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Small Molecules, Big Impact: A History of Chemical Inhibitors and the Cytoskeleton

Review

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Chemical inhibitors, whether natural products or synthetic, have had an enormous impact on the study of the eukaryotic cytoskeleton. Here we review the history of some of the most widely used cytoskeletal poisons and their influence on our understanding of cytoskeletal functions. We then highlight several new inhibitors and the targeted screens used to identify them and discuss why this approach has been successful.

Introduction

Few problems in biology have seen such a strong impact from the development of small molecule tools as the study of cell morphogenesis and the subsequent elucidation of the underlying anatomy of the cell that we now call the cytoskeleton. Compounds targeting the cytoskeleton are among the most commonly used chemical inhibitors in basic cell biological research. In addition, several of these have been developed into bona fide drugs widely used in the treatment of such diseases as cancers and gout. The goal of this review is to briefly review the history of a few of the most common inhibitors of the cytoskeleton with an emphasis on the impact these molecules had and continue to have on this field. By "impact," we mean either a significant conceptual leap in our understanding or a novel technique that becomes widely used. We will then highlight a few recent examples of novel small molecule inhibitors identified in screens targeting the cytoskeleton and discuss the promise that chemical approaches offer for the future of research on the cytoskeleton.

Colchicine and the Identification of Tubulin

The identification of the target of colchicine as tubulin, the subunit comprising the ubiquitous microtubule cytoskeleton of cells, is a remarkable example of forward chemical genetics. Indeed, the discovery of tubulin is intimately tied to the identification of the colchicine target. This tropolone derivative, found in the meadow saffron (genus *Colchicum*), has been used medicinally since at least the 18th century (and continues to be used) in the treatment of gout, and it is widely used as a research tool for the study of microtubules. Only in 1940 was the structure of the active component, colchicine, determined [1], and by the 1950s the effects of colchicine had been studied in cells and tissues of many types (for a comprehensive review of the early history of colchicine, see [2]).

Early investigation of the cellular effects of colchicine described the "explosion" of mitotic figures observed in tissues of colchicine-treated plants and animals (reviewed in [3]). Although we now understand that this arises from the arrest of the normal cell division cycle in mitosis, it was initially considered that colchicine could be inducing an altered mitosis in treated tissues that was called "c-mitosis." In plants, colchicine proved a rapid and convenient tool to generate agriculturally important polyploid strains, quickly replacing previous methods such as heat shock or treatment with other chemicals (extensively reviewed in [2]). In addition, the increased prevalence of mitotic figures in colchicine-treated cells was used to unambiguously determine that 46 chromosomes is the normal human diploid number rather than the previously believed 48 [3, 4]. Thus, even prior to the identification of the mechanism of action of colchicine, it was widely used in the areas of medicine, agriculture, and biology.

The determination of tubulin as the protein target of colchicine by Ed Taylor and colleagues in the late 1960s stands as a landmark in the identification of small molecule targets in complex mixtures as well as opening up the microtubule field by identifying the protein subunit that comprises these filaments. Using radiolabeled colchicine prepared by methylation of colchiceine in tritiated water, Borisy and Taylor biochemically characterized a colchicine binding activity in both intact cells and cell extracts [5, 6, 7]. This binding activity was found to be enriched in cells and tissues containing abundant microtubules, suggesting that the target of colchicine was the subunit of microtubules. Taylor and colleagues subsequently purified the colchicine binding protein from both sperm tails and mammalian brain and characterized it as a 120 kDa dimer containing 2 moles of bound GTP, thus identifying the molecular subunit of microtubules [8, 9]. The name "tubulin" was provided by Mohri [10], who determined the amino acid composition of the sea urchin sperm microtubule subunit. Thus, colchicine was at the same time the agent for tying microtubules to important cellular processes such as mitosis and the agent of protein (gene) discovery, fulfilling the requirements of forward chemical genetics.

Taxol and Nocodazole

In 1971, a natural product with antileukemic and antitumor activity was identified from an alcohol extract of the bark of the western yew (*Taxus brevifolia*) and named taxol [11]. Progress on taxol lagged due to its perceived low antitumor activity, the limited quantities of the compound, and scarcity of the source tree [12, 13]. Nevertheless, later observation of cells isolated from taxol-treated mice revealed the presence of abnormal mitotic figures [14]. Remarkably, in contrast to other microtubule poisons (colchicine, nocodazole, the *Vinca* alkaloids, eg.), taxol was shown to *stimulate* the polymerization of microtubules both in vitro [15] and in vivo [16, 17]. With this discovery, then, two distinct natural products had been identified with opposing activities on microtubule stability.

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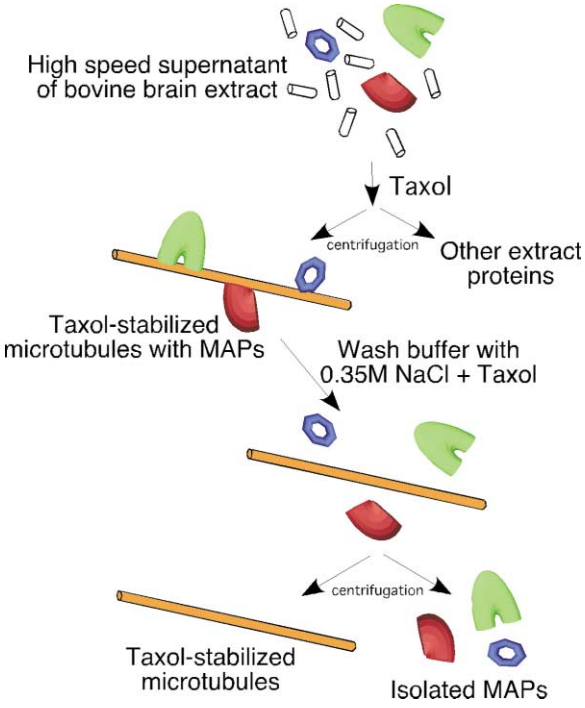

Figure 1. Use of Taxol-Stabilized Microtubules for the Isolation of MAPs

Richard Vallee isolated *m*icrotubule-*a*ssociated *p*roteins by adding taxol to induce the polymerization and stabilization of microtubules in a soluble extract of bovine brain. Taxol-stabilized microtubules were subsequently stripped of their associated proteins in a high-salt wash and pelleted, leaving the isolated MAPs in the supernatant [18].

