Dissecting cellular processes using small molecules: identification of colchicine-like, taxol-like and other small molecules that perturb mitosis

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Background: Understanding the molecular mechanisms of complex cellular processes requires unbiased means to identify and to alter conditionally gene products that function in a pathway of interest. Although random mutagenesis and screening (forward genetics) provide a useful means to this end, the complexity of the genome, long generation time and redundancy of gene function have limited their use with mammalian systems. We sought to develop an analogous process using small molecules to modulate conditionally the function of proteins. We hoped to identify simultaneously small molecules that may serve as leads for the development of therapeutically useful agents.

Results: We report the results of a high-throughput, phenotype-based screen for identifying cell-permeable small molecules that affect mitosis of mammalian cells. The predominant class of compounds that emerged directly alters the stability of microtubules in the mitotic spindle. Although many of these compounds show the colchicine-like property of destabilizing microtubules, one member shows the taxol-like property of stabilizing microtubules. Another class of compounds alters chromosome segregation by novel mechanisms that do not involve direct interactions with microtubules.

Conclusions: The identification of structurally diverse small molecules that affect the mammalian mitotic machinery from a large library of synthetic compounds illustrates the use of chemical genetics in dissecting an essential cellular pathway. This screen identified five compounds that affect mitosis without directly targeting microtubules. Understanding the mechanism of action of these compounds, along with future screening efforts, promises to help elucidate the molecular mechanisms involved in chromosome segregation during mitosis.

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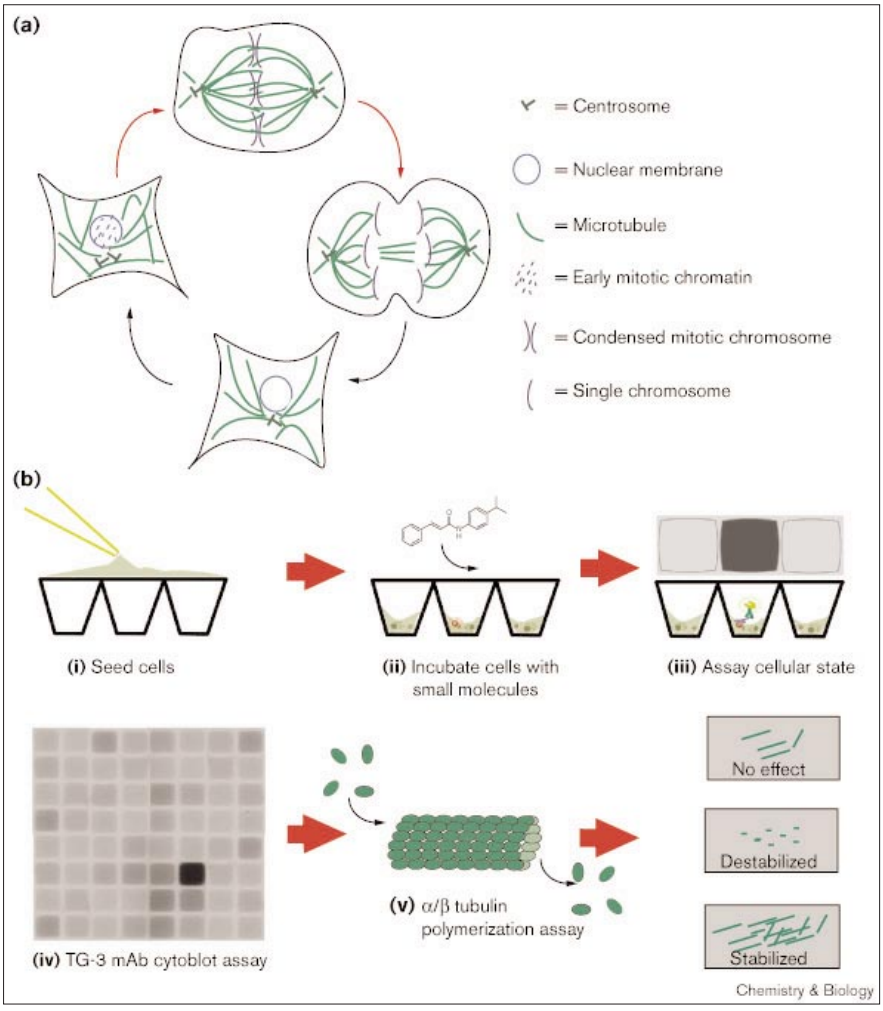
Introduction

High-throughput, phenotypic (based on observable material or behavioral properties) screening of small molecules in cells provides a powerful means to interface synthetic organic chemistry and cell biology. Because these assays can report on an overall cellular state, they provide an unbiased, system-based approach to exploring cellular pathways and processes. This situation differs from typical drug-screening efforts aimed at altering the function of a single, pre-selected target protein without immediate concern for the effect of compounds within cells. The approach described here emulates the logic of a classical (forward) genetic screen [1], but differs from genetics in that it relies upon small molecules, rather than mutations, to modulate conditionally the circuitry of biological processes [2,3] (see also http//www-schreiber.chem.harvard.edu/).

We applied this chemical genetic approach to an essential cellular process, seeking to identify small molecules that alter the progression of cells through the mammalian cell-division cycle. In particular, we focused on those pathways essential to chromosome segregation during mitosis (Figure 1a) [4–8]. Compounds were initially screened using a high-throughput cytoblot assay, in which an antibody is used to detect a post-translational modification characteristic of the process of interest (Figure 1b) [9]. This assay used TG-3, a monoclonal antibody (mAb) that recognizes a form of the protein nucleolin specifically phosphorylated during mitosis, to report indirectly on the progress of cells through mitosis [10–12]. Accordingly, small molecules that increase the reactivity of this mAb in cells are likely to cause the arrest of cells in the mitotic state (e.g., [13]). As many compounds that have previously been shown to arrest cells in mitosis directly affect the

Figure 1



Screening for small molecules that affect the progression of mammalian cells through mitosis. **(a)** Cell-cycle events involved in mitotic chromosome segregation. Cellular states linked by a red arrow show increased reactivity with the TG-3 mAb [11]. **(b)** Overview of the cytoblot assay and screening steps for identification of colchicine-like, taxol-like and other small molecules that perturb mitosis. See http://www-schreiber.chem.harvard.edu/shockwave/cytoblot.html for more details.

