Chemical genetics: tailoring tools for cell biology

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Chemical genetics is a research approach that uses small molecules as probes to study protein functions in cells or whole organisms. Here, I review the parallels between classical genetic and chemical-genetic approaches and discuss the merits of small molecules to dissect dynamic cellular processes. I then consider the pros and cons of different screening approaches and specify strategies aimed at identifying and validating cellular target proteins. Finally, I highlight the impact of chemical genetics on our current understanding of cell biology and its potential for the future.

It is an omnipresent challenge for every cell biologist to dissect a complex biological process within a cellular system. In general, it is not only the number of the participating components but also the complexity of their regulation and interactions that constitute this daunting task. Historically, genetic approaches have proven to be extremely powerful in elucidating the principles of a wide range of biological processes such as cell division in the budding yeast *Saccharomyces cerevisiae* [1], programmed cell death and organ development in the nematode *Caenorhabditis elegans* [2] or early embryonal development in the fruitfly *Drosophila melanogaster* [3]. These famous screens, honored by the Nobel Prize, are classical examples of forward-genetic approaches, which can be outlined by a three-step procedure: first, the genome of the chosen organism has to be mutagenized in a random fashion (e.g. EMS, X-ray, transposons), then the desired phenotype has to be detected within the large collection of generated mutants and, finally, the mutated gene that underlies the phenotype has to be identified. Whereas the forward-genetic method works its way from the desired phenotype 'forward' to the underlying mutated gene, reverse-genetics elucidates the function of a protein of interest by analyzing the phenotype when the encoding gene is mutated. In these studies, the introduced mutations can either affect the activity of the chosen protein (e.g. site-specific mutations) or entirely suppress its expression (e.g. 'knockout' mutations).

What is chemical genetics and why make use of it?
The term 'chemical genetics' has been coined for an approach that uses small organic molecules (see Fig. 1)

as probes to study protein functions in cultured cells or whole organisms. In accordance with the aforementioned genetic approaches, we can distinguish between forward- and reverse-chemical genetics. A forward-chemical genetic screen – like its genetic counterpart – can be subdivided into three discrete steps: first, the organism or cell type of interest has to be treated with a diverse collection of small molecules. This step mirrors the introduction of random genetic mutations in the forward-genetic approach. The goal of the second step is to identify the desired phenotype within the large collection of compound-treated organisms or cells and, finally, the cellular molecules (e.g. proteins, lipids, sugars) targeted by the bioactive compounds have to be identified. Similar to the genetic approach, the starting point for reverse chemical genetics is a protein of interest for which bioactive ligands are searched out of a large pool of small molecules.

An interesting combination of reverse genetics with chemistry is provided by a recent approach pioneered by Shokat and colleagues [4]. The underlying idea of their method is to genetically engineer a mutant nucleotide binding protein (e.g. kinase, motor protein), which becomes sensitive to a nucleotide analog but retains almost normal enzymatic activity in the presence of the wild-type nucleotide. In this way, the mutant allele can be inhibited by the nucleotide analog in a cellular context, whereas all other wild-type nucleotide binding proteins remain essentially unaffected (see below).

The genetic approach is powerful in that it can readily identify gene products involved in a specific process and then mutate the encoding gene with complete specificity within a complex cellular context. However, there are situations in which small molecules might be more useful. First, in many cases, one and the same small molecule can be applied to study a process of interest in a wide range of organisms, whereas the genetic approach logically demands that the desired genetic modification has to be regenerated every time the experimental system is switched. For example, brefeldin A was instrumental to study transport processes from the endoplasmic reticulum (ER) to Golgi in organisms as different as yeasts, plants and mammalian cells [5].