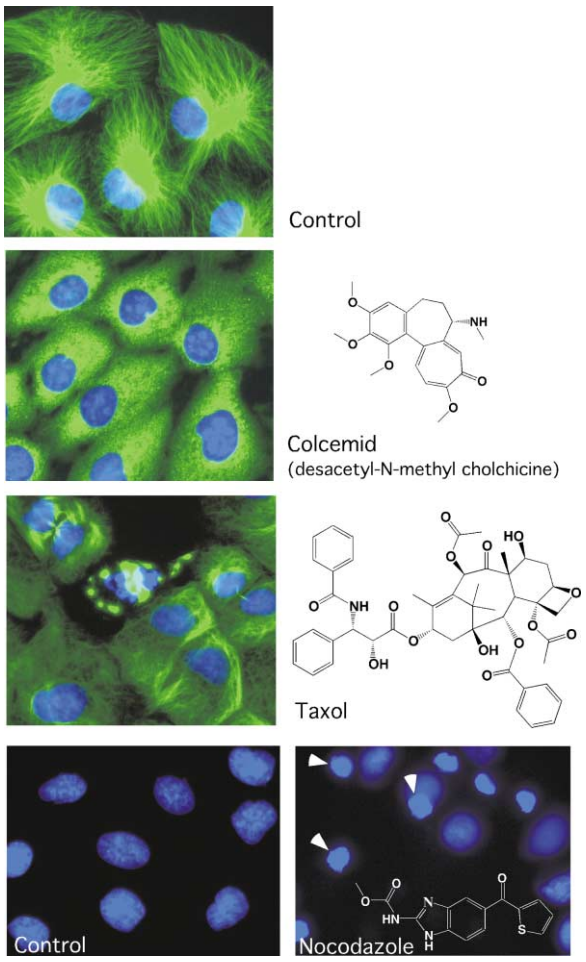

Figure 2. Structure and Effect of Classical Microtubule Poisons

In the upper panels, B-SC-1 cells treated with 2 μM colcemid or 10 μM taxol for 1 hr were fixed and stained with anti-tubulin antibodies (green), and DNA was stained with Hoechst (blue). In the lower panels, B-SC-1 cells treated for 24 hr with 3.3 μM nocodazole or vehicle control were stained with Hoechst. Note the increased prevalence of mitotic nuclei containing condensed DNA (several examples are marked with arrowheads).

The subsequent impact of taxol on basic biological research was dramatic. Vallee [18] exploited the strong stabilizing influence of taxol on microtubules to purify them and their bound microtubule-associated proteins (MAPs) from bovine brain (see Figure 1). Taxol was added to an extract of brain to polymerize microtubules and allow binding of endogenous MAPs. These filaments were then centrifuged and collected. A subsequent high-salt wash of the pellet stripped the MAPs, while the constant presence of taxol maintained the structural integrity of the microtubules, which could then be centrifuged away from the soluble MAPs. Thus, taxol allowed an affinity-based purification of MAPs that, because of the instability of microtubules to the high-salt extraction, would not have been possible otherwise. A similar microtubule affinity purification using taxol later aided the discovery and study of the microtubule-based motor protein kinesin [19]. Taxol-stabilized microtubules have also been used as the substrate to visualize gliding motility powered by both major microtubule-based motor families, kinesin and dynein, immobilized on glass coverslips.

A *synthetic* compound directly affecting microtubules was identified in Belgium (Janssen Pharmaceutica) in 1975 in a screen for antihelminthic compounds and was termed oncodazole (R 17934), presumably for an observed antitumor activity [20]. Two years later, this benzimidazole compound appears in the literature by the name nocodazole, and this term has endured to the present, perhaps because the early clinical promise against cancer was not realized. As with taxol, the microtubule disruption observed in nocodazole-treated cells led researchers to test directly if nocodazole bound to tubulin. De Brabander and colleagues showed that nocodazole indeed bound tubulin and in a manner competitive with colchicine [21].

What Have We Learned from Microtubule Poisons?

Immunofluorescence staining using anti-tubulin antibodies reveals a dramatic disruption of the microtubule network in cells treated with microtubule poisons (Figure 2). Nocodazole along with colchicine have been used to demonstrate functional roles for microtubules in numerous cell biological processes, including the anchoring of the Golgi complex at the microtubule-organizing center (reviewed in [22]), cell migration, and tumor inva-

sion (reviewed in [23]). Nocodazole is preferred over colchicine in basic research when reversible inhibition of microtubule polymerization is required. The dissociation rate of colchicine from tubulin is very slow [6], which helped in the identification of tubulin by Taylor and colleagues.