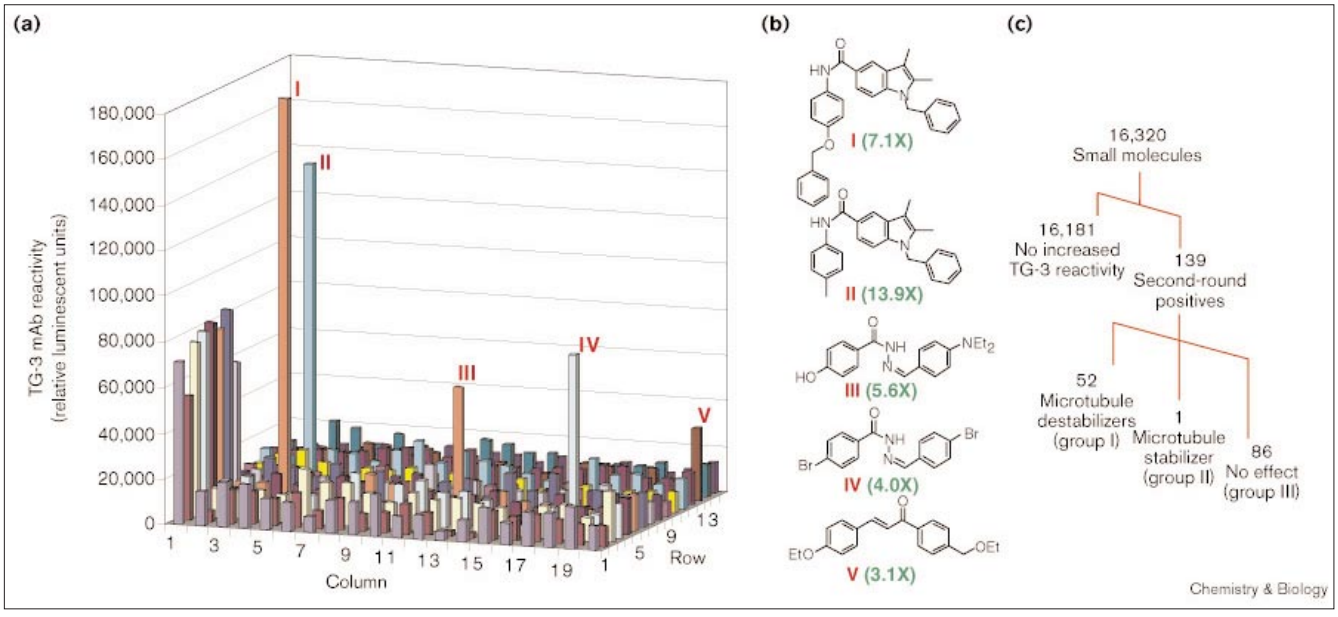
polymerization of α and β tubulin (the heterodimeric subunits of microtubules), and thereby alter the microtubule dynamics of the mitotic spindle [14,15] (Figure 1a), compounds that scored positively in this initial assay were subsequently tested in an *in vitro* tubulin polymerization assay (Figure 1b). To classify further compounds on the basis of their phenotypic effects, fluorescence microscopy was used to visualize the distribution of microtubules, actin and chromatin in cells treated with compounds of interest.

Results

Two rounds of screening of a 16,320 compound library (Diverse E set, Chembridge Corp.) at ~20–50 μM resulted in the identification of 139 compounds that increased (at least 2.5 times) the amount of phosphorylated nucleolin in asynchronous A549 lung epithelial cells, as measured by reactivity of the TG-3 mAb [9]. The structure of each of the 139 compounds, along with an approximate estimation of their potency, will be posted on our website at http://iccb.med.harvard.edu. An example of these data is shown in Figure 2a and b. These 139 compounds had no effect on the *in vitro* polymerization of actin (J. Peterson, unpublished observations), the *in vitro* degradation of a cyclin B–luciferase fusion protein in *Xenopus* extracts (R.W.K., unpublished observations) or the activation of a growth-factor-dependent reporter gene (B. Stockwell, unpublished observations), suggesting some level of specificity in their target interactions.

The ability of the 139 compounds to affect directly the polymerization of purified bovine brain tubulin was assessed using an *in vitro* tubulin polymerization assay. As summarized in Figure 2c, 52 compounds (group I) destabilized microtubules (data not shown) and one compound (group II) stabilized microtubules when assayed in this format. The remaining 86 compounds (group III) had no observable effect, and therefore appear to interfere with progression through mitosis by mechanisms not involving modulation of microtubule polymerization dynamics (see below).

Figure 2



Example and summary of screening data for small molecules that perturb the mitotic machinery of mammalian cells. Compounds (~50 μM) were pin-transferred into wells of a 384-well plate containing 50 μl media and ~4000 cells. After a 24 h incubation, cells were fixed and processed for a TG-3 cytoblot assay as described previously [9]. **(a)** Results of a TG-3 cytoblot assay on 314 of the 16,320 compounds screened. **(b)** Five compounds (**I–V**) were chosen from within this set and the values of their maximum fold-activation in TG-3 mAb reactivity (relative to a DMSO control) after retesting at ~100 μM are in green. As a positive control, column 1, rows 1–8 contained nocodazole (332 nM) and as a negative control, column 1, rows 9–16 received an equivalent concentration of DMSO. **(c)** Summary of complete screen showing the division of the initial 139 positive compounds (0.85 % of compounds screened) into three functional groups reflecting their effect on microtubule stability measured *in vitro* with purified bovine brain tubulin. Of the compounds picked in the first round 81% retested as positives in the second round. Compounds **I–V** all fell within group I. Compounds within group III were considered as candidates for mitosis-specific inhibitors that function through mechanisms not involving direct interaction with microtubules. Colors shown are only to delineate compounds within the same row.

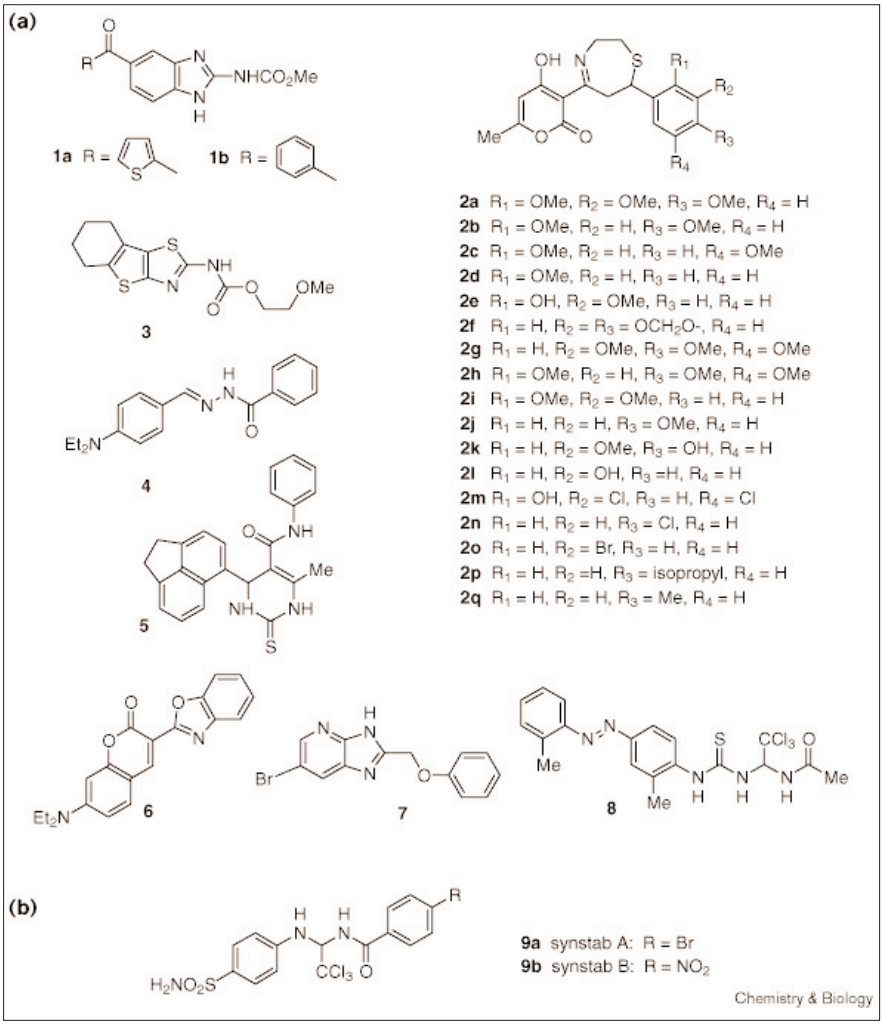
Group I: colchicine-like small molecules that destabilize microtubules