Second, most mutations are not conditional, which means that the activity of the protein of interest cannot be turned on or off at will. And even if conditional alleles are available (e.g. temperature-sensitive mutations or mutant alleles under the control of inducible promoters), it typically
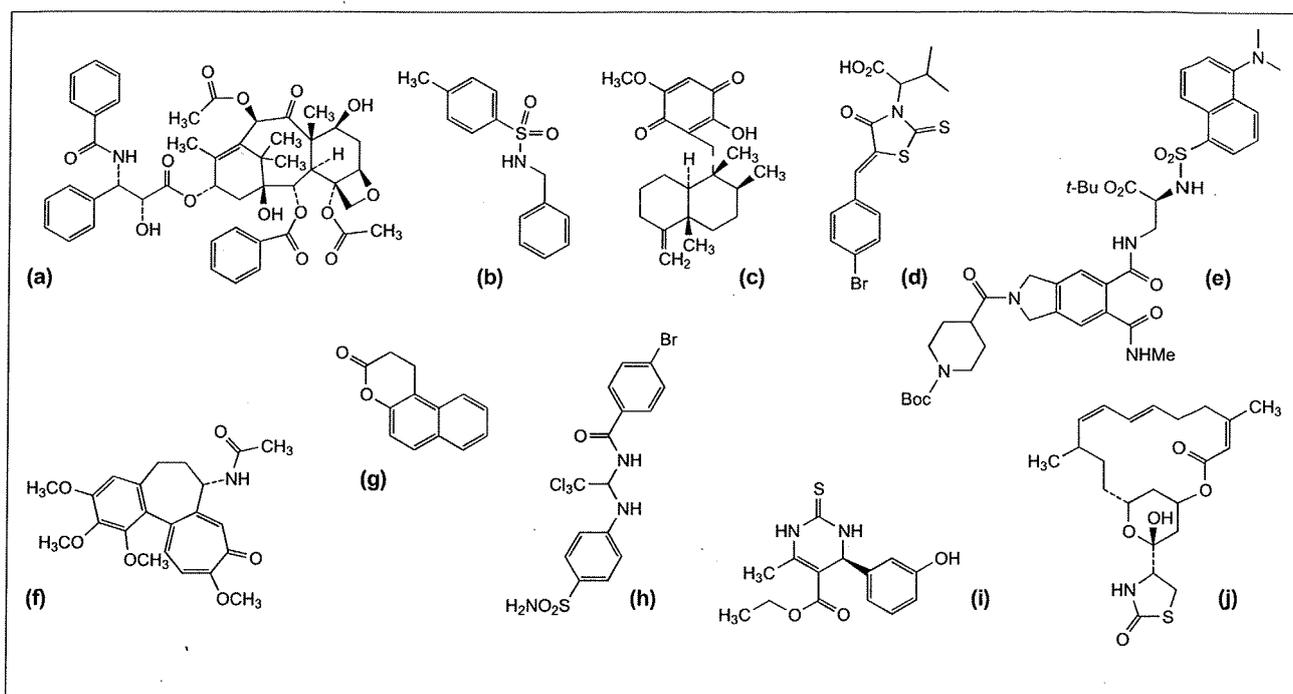
Fig. 1. Chemical structures of small molecules discussed in this review: (a) taxol, (b) BTS, (c) ilimaquinone, (d) BH3I-1, (e) IIA4B20, (f) colchicine, (g) splitomicin, (h) synstab A, (i) monastrol, (j) latrunculin A. In the context of this review, the term 'small molecules' is applied to all organic compounds with a molecular mass of less than 1500 Da, which have been either chemically synthesized or isolated from biological extracts. (For a review on peptide and peptidomimetic inhibitors, see [66]).

requires hours to change the protein complement of a cell. This lack of temporal control substantially limits the use of genetic approaches to study dynamic processes. By contrast, small molecules change directly the way proteins work, thereby representing tools with a uniquely high temporal resolution, capable of dissecting dynamic cellular processes. Notably, small molecules bear the potential to gain insights into fundamental biological processes not only by studying compound-treated cells but also by observing cells released from compound treatments. For example, the role of the microtubule-organizing center (MTOC) in nucleating microtubules was initially recognized by studies of cultured cells recovering from treatment with the microtubule poison colcemid [6].

Of particular interest, chemical genetics is predestined to pave the way for interdisciplinary research uniting biology, chemistry, pharmacology and medicine. In this way, basic research has the potential to create promising leads for the development of new therapeutic drugs. Of particular note, small molecules targeting microtubules represent impressive examples of synergies between basic research and drug development: colchicine was first used over 2000 years ago in the form of preparations of the meadow saffron *Colchicum autumnale* to treat pain associated with gout attacks. After the isolation of (−)-colchicine as the active constituent of the plant by the French chemists Pelletier and Caventon in 1820, this compound led to the discovery of tubulin by Taylor and colleagues in the late 1960s [7]. Today, colchicine is still one of the more effective treatments for gout, and, at the same time, it is a versatile probe to study microtubule functions in diverse cell-biological processes such as cell migration, trafficking

of intracellular vesicles or mitosis [8]. Taxol, the active compound from the bark of the Pacific yew tree (*Taxus brevifolia*), was identified in 1971 as a substance with antitumor activity and is today an approved drug to treat breast and ovarian cancer [9]. Unlike most other microtubule poisons (e.g. colchicine, nocodazole, the Vinca alkaloids), taxol actually promotes the polymerization of microtubules *in vitro* and *in vivo* [10,11] and therefore plays an essential role in almost all experiments requiring artificial microtubule polymerization (e.g. purification of microtubule-associated proteins or studies on microtubule-based motor proteins *in vitro* [12,13]).