An additional role for microtubules uncovered with the help of microtubule-disrupting agents is the trafficking of intracellular particles. The microtubule cytoskeleton is generally arrayed from a central organizing center termed the MTOC from which microtubules radiate throughout the cell (see Figure 2). Melanophores utilize this microtubule "highway" for the intracellular distribution of thousands of pigment granules that migrate along this network using microtubule-based motors. The dispersion of granules to the cell periphery produces an apparent "darkening" in the skin, whereas the aggregation of granules at the MTOC produces the opposite effect. In 1965, prior to the identification of tubulin, Stephen Malawista [24] demonstrated that colchicine perturbed the aggregation of frog melanocyte granules, which he interpreted in terms of a "decreased protoplasmic viscosity." Once the target of colchicine had been identified, subsequent work using colchicine and the *Vinca* alkaloids helped demonstrate that microtubules play a fundamental role in the movement of pigment granules (reviewed in [25, 26]). The importance of microtubules to the intracellular transport of other organelles and vesicles remains an important question in cell biology.

The Cell Cycle, Checkpoints, and Cancer

Since taxol, nocodazole, and the colchicine relative colcemid all block normal chromosome segregation, Schimke and coworkers tested the affect of microtubule disruption by these agents on cell cycle progression of numerous mammalian cell lines [27]. The authors observed that human cell lines generally arrested in mitosis in compound-treated cells, consistent with the postulate of a checkpoint that ensures that cells remain in mitosis until a proper spindle is assembled [28]. Indeed, inhibition of cell cycle progression is one of the most prominent features of cells treated with microtubule poisons, although cell lines vary in the effectiveness of this arrest [29]. The mitotic arrest induced by these compounds also provides a convenient manner to synchronize the cell cycle state of cultured cells. Cultured cells treated with microtubule-depolymerizing agents accumulate in mitosis (see Figure 2, lower panels) and can be synchronously released by removing the compound from the media. This feature is now widely exploited by those studying mitosis for the isolation of cells homogeneously arrested in the mitotic state.

Benomyl, an agricultural fungicide and microtubule-polymerization inhibitor structurally related to nocodazole, was used in a similar manner to explore this checkpoint genetically in the yeast *Saccharomyces cerevisiae* [30, 31]. Both groups identified yeast mutants that did not properly arrest cell cycle progression in response to disruption of microtubules by benomyl. These mutants, termed "bub" for "budding uninhibited by benomyl" and "mad" for "mitotic arrest deficient," identified several genes critical for ensuring the proper temporal order of cell cycle events, and understanding their function remains a central question in cell biology.

Their usefulness in arresting cell division has led to the assessment of many microtubule inhibitors for the treatment of cancer. Indeed, the microtubule-destabilizing *Vinca* alkaloids vinblastine (originally vincaleukoblastine [32]) and vincristine helped establish a link between microtubules and cancer. These closely related but chemically distinct compounds from leaves of the Madagascar periwinkle were originally isolated based on their ability to depress white blood cell counts (reviewed in [33]). This original observation has now matured into the current use of vinblastine and vincristine in the clinical treatment of Hodgkin's lymphoma and leukemia, respectively. The development and history of microtubule poisons for clinical use is outside the scope of this review, however, and the interested reader is referred to any of a number of reviews (e.g., [32, 12, 34]). Nevertheless, it deserves mentioning that taxol, colchicine, and the *Vinca* alkaloids are mature, modern pharmaceuticals and, because of the central role of the cytoskeleton in cell division, cytoskeletal proteins remain important anticancer targets [35]. Indeed, the next generation of tubulin-targeting, anticancer compounds is being developed to address limitations of the current arsenal, such as aqueous solubility, multidrug resistance, and the pronounced toxicity toward lymphocytes and peripheral neurons. One well-characterized example, the natural product epothilone, stabilizes microtubules with greater potency than taxol, is less sensitive than taxol to P-glycoprotein-mediated multidrug resistance, and remains active against taxol-resistant tumor models (reviewed in [36, 37]). Several total syntheses of epothilone have been achieved, and medicinal chemistry efforts have identified derivatives with improved pharmacological properties (reviewed in [38, 39]). Readers interested in a more comprehensive and mechanistic view of small molecules that target tubulin and their anticancer potential are directed to other reviews (see [23, 40]). Those interested in recent developments in the chemistry of taxol are referred to [41].

Cytochalasins, Phalloidin, and the Actin Cytoskeleton

The family of mold metabolites known as cytochalasins were independently isolated from distinct fungal species by Aldridge et al. [42] at Imperial Chemical Industries Ltd. and by Rothweiler and Tamm at the University of Basel [43]. Whereas Rothweiler and Tamm called their compound Phomin after the *Phoma* species from which they isolated the compound, Carter [44] provided the name cytochalasin from the Greek *cytos* (a cell) and *chalasis* (relaxation) to describe the effects of this compound on mouse fibroblasts. A preliminary article based on the work at ICI appeared in New Scientist, calling this compound family "one of the most remarkable groups of biologically active substances to be described in years," although perplexingly the name was spelled "cytocholasins" [45]. Cytochalasin inhibited whole-cell migration, ruffling of the cell margin, and cytoplasmic cleavage of dividing cells, but nuclear division continued, thus producing multinucleated cells over time.

Carter, knowing nothing about the target of cytochalasin, used this compound to probe two important biological questions: how do cells migrate and how do cells divide? Having recently proposed a mechanism for cell motility based on surface tension and differential adhesion of cells to substrates, which he termed 'haptotaxis' [46], it is not surprising that he wrongly interpreted the effects of cytochalasin on cell motility in terms of the compound increasing the adhesivity of the cell membrane to the substrate, thus preventing both forward movement and cell ruffling. Nevertheless, using cytochalasin he was able to imply a common molecular mechanism underlying cell ruffling, motility, and cytokinesis. Furthermore, this motility was distinguishable from the movement of spermatozoa, ciliates, and flagellates, which were not affected by cytochalasin and are now known to be actin-independent phenomena. Cytochalasin represented the first compound that could disrupt cytokinesis (cell division) without affecting karyokinesis (nuclear division), thus clearly establishing the independence of karyokinesis from cytokinesis. The usefulness of a specific inhibitor for studying the mechanism of cytokinesis had already been anticipated by Wolpert [47].

