript levels in a cell line lacking the putative target. Genetic, RNAi, or chemical genetic perturbations upstream or downstream of the putative target can also be used to assess 'synthetic' effects of the small molecule probe. In cases where a putative target for the small molecule is not readily apparent, bioinformatics-based approaches are emerging as a potentially powerful means of target identification. Transcriptional profiling analysis can be used to identify the transcriptional effects of the small molecule, which can then be used to propose possible targets. This will likely be most useful in cases where the target is relatively close to transcription or is, itself, a transcription factor. Transcriptional profiling analysis was used in 7 (13%) of the 56 studies presented herein. An exciting new approach is to use synthetic analysis in yeast to identify genetic knockouts that synergize with or abrogate the effects of the small molecule (Butcher and Schreiber, 2003; Zewail *et al.*, 2003). Again, analysis of these data should allow potential targets to be proposed.

Identification and applications of chemical genetic probes

Herein, we review 56 studies, reported from 1996 to 2003, in which new chemical genetic probes have been identified by screening combinatorial libraries or compound

collections. We have divided these into six groups based on the following biological target classes: cytoskeleton-associated targets, extracellular receptors, signal transduction proteins, transcriptional regulation targets, apoptosis targets and others. Of course, there is some overlap between these groups, but we have attempted to discuss each paper in the most relevant section. We have placed a special emphasis on subsequent applications of these molecules to improving our understanding of biological processes. Notably, over the 56 studies presented, the molecules were applied to cellular systems in 48 (86%) cases, and to organismal systems in 8 (14%) cases.

CYTOSKELETAL PROBES

Rearrangements of the cytoskeleton can occur on a timescale of seconds, making these processes impossible to study using genetic or RNAi approaches. In contrast, the fast diffusion and often reversible action of small molecule probes make chemical approaches to examining the cytoskeleton extremely powerful (Peterson and Mitchison, 2002). Although a significant number of natural products are already available to probe cytoskeletal processes, the vast majority of these target actin and tubulin. Thus, small molecules that target other proteins associated with cytoskeletal function are of great interest.

Blebbistatin and BTS, myosin II inhibitors to probe cytokinesis and muscle contraction

The cleavage furrow is a pucker on the cell surface that forms during cytokinesis and eventually deepens and spreads around the cell until two daughter cells separate (*Figure 2.2a*). Proper positioning of the cleavage furrow is specified by the mitotic spindle; however, the mechanism by which this occurs is poorly understood. To probe this dynamic process, Straight and co-workers screened an undisclosed compound collection for inhibitors of myosin II, a cytoskeletal protein that is involved in cleavage furrow ingression (Straight *et al.*, 2003).

The compounds were screened in a biochemical assay by incubation with human platelet non-muscle myosin II, actin, and ATP. Inhibition of myosin II ATPase activity was then detected by measuring the amount of residual ATP using luciferase and luciferin. One molecule, *(–)-blebbistatin* (*Figure 2.2b*), was active in both the primary ATPase inhibition assay and a secondary myosin II gliding motility assay ($IC_{50} = 2\ \mu M$). In contrast, blebbistatin did not inhibit the ATPase activities of human myosins Ib, Va or X. As hoped, blebbistatin blocked cleavage furrow ingression reversibly in dividing cells within five minutes, but did not affect assembly of the cleavage furrow machinery.

Blebbistatin was then used to probe spatial and temporal control of cytokinesis. In one set of experiments, HeLa cells were arrested in mitosis using monastrol, a small molecule inhibitor of the Eg5 kinesin (see below), then released into blebbistatin for one hour to allow initiation of cleavage furrow assembly. Other small molecules were then added to probe the connections between the mitotic spindle and the cleavage furrow machinery. Microtubule depolymerization with *nocodazole* led to delocalization of myosin II and anillin, another cleavage furrow component, indicating that continuous communication with the mitotic spindle is required to

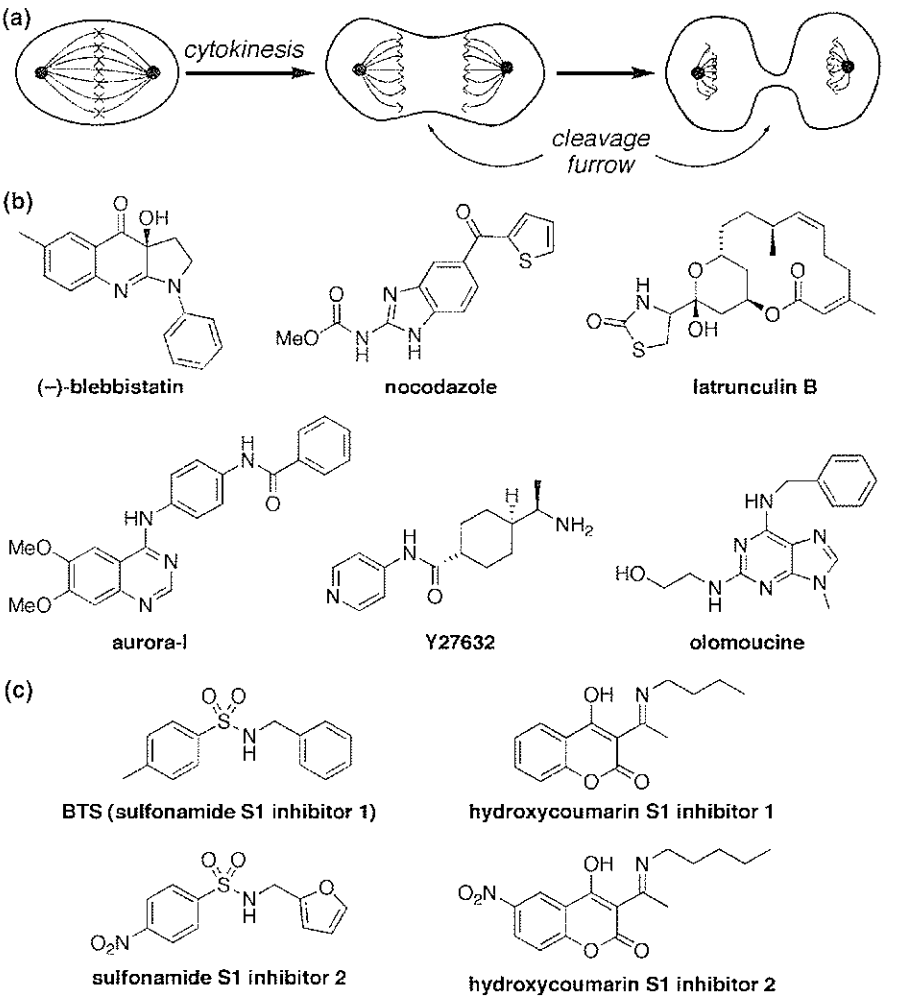


Figure 2.2. (a) Schematic representation of cleavage furrow formation process. (b) Structures of (–)-blebbistatin (the *S*-enantiomer has been proposed), a non-muscle myosin II inhibitor, and other small molecules used to probe cleavage furrow localization. (c) Structures of BTS and other rabbit muscle myosin II S1 inhibitors.

maintain localization of the cleavage furrow machinery. Actin depolymerization with *latrunculin* also led to delocalization of myosin II and anillin, but did not substantially affect the organization of cytokinesis-associated microtubules. These results suggest unidirectional communication from microtubules to the cleavage furrow machinery in this system. Addition of the aurora and Rho kinase inhibitors, *aurora-I* and *Y27632*, led to delocalization of myosin II but not anillin, indicating that localization of these cleavage furrow components is controlled independently. In contrast, addition of the cyclin-dependent kinase (CDK) inhibitor *olomoucine* did not perturb myosin II or anillin localization. This study clearly illustrates the utility of reversible, fast-acting small molecules for dissecting dynamic processes such as cytokinesis.

In a separate, earlier study, Straight and co-workers used the same strategy to screen 16 300 compounds from ChemBridge DiverSet E (100 nM) for inhibitors of rabbit muscle myosin II subfragment 1 (S1) (Cheung *et al.*, 2002). This led to the identification of four active compounds (*Figure 2.2c*), of which the sulfonamide *BTS* exhibited the highest activity ($IC_{50} \approx 5$ nM). In follow-up experiments, BTS was determined not to bind at the ATP binding site of S1, but rather to inhibit the interaction between S1 and F-actin. BTS was further shown to inhibit contraction in a number of specific isolated skeletal muscle fibre systems. This new, small molecule probe should be useful for mechanistic dissection of the connection between ATP hydrolysis and force production in muscles.

Monastrol and HR22C16, Eg5 kinesin inhibitors to probe mitotic spindle assembly

Assembly of the mitotic machinery is another dynamic process that is an attractive target for modulation by small molecules. However, most anti-mitotic small molecules bind directly to tubulin (Hamel, 1996). In an effort to identify small molecule probes that target other components of the mitotic machinery, Mayer and co-workers screened a compound collection for anti-mitotics using a cytoblot assay (Mayer *et al.*, 1999).

The 16 320 molecules in ChemBridge DiverSet E were screened in a whole-cell immunodetection (cytoblot) assay by incubation (≈45 μM) with A549 lung carcinoma cells for 18 hours, followed by treatment with an anti-phosphonucleolin antibody to detect increased nucleolin phosphorylation that correlates with mitotic arrest. This primary cytoblot screen yielded 139 active compounds. A secondary biochemical screen was used to eliminate 53 compounds that directly modulated tubulin polymerization. The remaining 86 compounds were analysed using a tertiary fluorescence microscopy screen, leading to the identification of five compounds that specifically targeted mitotic spindle assembly. One of these compounds, *monastrol* (cytoblot EC_{50} = 22 μM), induced a distinctive phenotype in which the normal bipolar mitotic spindle was replaced by a monoastral microtubule array surrounded by a ring of chromosomes (*Figure 2.3*).

A similar monoastral phenotype was known to be induced by treatment with antibodies specific for Eg5, a mitotic kinesin implicated in establishing spindle bipolarity (Rohani *et al.*, 1998). Based upon this information, a biochemical follow-up assay for Eg5-driven microtubule motility confirmed monastrol as a reversible inhibitor of Eg5 (IC_{50} = 14 nM). In contrast, monastrol did not inhibit conventional kinesin in a microtubule motility assay, and did not affect organelle localization mediated by other motor proteins in interphase cells.

Monastrol has been a valuable tool to probe mitotic spindle formation and cell division (Kapoor *et al.*, 2000; Kapoor and Mitchison, 2001; Faruki *et al.*, 2002; Hauf *et al.*, 2003; Khodjakov *et al.*, 2003). Recently, Salmon and co-workers used monastrol to test whether or not a bipolar spindle is essential for cytokinesis in mammalian cells (Canman *et al.*, 2003). Mammalian cells were treated with monastrol, resulting in mitotic arrest and formation of monopolar spindles. The cells were then microinjected with anti-Mad2 antibodies to induce entry into anaphase. Strikingly, cytokinesis occurred at a high frequency in the monopolar cells. Thus, a bipolar

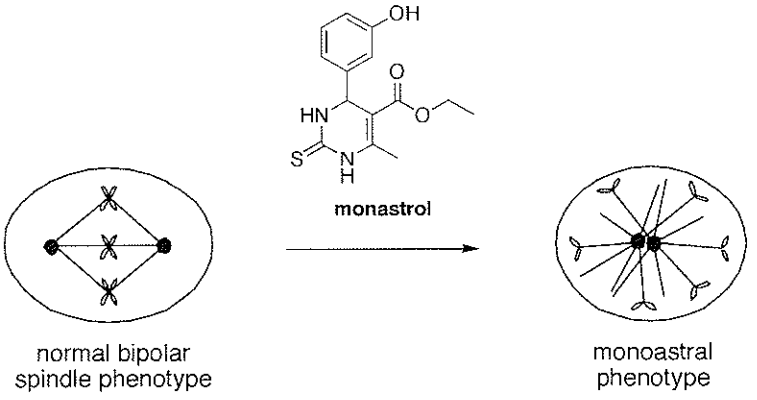


Figure 2.3. Schematic representation of monoastral microtubule array induced by monastrol.

spindle of opposed microtubule arrays is not required for cytokinesis in these cells. Careful examination of cleavage furrow positioning relative to the monopolar spindle suggested a novel, direct role for chromosomes in cleavage furrow positioning, in which localized formation of stable, chromosome-associated microtubules leads to spatially differential microtubule dynamics and stimulates contractility at the cell equator. These studies further highlight the utility of small molecules for dissecting complex, dynamic biological processes.

Kapoor and co-workers recently used a separate cell morphology-based screen to identify a second Eg5 inhibitor, *HR22C16* (*Figure 2.4*, $IC_{50} = 0.8$ nM) from the same ChemBridge compound collection (Hotha *et al.*, 2003). A small tuning library of 50 analogues was synthesized by solid phase parallel synthesis using a novel traceless cyclorelease reaction. These compounds were tested in an Eg5-driven microtubule motility assay, leading to the identification of *aminomethyl-HR22C16*, the most potent Eg5 inhibitor reported (IC_{50} = 90 nM). A photocaged variant of HR22C16 may provide increased temporal control over Eg5 function in cellular experiments.

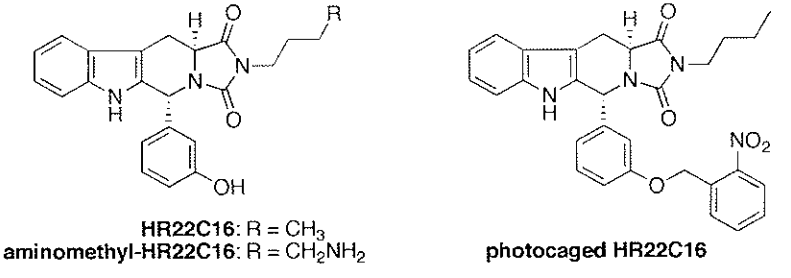


Figure 2.4. Structures of the Eg5 inhibitors HR22C16 and aminomethyl-HR22C16 and a photocaged variant of HR22C16.

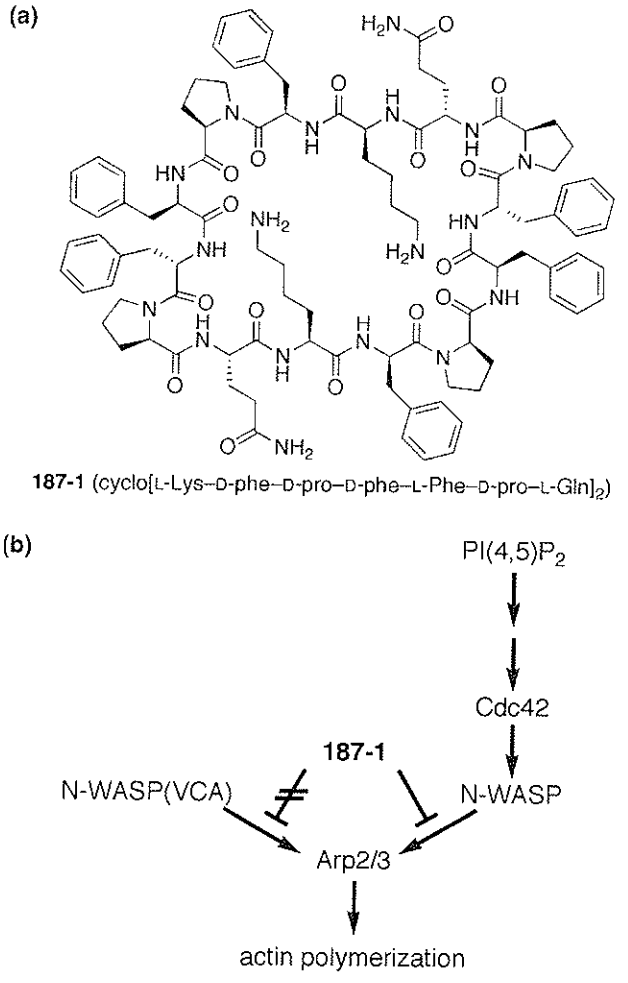


Figure 2.5. (a) Structure of the N-WASP inhibitor 187-1. (b) Arp2/3 activation by upstream signalling through N-WASP or by direct action of the VCA domain of N-WASP (N-WASP residues 392–505).

187-1, an N-WASP inhibitor to probe actin assembly signalling

Actin assembly is a dynamic process that is regulated by upstream signalling pathways. In one model, the Wiskott–Aldrich Syndrome protein (WASP) family integrates these upstream signals and activates the Arp2/3 complex, which directly promotes formation of new actin filaments in motile cells (*Figure 2.5*). While small molecules have been used typically to perturb cytoskeletal proteins directly, they should also be useful for modulating these upstream signalling pathways. To identify such molecules, Kirschner and co-workers screened a compound collection and a combinatorial library for inhibitors of actin assembly (Peterson *et al.*, 2001).

The combinatorial library of 384 cyclic peptides was synthesized by radio-

frequency encoded solid phase synthesis using Fmoc chemistry. The peptides were linked to the solid support via a glutamine side chain and macrocyclization was effected prior to cleavage. This library, in addition to 26 000 'drug-like' molecules from multiple other sources, was screened in a biochemical assay by addition of the compounds (≈60 nM) to *Xenopus* egg extracts, ATP, and pyrene-labelled actin, followed by induction of actin polymerization using phosphatidyl inositol-4,5-bisphosphate (PIP_2)-containing liposomes. Inhibition of actin polymerization was detected by the lack of an increase in pyrene fluorescence over 25 minutes. The most potent inhibitor, *187-1* (IC_{50} = 2 nM), was a 14-residue cyclodimeric peptide from the combinatorial library.

To identify the target of 187-1, the compound was re-tested for inhibition of actin polymerization initiated by factors downstream of PIP_2. The cyclic peptide did not inhibit *Xenopus* extract actin polymerization initiated by the Arp2/3-activating verprolin, cofilin, and acidic stretch homology (VCA) domain of N-WASP, indicating that 187-1 does not directly inhibit Arp2/3 or actin polymerization. In contrast, the 187-1 did antagonize N-WASP-upregulated actin polymerization in a purified protein system, suggesting N-WASP as the target. Direct association of 187-1 with N-WASP was confirmed in photo-cross-linking experiments. Subsequent binding experiments demonstrated that 187-1 stabilizes the interaction of the *C*-terminal VCA domain of N-WASP with its *N*-terminal autoinhibitory domain. Thus, 187-1 inhibits the N-WASP–Arp2/3 protein–protein interaction by an allosteric mechanism, stabilizing the inactive conformation of N-WASP.

Kirschner and co-workers propose that the identification of an N-WASP inhibitor in this broad forward chemical genetic screen implicates signalling through N-WASP as a rate-limiting step in the signalling pathway from PIP_2 to actin polymerization. Furthermore, N-WASP may be a viable therapeutic target for treating intracellular viral and bacterial infections that are dependent on N-WASP activation and X-linked severe congenital neutropenia, which is caused by constitutively active N-WASP. The cyclic peptide 187-1 should be a valuable tool for studying the role of N-WASP signalling in the dynamic process of actin assembly.

Tubacin, a selective inhibitor of the tubulin deacetylase HDAC6

Trichostatin A is a natural product that inhibits nuclear histone deacetylases (HDACs) leading to histone hyperacetylation (*Figure 2.6*). However, trichostatin treatment also leads to acetylation of cytoplasmic β-tubulin, complicating the interpretation of experiments using this small molecule probe. To separate the effects of tubulin acetylation from histone hyperacetylation, Schreiber and co-workers screened a combinatorial library to identify selective inhibitors of tubulin deacetylase activity (Haggarty *et al.*, 2003b,c).