The foundation of chemical screens: small-molecule libraries

In the past, biologically active small molecules have been mainly identified in complex biological mixtures derived from plants, marine sponges, fungi or bacteria. Obviously, this approach bears the risk that the initially observed activity is caused by the synergistic effect of numerous compounds rather than by a single small molecule. One way to circumvent this pitfall is to use large collections of defined, structurally diverse small molecules. However, until recently, this approach was restricted to pharmaceutical companies, who have assembled chemical libraries in the order of hundreds of thousands of compounds over the past century.

The enormous progress achieved in the field of combinatorial chemistry (see Box 1) [14], which has allowed the rapid production of a large number of chemically diverse molecules, was an important prerequisite to make chemical libraries available to academic researchers. As a result, a
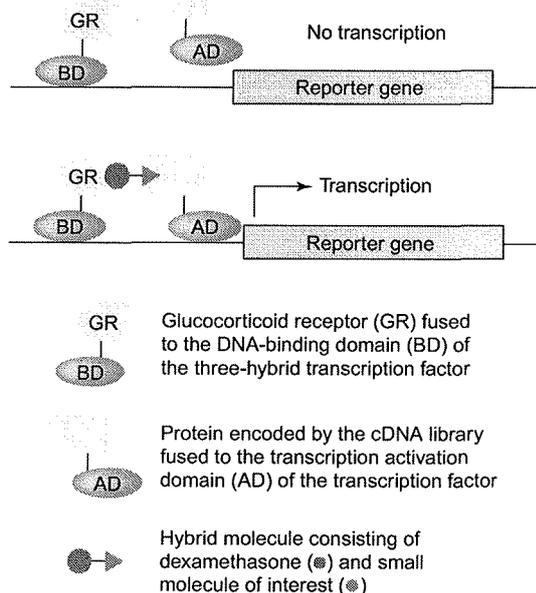
Fig. I.

constantly growing number of suppliers offer libraries of small molecules [15] that are suitable for use with screening platforms typically found in academia. Today, one of the biggest challenges for anyone interested in chemical genetics is to pick a library that fits best the intended screens, the available screening platform and, of course, the personal budget available. Many suppliers offer 'pre-selected' collections of 'drug-like molecules', known as

'pharmacophore' libraries [16]. Each of these commercially available libraries displays a high degree of structural diversity, whereas the individual compounds typically fulfil the following criteria: they are likely to cross cell membranes, they contain substructures resembling known bioactive molecules and they do not contain 'functional groups' (e.g. highly reactive groups) that are likely to cause cytotoxic effects. In addition to commercial suppliers, there are also nonprofit organizations offering small-molecule libraries such as the 'diversity set' of the National Cancer Institute, the 'NINDS collection' of the National Institute of Neurological Disorders and Stroke and the 'natural product pool' of the Hans-Knoell Institute [17].

The road to success: designing a suitable high-throughput screen

Given the challenge that typically tens of thousands of compounds have to be analyzed to find the desired bioactive molecules, it is self-evident that the intended screen has to be robust, automated as far as possible and miniaturized to an extent compatible with the 96-, 384- or even 1536-well format. In the process of designing a suitable screening approach, the first and major decision to be made is between a 'pure protein' and a 'cell-based' assay (see Fig. 2). Pure-protein screens are performed *in vitro* through binding or enzymatic assays using mainly optical readouts such as luminescence, absorbance or fluorescence (e.g. fluorescence intensity, fluorescence polarization, fluorescence resonance energy transfer [18]). By contrast, cell-based assays ensure that the target protein exists in its physiological context and that the identified molecules are membrane-permeable. A common cell-based approach is the reporter-gene assay, where the accumulation of an easily detectable enzymatic activity (e.g. luciferase) depends on the activity of the target protein (e.g. a transcriptional regulator). Alternatively, cell-based approaches can make use of functional readouts (e.g. cell viability) or of 'whole-cell immunoassays' (see Box 1), a luminescence-based method that uses a suitable antibody to detect an epitope in cells whose occurrence or disappearance can be used as a readout for a specific process of interest [19]. These latter two cell-based methods are not biased towards one particular protein but are conceptually broader, offering the potential to find compounds for poorly characterized or even unknown targets.