In order to understand the impact of cytochalasin in this area, it is necessary to review contemporary theories on the mechanism of cell cleavage. Previously, numerous ideas had been proposed to explain cytokinesis (reviewed in [47]). One of these, the cortical gel contraction hypothesis [48], proposed that contraction of a cortical network underlying the deepening cleavage furrow between daughter cells provided the force for cytokinesis. This model stood in opposition to surface expansion theories which suggested that plasma membrane expansion was an active process providing the energy for furrow ingression [49] or theories in which the mitotic apparatus itself or other subcortical components are responsible for force generation [50, 51].

Despite its postulate of a cortical gel composed of interlinked "elongate protein components" that could undergo a "forcible folding without relinquishing their intermolecular linkages" during contraction [48], the cortical contraction theory lacked a morphological structure that could be pointed to as the source of force. Using electron microscopy, several investigators subsequently identified circumferential filaments underlying the cleavage furrow which where proposed to represent the apparatus of the 'contractile ring' ([52] and references therein). It was Schroeder, however, who, by demonstrating that cytochalasin both disrupted this contractile ring and abolished cytokinesis [52, 53], directly implicated the filaments in cytokinesis and suggested a "purse string" mechanism for furrowing. This work, of course, also suggested a mechanism of action for cytochalasin with respect to the block of cytokinesis.

Morphologically similar microfilaments had already been observed in nondividing cells [54], although their relationship to the filaments of the ring remained speculative [52]. Ishikawa et al. [55] used a technique for decorating muscle actin filaments for electron microscopy using a proteolytic fragment of the myosin protein to probe the nature of microfilaments in nonmuscle cells. The characteristic arrowhead pattern they observed in animal fibroblasts as well as epidermal and epithelial cells suggested that these nonmuscle microfilaments could be related to muscle actin. Similar observations in *Acanthamoeba* [56], epithelial brush border [57], *Dictyostelium* [58], *Physarum* [59], and *Amoeba* [60] supported the universiality of these structures. These studies, as well as the biochemical characterization of actin-like filaments derived from nonmuscle cells, supported the ubiquity of actin. Still, the connection between these microfilaments, the filaments of the cytokinetic furrow, and the actin filaments of muscle was tenuous.

Wessells and coworkers studied the effects of cytochalasin and the microtubule inhibitor colchicine, whose target had been recently identified, on axonal outgrowth of cultured neurons [61]. They noted that cytochalasin rapidly disrupted microspikes and growth cone dynamics, whereas colchicine only affected the axon and on a much slower time scale. These important observations suggested a strong connection between the microfilaments and growth cone motility while establishing an important but distinct role for the microtubule system in axonal outgrowth. Similarly, work using cytochalasin on the glands of the oviduct and salivary epithelium showed that disruption of the microfilament network strongly perturbed gland morphogenesis [62, 63]. Mounting evidence seemed to suggest that the microfilaments themselves were the target of cytochalasin:

> The following processes are sensitive to cytochalasin: cytokinesis, cell movement, axonal growth cone and microspike activity, maintenance and change in shape of salivary gland epithelium, formation and maintenance of early glands in oviduct, and perhaps migration of nuclei in an epithelium preparatory to mitosis. Every such case can be explained if contractile filaments are rendered inoperative by the drug; and in every case so far examined, morphological alterations in microfilaments have resulted from application of cytochalasin. ...The common sensitivity to cytochalasin suggests a homology between those filaments comparable to that between microtubules from varying cell types in their sensitivity to colchicine [63].

The final demonstration that cytochalasin targets the microfilaments directly arrived in 1972. Parallels between the contractile ring and contraction in muscle had been made much earlier (reviewed in [47]), yet no molecular connection had been made between the two beyond the observation that the actin 'thin filaments' of muscle were similar in diameter to the 'microfilaments' observed in cells [64].

In a landmark paper, Spudich and Lin studied the effect of cytochalasin on purified actin and actomyosin from rabbit muscle [64]. These authors demonstrated that cytochalasin could decrease the viscosity of solutions of pure filamentous actin, thus revealing in one fell swoop that cytochalasin targets the actin protein of muscle, and actin therefore likely also comprises the contractile ring and other cellular microfilaments. This supported the prevailing hypothesis that actomyosin assemblies controlled aspects of the motility of nonmuscle cells and led to an explosion in the use of cytocha-

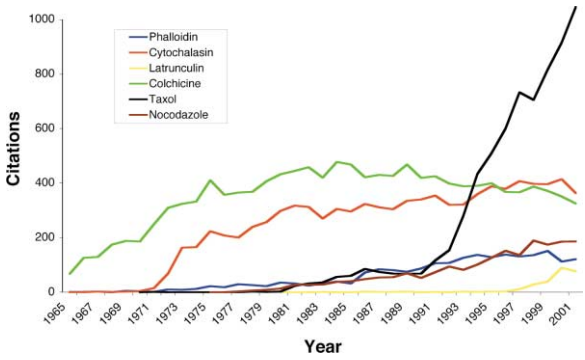

Figure 3. PubMed Citations of Cytoskeletal Inhibitors

The number of citations for each search term is plotted as a function of year.

lasin as a research tool for disrupting actin function (see Figure 3).