On the basis of their effectiveness in the cytoblot assay, the most potent antimitotic compounds identified are those compounds that destabilized microtubules *in vitro* (group I). These compounds include members of structural types **1**–**8** (Figure 3a). Compound **1a** is a well-known microtubule-destabilizer, nocodazole [14]. This compound was twice present within the library, as was analog **1b.**

The same three-ring skeleton is shared by six out of the 52 group I compounds (**2a**–**f**), although their dose response in the cytoblot assay varied (Figure 3a). This variation provided an opportunity to correlate the results obtained from the cytoblot and tubulin polymerization assays with the cellular effects of each compound. Cells were treated with compounds at a given concentration (see Figure 4), fixed, and the distribution of microtubules and chromatin was visualized using fluorescence microscopy (Figure 5). Compounds with effective concentrations required for half-maximal signal (EC_{50}) in the range of 0.5–1.0 μM in the cytoblot assay (e.g. **2b**, Figure 4b) completely destabilized microtubules in both interphase (e.g., Figure 5d) and mitotic (data not shown) cells, resulting in randomly arrayed mitotic chromosomes. Compounds having an EC_{50} in the range of 5–10 μM (e.g. **2e**) either partially destabilized interphase microtubules or had no visible effect on the microtubule cytoskeleton (data not shown). Regardless of their effects on interphase cells, these less potent compounds still caused abnormal mitotic spindle structures and altered chromosome distribution.

Fluorescence microscopy of cells treated with high concentrations (~50 μM) of 23 structural analogs of **2** (Diverse E set, Chembridge Corp.), which either scored negatively in the cytoblot assay or were not within group I, identified an additional 11 small molecules (**2g**–**q**) that destabilized microtubules in cells (Figure 3a). As compounds **2g**, **2i**, **2o** and **2q** were among those considered to be in group III, these compounds define a subset of group III that may also directly target tubulin, but may bind weakly and thus may be ineffective at targeting purified tubulin in an *in vitro* assay. For example, although compound **2g** had no effect on the stability of

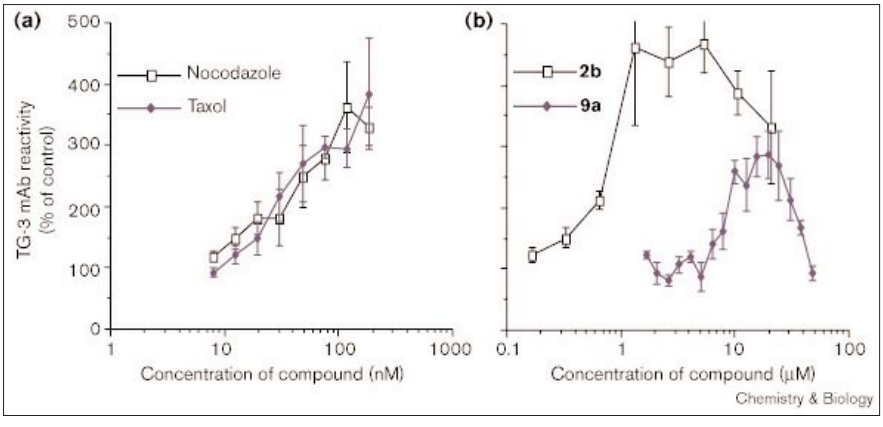
Figure 3



Chemical structures of small molecules that directly affect the stability of microtubules within cells. **(a)** Examples of compounds that destabilize microtubules. **(b)** Compounds that stabilize microtubules.

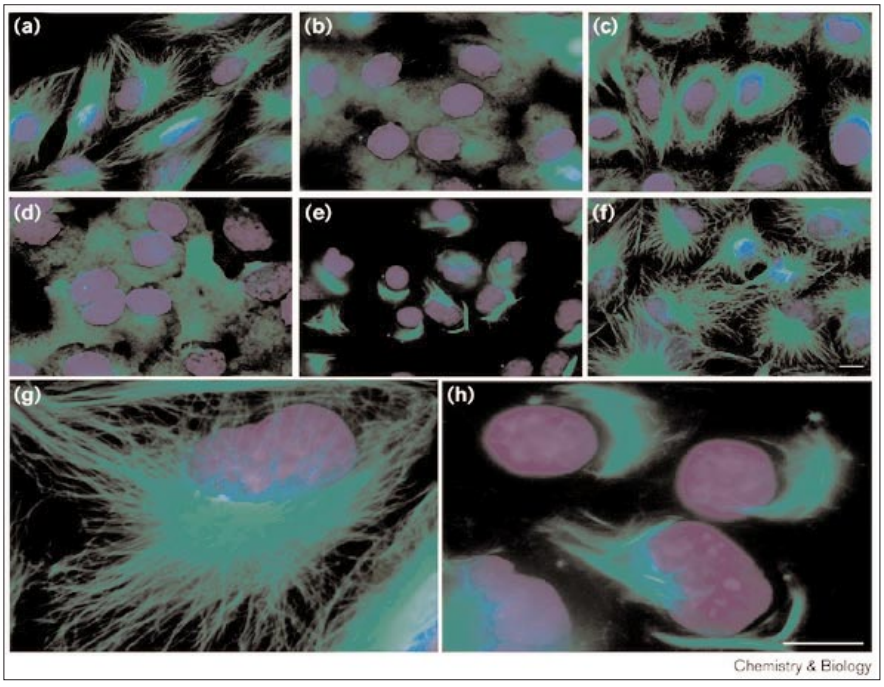
purified microtubules or on the microtubule cytoskeleton of interphase cells (data not shown), mitotic cells treated with this compound showed a shorter, disarrayed spindle and misaligned chromosomes (Figure 6c,d) compared

Figure 4



Dose response in A549 cells of small molecules directly affecting the stability of microtubules. **(a)** Treatment of cells with nocodazole and taxol. **(b)** Treatment of cells with **2b** and **9a**. Cells were treated for 22–24 h with compounds and a cytoblot assay using the TG-3 mAb was performed as described previously [8]. Results are depicted as the mean value (n = 2 for compound **2b**; n = 4 for compound **9a**, nocodazole, and taxol) with standard deviations indicated with bars. Data were normalized to a control treatment with DMSO (0.5% v/v). The concentration of each compound that caused half the maximum increase in TG-3 reactivity (EC_{50}) was estimated from these graphs.