Microscope-based approaches represent a new and exciting trend in cell-based screening towards acquiring more complex data. Unlike the aforementioned methods, this approach is capable of detecting subtle morphological changes (see Fig. 3). Moreover, microscope-based methods have the great advantage that they allow the collection not only of data on the specific process under investigation but also data on effects indirectly influencing the process of interest. For example, in a screen for inhibitors of cell migration (see Fig. 3), the total cell count can be applied to identify and sort out cytotoxic compounds, whose inhibitory activity on cell migration is indirectly caused by the fact that the cells are dying. Recently, this type of approach, which obviously demands the use of automated microscopes and image-analysis software [20], has been successfully applied to identify small molecules interfering
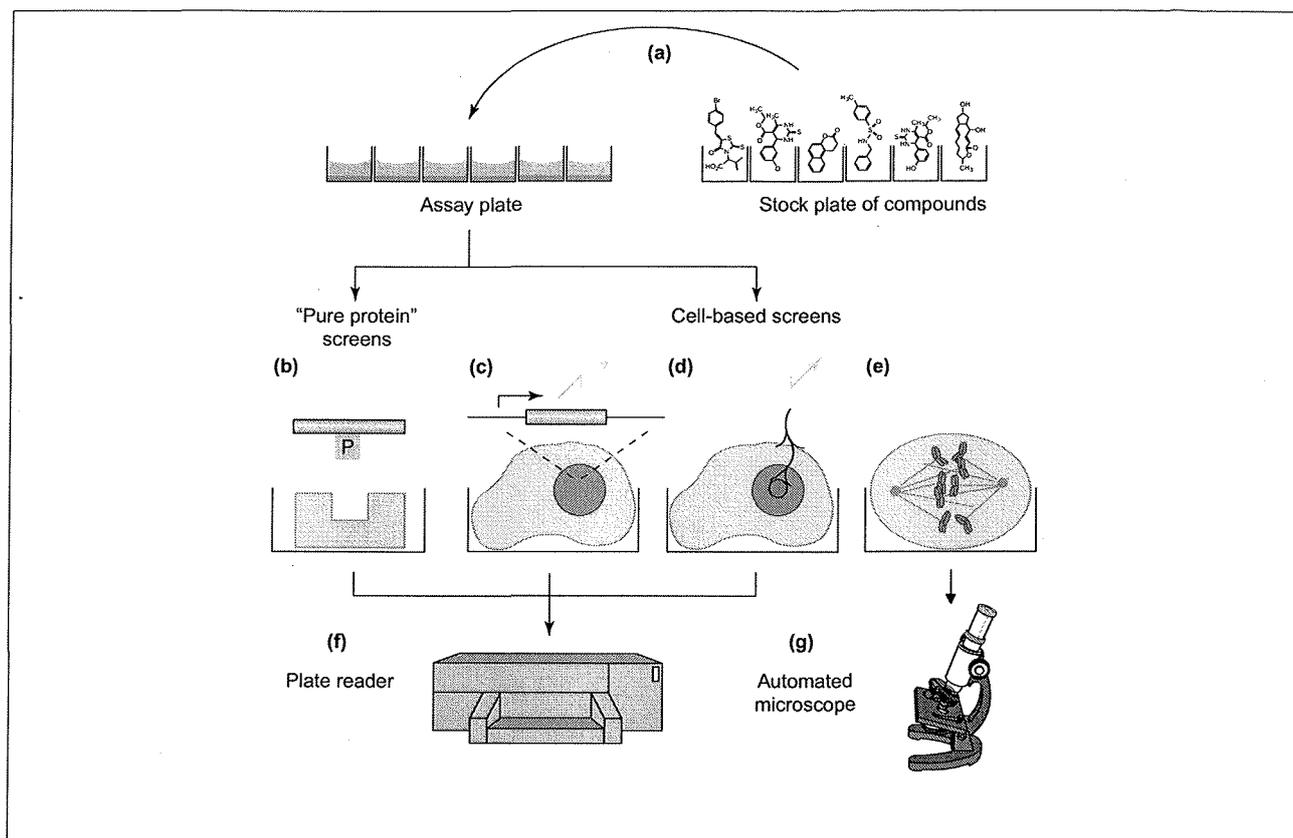
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Fig. 2. Small-molecule screens can be performed *in vitro* and *in vivo*. (a) A small volume of a single compound is added to each well of an assay plate containing protein solution or the cell line of interest. (b) Pure-protein assays can be applied to identify compounds modulating the activity of the protein of interest *in vitro* (e.g. the release of phosphate into the solution can be used as readout for the activity of a phosphatase). (c) Compounds targeting transcriptional regulators (e.g. p53) can be identified by measuring the expression level of a reporter gene (e.g. luciferase). (d) Whole-cell immunoassays apply specific antibodies to detect posttranslational changes within cells. (e) Microscope-based assays detect subtle morphological changes within cells – e.g. spindle morphology. (f) Plate readers measure optical signals (e.g. luminescence, absorbance) obtained by quantitative assays (b–d). (g) Morphological changes are detected by automated microscopes designed to acquire images at different wavelengths and at various positions within each well of a microtitre plate.

with a wide range of biological processes, including embryonic development [21], cell differentiation [22] and the transport of intracellular vesicles [23]. Table 1 highlights examples of bioactive compounds that have been recently identified via pure-protein or cell-based screens.