These studies connecting actin, microfilaments, and cytochalasin were reinforced by contemporary work on the fungal metabolite phalloidin. It was isolated in 1937 as one of the toxic peptides derived from mushrooms of the *Amanita* genus [65], whose toxicity has been studied since the early 1800s (reviewed in [66, 67, 68]). Morphological studies of intoxicated animals revealed only acute liver toxicity associated with vacuolization of the endoplasmic reticulum [69]. An important breakthrough resulted from the demonstration that phalloidin could induce the formation of microfilament-like filamentous structures both in vivo and in preparations of cytoplasmic membrane fragments [70]. Importantly, a nontoxic derivative of phalloidin, desmethylphalloinsulfoxide, did not induce these structures [70]. Two years later, following the lead of Spudich and Lin [64], it was shown that phalloidin could drive the polymerization of monomeric G-actin from rabbit muscle [71]. This polymerization could be inhibited by preincubation with cytochalasin, and the phalloidin-induced filaments produced both in vitro and in vivo could, like conventional actin filaments, be decorated with heavy meromyosin. Thus phalloidin, like cytochalasin, played an important role in connecting the morphologically defined microfilaments with the muscle actin protein.

Advances in Actin Biology Driven by Chemical Inhibitors

The number of literature citations of phalloidin or cytochalasin has increased steadily since the discovery of their mechanism of action (Figure 3). Although the general cell impermeability of phalloidin has limited its use in live cells, an important development has been the utilization of flourescently labeled phalloidin for staining filamentous actin in fixed tissues and cultured cells ([72]; Figure 4). Jasplakinolide, a natural product isolated from a marine sponge, also stabilizes actin polymers yet is cell permeable and has been used in live cells to investigate the importance of filament disassembly in, for example, lamellipodial extension [73]. The cytochalasins rapidly enter living cells, disrupt the actin cytoskeleton (see Figure 4), and have been used to implicate this structure in a various processes. Indeed, as Carter observed in his original description of the activity of cytochalasin:

> By interfering with specific cell activities such as cytoplasmic cleavage and cell movement, they should prove useful as research tools for investigating these important aspects of cell biology. [44]

The identification of the latrunculin family of actin monomer binding drugs deserves mention for its particular contribution to the study of the actin cytoskeleton of yeast. Originally identified as a toxic agent in the marine sponge *Latrunculia magnifica*, the latrunculins (A and B) were shown to disrupt the actin cytoskeleton in mammalian tissue culture cells [74]. Based on these initial observations, latrunculin was shortly thereafter shown to interact directly with monomeric actin in a 1:1 complex, preventing its incorporation into filaments [75].

Although actin is an essential gene in *Saccharomyces cerevisiae* [76], temperature-sensitive mutants in the actin gene have allowed some phenotypic analysis of actin mutations on an approximately 1 hr time scale [77]. The use of latrunculin in *S. cerevisiae*, however, allowed a first look at the acute phase of actin perturbation. Within minutes of addition, latrunculin caused the loss of filamentous actin in a reversible manner [78]. Cytochalasin, by contrast, had no effect, most likely due to cell permeability issues [79]. Using latrunculin, then, allowed the authors to demonstrate that even in the nonmotile yeast cell, the actin cytoskeleton exhibits dynamic de- and repolymerization like in mammalian cells, suggesting that dynamicity of the actin cytoskeleton is a universal phenomenon.

The rapid onset and effectiveness of latrunculin in yeast has led to its continuing wide use in exploring the role of the actin cytoskeleton of this organism in such areas as cell polarity [80], spindle orientation [81], and endocytosis [82]. Indeed, the widespread use of latrunculin when temperature-sensitive mutations also exist is a testament to the advantages offered by small molecule inhibitors (see below). Latrunculin has also become more widely used in mammalian cells. The observation that latrunculin and cytochalasin produce distinct effects in mammalian cells (Figure 4; [83]) is indicative of different mechanisms of action. Whereas cytochalasin binds both monomeric actin and filament 'barbed' ends, latrunculin binds exclusively to actin monomers, making the interpretation of latrunculin experiments somewhat more straightforward [75, 84]. Furthermore, cytochalasin B (but not cytochalasin D) was shown to also inhibit glucose uptake by cells [85, 86] raising questions of specificity, whereas the identification of mutants in actin that confer latrunculin resistance in *S. cerevisiae* have strongly suggested that the interaction of latrunculin and actin in yeast is highly specific [78]. Cytochalasin and latrunculin are each, therefore, unique probes of actin function offering distinct mechanisms of perturbation.

Small Molecules as Tools in Crystallography

Crystallographic analysis of cytoskeletal proteins is complicated by the tendency of individual subunits to polymerize into filaments at high concentrations. This