A combinatorial library of 7392 dioxane-containing compounds was synthesized using binary encoded solid phase split–pool synthesis (Sternson *et al.*, 2001b). The library was then biased toward deacetylases by incorporation of carboxylic acid, hydroxamic acid, and *o*-aminoanilide metal-binding head groups (Sternson *et al.*, 2001b). The library was screened (2–5 nM) in a cytoblot assay by incubation with A549 human lung carcinoma cells (Haggarty *et al.*, 2003b). Tubulin acetylation was detected using an anti-acetyltubulin antibody. In a parallel counterscreen, histone

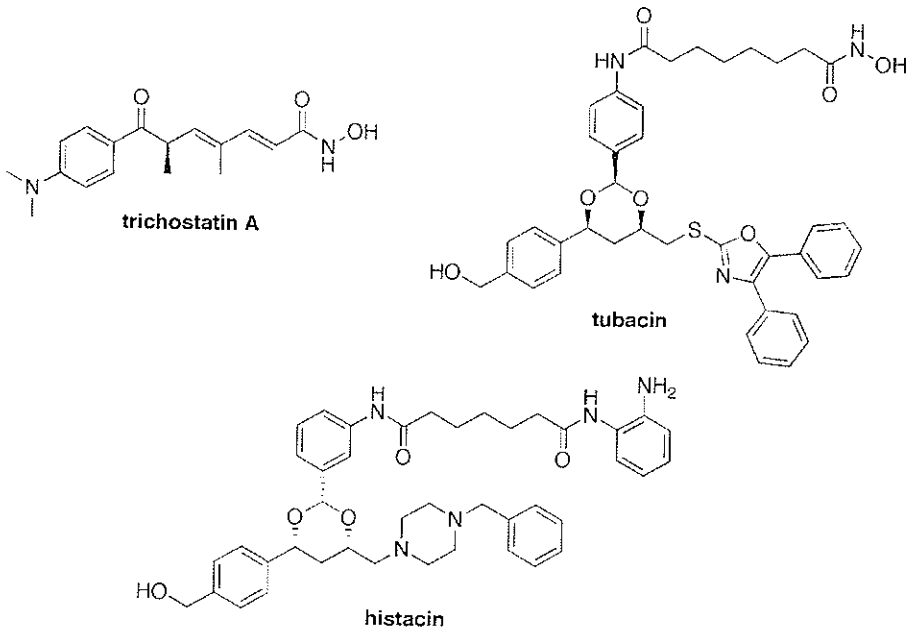


Figure 2.6. Structures of trichostatin A, a non-selective histone deacetylase and tubulin deacetylase inhibitor; tubacin, a selective tubulin deacetylase inhibitor; and histacin, a selective histone deacetylase inhibitor.

acetylation was detected using an anti-acetylhistone antibody. This primary screen revealed 617 active compounds, 273 of which were active only in the acetyltubulin assay, 142 of which were active only in the acetylhistone assay, and 202 of which were active in both assays. Extensive correlative statistical analysis of the chemical and biological properties of these compounds led to the identification of *tubacin* (*Figure 2.6*), a selective inhibitor of tubulin deacetylation (cytoblot EC_{50} = 2.5 nM), and *histacin* (Wong *et al.*, 2003), a selective inhibitor of histone acetylation (cytoblot EC_{50} = 34 nM). These selectivities were confirmed in follow-up immunofluorescence microscopy assays.

Transcriptional profiling experiments demonstrated that tubacin does not alter gene expression. Furthermore, fluorescence-activated cell sorting (FACS) analysis of tubacin-treated cells indicated no effect upon cell cycle progression. These results indicated that the effects of trichostatin on these processes are not due to its inhibition of tubulin deacetylase activity. Distinctive characteristics of HDAC6, a cytoplasmic tubulin-associated HDAC family member with tubulin deacetylase activity, suggested it as a possible target of tubacin. Inhibition of HDAC6 by tubacin was subsequently demonstrated by biochemical and immunofluorescence microscopy experiments with various HDAC6 constructs.

Tubacin was then used to probe the role of tubulin deacetylation in microtubule dynamics. A549 cells were treated with tubacin, resulting in increased tubulin acetylation, then treated with the microtubule depolymerizing agent nocodazole. In contrast to other studies with trichostatin, this experiment indicated that tubulin acetylation is not sufficient to stabilize microtubules, demonstrating the value of

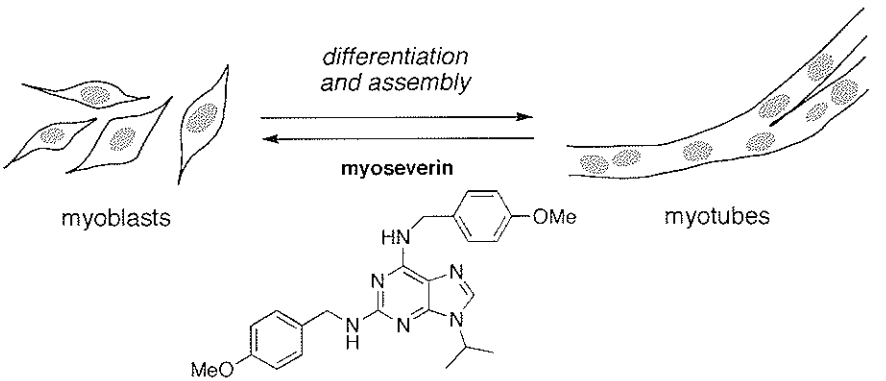


Figure 2.7. Assembly of myoblasts into myotubes and disassembly induced by myoseverin.

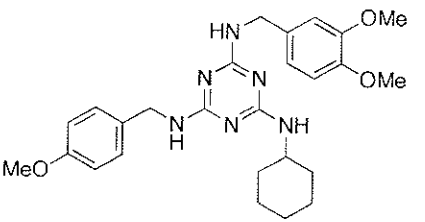
using highly specific small molecules to uncouple the effects of deacetylase activity on the cytoskeleton and chromatin. Notably, tubacin was also found to inhibit NIH 3T3 cell motility, implicating HDAC6 as a potential therapeutic target for antimetastatic and antiangiogenic cancer therapies. Furthermore, increased tubulin acetylation levels are observed in neurodegenerative disorders, such as Alzheimer's disease, suggesting additional potential for tubulin deacetylase inhibitors in treating these diseases.

Myoseverin, an inducer of myotube disassembly

A key feature of skeletal muscle cell differentiation is the fusion of mononucleated myoblasts into multinucleated myotubes. Conversely, during limb regeneration in amphibians, multinucleated myotubes disassemble into mononucleated fragments that grow and divide as individual cells (Brockes, 1997). To explore the possibility of modulating these processes with small molecules, Schultz and co-workers screened a combinatorial library for compounds that induce myotube disassembly (Rosania *et al.*, 2000; Perez *et al.*, 2002).

A library of 2,6,9-trisubstituted purines, originally targeted to CDKs as olomoucine analogues, was synthesized by solid phase and solution phase parallel synthesis (Chang *et al.*, 1999). The compounds were screened in a microscopy-based assay by addition to C2C12 murine muscle cells that had been induced to form myotubes in cell culture. Disassembly of long cylindrical myotubes into chains of smaller rounded cells was scored visually by phase contrast microscopy, leading to the identification of *myoseverin* (*Figure 2.7*) as an active myotube disruptor (24 hr EC_{50} = 11 nM).

Fluorescence microscopy follow-up experiments showed that actin microfilament structure was unaffected by myoseverin treatment while the microtubule cytoskeleton was disintegrated, indicating tubulin as a possible target. This was supported by an affinity labelling experiment with a biotinylated variant of myoseverin and by a biochemical microtubule depolymerization assay. Treatment of myotubes with other microtubule-targeted small molecules, nocodazole, colchicine, vinblastine, or Taxol, also induced myotube disassembly. Importantly, however, only myoseverin and Taxol induced myotube disassembly into viable non-apoptotic fragments, and



triazine microtubule depolymerizer

Figure 2.8. Structure of triazine inducer of microtubule depolymerization.

only myoseverin did so in a reversible manner. Myoseverin also had relatively little effect upon cell proliferation (GI_{50} = 12 μM). Transcriptional profiling experiments indicated that myoseverin modulates the expression of genes involved in cellular responses to tissue injury, such as growth factors and extracellular matrix remodelling proteins. Schultz and co-workers suggest that myotube disassembly may significantly enhance the ability of the resulting myoblasts to proliferate in response to growth stimuli by restoring nuclear autonomy and reconstituting the chromosome complement of normal diploid cells. Thus, small molecules that induce myotube disassembly can be used to probe this process, and may also have therapeutic potential in wound healing and tissue regeneration. This study also illustrates the importance of microtubules in the cytoskeletal structures of non-dividing cells.

Chang and co-workers recently screened a combinatorial library for more potent inducers of myotube disassembly using a *Dania rerio* zebrafish microscopy-based assay (Moon *et al.*, 2002). A library of 110 triazine-based analogues of myoseverin was synthesized by solid phase parallel synthesis. The compounds were incubated (10 nM) with zebrafish embryos at the 1K-cell stage (3 hours post-fertilization) or at the 17-somite stage (17 hours post-fertilization) for five hours. This led to the identification of 14 compounds that induced morphological changes similar to those observed with myoseverin and nocodazole. A secondary biochemical microtubule depolymerization assay was then used to confirm tubulin as a target. Active compounds were also tested in U937 human leukaemia cells to measure growth inhibitory activity. One of the most potent compounds was a *triazine microtubule depolymerizer* (*Figure 2.8*), with 4-fold greater potency than myoseverin in the microtubule depolymerization assay (IC_{50} = 5 nM) and 10-fold greater potency in the growth inhibition assay (GI_{50} = 1 nM). However, the activity of the triazine on myotube disassembly was not reported.

EXTRACELLULAR RECEPTOR PROBES

Extracellular targets are often readily addressed with antibodies since cell permeability is not required. However, small molecules can also be used to probe these targets and provide the additional opportunity to stabilize rather than block receptor–ligand interactions. In this section, we describe several examples of small molecules that target cell surface receptors involved in signalling and recognition. Although ligands for G-protein coupled receptors have generally been omitted,

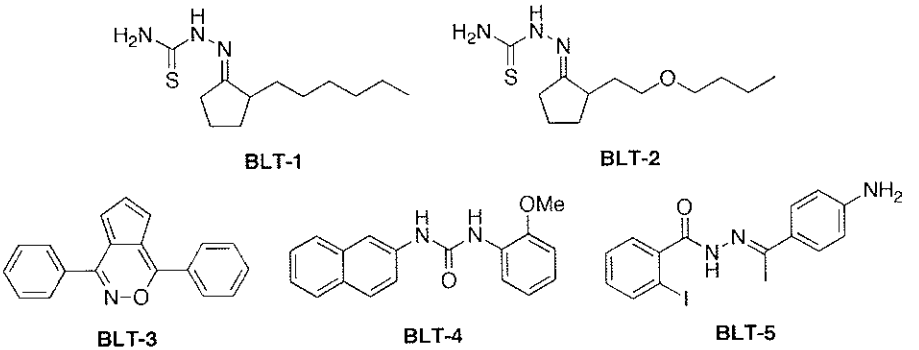


Figure 2.9. Structures of small molecules that inhibit lipid transport and efflux by stabilizing the HDL–SR-BI receptor interaction.

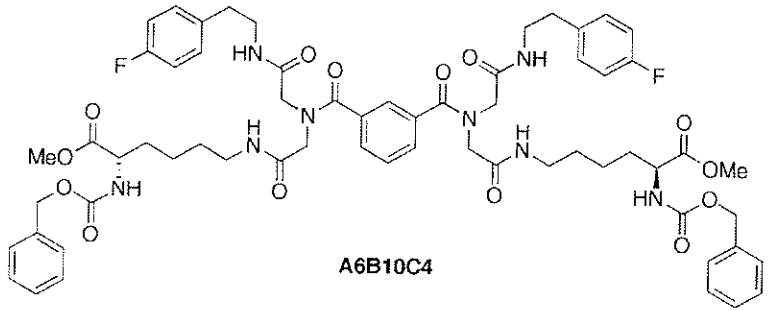
selected reports of novel selective and subtype-selective small molecules are included. In addition, a complementary 'dominant negative' approach involving small molecules that mimic cell surface receptors is described. Other examples involving cell surface receptors have been described in a recent review on small molecule modulation of protein–protein interactions (Berg, 2003).

BLTs, HDL receptor-mediated lipid transfer inhibitors

The high-density lipoprotein (HDL) receptor, scavenger receptor, Class B, type I (SR-BI) mediates both selective uptake of lipids, mainly cholesterol esters, from HDL to cells and efflux of cholesterol from cells to HDL. Alterations in SR-BI expression can affect a wide range of biological processes, including biliary cholesterol secretion, female fertility, red blood cell development, atherosclerosis, and the development of coronary heart diseases. To investigate the poorly understood mechanisms underlying this process, Kirchhausen and co-workers screened a compound collection for small molecule inhibitors of SR-BI-mediated lipid transfer (Nieland *et al.*, 2002).

A collection of 16 320 compounds from ChemBridge was screened (10 μM) in a cell-based biochemical assay for the ability to block cellular uptake of a fluorescent lipophilic dye from HDL into low-density lipoprotein receptor-deficient ldlA Chinese hamster ovary cells expressing stably transfected SR-BI. This led to the identification of five blockers of lipid transport (*Figure 2.9*), *BLT-1* (IC_{50} = 60 nM), *BLT-2* (IC_{50} = 0.35 μM), *BLT-3* (IC_{50} = 0.51 μM), *BLT-4* (IC_{50} = 2 μM), and *BLT-5* (IC_{50} = 7.1 μM). Activities were confirmed and shown to be reversible in a secondary assay for inhibition of ^{3}H-cholesteryl oleyl ether uptake.

Additional follow-up assays demonstrated that the BLTs are specific for SR-BI-mediated lipid transport and do not disrupt classical receptor-mediated endocytosis, the actin and tubulin networks, or the secretory pathway. Furthermore, BLT-1 and BLT-2 also potently inhibited SR-BI-mediated efflux of ^{3}H-cholesterol to HDL (IC_{50} = 0.15 μM and 0.47 μM). However, in comparison to their potencies in inhibiting uptake, BLT-3, BLT-4, and BLT-5 were significantly weaker inhibitors of efflux (IC_{50} = 17 μM, 55 μM, and 75 μM), indicating a possible difference between the



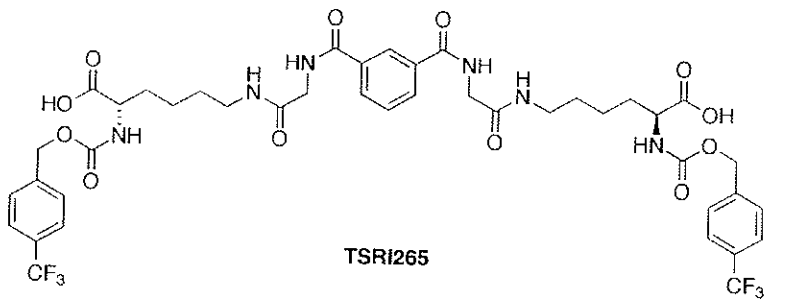


Figure 2.10. Structures of A6B10C4 and TSRI265, small molecule antagonists of the MMP2–$\alpha_v\beta_3$ protein–protein interaction.

uptake and efflux mechanisms. Notably, the BLTs were found to enhance binding of ^{125}I-labelled HDL to SR-BI-expressing cells and to decrease the dissociation rate.

These studies suggest a two-step mechanism for SR-BI-mediated lipid transfer, involving productive HDL binding, followed by coupled lipid transfer. Furthermore, the differential activities of BLT-3, BLT-4, and BLT-5 suggest differences in lipid uptake and efflux mechanisms. The BLTs should be valuable small molecule probes to study the role of SR-BI and HDL metabolism in a variety of physiological processes.

A6B10C4 and TSRI265, MMP2–$\alpha_v\beta_3$ antagonists to block angiogenesis

Association of matrix metalloproteinase 2 (MMP2, gelatinase A) with the endothelial cell surface integrin $\alpha_v\beta_3$ facilitates vascular invasion during tumour-induced angiogenesis. Known broad-spectrum MMP inhibitors block angiogenesis and suppress tumour growth, but also cause severe side effects. To identify selective antiangiogenic compounds, Cheresh, Boger and co-workers screened a combinatorial library for antagonists of the MMP2–$\alpha_v\beta_3$ protein–protein interaction (Boger *et al.*, 2001; Silletti *et al.*, 2001).

A combinatorial library of 600 symmetrical dimeric amides was synthesized by solution phase mixture synthesis (Boger *et al.*, 1998). The library was initially screened in a competitive binding assay as 60 mixtures of 10 compounds each. Purified integrins were immobilized onto microtitre plate wells, then treated with biotinylated MMP2 and library compounds (50 μM total, 5 μM per compound). The amount of bound MMP2 was quantified by HRP-antibiotin ELISA. Deconvolution of the initial hits led to the selection of *A6B10C4* (*Figure 2.10*) as a lead compound

(≈70% inhibition at 3 μM). Synthesis and evaluation of 77 analogues yielded *TSRI265* (IC_{50} ≈1 μM), which was demonstrated to bind directly to $\alpha_v\beta_3$, specifically inhibiting its interaction with MMP2. Notably, binding of $\alpha_v\beta_3$ to its extracellular matrix ligand, vitronectin, was not affected, indicating distinct binding sites.

In chick chorioallantoic membrane (CAM) assays, TSRI265 blocked growth factor-induced angiogenesis, and also inhibited growth of transplanted CS-1 hamster melanoma cells, leading to significant cell death within the tumour mass. These studies demonstrate that small molecules can be used to block specific protein–protein interactions and indicated that $\beta_v\beta_3$, rather than MMP2, may be a viable target for antiangiogenesis cancer therapy.

Peptidomimetic enediols, selective mu opioid receptor agonists

The opioid receptors are G-protein coupled receptors that are found in the central nervous system and mediate pain sensation (Standifer and Pasternak, 1997). Morphine and other morphine-like opioid agonists produce analgesia primarily through interaction with the mu opioid receptor (MOR). The delta (DOR) and kappa (KOR) opioid receptors also mediate analgesia, and can synergize with or antagonize the effects of MOR activation (Pan, 1998). The distinctions between the signalling pathways downstream of the various opioid receptors remain poorly understood. Thus, to develop new tools to dissect the functions of the opioid receptors, Verdine and co-workers screened two combinatorial libraries for selective non-peptidic MOR ligands (Harrison *et al.*, 2002; Shi *et al.*, 2003).

The first library consisted of 16 diastereomeric 1,5-enediols designed as non-peptidic analogues of *endomorphin-2* (*Figure 2.11*), an endogenous MOR ligand, and was synthesized by solid phase parallel synthesis (Harrison *et al.*, 2002). The compounds were screened (10 μM) in a competitive binding assay for displacement of ^{3}H-labelled diprenorphine from MOR-1 stably transfected CHO cells. A secondary assay for *^{3}H-DAMGO*-competitive binding to a MOR membrane preparation led to the identification of the *(S,S,S,R)-1,5-enediol* diastereomer as a MOR ligand (K_i = 8.8 nM) with affinity similar to that of endomorphin-2 (K_i = 1.2 nM). The stereochemical configurations of the aryl side chains in this 1,5-enediol are consistent with those in endomorphin-2, and any deviation led to significantly reduced binding affinity. The 1,5-enediol had good selectivity for MOR over DOR (57-fold, K_i = 0.51 μM versus *^{3}H-DPDPE*) and KOR (150-fold, K_i = 1.3 μM versus *^{3}H-U-69,593*). Several analogues were synthesized and tested, leading to the identification of an *(S,S,S,R)-1,5,10-enetriol* with similar MOR affinity (K_i = 10 nM) and enhanced selectivity (110-fold over DOR, 600-fold over KOR). These results represent encouraging progress toward matching the potency and selectivity of the peptide agonist endomorphin-2 (MOR K_i = 1.2 nM, 10 000-fold over DOR, 9000-fold over KOR).

Interestingly, when a second library of 16 diastereomeric 1,4-enediols (Shi *et al*, 2003) was screened, a greater diversity of stereochemical configurations was observed among the six highest affinity MOR ligands. Only one of the six had stereochemical configurations at both stereodiversified aromatic side chains that matched those in endomorphin-2. Furthermore, none of the stereochemical configurations at the four stereodiversified sites in the 1,4-enediol library was conserved

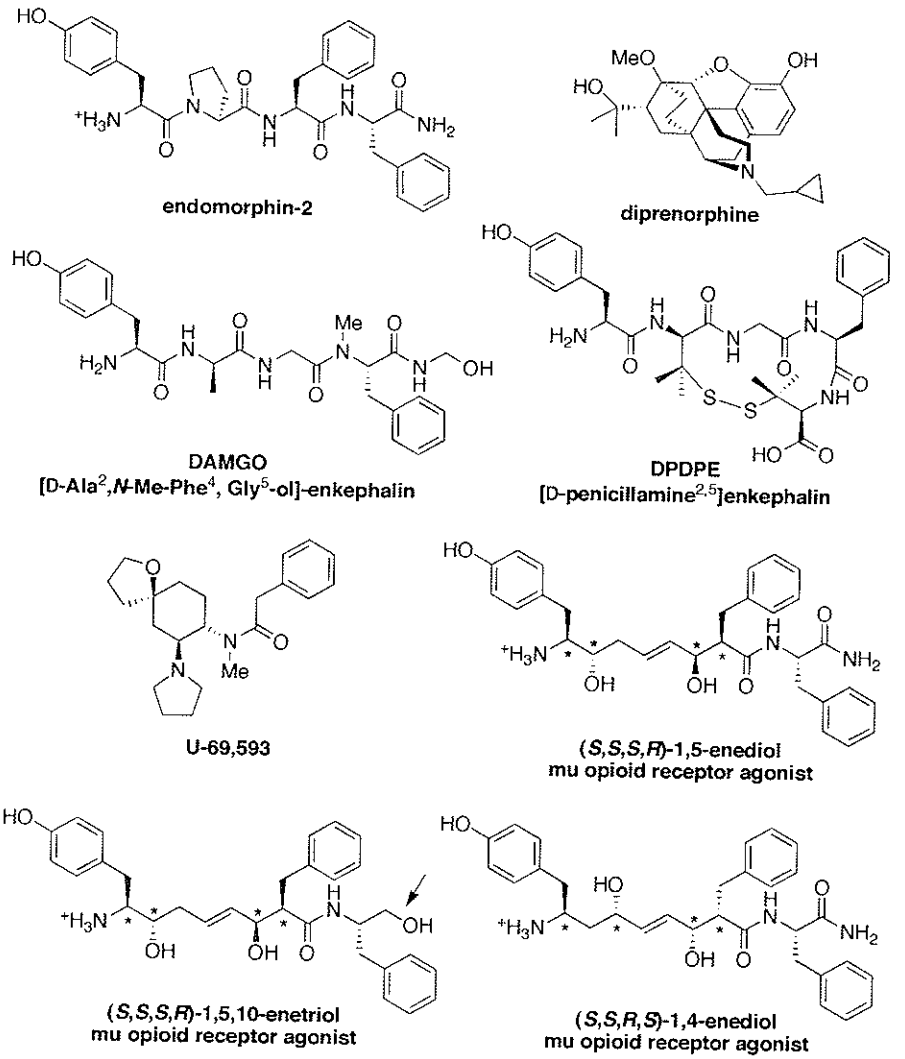


Figure 2.11. Structures of endomorphin-2, diprenorphine, DAMGO, DPDPE, U-69,593, and three non-peptidic MOR agonists.

across all six ligands. The most potent MOR ligand was the *(S,S,R,S)-1,4-enediol* (*Figure 2.11*, K_i = 14 nM), which exhibited moderate selectivity for MOR over DOR (82-fold) and KOR (32-fold). The active enediols were shown to be partial MOR agonists based on their ability to induce MOR-mediated GTP-γ-^{35}S binding by G-proteins in membrane preparations of MOR-1 stably transfected CHO cells. These studies underscore the important influence of stereochemical diversity in combinatorial libraries. New selective, non-peptidic opioid receptor agonists will be valuable tools for dissecting the roles of these receptors in analgesia and tolerance.