Target identification and validation

Target identification represents a major challenge for all forward-chemical genetic screens (see above). In the past, biochemical purifications have been the method of choice

for target identification. The two main routes are either to follow a traceable (radioactive or otherwise tagged – for example – biotin) derivative of the bioactive compound during fractionation of cell extracts or to immobilize the compound via a chemical linker on a solid phase support, followed by affinity purification of the cellular target [24]. Biochemical methods have been exceptionally successful in target identification (see Table 2). However, this approach reaches its limits when chemical modifications of the compound are not compatible with its biological activity or

Table 1. Pure-protein and cell-based small-molecule screens have yielded a wide range of bioactive compounds

| Compound name	Screening approach	Target protein	Year	Ref.
Pure-protein screen				
IIA4B20	Binding assay	Myc–Max dimer	2002	[53]
BH3I-1	Binding assay	Bcl-x$_L$	2001	[54]
BTS	Enzymatic assay	Skeletal muscle myosin II	2002	[55]
PD 098059	Enzymatic assay	MAP kinase kinase	1995	[56]
Cell-based screen				
Pifithrin-α	Reporter gene assay	p53	1999	[57]
SB 247464	Reporter gene assay	Granulocyte-colony stimulating factor receptor	1998	[58]
Synstab A	Whole-cell immunoassay	Tubulin	2000	[59]
Myoseverin	Microscope based	Tubulin	2000	[22]
Splitomicin	Functional assay	Sir2	2002	[40]
Monastrol	Whole-cell immunoassay	Mitotic kinesin Eg5	1999	[41]
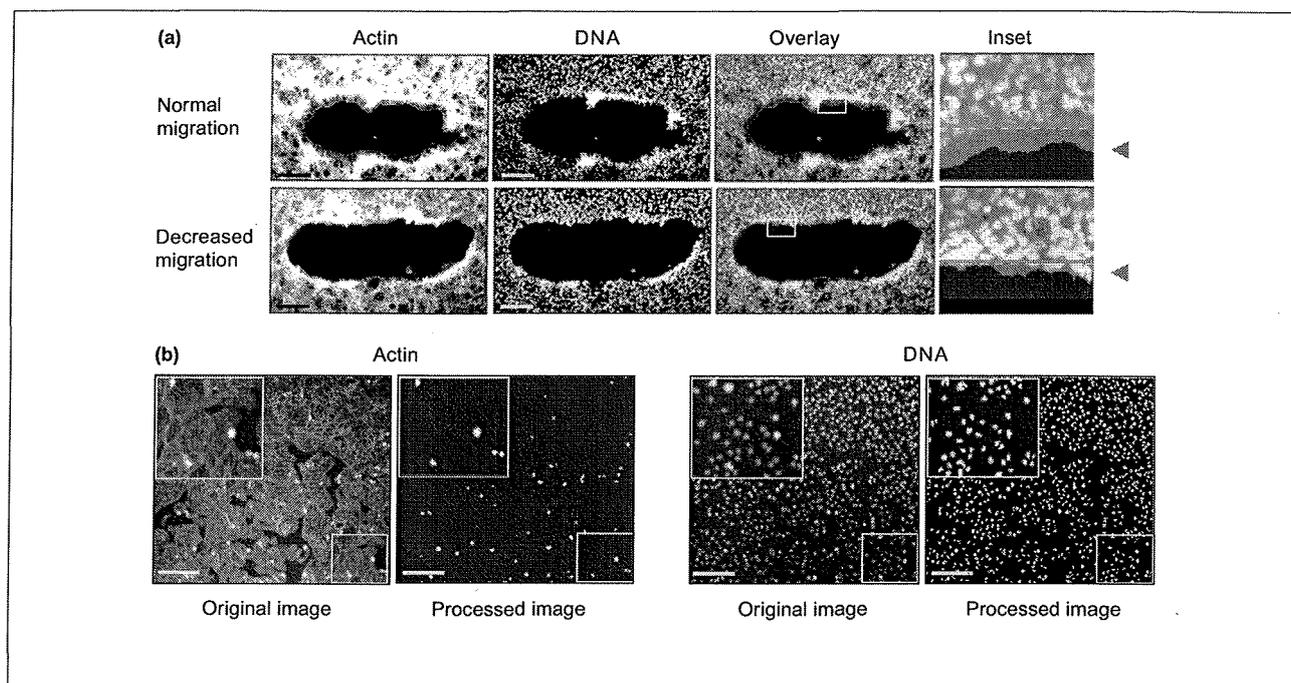
Fig. 3. Microscope-based approaches are capable of detecting subtle changes in cell morphology. (a) Immunofluorescence images of wounded cell monolayers. A confluent layer of cells in a microtitre plate was mechanically wounded, incubated with compounds and then fixed and stained for the actin cytoskeleton and DNA. The black area represents the induced wound. In the overlay panel, DNA is shown in green, actin in red and colocalization of DNA and actin in yellow. Most right panels show a higher magnification of the inset depicted in the overlay panels. Untreated cells (upper panel) should respond to the wound application by polarized growth, and thereby the distance between the cell periphery (red arrowhead) and the nuclei (blue line) should increase. If compound treatment (lower panel) inhibits polarized growth, this distance should remain constant. Image-analysis algorithms are available to calculate the surface area, which stains for actin but not for DNA (area of polarized growth), thereby providing a quantitative readout for cell migration. (b) Immunofluorescence images of an asynchronous cell population fixed and stained for the actin cytoskeleton and DNA. Computational analyses of the actin image allow the identification and quantification of mitotic cells, based on their round cell morphology. Additionally, the processed DNA image can be used to determine the total cell count per well, supplying information about cell loss owing to manipulation artifacts or putative cytotoxic compounds. Bar, 250 μm.