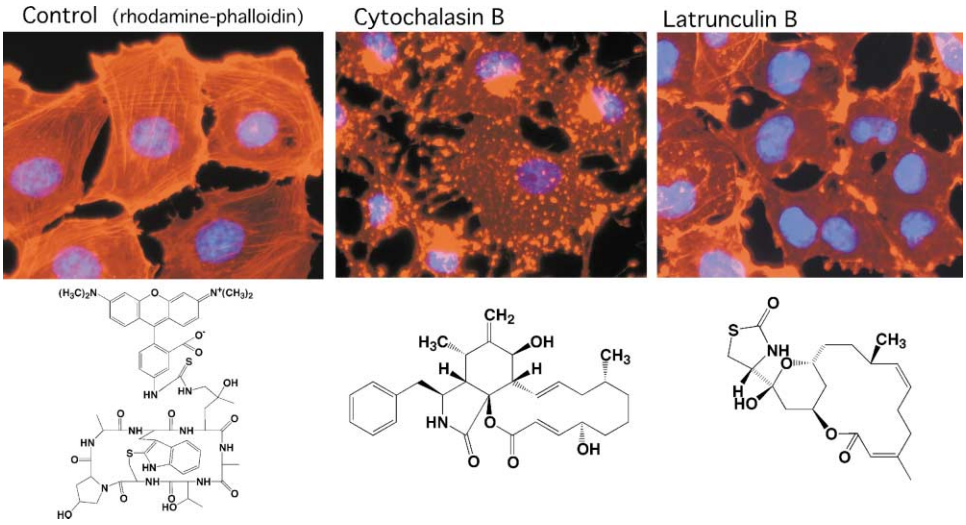

Figure 4. Structure and Effect of Classical Actin-Perturbing Agents

B-SC-1 cells treated with 10.4 μM cytochalasin B, 200 nM latrunculin B, or vehicle control were fixed and stained with rhodamine-conjugated phalloidin.

difficulty has been overcome in the case of actin by chemical modification of the actin monomer [87], by cocrystallizing actin with monomer binding proteins that prevent polymerization [88, 89, 90, 91], and by the use of the small molecule latrunculin [84]. Using the converse approach, Nogales et al. [92] used taxol and zinc to stabilize sheets of tubulin in order to derive an atomic model of the αβ tubulin dimer. Thus, small molecules have also been important tools for investigating the molecular architecture of actin and tubulin at the atomic level.

In and Out in the Blink of an Eye

Chemical inhibitors have illuminated cytoskeletal function not only through the study of compound-treated cells. Important observations have been made of cells during the "washout" or recovery period when compound-treated cells are washed into media lacking the inhibitor. Indeed, the rapid reversibility of colchicine and nocodzole was instrumental in revealing the microtubule-nucleating role of the microtubule organizing center (MTOC). Upon washout of cells treated with nocodazole or colcemid, microtubules are observed to preferentially regrow from this perinuclear structure, suggesting that the MTOC plays a normal role in the nucleation of new microtubules [93, 94, 95]. These observations were confirmed using cold-induced depolymerization of microtubules followed by rewarming [96].

Paul Forscher used a similar approach using cytochalasin to investigate actin dynamics in the neuronal growth cone [97]. Time-lapse video images revealed that on addition of cytochalasin, the actin cytoskeletal matrix within the growth cone disappeared by first receding away from the plasma membrane as an intact unit at a rate of 3–6 μm/min (see Figure 5). On cytochalasin washout, the matrix reappeared first at the plasma membrane and widened and extended toward the rear of the growth cone at an identical 3–6 μm/min. These observations strongly suggested that new actin assembly occurs proximal to the plasma membrane and that the entire actin network of the growth cone translocates centripetally back toward the axon. Importantly, this polymerization and retrograde actin flow from the leading edge is now thought to drive protrusion during cell migration [98].

New Screens, New Molecules

The majority of the most widely used cytoskeletal inhibitors today are natural products that initially drew interest for their toxicity or potential medicinal utility. The collective human experience can thus be seen as a rather "low-throughput" screen for bioactive molecules in the natural world. Recent advances in combinatorial chemistry and high-throughput bioassay screening, however, promise to rapidly increase the speed and efficiency of this process and allow it to be directed toward the identification of small molecules with a particular activity. The actin and tubulin networks of cells consist not only of the filaments and tubules themselves. A large number of regulatory and structural proteins, including motors, crosslinkers, depolymerizers, and filament bundlers, can act to create and organize these assemblies. Several researchers have begun to conduct screens for small molecules targeting components other than actin and tubulin themselves. New inhibitors and a few of these new screens are presented below and are intended to illustrate the diversity of target classes and approaches.

Motor Protein Inhibitors

The recent identification of the Eg5 kinesin inhibitor monastrol demonstrates the success of using whole-cell-based assays to identify inhibitors of the cytoskeleton. Mayer et al. [99] screened for compounds that would induce mitotic arrest in tissue culture cells as assayed by an antibody to the mitotic form of the nucleolin protein. Since compounds that perturb microtubule dynamics (e.g., nocodazole and taxol) can cause mitotic

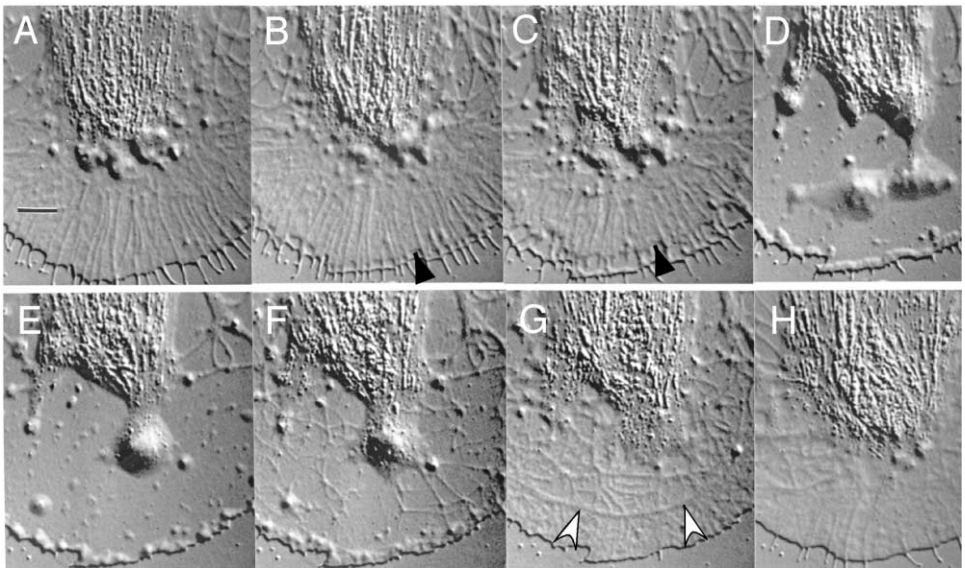

Figure 5. Demonstration that the Actin Network Exhibits Retrograde Flow from the Plasma Membrane, Where New Actin Polymerizes, Centripetally toward the Cell Body in *Aplysia* Neural Growth Cones