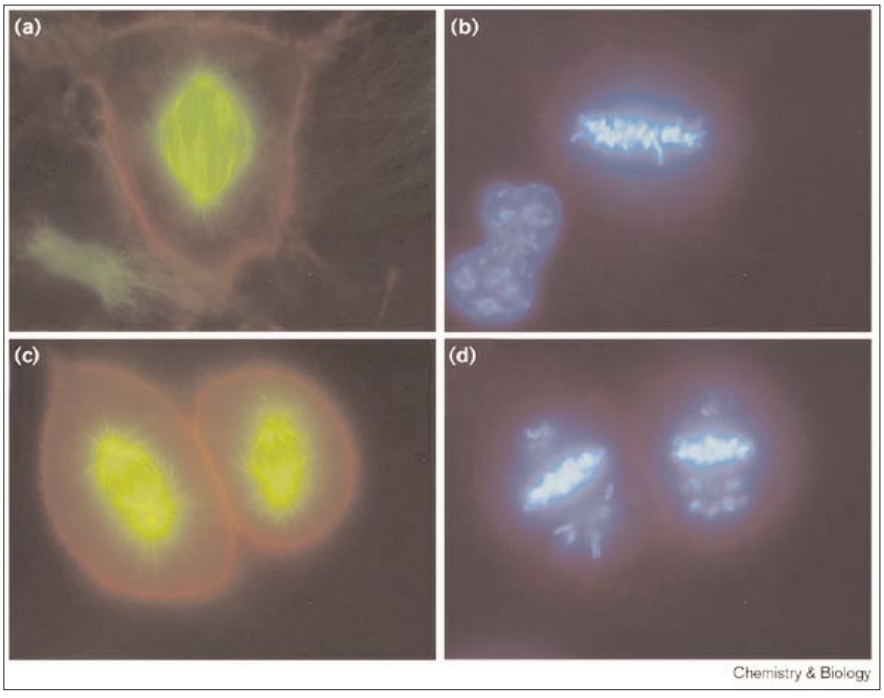
Figure 5



Interphase α-tubulin-staining patterns of BS-C-1 kidney epithelial cells after treatment with compounds that affect microtubule stability. Cells were treated for 4 h with **(a,g)** DMSO (0.1% v/v), **(b)** nocodazole (10 μM), **(c)** Taxol (100 nM), **(d)** compound **2b** (27 μM), **(e,h)** synstab A (25 μM) and for **(f)** synstab A (30 μM for 2 h, then 2 h recovery [following wash] without compound). Cells were fixed, stained for α tubulin (green) and chromatin (blue/purple), and fluorescence microscopy was used to visualize the distribution of microtubules. Treatment either with nocodazole (b) or with compound **2b** (d) causes a loss of the normal microtubule network. In contrast, treatment either with taxol (c), or with synstab A (e,h), causes a reversible (f), perinuclear accumulation of microtubule bundles. Scale bars, ~25 μm.

with the normal bipolar spindle and alignment of chromosomes (Figure 6a,b). These results, and the results described above, highlight the sensitivity of the mitotic spindle and checkpoint to relatively weak perturbations in microtubule dynamics that otherwise have no observable effect on interphase cells [6,15]. Although this sensitivity is remarkable, it is not unexpected given previous work with low doses of vinca alkaloids and taxol [16].

Figure 6



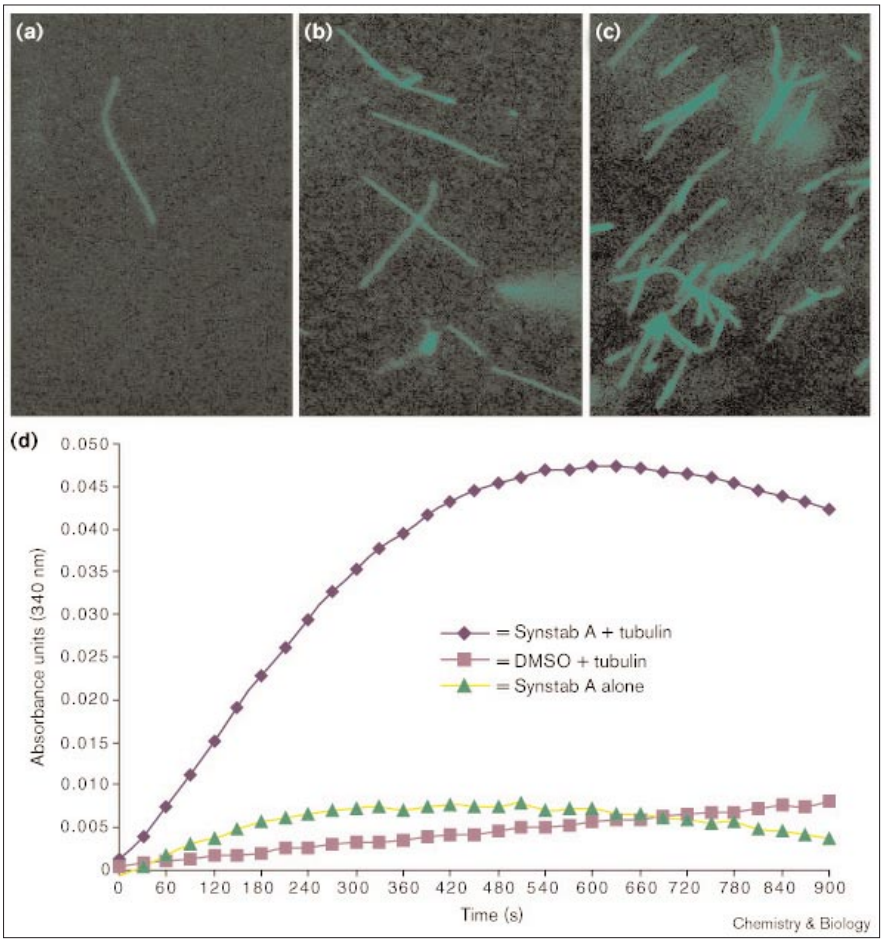
Effect of a weak microtubule depolymerizer on the mitotic spindle and chromosome distribution. BS-C-1 cells were treated for 4 h with DMSO (0.25 %) **(a,b)** or compound **2g** (49 μM) **(c,d)**, fixed and stained for α-tubulin (green), actin (red) and chromatin (blue).

Group II: taxol-like small molecules that stabilize microtubules

Compounds in group I share functional properties with many synthetic compounds and natural products that disrupt the interactions between the α/β tubulin subunits of microtubules, whereas small molecules having the opposite effect of stabilizing these interactions (e.g., taxol [17], discodermolide [18,19]) are more rare [14,20]. On the basis of the results from the visual assay of the effects of compounds on the *in vitro* polymerization of purified bovine brain tubulin (Figure 7), compound **9a** (group II; Figure 3b), here named synstab A (for synthetic stabilizer), stabilizes microtubules polymerized from purified bovine brain tubulin (Figure 7b), as does taxol (Figure 7c). As a second means of confirming the stabilizing effects of synstab A, we measured the change in the optical density upon the polymerization of a solution of tubulin in the presence of synstab A. In agreement with the visual assay, the addition of an excess of synstab A (100 μM) increased the rate and overall magnitude of the increase in optical density because of polymer-dependent light scattering, as compared with the addition of dimethyl sulfoxide (DMSO; Figure 7d). Because this increase in optical density reflects increased stability of microtubules in the absence of microtubule-associated proteins and other cellular factors, synstab A therefore stabilizes microtubules through a direct interaction with tubulin proteins.