when the small molecule lacks a sufficiently high binding affinity for the isolation of target proteins that are only present in low abundance.

By applying cDNA expression-based methods such as phage display [25], protein microarrays [26] or yeast 'three-hybrid systems' [27], the abundance of the target protein can be artificially increased. Phage display methods and protein microarrays follow the same basic concept (see Box 1): a tagged compound (see above) is used as a probe to identify its binding partner within a collection of spatially separated proteins. The basis of the yeast three-hybrid system is a synthetic hybrid molecule composed of a compound with a known binding partner (e.g. dexamethasone, which binds to the hormone-binding domain of the glucocorticoid receptor) and the small molecule of interest (see Fig. I in Box 1). Under conditions where one part of

Table 2. Examples of cellular targets of bioactive small molecules that have been identified by classical biochemical approaches

Compound name	Target protein	Ref.
Aminopurvalanol	Cyclin-dependent kinase	[32]
Dihydroeponemycin	20S proteasome	[60]
Fumagillin	Methionine aminopeptidase	[61]
Ilimaquinone	S-adenosylhomocysteinase	[62]
Trapoxin	Histone deacetylase	[63]
Lactacystin	20S proteasome	[64]
Rapamycin	FK506 binding protein 12 (FKBP12)	[65]

the hybrid molecule (dexamethasone) binds to its known ligand fused to the DNA-binding domain and the other part (small molecule of interest) binds at the same time to its target fused to the activation domain, transcription will be activated, enabling the expression of a reporter gene (e.g. an essential metabolic enzyme).

New genetic-based approaches for target identification became applicable by the advent of whole-genome sequence information. One possible approach, termed the haplo-insufficiency method, follows the logic that lowering the gene dosage from two copies down to one copy sensitizes yeast cells to the drug that acts on the gene product. Recently, this approach was validated on known targets of six small molecules [28]. An alternative approach, which is applicable to yeast as well as to higher eukaryotes, is based on the rationale that increasing the gene dosage of the target gene renders cells more resistant to the compound [29]. A key advantage of these genetic approaches is that they do not require chemical modifications of the selected compound.

At the end of any chemical screen, selected compounds have to pass the acid test of specificity and selectivity. In a first attempt, specificity can be assessed by testing whether the compound modifies the activity of target-related proteins *in vitro*. But no matter how many proteins are tested *in vitro*, the risk remains that the assumed target is not the relevant binding partner *in vivo*. Therefore, additional experiments are required to validate the target. In the case

of small molecules with an inhibitory mode of action, one possible strategy is to test whether the compound-induced phenotype can be either mimicked by shutting down the activity of the suspected target protein (by biochemical or genetic methods such as immunodepletion, antibody addition or RNA interference [30]) or rescued by artificially increasing the concentration of the suspected target in the assay [31,32].

Recent advances in transcriptional profiling paved the way for compound specificity studies in a global, cellular context [33]. Marton and colleagues applied this approach to show that 3-aminotriazole (3-AT), an inhibitor of His3p (an enzyme involved in histidine biosynthesis), causes more than 1000 changes in the expression profile of wild-type yeast cells. However, almost no changes in transcript level were observable when yeast cells deleted for *HIS3* were treated with 3-AT, indicating that 3-aminotriazole is a specific inhibitor of His3p in yeast cells [34].

What have we learned from use of small molecules?