Subtype-selective somatostatin receptor agonists

The somatostatins are peptide hormones that inhibit growth hormone secretion and also have neuromodulatory activity in the central nervous system. In addition, the somatostatins have been implicated as inhibitors of tumour cell growth. The somatostatin receptors are members of the G-protein coupled receptor superfamily. Five subtypes (sst1–sst5) have been cloned from human tissues, but antibody probes have been developed for only sst2 and sst5. The lack of subtype-selective ligands

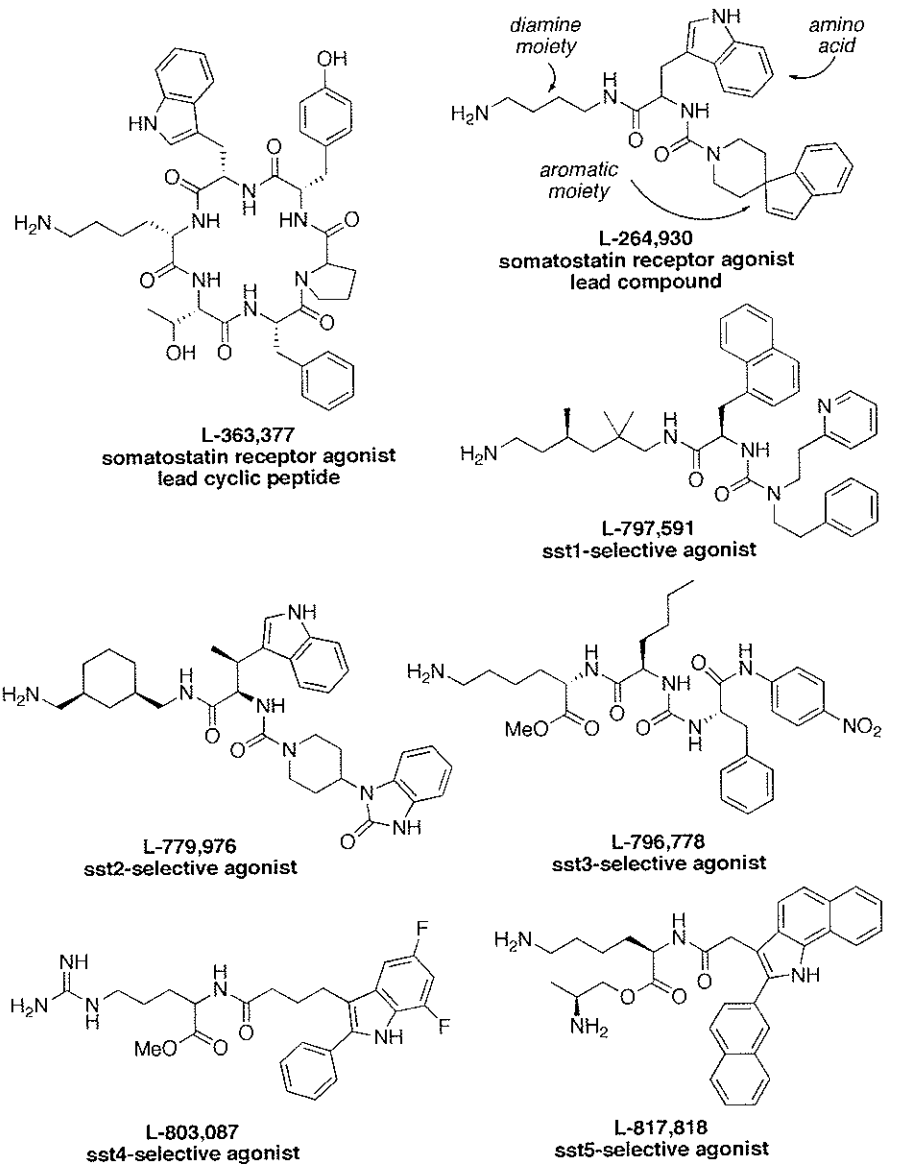


Figure 2.12. Structures of somatostatin receptor agonists.

has made it difficult to dissect the functions of these receptors. To address this problem, Rohrer and co-workers at Merck screened a series of combinatorial libraries for subtype-selective somatostatin receptor ligands (Rohrer *et al.*, 1998).

A known cyclic hexapeptide somatostatin receptor agonist, *L-363,377* (*Figure 2.12*), was used to select 75 similar compounds from approximately 200 000 in the Merck compound collection for evaluation in binding assays. Compounds were analysed for competitive binding to membrane preparations of sst-expressing CHO-K1 Chinese hamster ovary cells with ^{125}I-somatostatin. The most potent of these, *L-264,930* (sst2 K_i = 100 nM), was then used as a lead compound for the design of biased combinatorial libraries.

Four combinatorial libraries were synthesized in an undisclosed format and screened against all five receptors in competitive binding assays. The first combinatorial library was synthesized with 79 variants of the L-264,930 aromatic moiety, 20 variants of the amino acid module, and 20 variants of the diamine moiety. The incorporation of multiple stereoisomers resulted in a library of approximately 130 000 compounds, which was apparently screened as mixtures of 1330 compounds each. After deconvolution, *L-779,976* was identified as a potent, selective sst2 ligand (K_i = 50 pM, >6000-fold selectivity). This compound demonstrated agonist activity in inhibiting forskolin-stimulated accumulation of cAMP in CHO-K1 cells (IC_{50} = 50 pM) and in inhibiting growth hormone secretion from rat pituitary cells (RPC, IC_{50} = 25 pM).

The second combinatorial library was synthesized using 147 variants of the aromatic moiety, 22 variants at the amino acid module, and 21 variants of the diamine moiety, yielding 350 000 compounds that were screened as 147 mixtures. Subsequent deconvolution led to the identification of *L-797,591*, an sst1-selective ligand with agonist activity in a cAMP-responsive, element-driven β-galactosidase reporter gene assay (IC_{50} = 3 nM), and *L-796,778*, an sst3-selective ligand with partial agonist activity (CHO IC_{50} = 18 nM). A third combinatorial library of aryl indoles yielded *L-803,087*, an sst4-selective ligand with agonist activity (CHO IC_{50} = 0.2 nM). Interestingly, none of these three compounds was active in the RPC assay, or inhibited glucagon or insulin secretion from mouse pancreatic islet preparations.

Finally, a fourth combinatorial library, related to the first library, provided *L-817,818*, an sst5-selective ligand with agonist activity (CHO IC_{50} = 1.3 nM). Notably, this compound was also active in the RPC assay (IC_{50} = 3.1 nM) and inhibited mouse pancreatic islet insulin secretion (IC_{50} = 0.3 nM), but not glucagon secretion.

This study demonstrates that subtype-selective somatostatin receptor agonists can be identified from combinatorial libraries. These compounds should be extremely useful probes for dissecting the physiological functions of each of the five receptor subtypes.

AC-42, a subtype-selective M_1 muscarinic receptor agonist

Muscarinic receptors are G-protein coupled receptors in the parasympathetic nervous system that bind monoamine ligands, such as serotonin, adrenaline, dopamine, histamine, acetylcholine, and the prototypical agonist *muscarine* (*Figure 2.13*). The receptors regulate a wide range of physiological processes and, as such, are extensively studied therapeutic targets. In particular, the M_1 receptor subtype has been

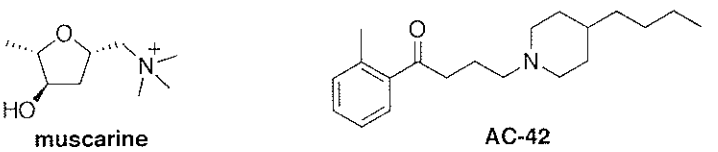


Figure 2.13. Structures of muscarine, a general muscarinic receptor agonist, and AC-42, an M_1 subtype-selective agonist.

targeted for the treatment of Alzheimer's disease. However, existing M_1 agonists bind a region of the receptor that is highly conserved across the five muscarinic receptor subtypes, leading to side effects such as sweating, nausea, and diarrhoea. Thus, Spalding and co-workers at ACADIA Pharmaceuticals screened a compound collection for subtype-selective M_1 agonists (Spalding *et al.*, 2002).

A collection of 145 000 structurally diverse compounds from an undisclosed source was screened in a cell growth assay. Based on the ability of many receptors to amplify NIH 3T3 cells, individual muscarinic receptor subtypes M_1, M_3, and M_5 were transiently co-transfected with a β-galactosidase marker (Braüner-Osborne and Brann, 1996). The compounds were then incubated with the cells for five days. β-Galactosidase activity was then measured to detect receptor activation. This led to the identification of *AC-42* as a potent and selective M_1 agonist (EC_{50} = 0.29 μM). Selectivity for M_1 over M_2 and M_4 was confirmed in secondary NIH 3T3 amplification assays with these receptor subtypes. Additional follow-up assays involving stimulation of phosphatidyl-inositol hydrolysis or inhibition of forskolin-stimulated cAMP production in M_1-expressing CHO cells further confirmed the unprecedented selectivity of AC-42 for M_1.

A series of M_1/M_5 chimeric receptors was then used to identify the regions required for AC-42 selectivity. These experiments showed that two regions (residues 1–45 and 418–460) of M_1 are required for AC-42 agonist activity. In contrast, classical muscarinic receptor agonists interact with two highly conserved residues in the central region of the receptors (Y381 and Q382). Notably, AC-42 maintained potent agonist activity with M_1 receptors that were mutated at these residues. Thus, this study demonstrates that G-protein coupled receptor function can be modulated in a subtype-selective fashion by small molecules that act through regions outside the conserved ligand binding domain.

Carbohydrate ligands for the Bauhinia purpurea *lectin*

Cell surface carbohydrates are involved in many pathological biological recognition processes such as chronic inflammation, tumorigenesis, and metastasis. To identify carbohydrates that could be used as soluble dominant negatives to block recognition processes, Kahne and co-workers screened a combinatorial library for ligands of the *Bauhinia purpurea* lectin (Liang *et al.*, 1996).

A library of approximately 1300 di- and trisaccharides was synthesized by binary encoded solid phase split–pool synthesis. The compounds were not cleaved, but instead left attached to the water compatible TentaGel resin to mimic carbohydrate display on a cell surface. The library was screened (6 copies) in a binding assay by

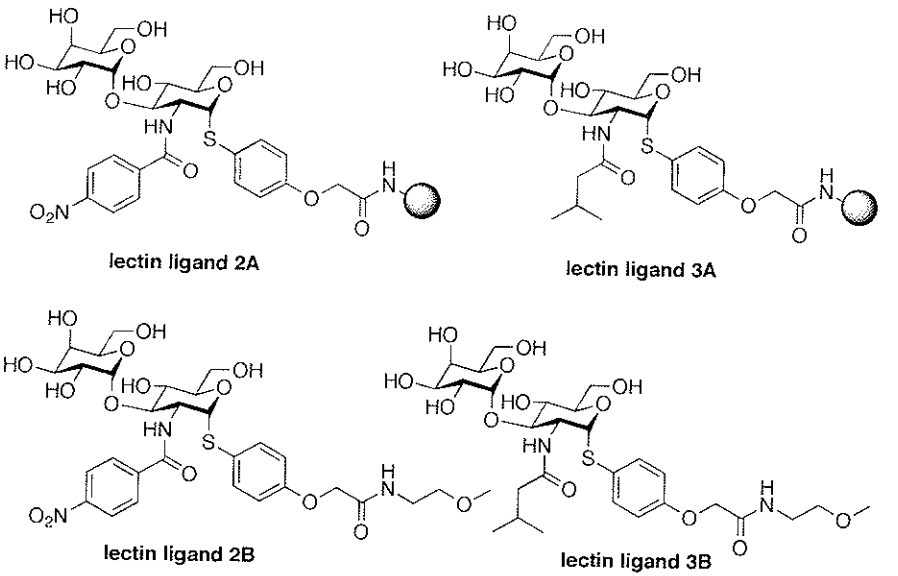


Figure 2.14. Structures of carbohydrate ligands for the *Bauhinia purpurea* lectin.

incubating the beads with biotin-labelled *Bauhinia purpurea* lectin, which contains multiple carbohydrate binding sites and causes erythrocyte agglutination. Beads carrying active ligands were identified by addition of streptavidin-linked alkaline phosphatase and staining. The resulting 25 coloured beads were decoded, and 13 were found to contain the same core disaccharide, *N*-acylated with a hydrophobic group. Five copies each of *lectin ligand 2A* and *lectin ligand 3A* were identified (*Figure 2.14*). The remaining 12 structures did not display any consensus and were discarded as false positives. Importantly, bead staining could be inhibited by addition of soluble analogues *lectin ligand 2B* and *lectin ligand 3B* (20 to 50 μg/mL). Thus, in contrast to the other studies described in this section, this work demonstrates a complementary approach to modulating receptor–ligand interactions with small molecules by binding the soluble component rather than the cell surface component.

SIGNAL TRANSDUCTION PROBES

Cell-permeable small molecules can be valuable tools for studying signal transduction pathways with many potential therapeutic implications, particularly in cancer. In addition to screening against individual signalling proteins, entire signalling pathways can be targeted. Small molecules that modulate a given pathway can then be used to identify new proteins that participate in the signalling pathway.

Uretupamine, a function-selective inhibitor of Ure2p signalling

Ure2p is a yeast signalling protein that is involved in the repression of genes associated with nutrient metabolism regulation, and is part of a signalling cascade

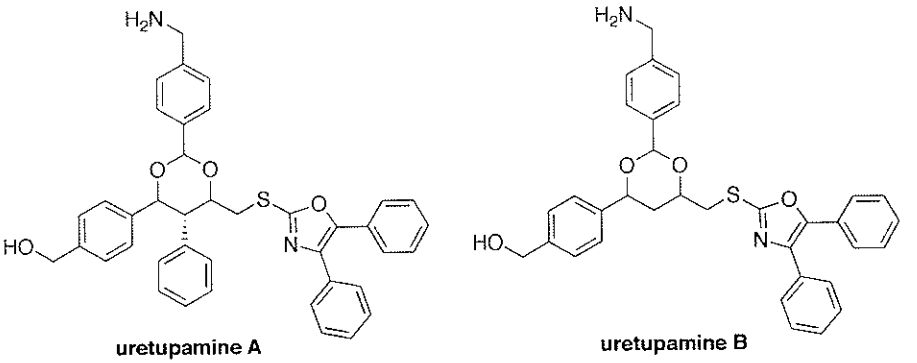


Figure 2.15. Structures of uretupamines A and B, carbon nutrient sensing pathway-selective Ure2p antagonists.

downstream of the Tor proteins. Despite extensive study using traditional techniques, the molecular basis for Ure2p function remains poorly understood, and the relevant binding sites have not been identified due to the lack of an endogenous ligand. Thus, to probe the functions of Ure2p, Schreiber and co-workers screened a combinatorial library for Ure2p ligands (Kuruvilla *et al.*, 2001).

A library of 3780 dioxane-containing compounds was synthesized using solid phase split–pool synthesis (Sternson *et al.*, 2001a). Compound identities were mass self-encoded by judicious selection of building blocks (Hughes, 1998). The compounds were printed onto glass microscope slides to generate small molecule microarrays (MacBeath *et al.*, 1999; Hergenrother *et al.*, 2000). In a binding assay primary screen, the microarray was treated with fluorescently labelled Ure2p, resulting in the identification of eight initial hits. A secondary *PUT1-lacZ* reporter gene assay was used to assess cellular activity since *PUT1* expression is known to be repressed by *URE2*. This led to the identification of *uretupamine A* (*Figure 2.15*), which bound to Ure2p with moderate affinity as determined by surface plasmon resonance ($K_d = 18.1\ \mu M$). Synthesis and evaluation of a small panel of analogues led to the identification of *uretupamine B*, which had improved solubility and affinity ($K_d = 7.5\ \mu M$).

To explore the effects and specificity of the uretupamines further, whole genome transcriptional profiling was performed in wild-type cells and a *ure2*Δ strain. Significantly, of the Ure2p-responsive genes, only glucose-sensitive genes were induced by uretupamines A and B, while those genes responsive to nitrogen nutrient quality were unaffected. Furthermore, the compounds had little or no effect on the *ure2*Δ strain, supporting the cellular specificity of uretupamines A and B. Importantly, the actions of uretupamines A and B are more selective than a genetic *URE2* deletion, since the Ure2p signalling pathway that is responsive to glucose can be modulated selectively, while the nitrogen sensing pathway remains unaffected. Thus, this study highlights a unique and powerful aspect of chemical genetics, the ability of small molecules to modulate a single function of a multi-functional protein selectively.

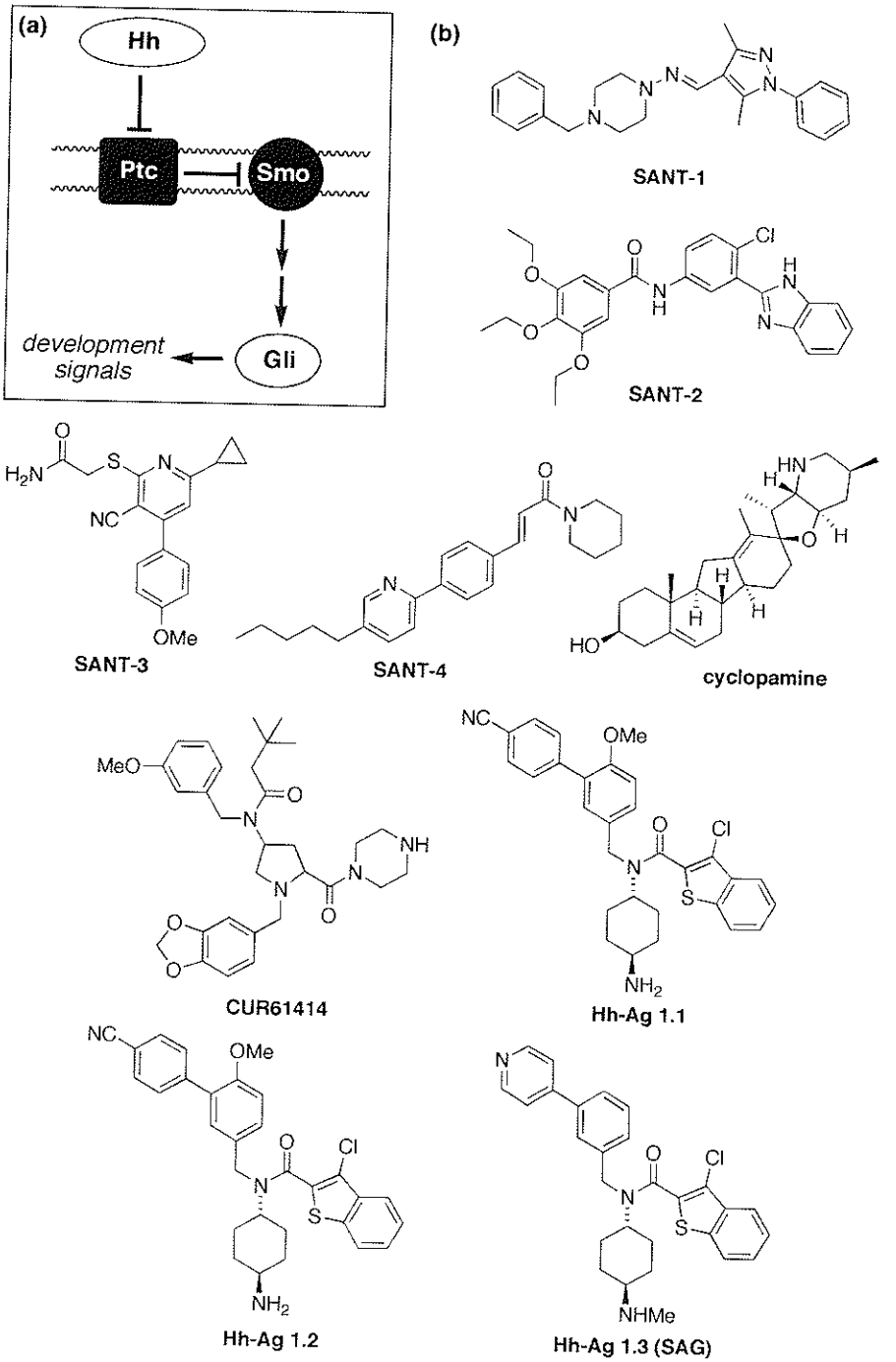


Figure 2.16. (a) Hedgehog signalling through Patched and Smoothened. (b) Small molecule modulators of Smoothened signalling.