To ensure that the observed increase in optical density was because of the formation of microtubules and not other structures, tubulin samples after polymerization were negatively stained and visualized under low (× 4000) and high magnification (× 50,000) using electron microscopy. As for samples treated with taxol (data not shown), there was a significant increase in the amount of microtubules observed in the presence of synstab A (Figure 8a) relative to treatment with DMSO (data not shown). Microtubules that were formed showed a longitudinal axis of symmetry and were of varying lengths. Under high magnification (Figure 8b), striations indicative of protofilament boundaries were observed and the overall morphology was indistinguishable from that of microtubules formed in the presence of either taxol or DMSO (data not shown). Thus, synstab A's mechanism of microtubule stabilization *in vitro*

Figure 7



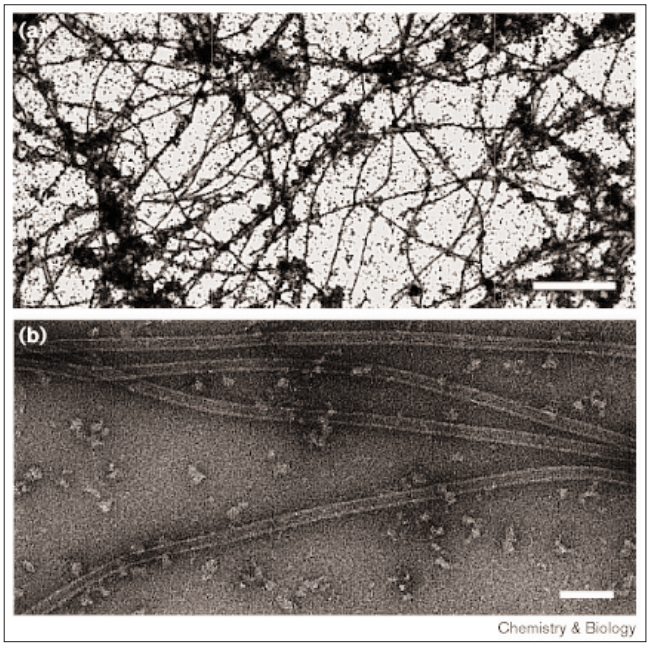
Visual and quantitative assays of the effect of synstab A on the polymerization of purified bovine brain tubulin. Representative fields of view from fluorescence microscopy of tetramethylrhodamine (TMR)-labeled tubulin (green) samples treated with **(a)** DMSO (0.22% v/v), **(b)** synstab A (22 μM) and **(c)** taxol (22 μM). **(d)** The polymerization of bovine brain tubulin was monitored by measuring the time-dependent change in optical density (absorbance units) at 340 nm in the presence either of synstab A (100 μM; blue) or of an equivalent concentration of DMSO (1% v/v; purple). In the absence of tubulin, the addition of synstab A alone (green) to BRB80 buffer had no significant effect over time.

does not involve a dramatic rearrangement of microtubule structure, as might be expected to occur if it caused a non-specific aggregation of tubulin.

In the cytoblot assay, synstab A had an EC_{50} of 10–15 μM (Figure 4b), whereas a compound lacking the sulphonamide group over a similar range of concentrations had no effect (data not shown). The value of this cytoblot EC_{50} is ~500-fold greater than that observed with taxol (Figure 4a). Consistent with the stabilizing effects of synstab A on purified microtubules (Figure 7), staining of kidney epithelial cells (BS-C-1) showed that synstab A treatment leads to microtubule bundles in interphase cells (Figure 5e,h) and to disrupted spindles and abnormal chromosome distribution in mitotic cells (data not shown). Testing of a series of 23 analogs of synstab A (Diverse E set, Chembridge Corp.), up to concentrations of 100 μM, revealed a second, but weaker compound (**9b**; Figure 3b) that caused similar phenotypic effects to those of synstab A (data not shown). The analog of synstab A lacking the terminal sulphonamide group, which had no effect on the *in vitro* polymerization of tubulin, also had no observable effect on cells up to 100 μM (data not shown). Because a 2 h treatment with synstab A followed by its removal by washing restored the normal microtubule staining pattern in both interphase (Figure 5f) and mitotic (data not shown) cells, the observed effects of synstab A are reversible and are not likely because of covalent modification of tubulin. The reversible bundling of interphase microtubules and the reversible effects on mitotic cells are reminiscent of those in cells treated with both discodermolide and taxol [19]. We note, however, that the bundling of microtubules induced by taxol is less pronounced than that induced by either synstab A in BS-C-1 cells or taxol in other cell lines (Figure 5c).

In agreement with the increased percentage of cells in mitosis observed by fluorescence microscopy, fluorescence-activated cell sorting (FACS; Figure 9) confirmed that, as with cells treated with nocodazole (Figure 9b) or taxol (Figure 9c), cells treated with synstab A (Figure 9d) had fully replicated chromosomes (4N DNA content) and increased TG-3 staining. In addition, total cell extracts derived from cells treated with taxol, or with synstab A at concentrations that do not affect viability, showed by immunoblotting increased TG-3-reactivity (Figure 10). As shown in Figure 4b, treatment of cells with synstab A at concentrations greater than 30 μM does not result in a dose-dependent increase in TG-3 reactivity even though the maximum signal is less than that of either taxol or nocodazole (Figure 4a). This suggests that at high concentrations either synstab A is less effective at inducing a mitotic arrest or it reduces cellular viability. In support of the latter notion, treatment of cells with synstab A for 24 h, but not with the analog lacking the sulphonamide group, reduced the viability of A549 cells by more than 80% at concentrations above 50 μM, as measured by the reduction of the tetrazolium salt MTS (data not shown).