The application of small molecules continues to have an enormous impact on our understanding of cell biology. There is an almost endless list of examples where small molecules, in combination with genetic screens, has facilitated the dissection of complex cellular processes. A famous example is the use of the microtubule poison benomyl to arrest yeast cells at the G2–M transition in a forward-genetic screen designed to identify genes involved in cell-cycle control [35,36]. However, the value of small molecules in answering cell-biological questions is clearly not limited to their ability to synchronize cultured cells [37]. Even in *Saccharomyces cerevisiae*, one of the most widely used model organisms for genetic manipulations, small molecules have provided insights into fundamental biological processes that have not been accessible by classical genetic approaches. For example, the actin-depolymerizing activity of latrunculin A, a natural product isolated from the sponge *Latrunculia magnifica*, has been used to unravel the role of the actin cytoskeleton in cell polarity and mitotic spindle orientation in budding yeast [38] (for review, see [39]). Similarly, the use of splitomicin demonstrated for the first time that continuous histone deacetylase activity of Sir2p is required for maintaining a transcriptionally silenced state in nondividing yeast cells [40]. In these two studies, small molecules were more successful than genetic approaches because the available conditional mutants did not possess the required temporal resolution (in the former case) or because conditional mutants were not available (in the latter case).

New and exciting insights into mitosis and its regulation in mammalian cells were facilitated by the discovery of the small molecules monastrol [41] and blebbistatin [42]. Monastrol, initially identified in a whole-cell immunoassay designed to identify compounds that arrest tissue-culture cells in mitosis (see Table 1), causes a remarkable phenotype in that the normal bipolar spindle is replaced by an array of microtubules surrounded by a ring of chromosomes (see Fig. 4). A strikingly similar phenotype had been previously observed when antibodies directed against the mitotic kinesin Eg5 were injected into tissue-culture cells [43]. This suggested that Eg5 could be a potential target of
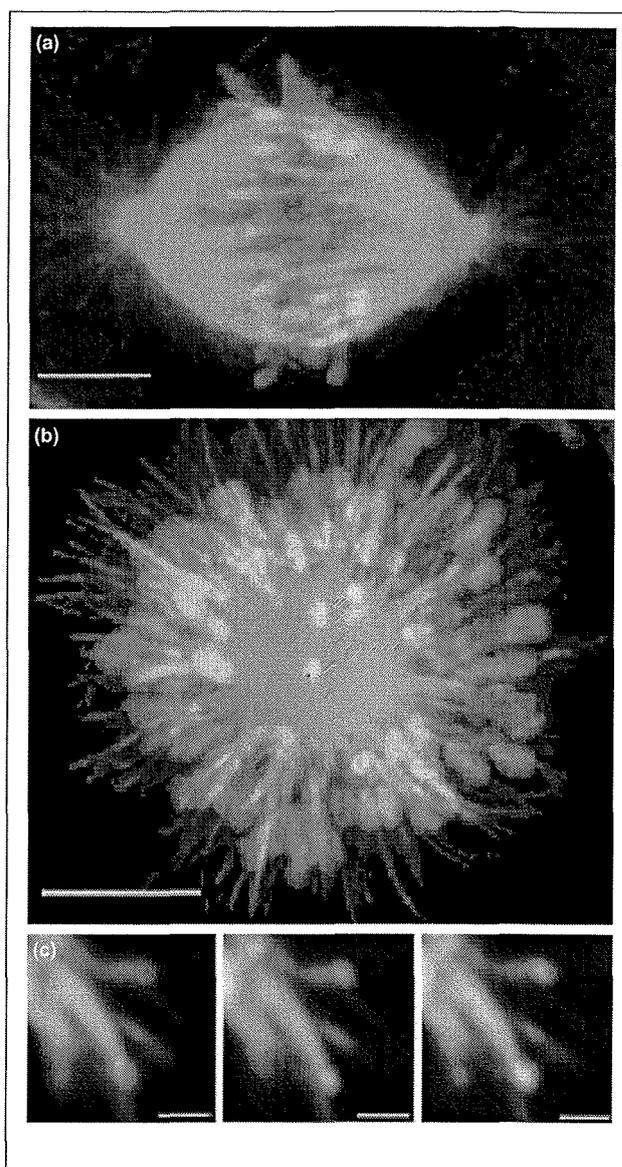

Fig. 4. The small molecule monastrol reveals new insights in the activation of the spindle-assembly checkpoint in tissue-culture cells. Immunofluorescence images: control-treated (a) and 68 μM monastrol-treated (b) African green monkey (BS-C-1) cells stained for DNA (blue) and tubulin (green). In monastrol-treated cells, bipolar spindles do not form but are replaced by monoastral spindles surrounded by a ring of chromosomes. Bar, 5 μm. (c) Monastrol-treated (50 μM) rat kangaroo (Ptk-2) cells permeabilized in the presence of 0.1 mM Ca²⁺ (to clear off all non-kinetochore microtubules) before fixation were stained for tubulin (green), kinetochores (red) and MAD2 (blue). Bar, 1 μm. Note that the checkpoint protein MAD2 localizes to kinetochores that are attached to microtubules in monoastral spindles. Images adapted, with permission, from Ref. [41] (a,b) and Ref. [44] (c).