SANTs, CUR61414, and Hh-Ag, small molecule modulators of Smoothened to probe Hedgehog signalling

The Hedgehog (Hh) family of secreted proteins are key regulators of cellular differentiation and proliferation during embryonic development. Hh proteins bind and inhibit Patched (Ptc), an integral membrane receptor, which in turn inhibits Smoothened (Smo), a G-protein coupled-like receptor. The mechanism by which Smo activation is coupled to downstream components is poorly understood. Furthermore, aberrant Hh activation has been linked to basal cell carcinoma and medulloblastoma (Oro *et al.*, 1997). Thus, to address these issues, Beachy and co-workers screened a compound collection for small molecule modulators of the Hh signalling pathway (Chen *et al.*, 2002).

A collection of 10 000 compounds from ChemBridge was screened for Hh pathway modulation using a luciferase reporter gene assay. Compounds were incubated (≈2 μM) with Shh-LIGHT2 cells, an NIH 3T3 line that stably incorporates both a Hh-responsive Gli-dependent firefly luciferase reporter gene and a constitutive *Renilla* luciferase reporter. Hh pathway-specific effects were detected by differential changes in the two luciferase activities. This led to the identification of six new Hh pathway antagonists (*Figure 2.16*). Of these, four molecules, *SANT-1* (IC_{50} = 20 nM, K_d = 1.2 nM), *SANT-2* (IC_{50} = 30 nM, K_d = 12 nM), *SANT-3* (IC_{50} = 100 nM, K_d = 44 nM), and *SANT-4* (IC_{50} = 300 nM, K_d = 71 nM), were found to bind directly to Smo in a follow-up competition binding assay with Smo-expressing Cos-1 cells and fluorescently labelled *cyclopamine*, a steroidal alkaloid previously identified as a Smo antagonist (Taipale *et al.*, 2000). The other two molecules appeared to act downstream of Smo, and have apparently not yet been fully characterized. Because the SANTs appear to antagonize Smo by subtly distinct mechanisms, these compounds should be valuable tools for studying regulation of the Smo signalling pathway.

Rubin and co-workers at Curis also recently screened a collection of approximately 100 000 small molecules (2–5 μM) from undisclosed sources for inhibitors of Hh signalling in an effort to identify lead compounds for treating basal cell carcinoma (Williams *et al.*, 2003). A similar Gli-driven luciferase reporter gene assay was used, in conjunction with a constitutive simian virus 40-luciferase reporter counterscreen. This led to the identification of *CUR61414* (*Figure 2.16*) as a Hh pathway antagonist (IC_{50} = 100–200 nM). In two follow-up assays using *in vitro* basal cell carcinoma models, CUR61414 induced apoptosis selectively in basaloid structures without affecting normal cells. Thus, CUR61414 acts as an anti-proliferative agent specifically affecting cells in which proliferation is driven by activation of the Hedgehog pathway. CUR61414 was also shown to antagonize Smo by direct binding (Frank-Kamenetsky *et al.*, 2002).

Porter and co-workers at Curis also used this reporter gene assay to screen 140 000 compounds (2–5 nM) from commercial sources for activators of Hh signalling (Baxter *et al.*, 2001; Frank-Kamenetsky *et al.*, 2002). This led to the identification of *Hh-Ag 1.1* (*Figure 2.16*), which arose from a library synthesized by Oxford Asymmetry International and acted as a moderate Hh pathway agonist (EC_{50} ≈3 nM). Synthesis and evaluation of over 300 Hh-Ag 1.1 analogues led the identification of more potent analogues, including *Hh-Ag 1.2* and *Hh-Ag 1.3*. In *in utero* assays with

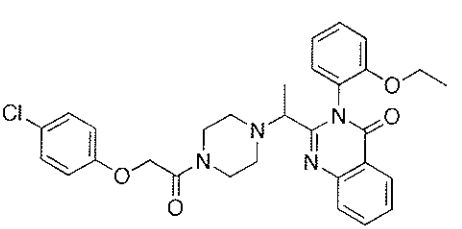
sonic Hh- and *Smo*-mutant mouse embryos, Hh-Ag 1.2 activity was shown to be dependent upon Smo, but not sonic Hh. Competition experiments with known Hh signalling antagonists indicated that Hh-Ag 1.2 acts downstream of the Hh–Ptc interaction. A tritiated form of another Hh-Ag analogue was also shown to bind directly to Smo. Beachy and co-workers also studied Hh-Ag 1.3 (designated SAG) in their reporter gene assay (EC_{50} = 3 nM) and demonstrated through photo-cross-linking that it binds directly to Smo (Chen *et al.*, 2002).

Overall, the opposing activities of the SANTs and cyclopamine compared to the Hh-Ags suggest that Smo activity may be regulated by conformational changes. The authors of these studies also make the intriguing proposal that Smo may, in fact, be regulated by endogenous small molecules. These new small molecule modulators of Smo function should be valuable tools to probe Hh signal transduction through Smo.

Erastin, a genotype-selective cytotoxic molecule

Molecules that exhibit genotype-selective lethality could serve as probes of signalling networks in tumour cells. By targeting specific molecular defects found in tumour cells, it may be possible to identify those critical pathways whose disruption leads to a tumorigenic phenotype. Toward this aim, Stockwell and co-workers screened a combinatorial library and compound collection for activity in a series of engineered tumorigenic cells and their respective precursors (Dolma *et al.*, 2003).

A combinatorial library of 20 000 compounds from Comgenex International, a collection of 1990 compounds from the NCI, and 1540 commercially available, biologically active compounds were screened using a cytotoxicity assay. Compounds were incubated (≈10 μM) for 48 hours with tumorigenic BJ-TERT/LT/ST/RAS^{V12} engineered cells resulting from the introduction of specific genetic elements into human primary foreskin fibroblasts (Hahn *et al.*, 1999). Cell viability was then measured using the dye calcein acetoxymethyl ester. Compounds exhibiting 50% or greater inhibition of staining were then re-tested in BJ and BJ-TERT/LT/ST/RAS^{V12} cells in a secondary screen for synthetic lethality, that is, lethality in tumour cells but not in isogenic primary cells. This led to the identification of nine compounds that were at least four times more potent in the transformed cells. Most were known anti-tumour drugs; however, one previously unknown compound, *erastin* (*Figure 2.17*), was identified (IC_{50} = 2.3 μM, >8-fold selectivity).



erastin

Figure 2.17. Structure of erastin, a tumour genotype-selective cytotoxic compound.

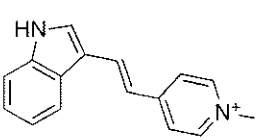
Follow-up experiments in other engineered cell lines indicated that erastin activity requires the presence of oncogenic RAS^{V12} and ST. To determine if erastin induces apoptosis, the nuclear morphology of erastin-treated tumorigenic cells was analysed by fluorescence microscopy. No karyorhexis or margination of chromatin was seen, no DNA fragmentation was seen, and a caspase inhibitor did not block cell death, indicating that apoptosis did not occur. Further efforts to elucidate the mechanism of action are under way. Thus, the small molecule approach led to the discovery of not only synergy between RAS^{V12} and ST, but also a rapid and selective non-apoptotic cell death pathway. Application of this synthetic lethal approach to other transforming genes may allow identification of the specific signalling networks that emanate from oncogenes and tumour suppressors. Notably, Schreiber and co-workers have recently extended this approach to testing multiple compounds on multiple genetic backgrounds for chemical genomic profiling of biological networks (Haggarty *et al.*, 2003a).

F16, a mitochondriotoxic molecule selective for neu*-overexpressing cells*

The *HER-2/erbB-2/neu* proto-oncogene is a member of a group of receptor tyrosine kinases that are important for the growth and differentiation of many tissues, and whose overexpression is linked to several different human cancers. As an alternative to targeting HER-2 itself to inhibit propagation of *HER-2*-overexpressing cells, perturbation of other pathways contributing to cellular transformation may also allow cell growth to be controlled. To probe the biological characteristics that distinguish transformed cells from normal cells, Leder and co-workers screened a compound collection for selective inhibitors of *HER-2*-overexpressing cells (Fantin *et al.*, 2002).

A collection of 16 000 compounds from ChemBridge was screened (10–15 μM) in a cytoblot assay for differential inhibition of cell proliferation in EpH4-A6 *HER-2*-overexpressing immortalized mammary epithelial cells and EpH4-EV control cells. Proliferation was measured by the level of 5-bromo-2′-deoxyuridine (BrdU) incorporation into DNA using an anti-BrdU primary antibody and an HRP-linked secondary antibody. The most potent compound, *F16* (*Figure 2.18*), partially inhibited BrdU incorporation at concentrations down to 100 nM.

In follow-up experiments, F16 inhibited growth in cancer cell lines that expressed *HER-2* at high levels, but a number of other cancer cell lines with moderate to undetectable levels of HER-2 were also affected. This suggests that other oncogenes in addition to *HER-2* can sensitize cells to F16. Notably, the extended π-electron



F16

Figure 2.18. Structure of F16, a selective inhibitor of cell proliferation in *HER-2*-overexpressing cells.

system and conformational rigidity of F16 result in characteristic energy absorption and emission bands that allow the molecule to be visualized directly. Autofluorescence experiments identified the mitochondria as the major target of F16. Studies of F16 in cells and with purified mitochondria showed that the small molecule disrupts the proton gradient across the inner mitochondrial membrane, causing an inner membrane channel to open, ultimately leading to mitochondrial dysfunction and cell death. F16 selectively targets the mitochondria of tumour cells due to their higher membrane potential relative to normal cells. Importantly, this study indicates a connection between *HER-2* overexpression and mitochondrial membrane potential.

Allosteric inhibitors of inducible nitric oxide synthase dimerization

Inducible nitric oxide synthase (iNOS) is a homodimer that catalyses NADPH-dependent oxidation of L-arginine to NO• and citrulline. NO• plays a central role in the central nervous, cardiovascular, and immune systems and iNOS is implicated in inflammatory and autoimmune diseases. Inhibition of the related enzyme endothelial nitric oxide synthase is undesirable, however, because of its role in maintaining vascular homeostasis. Thus, Devlin and co-workers at Berlex Biosciences and Pharmacopeia screened a combinatorial library in an effort to identify new selective iNOS inhibitors (McMillan *et al.*, 2000).

A combinatorial library of 8649 pyrimidineimidazole-based compounds was synthesized using binary encoded solid phase split–pool synthesis. Structurally related phenylimidazoles had previously been shown to inhibit iNOS selectively. Compounds were attached to the beads via a photolabile *o*-nitrobenzyl amide linker, allowing approximately 200 pmol of each compound to be photoreleased. Compounds were first screened (2 μM) in a biochemical assay for activity against partially purified recombinant iNOS; however, no active compounds were identified. In a second screen, compounds were assayed as pools of 20 molecules (1 μM each) for inhibition of cytokine-stimulated NO• production in human glioblastoma A-172 cells. NO• release was measured with the Griess reagent, which forms a dye in the presence of nitrite. Fifty-three different compounds exhibiting over 60% inhibition were found. Of these compounds, *iNOS inhibitor 1* (*Figure 2.19*) was composed

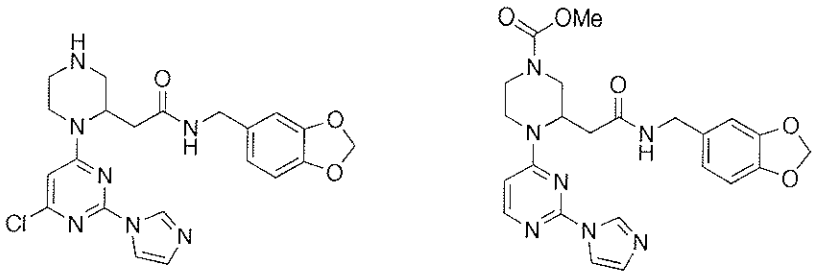


Figure 2.19. Structures of allosteric inhibitors of inducible nitric oxide synthase dimerization.

of the synthons most commonly found in active library members, and was one of the most potent (IC_{50} = 1.1 nM). A second compound, *iNOS inhibitor 2* (IC_{50} = 0.6 nM) was selected for further study because it lacked a potentially reactive chlorine group.

Because these compounds did not inhibit purified iNOS, Devlin and co-workers hypothesized that they might act as allosteric inhibitors of iNOS dimerization. This was supported by studies in RAW 264.1 mouse macrophage cells, in which treatment with LPS and IFN-γ results in expression of a mixture of iNOS monomers and dimers. Treatment with iNOS inhibitor 2 caused a dose-dependent increase in the amount of monomer present, as determined by low temperature SDS-PAGE and size exclusion chromatography. The compound was also shown to bind directly to the iNOS monomer by competitive binding experiments with a radiolabelled analogue (K_i = 2.2 nM). To elucidate the mechanism of inhibition and to gain insights into the dimerization mechanism, the cocrystal structure of iNOS inhibitor 2 with iNOS was determined. This analysis indicated that the compound occupies the monomer active site and causes conformational changes that may prevent dimerization by an allosteric mechanism. iNOS inhibitor 2 exhibited over 1000-fold selectivity for iNOS (IC_{50} = 28 nM) over endothelial nitric oxide synthase (IC_{50} = 32 μM) as assayed using crude cell lysates from transfected BSC-1 cells. This inhibitor (ED_{50} = 1.2 mg/kg i.p.) also suppressed NO• production in rats after systemic induction of iNOS by LPS. This study highlights the differences between biochemical and cell-based assays and demonstrates an important advantage of screening over rational design, the opportunity to identify previously unrecognized molecular targets for intervention by small molecules.

Dimeric molecules as selective inhibitors of c-Src tyrosine kinase

The tyrosine kinase c-Src is the parent member of a large tyrosine kinase family with wide-ranging biological functions. The majority of kinase inhibitors bind the conserved ATP-binding site, making it difficult to achieve selectivity among Src family members using small molecules (Bishop *et al.*, 1999). To address this problem, Ellman and co-workers used a novel library design strategy to identify selective c-Src inhibitors (Maly *et al.*, 2000).

The library design strategy requires neither lead compounds, nor knowledge of the structure of the biological target (*Figure 2.20*). First, a set of potential binding elements is prepared. Each molecule must be soluble in aqueous solution and incorporate a common linkage group. These compounds are then screened in a

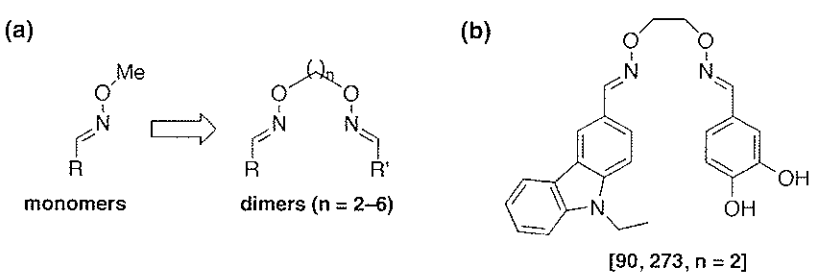


Figure 2.20. (a) Library design strategy. (b) Structure of c-Src selective tyrosine kinase inhibitor.

binding assay at high concentrations to identify even weak ligands. A combinatorial library is then prepared by connecting active binding elements using flexible linkers. Screening provides dimeric molecules that presumably bind the target at two sites, enhancing the probability of identifying a selective ligand.

Thus, a collection of 305 aldehyde-derived *O*-methyl oximes was screened (1 mM) to identify even weak inhibitors of constitutively active c-Src using a commercially available ELISA assay. This led to the identification of 66 monomers exhibiting >60% inhibition. Re-screening at lower concentration (0.5 mM) led to the selection of 47 monomers exhibiting >70% inhibition. Of these, 37 structurally diverse monomers were selected for inclusion in library synthesis. The 37 aldehyde monomers were coupled in all possible combinations via five different *O,O'*-diaminoalkanediol linkers using solution phase mixture synthesis. Screening (5 μM) and subsequent deconvolution led to the identification of *[90, 273, n = 2]* (*Figure 2.20*), a potent c-Src inhibitor (IC_{50} = 64 nM) with 75-fold selectivity for c-Src over family members Lyn and Fyn and >1000-fold selectivity for c-Src over Lck. This study demonstrates a novel approach to the challenging problem of achieving selectivity among similar proteins. Highly selective inhibitors should be powerful probes for dissecting the functions of various family members.

Dimeric molecules as selective inhibitors of protein tyrosine phosphatase 1B

A related approach has been used to identify dimeric molecules as selective phosphatase inhibitors. Protein tyrosine phosphatase 1B (PTP1B) is linked to type II diabetes and a number of cancers. The PTP1B catalytic domain is conserved among many tyrosine phosphatases; however, an adjacent non-conserved secondary binding site has also been discovered (Puius *et al.*, 1997). Lawrence, Zhang and co-workers first used this information to identify dual site-binding PTP1B-specific phosphatase inhibitors from a phosphotyrosine-biased library of non-natural peptides (Shen *et al.*, 2001). Szczepankiewicz and co-workers recently extended this approach to non-phosphate-containing inhibitors using an NMR-based screening approach (Szczepankiewicz *et al.*, 2003).

A collection of 10 000 compounds from an undisclosed source was initially screened in an NMR-based assay for binding to the catalytic site of labelled PTP1B as detected by perturbation of the $^{1}H/^{15}N$- and $^{1}H/^{13}C$-HSQC spectra. This led to the identification of *PTP1B inhibitor 1* (*Figure 2.21*), which bound to the active site weakly (K_d = 100 μM) and was a weak inhibitor of PTP1B-mediated *p*-nitrophenylphosphate hydrolysis (K_i = 293 μM). Subsequent structure-based design modifications led to *PTP1B inhibitor 12* (K_i = 1.1 μM), which included an appropriately placed tether to attach a second ligand for the nearby non-catalytic binding site. NMR-based screening of the same 10 000-compound collection at this secondary site led to the identification of several fused aromatic acid ligands (K_d >1 mM). Coupling of a structurally similar napthoic acid moiety to the catalytic site ligand yielded a potent dimeric *PTB1B inhibitor 23* (K_i = 22 nM). Moreover, this compound showed excellent selectivity for PTB1B over other phosphatases, LAR (K_i = 1.3 μM), SHP-2 (K_i = 2.49 μM), CD45 (K_i = 53.6 μM), and calcineurin (K_i >300 μM). Modest selectivity was also achieved for PTP1B over the closely related T cell protein tyrosine phosphatase (K_i = 49 nM). In contrast, the selectivity of the monomeric

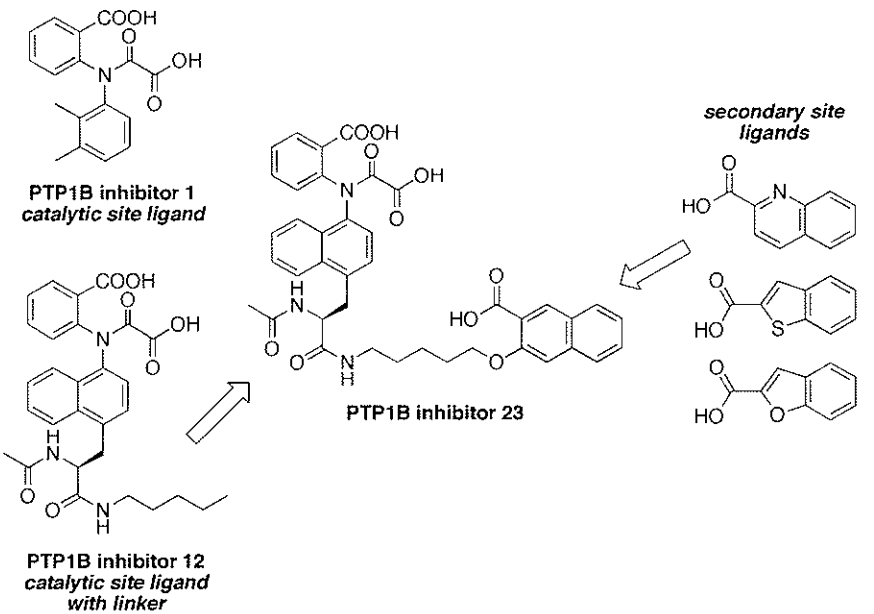


Figure 2.21. Structures of catalytic and secondary site PTP1B ligands leading to a dimeric inhibitor with high potency and selectivity.