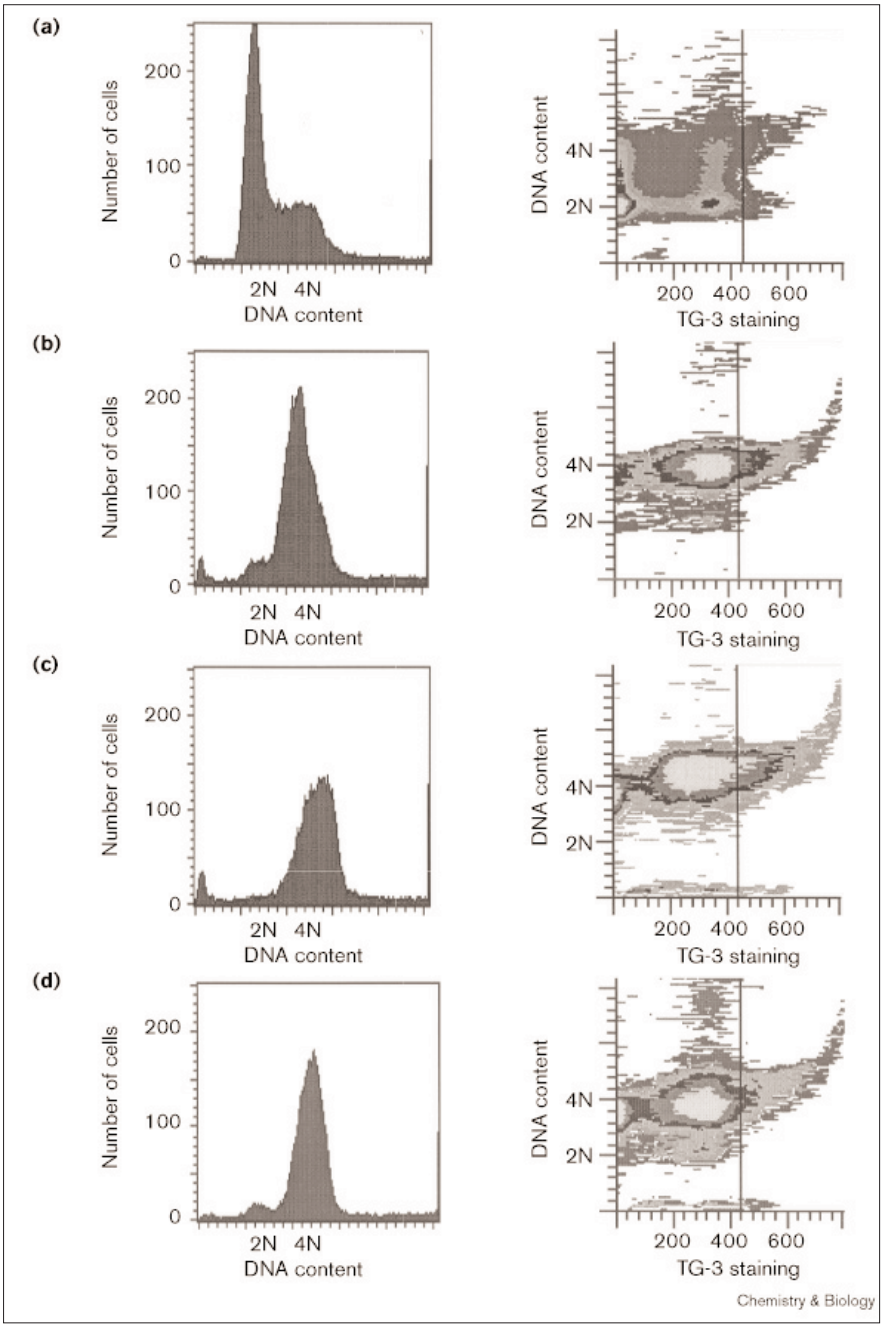
Figure 8



Effect of synstab A on the *in vitro* polymerization of purified bovine brain tubulin. Electron micrographs of negatively stained microtubules formed in the presence of synstab A (100 μM). **(a)** Low magnification (×4000), scale bar = 400 μm. **(b)** High magnification (×50,000), scale bar = 200 μm.

Taxol is thought to stabilize microtubules by counteracting the effects of GTP hydrolysis through its binding to a globular domain on β tubulin [21]. To address whether synstab A mimics the taxol mechanism of microtubule stabilization, we determined whether synstab A could compete with fluorescently labeled (Oregon Green) taxol (OGtx) for binding to microtubules previously stabilized with guanylyl-α,β-methylene diphosphonate (GMPCPP, a non-hydrolysable analogue of GTP). Using a fluorescent microscopy assay, an equivalent concentration of DMSO had no effect (Figure 11a), whereas synstab A displaced OGtx from the microtubules when added at a concentration of 500 μM (Figure 11b). In the same assay, unlabeled taxol caused a similar displacement when added at a concentration of 1 μM (data not shown). This mutually exclusive binding of synstab A and OGtx is consistent with the observed taxol-like effects of synstab A on cells (Figure 5e,h), and suggests that the effects of synstab A are either because it binds to the same or overlapping sites on microtubules as taxol, or as a result of its ability to induce a conformational change that prevents taxol binding. Because the addition of synstab A did not decrease the stability of GMPCPP-stabilized microtubules (Figure 10d), and because synstab A did not alter the overall microtubule

Figure 9

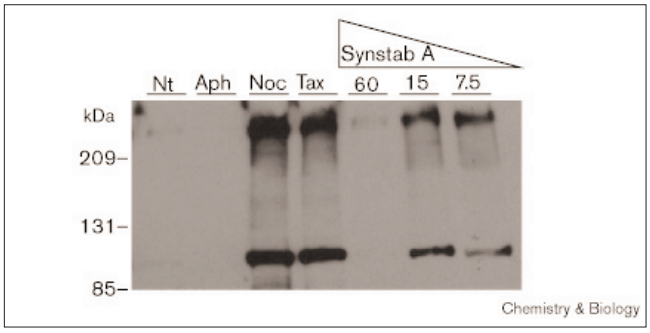


Cell-cycle distribution upon treatment of cells with synstab A. Fluorescence-activated cell sorting (FACS) analysis of A549 human lung carcinoma cells treated for 22 h with **(a)** 0.2% DMSO, **(b)** 332 nM nocodazole, **(c)** 2 μM taxol or **(d)** 15 μM synstab A. Cells were prepared for FACS analysis as described previously [11] and the data were processed using ModFiLT (V2.0). Depicted are the relative number of cells, DNA content measured from propidium iodide staining (2N or 4N) and TG-3 mAb reactivity measured through fluorescein-isothiocyanate labeling.

structure, as seen from the electron micrographs (Figure 8), the displacement of OGtx by synstab A does not occur by inhibiting formation of polymers to which taxol binds.

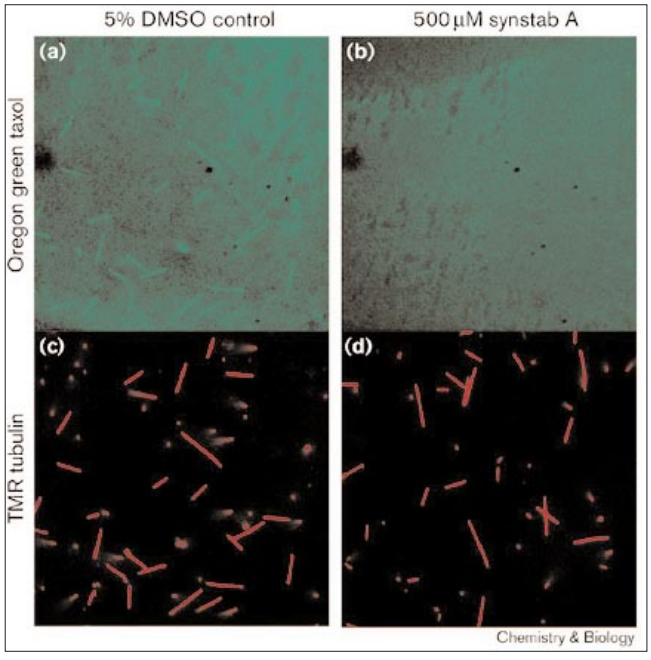
Given the similarities between the effects of synstab A and taxol on cells, and synstab A's ability to compete with taxol for binding to microtubules, we next determined whether synstab A treatment mimics other properties of known microtubule stabilizers. Because taxol and discodermolide prevent cold depolymerization of microtubules [19], we tested whether treatment of cells with synstab A would cause a similar effect. Although taxol treatment (10 μM, 4 h) noticeably stabilized microtubules, synstab A treatment (25 μM, 4 h) only partially protected cells from cold depolymerization (4°C, 0.5 h), resulting in a slight increase in the number of remaining microtubules as compared with untreated cells (data not shown). This suggests that either synstab A stabilizes microtubules in a manner

Figure 10



Increased TG-3 reactivity upon treatment of cells with synstab A. A549 cells were treated for 22 h with 0.2% DMSO (Nt), 12 μM aphidicolin (Aph), 332 nM nocodazole (Noc), 2 μM taxol (Tax) or synstab A, as indicated (in micromolar). Equivalent amounts of total cell extract were immunoblotted using the TG-3 mAb. Molecular weight standards shown are in kDa. The high-molecular weight proteins, other than nucleolin (105 kDa), that show increased detection with TG-3 upon treatment with nocodazole, taxol and synstab A remain unknown.