Reproduced from The Journal of Cell Biology, 1988, *107*, 1505–1516 by copyright permission of The Rockefeller University Press. Time-lapse differential interference contrast video images of a growth cone after treatment with 10 μM cytochalasin. (A)–(E) represent 0, 0.5, 1, 3, and 9 min of treatment, respectively. The actin network is seen to recede from the plasma membrane at 3–6 μm/min (arrowheads in B and C). Cytochalasin was then removed from the culture media, and cells were allowed to recover for 1, 3, and 17 min (F–H, respectively). The border of the new, advancing actin network (empty arrowheads in G) also migrates at 3–6 μm/min toward the axon.

arrest, active compounds were subsequently counterscreened to eliminate molecules that directly affect microtubule dynamics in vitro. One of the resulting compounds produced a remarkable reorganization of the mitotic spindle of treated cells. Instead of bipolar spindles, monastrol-treated cells produced monoastral spindles. A similar phenotype had been previously observed both in vitro [100] and in vivo [101] on inhibition of the mitotic kinesin Eg5 using anti-Eg5 antibodies. Indeed, in vitro experiments showed that monastrol inhibited microtubule motility powered by Eg5, whereas a structurally related compound that did not cause monoasters in cells did not [99].

Importantly, monastrol appears to show remarkable specificity despite its low micromolar IC_{50} against Eg5. Microtubule arrays in interphase cells appear to be completely unaffected, and effects of monastrol are rapidly reversed on washout of the compound. The specificity, reversibility, and cell permeability of monastrol promises that this compound will be an invaluable tool to help reveal the functions of Eg5 during mitosis. Indeed, using monastrol it was shown that the motor activity of Eg5 was not required for its normal spindle localization [102]. In addition, perturbation of spindle function by monastrol allowed Kapoor et al. [103] to probe the spindle-assembly checkpoint without directly affecting microtubule dynamics. An Eg5 inhibitor with nanomolar affinity was recently reported [104], and this or related compounds will be tested as anticancer drugs in humans.

Recently, two new cell-permeable myosin motor inhibitors were identified in pure protein screens for inhibitors of skeletal muscle myosin II [105] and nonmuscle myosin II [106]. The myosin superfamily of actin-based motors is large and diverse [107], and small molecules that can discriminate between members will allow detailed study of their unique functions in vivo.

A conceptually different approach to chemical inhibition of motor proteins has now been used with both kinesin and myosin motors. Pioneered by Shokat [108], this method involves expression of mutated nucleotide binding proteins with engineered sensitivity to a nucleotide analog. The first proof of principle of this approach for motor proteins involved a single amino acid mutation in the nucleotide binding pocket of kinesin [109]. This mutation conferred sensitivity to the nonhydrolyzeable ATP analog cyclopentyl-adenylyliminodiphosphate, which does not inhibit the wild-type protein. Thus, these authors demonstrated a new experimental approach for the specific inhibition of motor proteins. Holt et al. [110] have recently utilized this approach to address the function of the myosin-1c protein in adaptation of the hair cells in the sensory epithelium of the inner ear. A mutation of the myosin-1c nucleotide binding pocket was generated that would accommodate an N^6-modified ADP analog but that would not prevent its utilization of ATP [111]. Sensory epithelia isolated from mice expressing the mutant protein behaved normally electrophysiologically. When exposed to the ADP analog, however, a loss of the adaptive response to hair cell deflection was observed, demonstrating a crucial role for myosin-1c in hair cell adaptation.

Signaling Protein Inhibitors

Using an approach intermediate between cell-based screens and pure protein assays, Peterson et al. [112] screened for small molecules that would inhibit a signal-

ing pathway controlling the nucleation and polymerization of actin filaments in a cytoplasmic extract. By screening for inhibitors of an entire pathway, these authors screened multiple potential targets, both known and unknown, allowing the biology to dictate the best targets. Interestingly, two inhibitors of a signal integrating protein, the neural Wiskott Aldrich Syndrome protein (N-WASP), were identified using this screen ([112]; J.R.P., L. Bickford, A. Kim, M. Kirschner, and M. Rosen, unpublished data). N-WASP exists in an autoinhibited state that can be activated by binding to signaling molecules such as active cdc42, Nck, or phosphatidylinositol 4,5-bisphosphate [113]. On binding its activators, N-WASP undergoes a conformational change that allows it to activate the Arp2/3 complex, an actin nucleating protein complex [113]. Both of the inhibitors, 187-1, a 14 amino acid cyclic peptide, and wiskostatin, an N-alkylated carbazole derivative, appear to inhibit N-WASP by stabilizing the autoinhibited conformation, thus preventing subsequent activation of the Arp2/3 complex (Figure 6). The identification of two chemically distinct inhibitors of N-WASP suggests that this protein is not only an important signaling node but potentially also an important locus for inhibitors of this pathway.