PTP1B inhibitor 12 was an order of magnitude lower in each case. This study demonstrates a structure-based approach to dimeric inhibitors with high selectivity. Such inhibitors should be valuable probes to dissect phosphatase functions in cellular processes.

Purvalanols, selective inhibitors of cyclin A–CDK2

The CDKs play key roles in regulating the cell cycle, and downregulation of CDK inhibitor proteins is often a factor in the development of cancer. To develop new selective probes of CDK function, Schultz and co-workers screened a combinatorial library for small molecule CDK2 inhibitors (Gray *et al.*, 1998).

Several combinatorial libraries of 2,6,9-trisubstituted purines were synthesized by parallel solid phase or solution phase synthesis. These compounds were designed as analogues of *olomoucine* (*Figure 2.22*), which exhibits good selectivity but only moderate inhibitory activity ($IC_{50} = 7\ \mu M$) against a subset of the CDK family. Based on the orientation of the purine ring of olomoucine within the ATP-binding site of CDK2, new substituents were introduced at the 2, 6, and 9 positions of the purine ring in an attempt to improve the binding affinity and selectivity. Compounds were screened in a biochemical assay for inhibition of histone H1 phosphorylation by cyclin A–CDK2. This led to the identification of *purvalanol B*, a potent inhibitor of cyclin A–CDK2 ($IC_{50} = 6$ nM). A more cell-permeable analogue, *purvalanol A* ($IC_{50} = 70$ nM), was also identified.

Purvalanol B showed excellent selectivity for CDK inhibition over a variety of unrelated kinases. Furthermore, only a subset of the CDK family (cyclin B–cdc2,

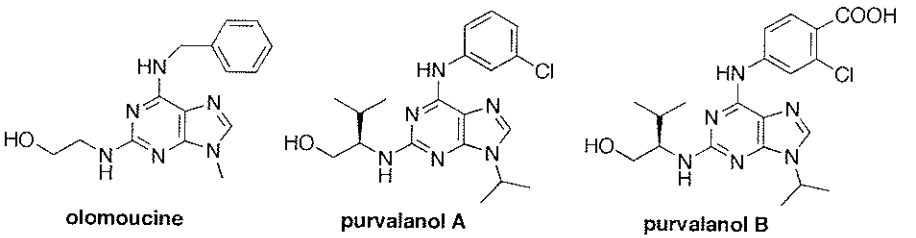


Figure 2.22. Structures of selective CDK inhibitors.

IC_{50} = 6 nM; cyclin A–CDK2, IC_{50} = 6 nM; cyclin E–CDK2, IC_{50} = 9 nM; p35–CDK5, IC_{50} = 6 nM) was inhibited at significant levels. Purvalanol A showed moderate activity against a panel of 60 human tumour cell lines (mean GI_{50} = 2 μM) with potent activity against a KM12 colon cancer cell line (GI_{50} = 76 nM) and a NCI-H522 non-small cell lung cancer line (GI_{50} = 347 nM). Transcriptional profiling analysis in yeast was also carried out to delineate the differences between purvalanol analogues and flavopiridol, another CDK inhibitor. The identification of small molecule inhibitors with selectivity between CDK family members remains an ongoing challenge. Such molecules will be valuable probes for dissecting the roles of the CDKs at various stages of the cell cycle.

TWS119, a GSK-3β inhibitor to probe neurogenesis

Stem cells hold promise for treating degenerative diseases such as Parkinson's disease and diabetes. However, better understanding of the signalling pathways that control stem cell fate is required to evaluate the prospects for these new therapies. Since small molecules that induce differentiation of stem cells could help to investigate the molecular mechanisms of this process, Schultz and co-workers screened combinatorial libraries for small molecules that induced the selective differentiation of stem cells to neurons (Ding *et al.*, 2003).

A library of 45 140 heterocycles directed toward kinases was synthesized using solid phase synthesis (Ding *et al.*, 2002). Mouse P19 embryonal carcinoma cells were used for the screen because they are pluripotent with a broad range of embryonic developmental potentials. A luciferase reporter gene driven by a specific neuronal marker, neuronal Tα1 tubulin, was used to screen the compounds (5 μM).

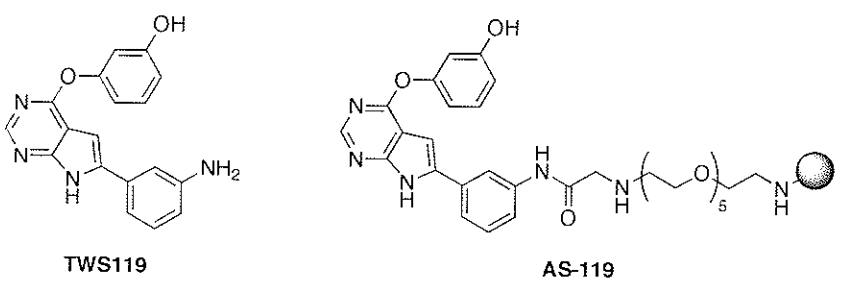


Figure 2.23. Structures of TWS119, a GSK-3β inhibitor that induces neuronal differentiation, and AS-119, an affinity matrix used for target identification.

Primary hits were confirmed by immunofluorescence microscopy and observation of characteristic neuronal morphology. Subsequent synthesis and evaluation of a tuning library based on the 4,6-disubstituted-pyrrolopyrimidine core led to the identification of *TWS119* (*Figure 2.23*), which induced neuronal differentiation in P19 cell monolayers (30–40% at 1 μM).

The target of TWS119 was identified as glycogen synthase kinase-3β (GSK-3β) by affinity chromatography. GSK-3β is a multifunctional serine/threonine kinase that plays a role in pattern formation during embryonic development, cell fate determination, and transcriptional control. Western blots of P19 cells treated with active TWS analogues revealed increased levels of β-catenin, a downstream substrate of GSK-3β in the Wnt signalling pathway. Wnt signalling has been implicated in neuronal induction from pluripotent embryonic stem cells. Thus, TWS119 should be a useful probe to gain new insights into the molecular mechanisms of stem cell differentiation.

NodH sulfotransferase inhibitors to probe sulfotransferase function

Carbohydrate sulfotransferases are a large family of enzymes with overlapping tissue distribution and substrate specificities. They are involved in a variety of biological processes, including molecular recognition, detoxification, hormone regulation, drug processing, and modulation of receptor binding. They have also been implicated in inflammatory disease, cancer metastasis, and HIV and herpes viral entry. As a first step toward deconvoluting the role of each sulfotransferase gene product, Bertozzi and co-workers screened a compound collection for carbohydrate sulfotransferase inhibitors (Armstrong *et al.*, 2000).

Carbohydrate sulfotransferases catalyse the transfer of a sulfonyl group from the ubiquitous donor adenosine-3′-phosphate-5′-phosphosulfate (*PAPS*) to a hydroxy or amino group of an oligosaccharide (*Figure 2.24a*). Since kinases catalyse a similar anionic group transfer reaction, a collection of 139 compounds were selected from previously reported kinase-directed combinatorial libraries (Gray *et al.*, 1998; Chang *et al.*, 1999) and commercially available protein kinase inhibitors from Calbiochem for screening. Compounds were screened in a medium-throughput radiolabel transfer biochemical assay by incubation (200 μM) with NodH, a *N*-acetylglucosamine (GlcNAc)-6-sulfotransferase from *Rhizobium meliloti*, in the presence of ^{35}S-labelled PAPS and a chitobiose substrate. Inhibitory activity was assessed by thin layer chromatography separation of ^{35}S-labelled product from ^{35}S-PAPS. This led to the identification of six *NodH sulfotransferase inhibitors* (*Figure 2.24b*) with moderate activity (IC_{50} = 20–40 μM). Notably, in follow-up assays to probe the specificity of these inhibitors, none of these compounds showed measurable inhibitory activity against a GlcNAc-6-sulfotransferase expressed in human high endothelial venules or a widely expressed keratan sulfate sulfotransferase. However, in keeping with the original library designs, these compounds also inhibit p38/MAP kinase and CDK2.

Specific β-arylsulfotransferase-IV inhibitors

In an effort to improve upon the modest activity and specificity of previously

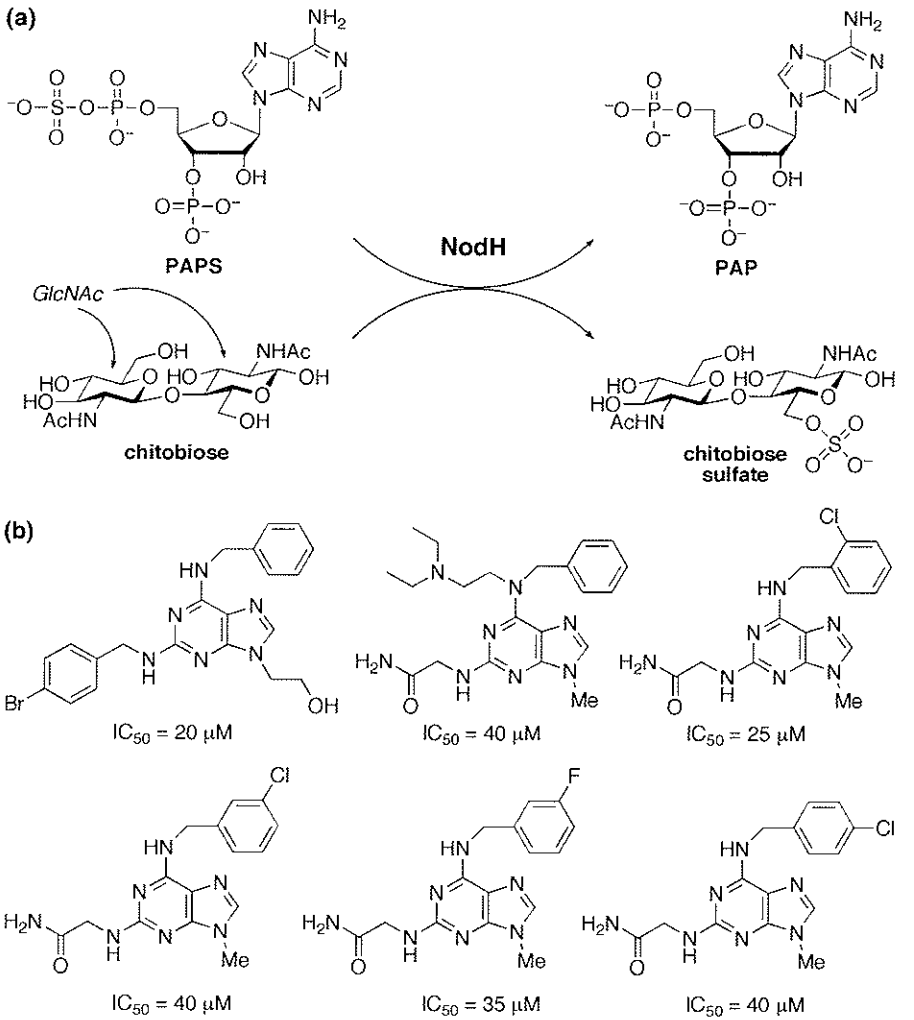


Figure 2.24. (a) Biochemical assay for NodH sulfotransferase inhibitors. (b) Structures of NodH sulfotransferase inhibitors.

discovered sulfotransferase inhibitors, Wong and co-workers developed a high-throughput screen for inhibitors of β-arylsulfotransferase-IV (β-AST-IV) (Chapman *et al.*, 2002).

β-AST-IV is a detoxification enzyme originally isolated from rat liver. Importantly, it accepts a wide range of aromatic alcohol and amine substrates, facilitating the development of a high-throughput biochemical assay with a fluorescent readout using *methylumbelliferyl sulfate* and adenosine-3′,5′-bisphosphate (PAP) in a reverse sulfotransferase reaction (*Figure 2.25*). The fluorescent product is readily monitored using a standard plate reader. A combinatorial library of 35 000 purine and pyrimidine analogues, originally directed toward kinases, was generated using solid phase and solution phase synthesis (Ding *et al.*, 2002). Compounds were

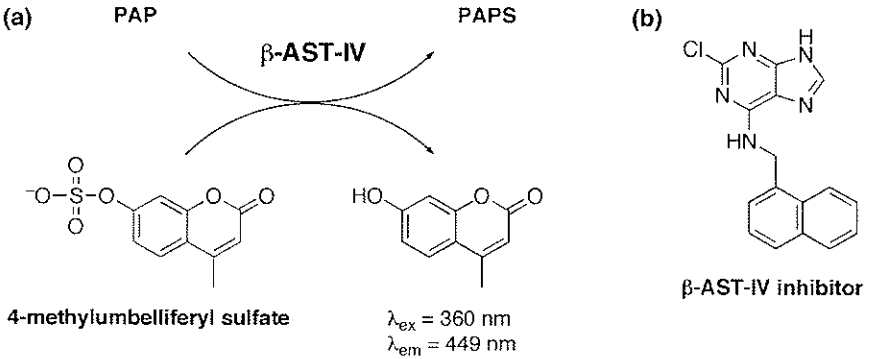


Figure 2.25. (a) Fluorescence-based high-throughput assay for reverse sulfotransferase activity. (b) Structure of β-AST-IV inhibitor.

incubated (10 μM) with the sulfotransferase, PAP, and 4-methylumbelliferyl sulfate, leading to the identification of 13 hits that displayed at least 50% inhibition. These compounds were re-screened at lower concentration (4 μM), leading to the identification of a *β-AST-IV inhibitor* (*Figure 2.25*) with potent activity (K_i = 96 nM). Follow-up biochemical assays with a variety of other nucleotide binding proteins indicated no cross-reactivity, with the exception of human Sult1A1, a cytosolic sulfotransferase. However, the inhibitory activity for Sult1A1 (IC_{50} = 770 nM) was almost an order of magnitude weaker than that for β-AST-IV. Cell-permeable sulfotransferase inhibitors with high specificity should be powerful tools for dissecting the functional implications of post-translational sulfation.

A role for copper in TGF-β pathway activation

The transforming growth factor-β (TGF-β) family of cytokines are key regulators of cell proliferation and differentiation. They bind to cell surface TGF-β receptors (TGF-βR), which, in turn, phosphorylate the SMAD transcription factors, activating them for translocation to the nucleus. TGF-β signalling is modulated by an intricate network of regulatory signals. To develop new tools to study these processes, Schreiber and co-workers screened a compound collection for small molecule modulators of TGF-β signalling (Stockwell *et al.*, 1999b).

A collection of 16 000 compounds from ChemBridge was screened for activation of a TGF-β-responsive luciferase reporter gene in a Mv1Lu mink lung epithelial cell line. This led to the identification of *TGF-β-responsive reporter gene activator 1a* and *TGF-β-responsive reporter gene activator 2* (*Figure 2.26*) that activated the

Figure 2.26. Structures of small molecule activators of a TGF-β-responsive reporter gene.

reporter gene in a dose-dependent manner (EC_{50} ≈2 μM and 30 μM). Transcription profiling experiments indicated that activator 1a induced upregulation of genes associated with metal ion homeostasis. Zn^{2+}, Cu^{2+} and Al^{3+} were shown to suppress the activity of activator 1a, but failed to suppress activator 2 or TGF-β. Subsequently, Cu^{2+} was found to activate the reporter gene directly, and to synergize potently with activator 2, suggesting that this compound may act as a copper transporter. A known copper chelator partially suppressed the activity of activator 2, TGF-β, and copper, but not activator 1a. Although the targets of activators 1a, 2, and copper have not been determined, this study led to the serendipitous identification of a new role for metal ions in TGF-β-responsive signal transduction.

TRANSCRIPTIONAL REGULATION PROBES

Gene transcription in eukaryotes is a complex process that involves integration of a variety of regulatory signals resulting from multiple protein–DNA and protein–protein interactions. Packaging of DNA into chromatin provides an additional layer of transcriptional regulation. Since individual proteins may be involved in multiple regulatory complexes, small molecule modulators can act with greater specificity than genetic mutations or siRNAs, making them powerful tools for dissecting transcriptional regulation.

ITSAs, suppressors of the HDAC inhibitor trichostatin A to dissect the roles of histone and tubulin acetylation

Increased histone acetylation is caused by HDAC inhibitors and correlates with transcriptional activation and cell cycle arrest. To study cell cycle arrest induced by the HDAC inhibitor trichostatin A further, and to examine the role of trichostatin-promoted tubulin acetylation in other cellular processes, Schreiber and co-workers screened a compound collection in a chemical genetic modifier screen to identify small molecule suppressors of trichostatin (Koeller *et al.*, 2003).

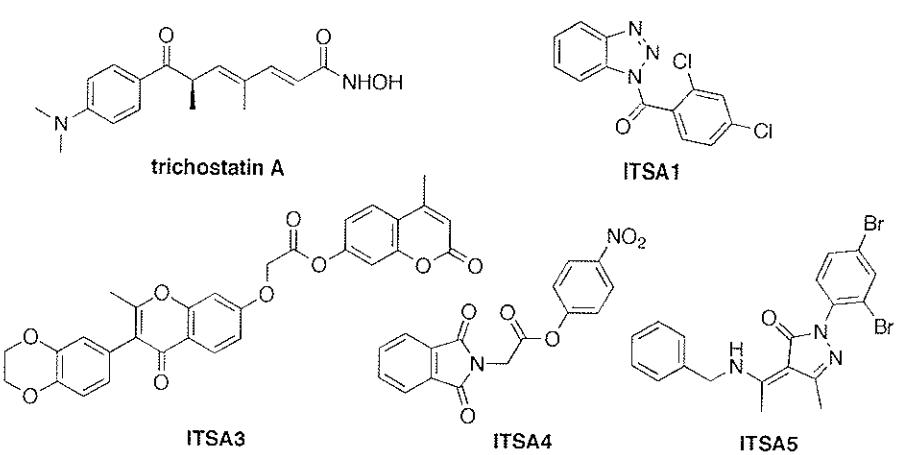


Figure 2.27. Structures of the deacetylase inhibitor trichostatin A and four suppressors of trichostatin A.

A collection of 9600 compounds from ChemBridge was screened for suppression of trichostatin's effects upon the cell cycle using a cytoblot assay. Cells were arrested in the G1 and G2 phases of the cell cycle with trichostatin, then incubated with the compounds (20 μM). Nocodazole was then used to capture in mitosis those cells that were able to bypass arrest in G1 or G2. These cells were detected by increased phosphonucleolin levels observed in the cytoblot, leading to the identification of a series of trichostatin inhibitors (ITSAs). Further studies focused on *ITSA1*, *ITSA3*, *ITSA4*, and *ITSA5* (*Figure 2.27*).

Treatment of A549 lung carcinoma cells with trichostatin, and subsequent incubation with individual ITSAs (50 μM, 2 h) showed that these compounds suppressed trichostatin-induced (300 nM, 2 h) histone hyperacetylation. Notably, ITSA1 does not appear to act directly upon HDAC and was active only when added at the same time as or after trichostatin, indicating that the target of ITSA1 is either not present or is not correctly localized in the absence of trichostatin. The ability of the ITSAs (50 μM, 1 h) to suppress trichostatin-induced (300 nM, 2 h) tubulin acetylation in A549 cells was then examined using immunofluorescence microscopy. While ITSA1 and ITSA3 downregulated tubulin acetylation, ITSA4 and ITSA5 did not, demonstrating that suppression of tubulin acetylation is not required to bypass trichostatin-induced cell cycle arrest. This study highlighted the fact that tubulin acetylation is an additional consequence of treatment with trichostatin-like HDAC inhibitors. The differential activities of the ITSAs make them valuable tools for uncoupling the effects of histone and tubulin acetylation. Moreover, this study demonstrates successful application of a chemical genetic modifier screen to identify new small molecule probes.

A3, sirtinol, and splitomicin, inhibitors of Sir2p, an NAD-dependent deacetylase

Sir2p is the founding member of a large family of NAD-dependent deacetylase enzymes called sirtuins. These deacetylases are conserved from prokaryotes to eukaryotes, although many remain uncharacterized. Deacetylation of histones by Sir2p results in chromatin condensation and transcriptional silencing. To study the biological functions of the sirtuins in a variety of experimental systems, Schreiber and colleagues screened a compound collection for small molecule inhibitors of sirtuins (Grozinger *et al.*, 2001).