monastrol, and indeed motility assays demonstrated that monastrol inhibits Eg5-dependent movement of microtubules *in vitro*. Owing to its fast mode of action, specificity and reversibility [41,44], monastrol opened up new opportunities to elucidate the function of Eg5 in mitosis. Particular, the provocative idea of the existence of a static spindle matrix re-emerged with the observation that, in *Xenopus* extracts, Eg5 remains static within the spindle when its motor activity is inhibited by monastrol, whereas tubulin, building up the mitotic spindle, constantly moves

polewards [45]. Experiments addressing the question of why monastrol-treated cells arrest in mitosis showed that the checkpoint protein MAD2 localizes to a subset of kinetochores in monastrol-treated cells, suggesting the spindle assembly checkpoint is activated in these cells [44]. Moreover, closer examination of monastrol-treated cells revealed MAD2 staining of kinetochores that are clearly attached to microtubules (see Fig. 4). This observation challenges the proposal that microtubule attachment rather than tension regulates association of MAD2 with mammalian kinetochores [46].

Blebbistatin was identified in a pure-protein screen for inhibitors of non-muscle myosin II, which provides the force for the contraction of the cleavage furrow (a ring composed of actin and actin-binding proteins such as myosin II and anillin) during cytokinesis. *In vivo*, blebbistatin rapidly inhibits the ingression of the cleavage furrow, causing cells to arrest at the end of mitosis with unseparated daughter cells. By adding nocodazole to blebbistatin-treated cells, Straight *et al.* could demonstrate that microtubule-to-cortex communication is continuously required to maintain the localization of myosin II and anillin at the cleavage furrow. Further experiments using blebbistatin in combination with proteasome inhibitors revealed that exit from cytokinesis in mammalian cells depends on ubiquitin-dependent proteolysis.

Novel insights into the regulation of programmed cell death (apoptosis) were facilitated by the recent discovery of the small molecule PETCM [α-(trichloromethyl)-4-pyridine-ethanol] [47]. Wang and coworkers identified PETCM in a high-throughput screen for activators of caspase-3 in an *in vitro* cell death assay. Searching for the proteins that mediate the PETCM pro-apoptotic effect, Wang and colleagues identified the oncoprotein prothymosin-α (ProT) and the tumor-suppressor putative HLA-DR-associated proteins (PHAPs) as important regulators of caspase-3 activation, albeit with opposing effects. PHAP proteins appear to be positive regulators of apoptosis, whereas ProT has an anti-apoptotic function by inhibiting the formation of the apoptosome (a macromolecular complex required for the activation of caspase-9, which in turn activates caspase-3). Further experiments demonstrated that PETCM inhibits the anti-apoptotic activity of ProT and thereby promotes the formation of active caspase-3 in the *in vitro* cell-death assay. The anti-apoptotic activity of ProT was confirmed by the observation that depletion of ProT by RNA interference sensitizes cultured cells to apoptosis.

A whole collection of small molecules, including brefeldin A, nocodazole, ilimaquinone and okadaic acid, has facilitated important insights into the biology of the Golgi apparatus (reviewed in [48]). These compounds were instrumental, for example, in establishing the cytoskeletal anchoring of the Golgi apparatus close to the MTOC (nocodazole) and in unraveling retrograde flow mechanisms in the secretory pathways (brefeldin A). Similarly, tunicamycin and related inhibitors facilitated the dissection of glycoprotein biosynthesis in a wide range of experimental systems (reviewed in [49]). In these and many other fields of cell biology, small molecules constitute well-established experimental tools to address important

problems that are often difficult to approach by other means. In other fields, however, such as neurobiology, developmental biology or cellular microbiology, the use of small molecules has only just begun [50,51]. One example showing the value of chemical genetics in neurobiology is provided by the recent study on the function of myosin-1c in adaptation in hair cells (the process by which the ear's sensitivity to sound is changed by the presence of a constant sound) [52]. By expressing a mutant form of myosin-1c susceptible to inhibition by N6-modified ADP analogs in utricular hair of transgenic mice, Holt *et al.* were able to show that the ADP analog largely blocks the ability of these cells to adapt to mechanical stimuli, suggesting that myosin-1c participates in adaptation in hair cells. This inhibition was specific for the engineered mysoin-1c because adaptation in transgenic mice expressing wild-type Myo-1c was entirely normal, even in the presence of the ADP analog.

Concluding remarks

There is no doubt that small molecules have proven themselves as invaluable tools for cell biology. However, it is important to bear in mind that the value of chemical genetics is inseparably tied to the quality of the applied molecules, with compound specificity representing the most crucial issue. In the light of this challenge, a combined effort on the part of chemists and biologists is required to generate novel, highly diverse libraries of small molecules, to design suitable screening approaches and finally to validate the binding partners of active compounds as the relevant targets *in vivo*. Given the enormous progress that has been made in each of these fields, chemical genetics will have a rapidly growing impact on modern biology.

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