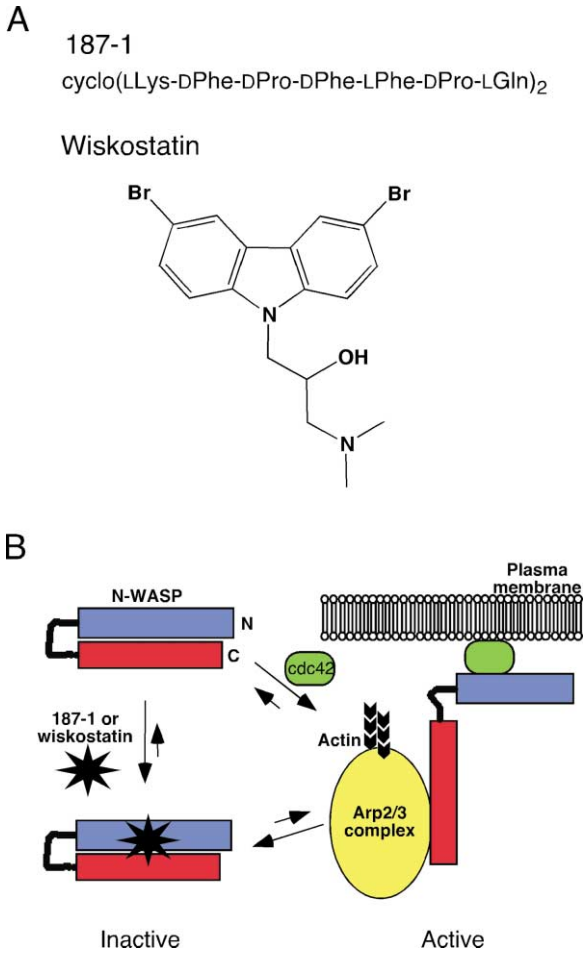

Figure 6. Inhibitors of N-WASP Block Actin Filament Assembly by Stabilizing the Autoinhibited Conformation of N-WASP

(A) Structures of two N-WASP inhibitors, the 14 amino acid cyclic peptide 187-1 and wiskostatin.

(B) Signaling molecules including Cdc42 bind N-WASP, relieving its intrinsic autoinhibition and exposing a C-terminal domain that can bind and activate the Arp2/3 complex to nucleate a new actin filament. 187-1 and wiskostatin attenuate this signaling cascade by stabilizing the autoinhibited conformation of N-WASP, antagonizing activation by upstream signaling molecules (based on [112] and J.R.P., L. Bickford, A. Kim, M. Kirschner, and M. Rosen, unpublished data).

Conclusions

Why have chemical approaches to study the cytoskeleton been so successful? One answer must certainly be the swift action of small molecules. Cytoskeletal rearrangements typically occur over seconds, a time scale inaccessible to traditional genetic approaches but addressable by the rapid diffusion of small molecules. This avoids the complications of cellular adaptation/compensation that can arise when using genetic knockout approaches. An alternative to the knockout of genes of interest is the use of temperature-sensitive mutants. This approach sometimes allows relatively rapid protein inactivation and also allows the study of the loss of function of essential genes. Temperature shifts alone, however, have the potential for nonspecific perturbation [114]. Indeed, transcriptional profiling of *Saccharomyces cerevisiae* has demonstrated "massive and rapid alterations in genomic expression" of wild-type strains on temperature shift from 25° to 37°C [115, 116]. The quick reversibility of many inhibitors allows acute temporal control over the inhibition as well as investigation of the 'recovery phase.' Additionally, the apparent target specificity shown by inhibitors like nocodazole and latrunculin allows almost genetic knockout-like inactivation of individual components of the complex cytoskeletal network. Finally, the functional roles of actin and tubulin appear to be broadly conserved across the eukaryotes. Therefore, the study of different experimental systems is greatly benefited by reagents that are neither species- nor cell-type dependent.

More speculatively, perhaps the cytoskeleton has been well served with small molecule inhibitors simply because many of its components are "druggable." A remarkable number of small molecules have been identified that directly target microtubules [40]. In one unbiased screen for compounds that would induce mitotic arrest, 38% of the initial 'hits' proved to directly affect microtubule polymerization and/or stability [99, 117]. These results raise an important question: what makes tubulin such a good drug target? Further study of the structural basis for the binding and mechanism of action of these inhibitors should help shed light on this issue. In addition, the mitotic checkpoint may be sensitive to and amplify even subtle perturbations of the spindle.

Intriguingly, of the small molecule targets discussed here (actin, tubulin, Eg5, muscle myosin, nonmuscle myosin, N-WASP), all are proteins that undergo reversible conformational changes as part of their functional cycles. Indeed, several of their inhibitors (latrunculin, 187-1, wiskostatin) appear to act by blocking these conformational changes, suggesting that target "inhibitability" and conformational flexibility may be related [84, 112]. In this context, it is interesting to note the lack of

small molecules that target the third major cytoskeletal system, the 'intermediate' filaments. These structures are thought to play predominantly a more rigid, structural role, and their inherent stability, then, may be less amenable to disruption by small molecules.

The newly discovered inhibitors discussed above represent only the tip of the iceberg of molecules yet to be identified using high-throughput screening technology. The evolving technology coupling combinatorially synthesized compound libraries with cytoskeleton-oriented screens, whether pure protein, extract-, or cell-based, promises to rapidly deliver to chemical genetics the equivalent of "saturation mutagenesis:" all cytoskeletal targets screened versus a vast universe of small molecules. The greatest challenge to the chemical approach remains the issue of specificity, ensuring that the phenotype caused by a compound is indeed due the inhibition of only its supposed "target." Confirmation using independent approaches will help address this concern. Yet nature has provided us with remarkable examples of small molecules that appear to act on particular cytoskeletal proteins with exquisite specificity. This has been demonstrated directly by the fact that mutations conferring resistance to taxol and latrunculin can be identified in the tubulin and actin genes, respectively [118, 77]. These encouraging examples suggest that if we seek, we shall find.

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