A collection of 1200 compounds from ChemBridge and 400 compounds from the

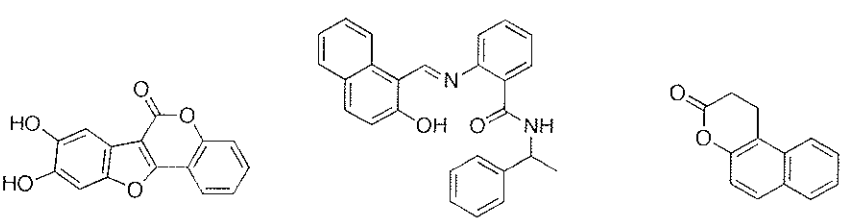


Figure 2.28. Structures of small molecule inhibitors of the sirtuin family NAD-dependent deacetylase Sir2p.

Harvard Institute of Chemistry and Cell Biology Diversity Set were screened in a yeast reporter gene assay. A *URA3* reporter gene was integrated into a telomeric locus where it is silenced by Sir2p function. Compounds were incubated (10–20 μM) with the yeast in the presence of 5-fluoroorotic acid (5-FOA), and inhibition of Sir2p leading to URA3 expression was detected by cell death. A counterscreen in the absence of 5-FOA was used to eliminate cytotoxic compounds. This led to the identification of *A3* and *sirtinol* (*Figure 2.28*), which inhibited both yeast Sir2p (IC_{50} = 70 μM and 70 μM) and the human sirtuin family member SIRT2 (IC_{50} = 45 μM and 40 μM).

Further studies showed that A3 inhibits human HDAC1 activity, while sirtinol does not, suggesting that sirtinol specifically inhibits the sirtuin family of deacetylases. The ability of sirtinol to inhibit sirtuins from both yeast and humans raised the possibility that it could also be applied to sirtuins from other organisms. Preliminary investigation of sirtinol activity in the plant *Arabidopsis*, which contains at least two sirtuins, suggested that it had an effect on apical–basal body axis development and vascularization. The observed phenotype closely resembled that resulting from inhibition of auxin transport.

Based on these preliminary screens, Zhao and co-workers investigated the effects of sirtinol on auxin signalling in *Arabidopsis* (Zhao *et al.*, 2003). Auxin is a plant hormone that regulates plant growth and development. Analysis of auxin signalling is complicated by auxin polar transport, a process whereby an auxin concentration gradient is maintained among neighbouring cells. This concentration gradient limits the cellular accessibility of exogenous auxin. As a result, auxin signalling components may be missed in genetic screens for mutants that are resistant to exogenous auxin. To complicate matters further, auxin polar transport and auxin signalling are interdependent processes, making isolation and characterization of either one difficult.

Sirtinol (25 μM) caused upregulation and ectopic expression of auxin in a DR5-GUS line containing an auxin-responsive reporter gene. Microarray analysis with an *Arabidopsis* whole genome chip indicated that sirtinol induced a gene expression pattern similar to that in auxin-treated plants. Sirtinol also promoted auxin-related developmental changes, consistent with the observation that sirtinol activates auxin-inducible genes. The response of known auxin mutants to sirtinol was then tested. All auxin signalling mutants were less sensitive to sirtinol than wild-type plants. In contrast, auxin polar transport mutants and wild-type plants responded similarly to sirtinol. This indicated that sirtinol targets auxin signalling, and not auxin polar transport. A genetic screen for sirtinol resistant mutants led to the identification of a mutant that was named *sir1* (*sir*tinol *r*esistant *1*). PSI-BLAST searches revealed that SIR1 is actually the *Arabidopsis* homologue of yeast Uba4, a ubiquitin activating enzyme (E1)-like protein. Given that *sir1* is hypersensitive to auxin and that SIR1 appears to be a direct target of sirtinol, Zhao and co-workers propose that SIR1 functions as a negative regulator of auxin signalling. Sirtinol inhibition of SIR1 therefore leads to high auxin phenotypes. Although sirtinol was originally identified as an inhibitor of the sirtuin family of deacetylases, this study led to the serendipitous discovery of SIR1, an E1-like protein in *Arabidopsis* that is also inhibited by sirtinol and that regulates auxin signalling.

Gottschling, Simon and co-workers have also identified a yeast Sir2p inhibitor

using a similar *URA3* reporter gene assay (Bedalov *et al.*, 2001). However, in this case, a positive selection approach was used to identify Sir2p inhibitors based on the ability of yeast to grow on uracil-free media. A collection of 6000 compounds from the NCI repository was screened (0.5, 5, and 50 μM), leading to the identification of 11 active molecules. A follow-up screen was used to identify specific Sir2p inhibitors using a *TRP1* reporter gene integrated into a different Sir2p-regulated locus. This led to the identification of *splitomicin* (*Figure 2.28*), which also induced growth of the *TRP1* reporter gene strain in the absence of tryptophan.

Whole genome microarray analysis was then used to compare the transcriptional profiles of wild-type cells treated with splitomicin, a *sir2* deletion mutation, and four other deletion mutants of *SIR2* homologues. Importantly, the transcriptional effects of splitomicin correlated most strongly with those of the *sir2* deletion mutation. Splitomicin also inhibited the histone deacetylase activity of Sir2p *in vitro* (IC_{50} = 60 μM). The temporal control provided by the small molecule splitomicin was then used to demonstrate that continuous Sir2p deacetylase activity is required to maintain a silent state in non-dividing yeast cells.

Quercetin, piceatannol, and resveratrol, activators of SIRT1 and Sir2p, NAD-dependent deacetylases

More recently, Sinclair and co-workers have identified small molecule activators of the sirtuins SIRT1 and Sir2p to study longevity regulation in response to calorie restriction (Howitz *et al.*, 2003). In yeast, the calorie restriction response is mediated by the nicotinamidase *PNC1*, which depletes nicotinamide, a physiological inhibitor of Sir2p. In mammalian cells, SIRT1, in turn, deacetylates and negatively regulates p53, increasing cell survival.

Compound collections from BIOMOL were screened (100 μM) using a biochemical assay that detects deacetylation of an *N*-acetylated BIOMOL 'Fluor de Lys' substrate, which sensitizes the substrate for conversion to a fluorophore. The initial screen led to the identification of SIRT1 stimulators, *piceatannol* and *quercetin* (*Figure 2.29*), plant polyphenols that have previously been identified as kinase inhibitors. A secondary screen of collections of similar compounds led to the identification of *resveratrol* as an effective SIRT1 stimulator (13.4-fold vs. control). Follow-up studies demonstrated that resveratrol does not affect the V_{max} of SIRT1, but does cause a 35-fold decrease in the K_m for acetylated peptide substrate and a 5-fold decrease in K_m for NAD^+. Additional experiments demonstrated that resveratrol also stimulates the yeast sirtuin Sir2p and increased the average lifespan of

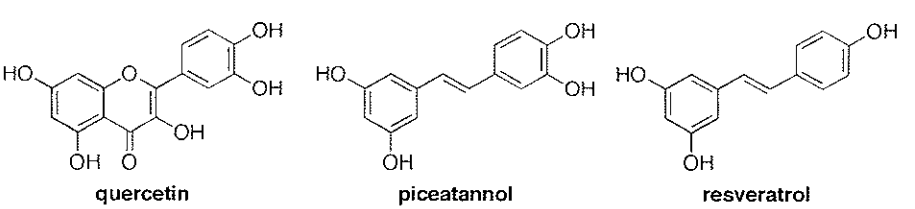


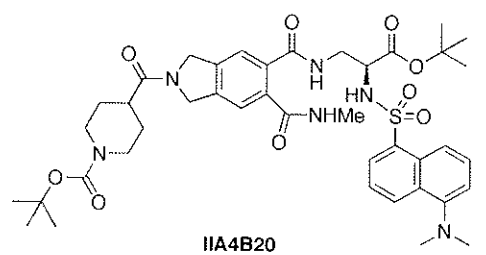
Figure 2.29. Structures of polyphenolic small molecule activators of the sirtuins SIRT1 and Sir2p.

S. cerevisiae by 70% (10 μM). Resveratrol had no effect upon a *sir2*Δ targetless strain, but was active in a *pnc1*Δ strain, indicating that it acts downstream of *PNC1* and requires *SIR2*. In mammalian cells, resveratrol (0.5 μM) was also shown to activate SIRT1 and to increase cell survival after ionizing radiation. This study provides an intriguing connection between the beneficial effects of the plant polyphenols on human health and the mimicking of calorie restriction conditions through sirtuin activation.

IIA4B20 and IIA6B17, antagonists of Myc–Max transcription factor heterodimerization

Myc is a basic helix–loop–helix leucine zipper (bHLHZip) transcription factor that promotes oncogenic transformation and tumorigenesis by regulating genes that drive cell proliferation and stimulate angiogenesis. Transcriptional activation of Myc target genes requires heterodimerization of Myc with Max, another bHLHZip protein. Inhibitors of Myc–Max dimerization could be useful to treat cancers that depend on sustained activation of Myc. To identify such compounds, Vogt and co-workers screened a combinatorial library for Myc–Max dimerization antagonists (Berg *et al.*, 2002).

An unbiased combinatorial library of 7000 anhydride-derived peptidomimetic compounds was synthesized by solution phase mixture synthesis (Boger *et al.*, 2000). A FRET-based, cell-free binding assay was used to screen for antagonists of Myc–Max heterodimerization. The bHLHZip domains of Myc and Max were fused to the *N*-termini of cyan fluorescent protein (CFP) and yellow fluorescent protein



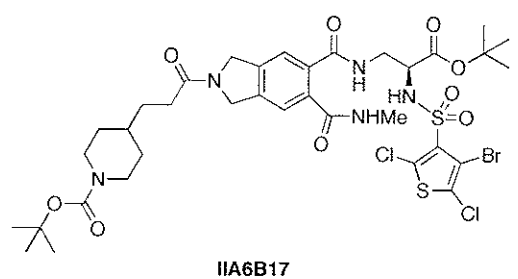


Figure 2.30. Structures of IIA4B20 and IIA6B17, small molecule antagonists of Myc–Max heterodimerization.

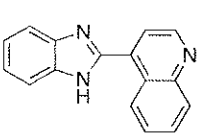
(YFP), respectively, and the fusion proteins were allowed to dimerize. The heterodimers were then incubated with library compounds (25 μM), and FRET analysis was used to identify compounds that caused dissociation of the dimer into its monomeric components. After deconvolution, two compounds, *IIA4B20* and *IIA6B17* (*Figure 2.30*), were identified as active inhibitors (26% and 38% inhibition at 25 μM). Activity was confirmed (IC_{50} = 75 μM and 125 μM) with a follow-up ELISA involving immobilized Max bHLHZip and GFP-labelled Myc detected using an HRP-conjugated anti-GFP antibody. However, only IIA6B17 was able to block DNA binding by MycGFP–Max in an electrophoretic mobility shift assay (IC_{50} = 50 μM), suggesting that the binding site for IIA4B20 may be inaccessible in the presence of DNA. Notably, both compounds blocked Myc-induced oncogenic transformation of chicken embryo fibroblasts in cell culture (IC_{50} = 20 μM and 20 μM), although IIA6B17 also blocked Jun-induced transformation. Although further analysis and optimization of these antagonists is needed, this study illustrates the exciting possibility that small molecules can be used to modulate protein–protein interactions that play a key role in transcriptional regulation.

Mellillo and co-workers have also carried out a screen for small molecule modulators of HIF-1 (hypoxia inducible factor-1), a related bHLHZip transcription factor (Rapisarda *et al.*, 2002). The NCI Training and Diversity Sets were screened using a HIF-1-driven luciferase reporter gene assay. Three known camptothecin analogues (EC_{50} ≈32.7, 181, and 477 nM) were identified in the screen, suggesting a particular connection between topoisomerase-I function and HIF-1-dependent gene expression.

A combined genetic–chemical genetic approach to specific inhibitors of mutant zinc finger transcription factors

Zinc finger proteins are the most ubiquitous family of transcription factors in the human genome, with over 1000 members. To modulate the functions of specific zinc finger transcription factors, Schultz and co-workers have recently screened a combinatorial library for small molecules that can rescue the function of zinc fingers carrying specific deleterious mutations (Lin *et al.*, 2003). This combined genetic–chemical genetic approach is a potentially general means to modulate zinc finger function specifically. (Although technically outside the parameters of this review, we elected to include this report since it is relatively recent and used screening to identify an appropriate small molecule activator.)

To identify molecules that would rescue the activity of a mutated transcription factor, cavity-generating mutations (F116A/H125G) were introduced into the murine



2-(4'-quinoline)benzimidazole

Figure 2.31. Structure of small molecule that partially rescues the function of a mutant zinc finger transcription factor.

zinc finger zif268, C7. This structurally compromised C7 mutant lacks transcriptional activity. A reporter gene assay was then used to screen for molecules that could induce expression of a C7 recognition element-driven luciferase reporter gene in the presence of the mutant C7 in HeLa cells. A combinatorial library of 250 heterocycles, originally directed to kinases (Ding *et al.*, 2002), was used in the screen (100 μM). Based upon the initial hits, a small tuning library was synthesized and screened, leading to the identification of *2-(4'-quinoline)benzimidazole* (*Figure 2.31*) as a moderate activator of the reporter gene (EC_{50} = 35 μM). Notably, this compound had no effect upon reporter gene expression in the presence of wild-type C7, indicating specificity for the mutant. Surface plasmon resonance was also used to demonstrate that the small molecule causes a 16-fold increase in the binding affinity of the mutant C7 for a cognate C7 recognition element DNA sequence (K_d = 5.7 μM increased to 350 nM). In comparison, however, the wild-type C7 binds with much greater affinity (K_d = 1.0 nM). This study extends the utility of combined genetic–chemical genetic approaches to zinc finger transcription factors. Due to the specificity of the genetic component of this approach, potent small molecule activators are not necessarily required for high specificity among zinc fingers. However, if they can be identified, such molecules will likely have greater overall cellular specificity, making them general and powerful tools for studying zinc finger function.

Pifithrin α, a small molecule suppressor of p53-mediated apoptosis

The transcription factor p53 is a tumour suppressor that induces growth arrest or apoptosis in response to a variety of stress signals, thereby eliminating damaged and potentially oncogenic cells from the organism. Conversely, inhibition of p53 may also be a means to protect normal tissues from the side effects of cancer therapies involving p53-deficient tumours. Along these lines, p53-dependent apoptosis occurs in sensitive tissues after gamma-irradiation (Komarova *et al.*, 1997), and p53-deficient mice have been shown to survive higher doses of gamma irradiation than wild-type animals (Westphal *et al.*, 1997). To investigate the feasibility of this approach, Gudkov and co-workers screened a compound collection for small molecule inhibitors of p53-mediated apoptosis (Komarov *et al.*, 1999).

A collection of 10 000 compounds from ChemBridge was screened in a *lacZ* reporter gene assay. Compounds were incubated (10–20 μM) with a ConA mouse

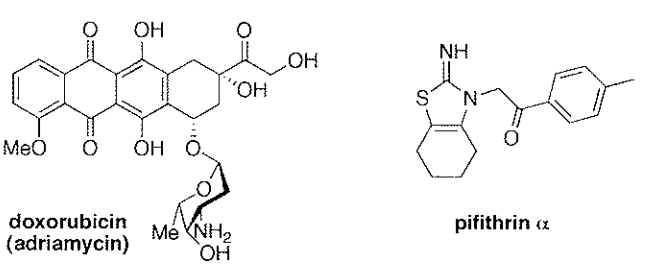


Figure 2.32. Structures of doxorubicin, a potent inducer of p53 function, and pifithrin α, a p53 antagonist.

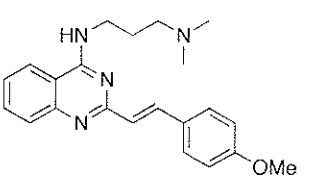
cell line that carries wild-type p53 and a *lacZ* reporter driven by a p53-responsive promoter. *Doxorubicin* (*Figure 2.32*) was used to induce p53. This led to the identification of *pifithrin* α, a compound that attenuated p53-responsive reporter gene expression without affecting cell growth or survival. Pifithrin α also antagonized reporter gene expression induced by UV light and gamma irradiation in a dose-dependent manner. Northern blot analysis confirmed that pifithrin α downregulates expression of p53-responsive genes.

The effects of pifithrin α on p53-mediated apoptosis were then analysed in an *E1a+ras*-transformed mouse embryo fibroblast line that undergoes rapid p53-dependent apoptosis in response to a variety of treatments. Pifithrin α (10 μM) inhibited apoptotic death of these cells in response to doxorubicin, etoposide, Taxol, cytosine arabinoside, UV light, and gamma irradiation. In contrast, pifithrin α had no effect on UV-treated cells in which p53 is suppressed by a dominant negative mutation. Pifithrin α also markedly inhibited apoptosis induced by direct overexpression of transiently transfected p53 in p53-deficient human sarcoma Saos-2 cells. This suggests that pifithrin α acts downstream of p53. Further, pifithrin α did not alter the phosphorylation state or sequence-specific DNA binding of p53 in ConA cells after DNA-damaging treatments. However, pifithrin α was shown to increase the ratio of cytoplasmic to nuclear p53 after induction by UV irradiation. Thus, pifithrin α may act by modulating the nuclear import and/or export of p53, or by decreasing the nuclear stability of p53.

In C57Bl and Balb/c mouse strains, pifithrin α treatment (2.2 mg/kg i.p.) completely protected mice from 60% lethal doses of gamma irradiation (8 Gy and 6 Gy). Importantly, despite the fact that p53-deficient mice are extremely sensitive to radiation-induced tumorigenesis (Kemp *et al.*, 1994), no tumorigenesis was observed in any of the pifithrin α-treated mice that survived gamma irradiation (n = 30, 7 mo). These results support the exciting possibility that p53 antagonists can be used to reduce the side effects of radiation therapy or chemotherapy for p53-deficient cancers.

CP-31398, a small molecule stabilizer of the active conformation of the p53 DNA binding domain

The activity of p53 is dependent on the ability of the protein to maintain a correct DNA binding conformation. In cancer cells, mutant p53 proteins may have decreased conformational stability and, hence, decreased activity. To explore the possibility of rescuing mutant p53 function, Rastinejad and co-workers at Pfizer



CP-31398

Figure 2.33. Structure of CP-31398, a small molecule that stabilizes the active conformation of the p53 DNA binding domain.

screened a compound collection for molecules that upregulate p53 by stabilizing the active conformation of the DNA binding domain (Foster *et al.*, 1999).

A collection of >100 000 synthetic compounds, presumably from the Pfizer compound collection, was screened in an ELISA using a monoclonal antibody, MAb1620, that binds selectively to the active conformation of the p53 DNA binding domain. Recombinant p53 DNA binding domain was immobilized in microtitre plate wells and heated in the presence of the compounds (45°C, 30 min). The wells were then probed with MAb1620 and an HRP-linked secondary antibody to identify small molecules that stabilized the active conformation of p53. Active compounds were then tested in a secondary screen for their ability to stabilize the active conformation of mutant p53 proteins, which are less stable than wild-type p53 to heating. In a tertiary screen, active compounds were tested for the ability to stabilize transfected mutant p53 in p53-null H1299 lung carcinoma cells. This led to the identification of *CP-31398* (*Figure 2.33*), a lead compound with activity at micromolar concentrations.

Subsequent follow-up experiments demonstrated that CP-31398 (100 mg/kg i.p.) stabilizes the active conformation of p53 in mouse xenografts derived from transfected H1299 cells. CP-31398 also upregulates mutant p53-driven expression of a luciferase reporter gene in H1299 cell culture and in mouse xenografts. Additional studies have been directed toward elucidating the exact mechanism of CP-31398 action (Rippin *et al.*, 2002; Wang *et al.*, 2003). This report represents another example of allosteric regulation of protein function using small molecules, and may lead to a new strategy for cancer therapy.

Haptamides, inhibitors of Hap2/3/4/5p mediated transcription

The Hap2/3/4/5p transcription factor complex is involved in regulation of aerobic respiration, nutrient response signalling, and mitochondrial function and, hence, is connected to numerous processes, such as circadian rhythm and various disease states, including cancer and diabetes. To probe the functions of this transcription factor complex, Schreiber and co-workers screened combinatorial libraries for small molecules that modulate the transcriptional activity of the Hap3p subunit (Koehler *et al.*, 2003).