Figure 11



Mutually exclusive binding of synstab A and taxol to bovine brain microtubules. Fluorescence microscopy was used to visualize the binding of an Oregon-Green-labeled taxol derivative (OGtx) to TMR-labeled (red), GMPCPP-stabilized microtubules. When incubated in the presence of 5% DMSO, but in absence of a competitor, the OGtx signal **(a)** colocalizes with the TMR signal from microtubules **(c)**. In contrast, when incubated in the presence of synstab A (500 μM), the OGtx signal **(b)** does not colocalize with the TMR signal from microtubules **(d)**.

different than that either of taxol or of discodermolide, or that the stabilization of microtubules upon treatment with synstab A is sufficient to perturb interphase and mitotic microtubule dynamics, but not sufficient to counteract the destabilizing effects of cold-induced depolymerization due to differences in binding affinity or cell permeability.

Group III: small molecules targeting mitotic machinery other than tubulin

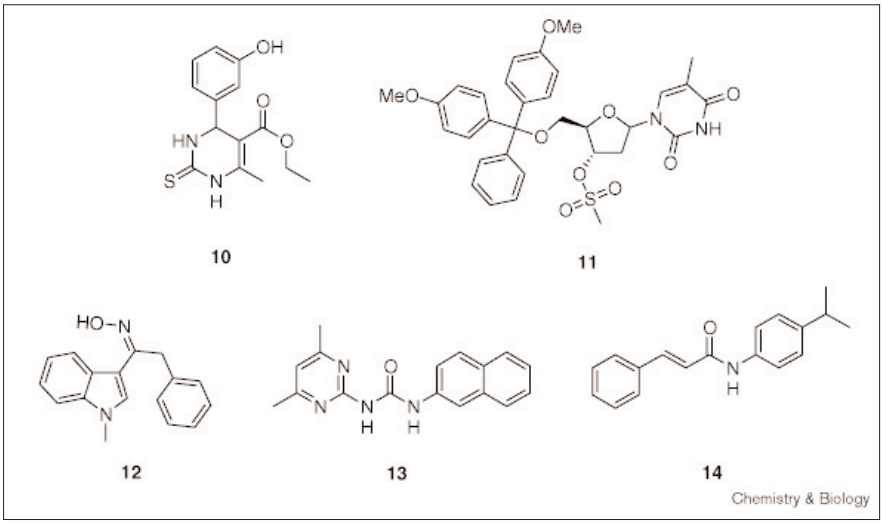
To begin to determine the mechanism of action of the 86 remaining group III compounds, each compound was tested in a TG-3 cytoblot assay using cells that had previously been arrested in interphase by the histone deacetylase inhibitor trichostatin A [22] or the topoisomerase II inhibitor ICRF-193 [23]. Under these conditions, none of the group III compounds stimulated cells to accumulate in mitosis, indicating that they require active cell-cycle progression to achieve their effects (data not shown).

Visual comparison of the chemical structures of group III compounds to those of group I compounds revealed that seven of 86 compounds show close structural homology to compounds that act as destabilizers of purified tubulin *in vitro*. Furthermore, although a number of these compounds had no observable effect on interphase cells, the effects of these compounds on microtubule organization in mitotic cells are similar to those observed for known weak microtubule depolymerizers (e.g., Figure 6c,d). This suggests that a subset of compounds in group III are likely to be weak microtubule destabilizers, ineffective at destabilizing purified microtubules under the conditions used to identify compounds in group I.

As observed with the variation in strength of microtubule depolymerization, the phenotype induced by many of the group III compounds, in terms of chromosome distribution, microtubule morphology and actin distribution in interphase and mitotic cells, depended upon both the concentration tested and duration of treatment. Treatment with 12 group III compounds at ~50 μM led to cell death or detachment after 4 h; these compounds were not examined further in this study. Given the similarity between aspects of mitotic mechanisms and apoptosis, these compounds may target components common to both pathways [24,25].

As a more stringent test of whether compounds had visible effects on chromosomes and the cytoskeleton, cells were treated for 4 h with of each of the remaining group III compounds at ~100 μM. Under these conditions, 69 compounds either had no observable effect on interphase cells (although still increased the number of apparently normal mitotic cells) or caused disorganization of otherwise straight microtubules in interphase cells and partially or completely disrupted spindles and chromosome distribution in mitotic cells. The compounds that produced the latter effects may target cellular regulators of microtubule dynamics, such as the centrosome that nucleates microtubules or the microtubule-associated proteins that can

Figure 12

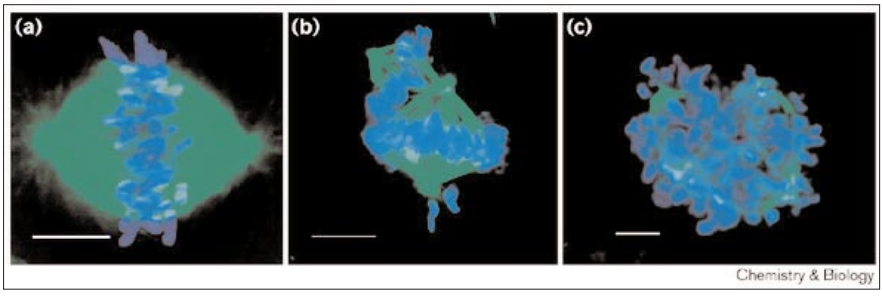


Chemical structures of mitosis-specific small-molecule inhibitors that do not interact directly with microtubules and have no observable morphological effect on the cytoskeleton or chromatin of interphase cells. Compound **10** (monastrol) is an inhibitor of the kinesin-related motor protein Eg5 [29]. The cellular targets of compounds **11**–**14** remain unknown.

either stabilize or destabilize microtubules in cells [26]. The similarity in phenotype of these compounds to weak destabilizers, however, makes it difficult to determine their mechanism of action without further tests of resynthesized compounds. In general, those compounds that increased the number of cells in mitosis without an obvious morphological effect did so weakly, even at high concentrations, compared with compounds that were found to directly target microtubules (e.g., Figure 3). These compounds may affect the activity of cell-cycle regulators, the activity of which is required for exit from mitosis (e.g., the activity of the anaphase-promoting complex CDC5,14,15,20) [27]. On the basis of the localization of MAD2 and the phosphorylation status of the kinetochore (the macromolecular structures that links mitotic chromosomes to microtubules) epitope 3F3 [28], it should be possible, in future studies, to determine whether the action of these compounds involves the activation of the spindle assembly checkpoint or some other mechanism.

From fluorescence microscopy of cells stained for microtubules, chromatin and actin, the remaining five compounds (**10**–**14**; Figure 12) were determined to be mitosis specific. Phenotypically, all five compounds had no observable effect upon interphase cells, but disrupted mitotic spindle morphology, altered chromosome distribution, or both, in mitotic cells. As discussed by Mayer *et al.* [29], the unique monopolar phenotype of one of these compounds (**10**, named monastrol) led to the discovery that monastrol specifically inhibits the kinesin-related protein Eg5. Treatment of BS-C-1 cells with compound **11** caused a similar monopolar phenotype as monastrol (data not shown), suggesting that **11** targets Eg5 or another component of the pathway required for spindle bipolarity. The cellular targets of the remaining three compounds remain unknown. In support of the hypothesis that each of these compounds target different components of the mitotic machinery, the phenotypic effects upon treatment of cells with these compounds were each distinct (Figure 13). For example, treatment with compound **12** (Figure 13b) caused the formation of a double spindle and minor chromosome misalignment, whereas compound **14** had no apparent effect on the mitotic spindle but caused severe chromosome misalignment (Figure 13c). This later phenotype

Figure 13



Example of mitotic abnormalities induced by compounds that have no observable effect on interphase cellular morphology or chromatin distribution. BS-C-1 kidney epithelial cells were treated for 4 h with **(a)** DMSO (0.1%v/v), **(b)** compound **10** (70 μM) and **(c)** compound **14** (70 μM) and stained for α tubulin (green) and chromatin (blue). Scale bar, ~15 μm.

suggests that compound **14** may target a component of the kinetochore and thereby prevent the alignment of chromosomes at the metaphase plate.