Three different diversity-oriented synthesis pathways were used to generate combinatorial libraries by binary encoded, solid phase split–pool synthesis (Stavenger and Schreiber, 2001; Sternson *et al.*, 2001a; Spring *et al.*, 2002). A total of 12 396

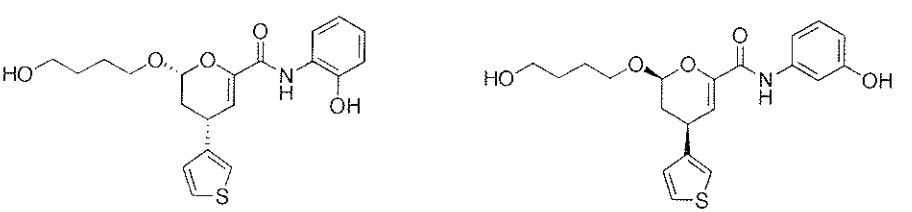


Figure 2.34. Structure of the haptamides, small molecule inhibitors of the Hap3p transcription factor.

compounds were then printed onto glass microscope slides for screening in a small molecule microarray binding assay. A purified Hap3p-glutathione *S*-transferase (GST) fusion protein was used to probe the microarray, and binding was detected using a Cy5-labelled anti-GST antibody. This led to the identification of *haptamide A* (*Figure 2.34*), which was derived from a library of dihydropyrancarboxamides (Stavenger and Schreiber, 2001) and bound Hap3p with moderate affinity (K_d = 5.03 μM), as determined by surface plasmon resonance. In a follow-up experiment, haptamide A was shown to inhibit expression of a Hap2/3/4/5p-driven *GDH1-lacZ* reporter gene in yeast (IC_{50} = 42 μM).

Subsequent synthesis and evaluation of haptamide A analogues led to the identification of *haptamide B*, which had greater affinity for Hap3p (K_d = 0.33 μM) and increased inhibitory activity in the reporter gene assay (IC_{50} = 23.8 μM). Transcriptional profiling experiments indicated that both treatment with haptamide B (50 μM) and deletion of *HAP3* led to downregulation of Hap2/3/4/5p-regulated genes. While the precise mechanism of haptamide B activity remains to be determined, this study represents another example of the use of a small molecule, microarray-based binding assay to identify cell-permeable modulators of protein function. Haptamide B should be a valuable tool to study Hap2/3/4/5p-mediated transcription and regulation of this complex.

Small molecule agonists of the farnesoid X receptor to probe cholesterol metabolism

The farnesoid X receptor (FXR) is a specialized sensor for bile acids and controls transcription of networks encoding key cholesterol metabolism enzymes. To probe

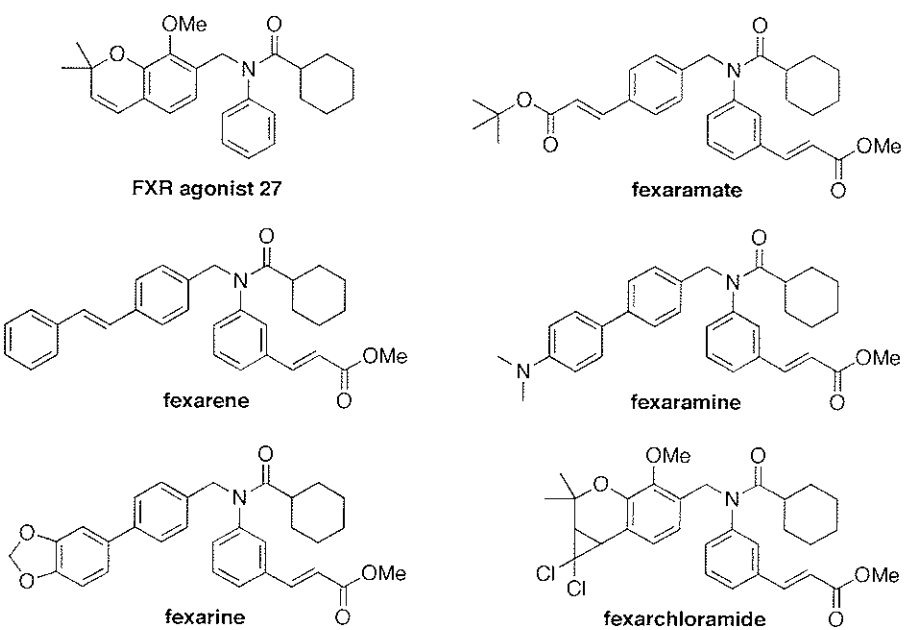


Figure 2.35. Structures of small molecule FXR agonists.

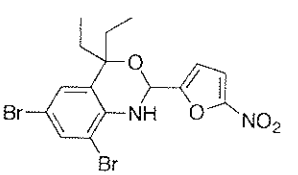
the role of FXR in cholesterol metabolism and its potential as a therapeutic target, Nicolaou and co-workers screened a combinatorial library for FXR agonists (Nicolaou *et al.*, 2003).

A combinatorial library of 10 000 natural product-like 2,2-dimethylbenzopyrans was synthesized using radiofrequency-encoded split–pool synthesis. The compounds were screened using an FXR-responsive luciferase reporter gene assay, providing several lead structures. A focused library of approximately 200 analogues was then synthesized using solid phase techniques and screened, leading to the identification of *FXR agonist 27* (*Figure 2.35*) that had moderate agonist activity (EC_{50} = 5–10 μM). Extensive SAR analysis through the synthesis of multiple focused libraries by parallel solution phase synthesis led to the identification of several potent FXR agonists, including *fexaramate* (EC_{50} = 127 nM), *fexarene* (EC_{50} = 36 nM), *fexaramine* (EC_{50} = 25 nM), *fexarine* (EC_{50} = 38 nM), and *fexachloramide* (EC_{50} = 188 nM). These compounds should prove valuable new probes to study FXR function, and may also serve as therapeutic leads in the future.

CG-18, an inducer of interferon-β enhanceosome assembly

Assembly of enhanceosomes on DNA enhancer sequences plays an important role in transcriptional regulation. However, little is known about the signal transduction pathways that lead to enhanceosome assembly. To probe this process, Kim and co-workers screened a compound collection for small molecules that activated interferon-β enhancer-driven transcription (Kim *et al.*, 2000).

A collection of 400 compounds from ChemBridge was screened (10–20 μg/mL) using a HeLa cell reporter gene assay involving a GFP reporter linked to the interferon-β enhancer. This led to the identification of *CG-18* (*Figure 2.36*), which also induced a second reporter gene in a dose-dependent manner (EC_{50} ≈10 μM). Subsequent follow-up assays measured the ability of CG-18 to activate reporter genes downstream of specific positive regulatory domains of the interferon-β enhancer. These experiments indicated that CG-18 upregulates the transcription factors that bind to each of the domains of the enhancer. This led to the hypothesis that CG-18 acts upstream of the interferon-β enhancer-binding proteins by stimulating mitogen-activated protein kinase kinase kinase (MEKK) family members. This was confirmed in biochemical experiments with immunoprecipitated MEKK1. Further experiments demonstrated that upregulation of MEKK1, indeed, leads to activation of all of the transcription factors involved in the interferon-β enhanceosome, delineating a signalling pathway leading to interferon-β enhanceosome assembly. Although the



CG-18

Figure 2.36. Structure of CG-18, a small molecule activator of the interferon-β enhanceosome.

exact mechanism of CG-18 activity has not yet been determined, it has already proven a useful tool for studying enhanceosome assembly.

A natural product inhibitor of the bacterial RNA polymerase–σ70 protein–protein interaction

Binding of sigma factor proteins to the core RNA polymerase is essential for specific initiation of transcription in bacteria. To explore the possibility of targeting these protein–protein interactions as a new antibiotic target, Burgess and co-workers screened a collection of crude sponge extracts using a luminescence resonance energy transfer (LRET) binding assay, related to FRET (Bergendahl *et al.*, 2003). The major sigma factor of *E. coli*, σ70, was labelled with an europium-diethylenetriaminepentacetic acid/7-amino-4-methylcoumarin-3-acetic acid complex as a donor. A fragment of the β' subunit of *E. coli* RNA polymerase was labelled with the Cy5 analogue IC5 as an acceptor. The LRET assay was then used to screen 100 marine sponge extracts (≈1 μM), leading to the identification of a single extract that reduced the LRET signal by 90%. Activity was confirmed with an *in vitro* transcription follow-up assay (IC_{50} ≈1 μM). Unfortunately, additional follow-up studies for inhibition of *E. coli* growth were inconclusive due to the limited amount of the extract available. Nonetheless, this study demonstrates the utility of the LRET assay for identifying inhibitors of protein–protein interactions, and future screens may identify suitable leads for antibiotic drug development.

APOPTOSIS PROBES

Apoptosis is a key cellular process that is critical for normal development and function of multicellular organisms (Kaufmann and Hengartner, 2001). Increased rates of apoptosis are associated with neurodegenerative diseases, including Alzheimer's and Parkinson's diseases, AIDS, and cardiovascular disease. Conversely, abnormally low rates of apoptosis are linked to human cancers and autoimmune disorders. The development of small molecules that target proteins involved in apoptosis has been of great interest, both to probe this complex, tightly regulated process, and to identify new avenues for therapeutic intervention. Moreover, this area presents an exciting challenge since many protein–protein interactions, traditionally intractable targets for modulation by small molecules, are involved in apoptotic signalling (Huang, 2002; Rutledge *et al.*, 2002). In this section, we present several examples of small molecules that target apoptotic processes.

Small molecule inhibitors of the Bak-BH3–Bcl-2 protein–protein interaction

The Bcl-2 protein family regulates the response of mitochondria to upstream apoptotic signals (Huang, 2002). While the exact mechanism of action is unknown, several studies indicate that Bcl-2 homology 3 domain (BH3)-mediated homodimerization and heterodimerization plays a significant role in the regulation process. To investigate this idea further, Yuan and co-workers screened a compound collection for small molecules that disrupt the binding of the BH3 domain of Bak to Bcl-x_L (Degterev *et al.*, 2001).

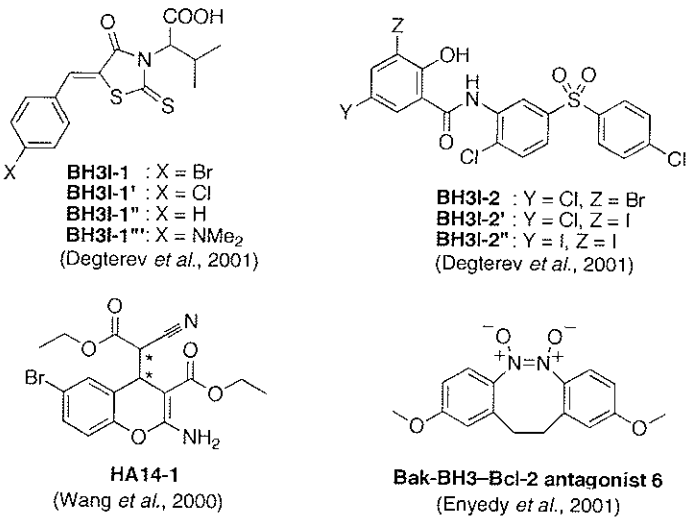


Figure 2.37. Structures of small molecule of the Bak-BH3–Bcl-x_L and Bak-BH3–Bcl-2 protein–protein interactions.

A collection of 16 320 compounds from ChemBridge was screened using a fluorescence polarization binding assay to monitor displacement of fluorescently labelled Bak-BH3 domain from a recombinant GST–Bcl-x_L fusion protein. This led to the identification of *BH3I-1*, *BH3I-1'*, and *BH3I-2* (*Figure 2.37*), which blocked the Bak-BH3–Bcl-x_L protein–protein interaction (K_i = 2.4 μM, 3.6 μM, and 4.1 μM). These compounds, in addition to four commercially available analogues (*BH3I-1''*, K_i = 12.5 μM; *BH3I-1'''*, K_i n.d.; *BH3I-2'*, K_i = 3.3 μM; *BH3I-2''*, K_i = 6.4 μM) were studied in further experiments.

Treatment of cells with the BH3Is resulted in DNA fragmentation, a sub-G1 DNA content indicative of apoptosis, and the appearance of Annexin V binding without an increase in staining with propidium iodide. These results indicated that the inhibitors were capable of inducing apoptosis. Subsequent studies with caspase-3 and caspase-9 fluorogenic substrates demonstrated that the BH3Is induce the activation of caspases in the mitochondrial pathway. The extent of cell death after treatment with the BH3Is correlated with the K_i values determined *in vitro*, suggesting that the BH3Is act through direct disruption of BH3 domain interactions. FRET experiments confirmed BH3I inhibition of heterodimerization of Bcl-x_L and Bax, another Bcl-2 family member, in intact cells. These findings showed that BH3-mediated heterodimerization plays a crucial role in the anti-apoptotic functions of Bcl-x_L, and is required for the maintenance of cellular homeostasis.

Huang and colleagues recently used an *in silico* binding assay to identify small molecule probes of Bcl-2-regulated apoptotic pathways (Wang *et al.*, 2000). The NMR structure of Bcl-x_L was used to generate a homology model of Bcl-2. The DOCK molecular modelling programme was then used to screen a collection of 193 833 compounds from the MDL/ACD 3D database. The 1000 compounds that

displayed the best binding to the BH3 binding region of the Bcl-2 model were analysed further. The orientation of each molecule was optimized, and the binding energy was calculated. Subsequently, 28 of these molecules were actually obtained for study in biological systems.

A fluorescence polarization assay was used to screen for competitive binding with Bcl-2 and a fluorescently labelled peptide ligand. This led to the identification of *HA14-1* (*Figure 2.37*), which bound with the highest affinity ($IC_{50} \approx 9$ μM). HA14-1 induced apoptosis in HL-60 human myeloid leukaemia cells in a dose-dependent manner, and the cells displayed the characteristic DNA fragmentation effect indicative of apoptosis. These effects could be suppressed by preincubation with the broad-spectrum caspase inhibitor zVAD-fmk. Western blot analysis confirmed the activation of caspase-9, caspase-3, and PARP in response to HA14-1. This apoptotic effect depends on the action of Apaf-1, but is distinct from the Fas pathway.

Wang and co-workers used a similar *in silico* binding assay to identify inhibitors targeted to the BH3-binding region of Bcl-2 (Enyedy *et al.*, 2001). They screened the NCI's public compound database, which contains 206 876 small molecules and natural products. This led to the identification of *Bak-BH3–Bcl-2 antagonist 6* (*Figure 2.37*), which bound to the BH3 binding region of Bcl-2, as determined by a fluorescence polarization assay (IC_{50} = 10.4 nM). This compound also induced apoptosis in HL-60 cells in a dose-dependent manner (IC_{50} = 4 nM).

These latter two studies demonstrate that *in silico* screening is a useful technique for reverse chemical genetic screens. Although it requires the availability of structural information and identification of a putative binding site, this approach can be used to identify small molecule ligands that have binding affinities comparable to those of ligands identified by 'wet' screening approaches.

TWX024, TWX006, and NSC 321206, inhibitors of the XIAP–caspase protein–protein interaction

The caspase family of proteases are downstream effectors of apoptotic signalling that are negatively regulated by direct binding of inhibitor of apoptosis proteins (IAPs) (Salvesen and Duckett, 2002). Conversely, appropriate apoptotic stimuli lead to mitochondrial release of proapoptotic proteins, such as second mitochondria-derived activator of caspase/direct IAP binding protein with low p*I* (Smac/DIABLO), which bind competitively to IAPs and release caspases. Notably, IAPs are overexpressed in many cancer cells. To probe the activities of IAPs and caspases, Deveraux, Schultz, and co-workers screened a combinatorial library for inhibitors of the protein–protein interaction between X-linked IAP (XIAP) and caspase-3 (Wu *et al.*, 2003).

Initially, 160 000 compounds from an undisclosed source were screened (10 μM) using a biochemical assay for caspase-3 activity on a fluorogenic substrate in the presence of recombinant XIAP. Subsequently, a combinatorial tuning library of 7040 compounds was synthesized using solid phase parallel synthesis. Screening using the biochemical assay led to the identification of *TWX006* and *TWX024* (*Figure 2.38*), which were moderate XIAP antagonists (IC_{50} = 10 μM and 25 μM).

TWX024 exhibited higher aqueous solubility and was used in cell-based follow-up assays. The compound (25 μM) successfully restored CD95-stimulated apoptosis

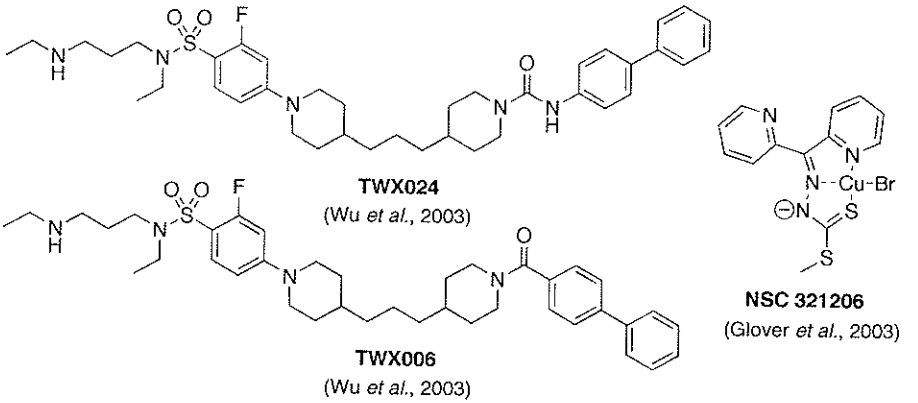


Figure 2.38. Structures of TWX006, TWX024, and NSC 321206, small molecule ligands for XIAP.

in XIAP co-transfected 293 cells. Meanwhile, the compound alone (40 μM) did not induce apoptosis. Co-precipitation experiments indicated that TWX024 specifically disrupts the binding of XIAP to caspase-3. TWX024 also synergistically stimulated apoptosis in HCT116 cells that were deficient in Bax, which promotes the release of Smac from the mitochondria. This experiment, coupled with molecular modelling studies, supports a model in which TWX024 is a functional mimic of Smac, binding to XIAP and displacing caspase-3. TWX024 is a valuable new tool to probe apoptotic regulation pathways, and can also be used to validate XIAP as an anticancer target.

Glover and co-workers have also screened the NCI Training Set and Diversity Set for Smac-mimetic XIAP ligands (Glover *et al.*, 2003). A fluorescence polarization binding assay was used to screen for molecules (1 μM and 50 μM) that compete with a labelled peptide corresponding to the *N*-terminus of Smac. Follow-up serial dilution experiments were used to eliminate highly fluorescent and fluorescence-quenching compounds, leading to the identification of *NSC 321206* (*Figure 2.38*) as a moderate inhibitor (IC_{50} = 10.32 μM). In light of the presence of copper, the molecule was not deemed a promising lead compound for further cell-based experiments. However, the fluorescence polarization assay provides an efficient method to screen larger libraries for new inhibitors.

N-*Acyl aromatic amines, selective inducers of apoptosis in cancer cells*

Members of the *N*-acyl aromatic amine class of molecules have been shown to exhibit pro- and anti-apoptotic activity. Using an anti-apoptotic natural product from the whole plant extract of *Isodon excisus* (*Figure 2.39*) as a lead compound, Hergenrother and co-workers recently screened a combinatorial library for molecules that selectively induce apoptosis in cancer cells (Nesterenko *et al.*, 2003).

A combinatorial library of 88 amides was synthesized from 11 aromatic ethylamines and 8 cinnamic acids using a polymer-supported carbodiimide. The library (100 μM) was first screened for cytotoxicity in HL-60 leukaemia and U-937 lymphoma cell lines using a dye bioreduction assay. Three compounds, *apoptosis*

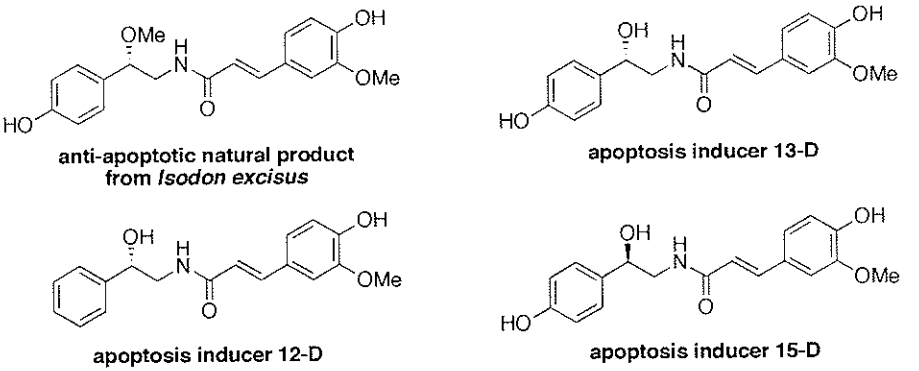


Figure 2.39. Structures of an anti-apoptotic natural product from *Isodon excisus* and analogues that selectively induce apoptosis in cancer cells.

inducers 12-D, *13-D*, and *15-D*, had activity against both cell lines (U-937 IC_{50} = 61 μM, 44 μM, 109 μM). Secondary experiments were used to differentiate between apoptotic and necrotic cytotoxicity, and demonstrated that 13-D induced apoptosis, as evidenced by induction of caspase-3, mitochondrial depolarization, membrane blebbing, and cell shrinkage. A tertiary experiment then assessed the selectivity of 13-D for cancer cells by comparison to non-cancerous mouse splenocytes. Strikingly, even at high concentrations (500 μM), 13-D exhibited no toxicity against these non-cancer cells. This study provides a new small molecule to study selective induction of apoptosis in cancer cells, and may ultimately lead to a new therapeutic target for cancer.

SMALL MOLECULE PROBES FOR OTHER SYSTEMS

The chemical genetic approach has also been applied to a variety of other biological systems that fall outside the above classifications. These include dynamic processes such as development and protein trafficking, where the fine temporal control afforded by small molecules is extremely useful.

Small molecule modulators of zebrafish development

The zebrafish *Danio rerio* is an attractive model organism for studying developmental processes due to its small embryo size, large clutch sizes, and *ex utero* development in a transparent embryo. Furthermore, zebrafish embryos are highly permeable to small molecules, facilitating the identification of small molecules to probe developmental processes. Small molecule modulators provide a powerful means to determine the timing of critical developmental processes. Schreiber and co-workers have identified a number of such small molecule probes using a microscopy-based forward chemical genetic screen of a compound collection (Peterson *et al.*, 2000).

A collection of 1100 compounds from ChemBridge was incubated (1 μM) with fertilized zebrafish eggs in 96-well microtitre plates. The embryos were examined visually for developmental defects at one, two, and three days post-fertilization (dpf). Special attention was paid to the central nervous system, the cardiovascular

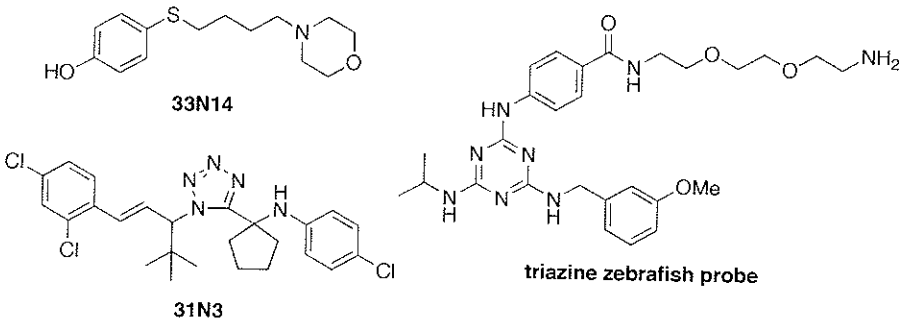
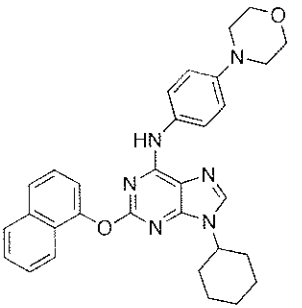


Figure 2.40. Structures of small molecules used to probe zebrafish melanocyte, otolith, and brain/eye development.

system, pigmentation, and the ear. Approximately 2% of the compounds were lethal, or caused general necrosis, while approximately 1% of the compounds appeared to affect a specific aspect of one of the systems examined. Many of the phenotypes could be correlated with those caused by known genetic mutations, indicating that the targets are likely involved in the same biological pathways.

In particular, two molecules were used to study the timing of developmental processes. The molecule *33N14* (*Figure 2.40*) inhibits pigmentation in the zebrafish body, but not in the pigmented retinal epithelium. This molecule was used to determine that specification or proliferation of melanocytes in the zebrafish body can occur up to 2 dpf, but it is limited thereafter. Another compound, *31N3*, modulated ear development by blocking the formation of otoliths but not the surrounding otic vesicles. Otoliths are small bony structures that allow zebrafish to sense vibrations and maintain balance. As a result, 31N3-treated fish are unable to maintain balance and often swim on their sides or upside down. This molecule was used to demonstrate that the critical stage of otolith development occurs between 14 and 26 hours post-fertilization. This study demonstrates that large-scale developmental screens can identify small molecules that modify developmental events with specificity approaching that of genetic mutations. The unique temporal control afforded by small molecules makes the dissection of developmental processes possible with a precision not possible with gene knockouts.

Recently, Chang and co-workers used this visual microscopy-based assay to screen a combinatorial library of 1536 triazines (50 μM) for brain/eye morphological changes (Khersonsky *et al.*, 2003). The compounds were all tagged with a linker terminating in an amino group to facilitate subsequent identification of the biological targets by affinity chromatography. This led to the identification of a *triazine zebrafish probe* (*Figure 2.40*) that caused morphological changes in head development and retarded eye development. The compound was then immobilized on AffiGel 10 beads and treated with zebrafish protein extracts. Two strong bands were identified following washing and SDS-PAGE analysis. Excision and LC-MS/MS analysis identified the 40S ribosomal subunit protein S5 from the 23 kDa band. Three proteins were identified from the 18 kDa band, the 40S ribosomal subunit protein S18, the *Danio rerio* EST sequence similar to human 40S ribosomal subunit protein S13, and mouse 60S ribosomal subunit protein L28. These studies implicate a protein complex that includes S5, S13, S18, and L28 in zebrafish brain/eye



purmorphamine

Figure 2.41. Structure of purmorphamine, a small molecule inducer of mesenchymal stem cell osteogenesis.

development, and demonstrated that screening of tagged libraries can facilitate target identification.

Purmorphamine, a small molecule inducer of stem cell differentiation

Mesenchymal stem cells are capable of differentiating into the mesenchymal cell lineages, such as bone, cartilage, adipose, muscle, stroma, and tendon, and play important roles in repair and regeneration (Pittenger *et al.*, 1999). Misregulation of these processes is associated with several bone-related diseases. To develop new tools to study this mesenchymal stem cell differentiation, Schultz and co-workers screened a combinatorial library for small molecules that induce osteogenesis in mesenchymal stem cells (Wu *et al.*, 2002).

A combinatorial library of 50 000 heterocycles, originally directed to kinases (Ding *et al.*, 2002), was screened in C3H10T1/2 multipotent mesenchymal progenitor cells, which can differentiate into various mesenchymal cells and are typically used as a model system for osteoblast differentiation. In this cell-based biochemical assay, osteogenesis was detected by expression of a specific marker, alkaline phosphatase (ALP), which hydrolyses inorganic pyrophosphate to phosphate, and promotes the formation of hydroxyapatite crystals in bone matrix. ALP also hydrolyses 2'-(2'-benzothiazoyl)-6'-hydroxybenzotiazole phosphate, generating a highly fluorescent product. This led to the identification of *purmorphamine* (*Figure 2.41*), which induced ALP expression (EC_{50} = 1 μM). In a follow-up assay, the bone-specific transcription factor Cbfa1/Runx2 was used in a luciferase reporter gene assay to confirm that purmorphamine induces differentiation into osteoblasts. Treatment of C3H10T1/2 cells with purmorphamine led to a more than 6-fold increase in reporter activity, and histochemical staining showed that more than 80% of cells expressed ALP after purmorphamine treatment. Purmorphamine also synergized with BMP-4, a known inducer of C3H10T1/2 osteoblast differentiation. The effect was approximately 3-fold greater than a simple additive effect, suggesting purmorphamine does not act as a BMP-4 analogue. This synergistic effect can also induce the transdifferentiation of preadipocytes into an osteoblast lineage. This

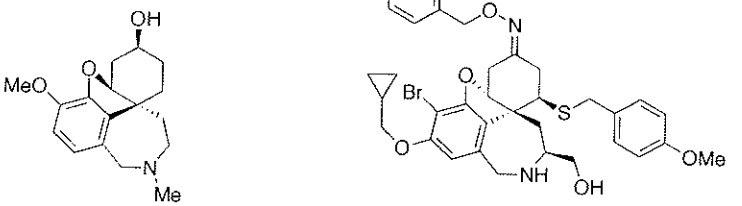


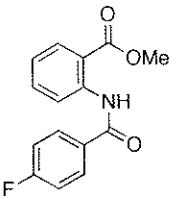
Figure 2.42. Structures of galanthamine, a natural product acetylcholinesterase inhibitor, and secramine, a combinatorial library-derived modulator of Golgi–plasma membrane protein trafficking.

study identified a new small molecule inducer of stem cell differentiation. Elucidation of the biological target should provide new insights into the molecular mechanisms of differentiation.

Secramine, a small molecule modulator of Golgi–plasma membrane protein trafficking

The secretory pathway involves protein trafficking from the endoplasmic reticulum (ER), through the Golgi apparatus, to the plasma membrane. Most of the steps involved in secretion are dynamic and highly regulated, making this process difficult to study using traditional genetic and biochemical methods. As a result, specific, reversible, fast-acting small molecule modulators of the secretory pathway are highly desirable. While the natural product secretion inhibitor brefeldin A has been useful for studying Golgi function, it has pleiotropic effects in mammalian cells whose mechanisms are often hard to differentiate. Thus, to develop new tools to study the secretory pathway, Shair and co-workers screened a combinatorial library for small molecules that modulate protein trafficking (Pelish *et al.*, 2001).

An unbiased combinatorial library of 2527 *galanthamine* (*Figure 2.42*) analogues was synthesized by solid phase parallel synthesis. The galanthamine scaffold was not selected on the basis of its activity as an acetylcholinesterase inhibitor (Coyle and Kershaw, 2001), but rather as a rigid core structure for generating diverse, complex molecules. In the primary microscopy-based screen, a GFP fusion protein of the surface glycoprotein of vesicular stomatitis virus (VSVG) was used to monitor the ability of library members (750 nM) to modulate protein trafficking. This led to the identification of *secramine*, which blocked trafficking from the Golgi apparatus to the plasma membrane (EC_{50} = 2 μM). Notably, galanthamine has no observable effects on the secretory pathway at concentrations up to 100 μM. Although the target of secramine has not yet been identified, this study yielded a new small molecule to probe the secretory pathway, and supports the idea that natural products can be used as library core structures to identify small molecules with unrelated biological activities.



Exo1

Figure 2.43. Structure of Exo1, a small molecule inhibitor of ER–Golgi protein trafficking.

Exo1, a small molecule inhibitor of ER–Golgi protein trafficking

In related efforts, Feng, Kirchhausen and co-workers screened a compound collection for small molecules that modulate specific steps in protein trafficking (Feng *et al.*, 2003).

The phenotype-based assay used a temperature-sensitive mutant of VSVG–GFP (VSVG^{ts}–GFP) that is exported to the Golgi apparatus, then the plasma membrane at 32°C, but that remains in the ER at 40°C. Automated fluorescence microscopy was used to screen a collection of 10 240 compounds from ChemBridge (≈67 μM) for the ability to modulate VSVG^{ts}–GFP localization at these temperatures. This led to the identification of *Exo1* (*Figure 2.43*), which blocked VSVG^{ts}–GFP exocytosis to the ER at 32°C (IC_{50} ≈20 μM). Subsequent fluorescence microscopy experiments indicated that Exo1 disrupts vesicular traffic from the ER to the Golgi apparatus by disrupting Golgi structures, inducing massive tubulation. Although brefeldin A induces similar effects, Exo1 does not appear to act directly on ARF1 GTPase, the target of brefeldin A. Exo1 also does not affect the trans-Golgi network or endosomes. Although the biological target of Exo1 remains to be determined, it is a valuable addition to the repertoire of small molecules available to study the dynamic process of protein trafficking.

Isatin O-acyl oximes, selective inhibitors of the neuronal ubiquitin hydrolase UCH-L1

The ubiquitin *C*-terminal hydrolase UCH-L1 is believed to play a key role in processing polyubiquitin and/or ubiquitinated proteolytic peptides by catalysing ubiquitin hydrolysis or transfer. UCH-L1 has been linked to Parkinson's disease, the progression of certain non-neuronal tumours, and neuropathic pain. To probe the role of UCH-L1 in these processes, Lansbury and co-workers screened a set of compound collections for UCH-L1 inhibitors (Liu *et al.*, 2003).

Approximately 42 000 commercially available compounds from Prestwick, Specs, Biospecs, Bionet Research Ltd., Maybridge Plc., Cerep, Peakdale, and Chemical Diversity Labs were screened (50 nM) using a biochemical assay for the UCH-L1-catalysed release of 7-amino-4-methylcoumarin from the *C*-terminus of ubiquitin. A secondary screen was used to determine selectivity for UCH-L1 over a systemic isoform UCH-L3. This led to the identification of a series of isatin *O*-acyl oximes,

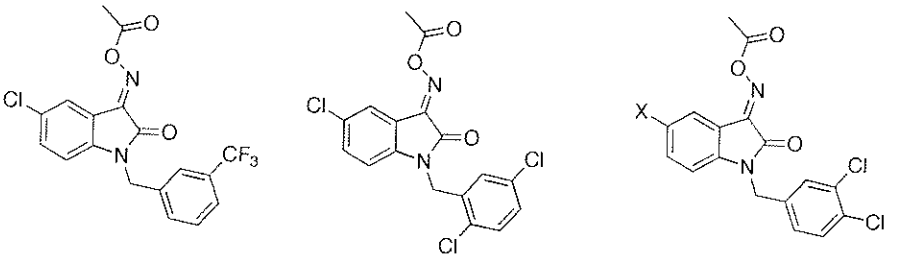


Figure 2.44. Structures of isatin *O*-acyl oximes UCH-L1 inhibitors 1, 30, 50, and 51.

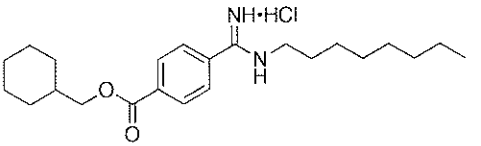
including *UCH-L1 inhibitor 1* (*Figure 2.44*), with micromolar inhibitory activity (IC_{50} = 6 nM) and good selectivity for UCH-L1 over UCH-L3 (5.3-fold vs. IC_{50} = 32 nM). Synthesis and testing of a series of approximately 50 analogues provided *UCH-L1 inhibitors 30*, *50*, and *51*, with submicromolar activity (IC_{50} = 0.88 nM, 0.82 nM, 0.94 nM) and improved selectivity (28-fold, 21-fold, 22-fold). UCH-L1 inhibitor 30 was found to act as a reversible, competitive inhibitor of UCH-L1 (K_i = 0.40 nM). UCH-L1 inhibitor 50 also promoted proliferation of H1299 non-small cell lung cancer cells that express UCH-L1, but not H358 non-small cell lung cancer cells that do not express UCH-L1. Subsequent experiments indicated an inverse correlation between UCH-L1 expression levels and proliferation of H1299 cells. The new UCH-L1 inhibitors identified in this study should be useful tools to probe the role of UCH-L1 in Parkinson's disease, cancer, and neuropathic pain.

SFK1, a small molecule suppressor of FK506 to probe mitochondrial function

The immunosuppressant FK506 inhibits the Ca^{2+}-dependent protein phosphatase calcineurin, which is required for the growth of yeast under certain stress conditions, such as high Na^+ concentration. To identify new small molecules to probe calcineurin function, Schreiber and co-workers screened compound collections and combinatorial libraries for molecules that would permit growth of calcineurin-deficient cells under high NaCl concentration (Butcher and Schreiber, 2003).

A collection of approximately 100 000 compounds from ChemBridge, and the NCI and a combinatorial library (Stavenger and Schreiber, 2001) were screened (20–40 μM) for their ability to enable growth of calcineurin-deleted or FK506-treated wild-type yeast in high-NaCl media. This led to the identification of *SFK1* (*Figure 2.45*), which promotes growth of FK506-treated cells (EC_{50} = 1.5–2.5 μM). However, SFK1 also inhibited growth under low NaCl conditions, suggesting that SFK1 causes the level of sodium in the cell to be reduced. Affinity chromatography was then used to identify possible targets of SFK1. An amino derivative of SFK1 was synthesized and immobilized on a sepharose resin, then a yeast lysate was incubated with the SFK1 resin. Por1p, a pore protein in the outer mitochondrial membrane, was identified as an SFK1 target, suggesting that SFK1 may be localized to the mitochondria.

To characterize the interactions of SFK1 on a genome-wide scale, the haploid



SFK1

Figure 2.45. Structure of SFK1, a small molecule modulator of mitochondrial function.

yeast deletion mutants generated by the *Saccharomyces* Genome Deletion Project were screened for strains that showed synthetic effects of selective sensitivity or resistance to SFK1. Several deletion strains with impaired mitochondrial function or impaired ion homeostasis were selectively sensitive to SFK1 treatment, providing additional evidence that SFK1 affects both mitochondrial function and ionic balance. In addition, transcriptional profiling analysis of SFK1-treated cells showed that the small molecule inhibits the transcription of genes associated with mitochondrial functions. These results suggest that ionic balance is tied to mitochondrial function, and that calcineurin acts as a mediator of this connection.

A small molecule cell migration inhibitor identified using high-throughput cell imaging

Cell migration is a critical process in embryonic development and adult physiology and has also been implicated in tumour metastasis and angiogenesis. Mitchison and co-workers recently reported a microscopy-based screen for cell migration inhibitors using a tissue culture wound healing assay adapted for microtitre plates (Yarrow *et al.*, 2003). Confluent BS-C-1 monkey epithelial cell monolayers were scratched with a floating pin array, followed by treatment with approximately 16 000 different compounds, presumably from a ChemBridge compound collection. After 6 h, the cells were fixed and stained for DNA and actin. Approximately 300 compounds blocked cell migration as determined by high-throughput microscopy. A secondary screen for cell viability led to the identification of a single compound (structure not disclosed) that blocks cell migration without causing general toxicity. This study demonstrates an efficient method of screening for cell migration inhibitors using high-throughput cell imaging.

An organelle-targeted fluorescent library

In an interesting twist on chemical genetic screening, Chang and co-workers recently synthesized a combinatorial library of 276 fluorescent styryl compounds by solution phase synthesis and screened them for localization to specific organelles or macromolecules (Rosania *et al.*, 2003). First, the absorption and emission spectra of each compound were determined and were shown to span a colour range from blue to long red, due to the structural diversity incorporated. The molecules were then incubated with UACC-62 human melanoma cells, and subcellular localizations of the compounds were determined using fluorescence microscopy. Since the library compounds are positively charged, it is not surprising that 64 out of the 276 tested compounds localize to the mitochondria. Other compounds localized to organelles

other than the mitochondria, such as the nucleolus, nucleus, endoplasmic reticulum, and some vesicles and granules in the cells. Chang and co-workers are currently investigating whether these molecules are targeted to specific macromolecules. Already, these small molecules can be used as new reagents to visualize specific subcellular structures.

Future directions

As described in this review, chemical genetic screens have provided a wide range of new small molecule probes to study biological processes. Broader acceptance of the chemical genetic approach, coupled with the availability of commercial compound collections, has led to a significant rise in the number of new small molecule probes reported over the last three years. Of the 56 studies presented in this review, 7 (13%) were published in 2001, 13 (23%) were published in 2002, and 22 (39%) were published in 2003. Several exciting initiatives bode well for the increased use of chemical genetic approaches in the future.

First, continuing efforts in diversity-oriented synthesis should provide new combinatorial libraries of complex molecules for screening. Many of the molecules in this review were identified from the ChemBridge compound collection at the Harvard Institute of Chemistry and Cell Biology, or from a kinase-directed heterocycle library synthesized by Schultz and co-workers. Doubtless, these will continue to be fruitful sources of new small molecule probes in the future. However, new combinatorial libraries should provide complementary chemical structures that may be able to address a wide range of targets with high cellular specificity. Importantly, the recently released NIH Roadmap for Medical Research (http://nihroadmap.nih.gov) includes a Molecular Libraries Initiative to assemble over 500 000 compounds that will be made available to public sector biomedical researchers for screening.

Second, continuing development of informatics-based approaches to target identification is facilitating this traditionally daunting process. Transcriptional profiling has been used increasingly for this purpose in recent years, and we expect that genetic–chemical genetic synthetic analysis, using the haploid yeast deletion mutants generated by the *Saccharomyces* Genome Deletion Project, will follow a similar trend. An intriguing further possibility is the idea that access to a complete set of siRNAs to silence every gene in the human genome would allow synthetic analysis to be extended to mammalian cells. Moreover, ongoing advances in computational biology should allow the analysis of bioinformatic data to become increasingly routine, further speeding the process of target identification.

Third, a new NIH-sponsored initiative, ChemBank (http://chembank.med.harvard.edu), is currently being developed to make data on chemical genetic probes freely available to the public. As the number of established chemical genetic probes continues to increase, this will serve as a useful destination for researchers seeking a small molecule to study their biological system of interest.

In view of the increasing impact of the chemical genetic approach in improving our understanding of biological systems, coupled with ongoing efforts to facilitate efficient, systematic identification of small molecule probes having high cellular specificity, chemical genetics is poised to provide a powerful complement to genetics and RNAi in the coming years.

Note added in proof

Three articles cited in this review included disclosures of new small molecule probes that were not discussed explicitly herein: Barnes–Seeman *et al.*, 2003; Spring *et al.*, 2002; Sternson *et al.*, 2001a.

Furthermore, we have identified several additional relevant papers from the period 1996–2003 describing new small molecule probes:

CHANG, Y.-T., CHOI, J., DING, S. *ET AL.* (2002). The synthesis and biological characterization of a ceramide library. *Journal of the American Chemical Society* **124**, 1856–1857.

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PART 2

Microbial & Cellular Biotechnology