Future directions and challenges

Since the first description in 1889 of the effects of colchicine on mitosis, small molecules have played an essential role in the dissecting of the molecular mechanisms of chromosome segregation [30]. Future work from this screen will be aimed at the identification of the cellular targets of compounds **11**–**14** using biochemical or genetic methods. The success of the first approach is dependent upon both the specificity of the compound and the affinity, whereas the success of the latter approach depends on specificity and on the availability of existing mutant phenotypes to match observed phenotypic defects. In this regard, comparison of the effects of these compounds to known mutants of yeast and to developing embryos of the nematode *Caenorhabditis elegans* may provide a means either of identifying the exact target or of suggesting a mechanism of action [31]. This comparison can be based upon the observation of cellular morphology, the use of DNA microarrays and proteome-wide analysis.

One of the main challenges in high-throughput screening is to obtain the largest amount of useful information while using the smallest amount of compound. In this regard, a negative result obtained with a compound in one assay may be highly instructive in the context of another assay. Often the amount of compound available to validate whether its mechanism is distinct from previously known compounds is limited. In the case at hand, our ability to assess with a small amount of compound whether compounds targeted tubulin directly, using purified tubulin from bovine brain, was instrumental to the identification of compounds affecting the mitotic machinery through novel mechanisms.

Significance

Although the discovery of small-molecule inhibitors of protein–protein interactions is generally demanding, we noted the significant occurrence (~0.3% of compounds screened) of direct inhibitors of α/β tubulin interactions in this study. This illustrates the use of a system-based, phenotype-directed screen to identify components in a pathway that are most easily targeted by small molecules. It also suggests that the toxicity associated with many compounds may be due to their ability to destabilize microtubules. Small molecules capable of stabilizing protein–protein interactions are of interest both from the perspective of understanding the molecular basis of protein–protein interactions and in the specific case of α/β tubulin, because of the demonstrated use of taxol as an anticancer agent. Although synstab A shares many of the functional properties of taxol, it does not share structural features of known stabilizers of microtubules both natural (taxoids, discodermolide, epothilone and eleutherobin) and synthetic (e.g., GS-164 [32]). The ease with which synstab A was identified and its simple structure suggests to us that screening-based approaches to the discovery of taxol-like compounds will prove more effective than design-based approaches using 'pharmacophore' models [33,34]. In the context of other complex cellular processes, these results suggests that cell-based assays, initially using an antibody to select for small-molecule-induced post-translational modifications, in conjunction with directed cytology and *in vitro* assays, will provide a fruitful means of exploring many biological processes.

Materials and methods

Cell cultures

A549 (human, lung carcinoma) and BS-C-1 (African green monkey, kidney epithelial) cells were maintained at 37°C with 5% CO_2 in Dulbecco's modified eagle medium containing 10% fetal bovine serum, 100 units/ml penicillin G sodium, 100 μg/ml streptomycin sulphate and 2 mM glutamine (DMEM+; GibcoBRL).

TG-3 cytoblot assay

Cells were seeded in 50 μl of DMEM+ at a density of 4,000 cells per well of 384-well assay plates (Nalge Nunc, white, tissue culture treated) and cultured for 12 h at 37°C with 5% CO_2. Library compounds (Diverse E set, Chembridge Corp.) dissolved in DMSO (5 mg/ml) were arrayed in 384-well plates and 50–200 nl of each compound was pin-transferred into 384-well assay plates. After 22–24 h, the percentage of cells in mitosis was assayed using the TG-3 mAb (11–13) as described [9]. Data were collected on an Analyst plate reader (LJL Biosystems) with 0.2 s integration time. After two rounds of screening, compounds that increased TG-3 reactivity by 2.5 times or greater than control cells treated with DMSO were chosen for further analysis. To test for the requirement of active cell-cycle progression, cells were pretreated for 4 h, with either 300 nM trichostatin or 14 μM ICRF-193, and group III compounds were added to a final concentration of ~50 μM. After 16–18 h, a TG-3 cytoblot assay was performed.

In vitro *tubulin polymerization and taxol competition assays*

Purification of bovine tubulin and labeling of tubulin with tetramethylrhodamine (TMR) or Oregon Green (OG) fluorescent dye were performed as described [35] (see also http://iccbweb.med.harvard.edu/mitchisonlab/Pages/tubprep.html for more details). The extent of microtubule polymerization was determined in the presence of each compound at ~50 μM using a standard *in vitro* tubulin polymerization assay (see http://iccbweb.med.harvard.edu/mitchisonlab/Pages/poly.html for more details). After incubation for 20 min at 37°C, samples of reactions were spotted on a glass slide and the extent of tubulin polymerization was visually assessed using fluorescence microscopy of OG-labeled microtubules. To quantitatively assess the effect of synstab A on the dynamics of microtubule polymerization, either synstab A (100 μM), or an equivalent concentrations of DMSO, was added to 10 μM tubulin and incubated at 37°C, in the presence of 1 mM GTP, in degassed PEM buffer (80 mM PIPES, pH 6.9, 1 mM EGTA, 1 mM $MgCl_2$). After 2 min, the optical density of the sample (340 nm) was measured at 30 s intervals using a Hewlett Packard 8452A diode array spectrophotometer. For competition experiments between synstab A and taxol, 500 μM synstab A, or an equivalent concentration of DMSO (5%), was added to TMR-labeled, GMPCPP-stabilized microtubules (400 nM) in PEM buffer containing 240 nM of a fluorescently labeled (OG) taxol derivative (OGtx) (RF, unpublished results). Following incubation at room temperature for 10 min, 1.5 μl samples of each reaction were spotted onto a glass slide, covered with a coverslip and the localization of OGtx to the GMPCPP-stabilized, TMR-labeled microtubules was visualized using a

Nikon E-800 fluorescence microscope. Control treatments in the absence of DMSO showed OGtx staining of TMR-labeled microtubules, whereas the addition of 1 μM unlabeled taxol prevented the co-localization of OGtx to TMR-labeled microtubules.

Immunofluorescence and negative stain electron microscopy
BS-C-1 cells were seeded on glass coverslips at 80–90% confluency and compounds in DMSO were added to final concentrations as indicated. Cells were fixed with 0.2% glutaraldehyde and then stained for microtubules with an anti-α-tubulin antibody (Sigma), for chromatin with Hoecsht 33342 dye (Molecular Probes), and for actin with tetramethyl rhodamine isothiocyanate-conjugated phalloidin (Sigma). Images were obtained using a Nikon E-800 or Leitz microscope. For electron microscopy of microtubules, samples of tubulin after polymerization in the presence of either synstab A (100 μM) or an equivalent concentration of DMSO were spotted on to carbon-coated copper grids (200 mesh) and negatively stained for 1 min with 0.5% (w/v) uranyl acetate. After washing with 5 mM EGTA, images were obtained under low (× 4000) and high magnification (× 50,000).

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