

Identification of a Novel Protein Regulating Microtubule Stability through a Chemical Approach

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Summary

To identify novel proteins regulating the microtubule cytoskeleton, we screened a library of purine derivatives using mitotic spindle assembly in *Xenopus* egg extracts as an assay. Out of a collection of 1561 compounds, we identified 15 that destabilized microtubules without targeting tubulin directly, resulting in small spindles. Affinity chromatography with one compound, named diminutol, revealed a potential target as NQO1, an NADP-dependent oxidoreductase. A role for NQO1 in influencing microtubule polymerization was confirmed through inhibition studies using known inhibitors and immunodepletion. Therefore, this chemical approach has identified a novel factor required for microtubule morphogenesis and cell division.

Introduction

The goal of modern cell biology is to understand complicated cellular processes at a molecular level. One vital process is cell division, which requires the mitotic spindle, a structure composed of dynamic tubulin polymers (microtubules) and many associated proteins that accurately segregate chromosomes to two daughter cells. Small molecules can be extremely valuable tools to probe and modulate such complex cellular events. Recent advances in phenotypic screening methods have enabled large, diverse collections of natural products, combinatorial libraries, and historically archived syn-

thetics to be screened in order to find specific inhibitors of both known and novel proteins, including kinases and molecular motors [1]. These small molecules can modulate cellular functions by inhibiting or inducing protein-protein interactions, disrupting signaling cascades, or blocking specific enzymatic reactions.

Typically, one of two screening approaches is employed to identify useful small molecules. Target-based screens are often performed, particularly in the pharmaceutical industry, with the goal of identifying inhibitors of known proteins involved in human disease. These screens are usually performed in vitro and test for direct binding between members of a compound library and the purified protein of interest. While many valuable drugs have emerged from this type of screening, the resulting inhibitors are limited to targeting previously identified, well-characterized proteins. An alternative approach is the use of phenotypic screening to identify inhibitors of specific cellular events. This approach is powerful, since it can be used to probe complex processes and can also be used as a tool to identify novel proteins.

In the current study, we have applied a chemical strategy to identify inhibitors of mitotic spindle assembly in *Xenopus* egg extracts, a powerful system that mimics many cellular processes in vitro. Affinity chromatography is then used to identify potential targets by derivatizing inhibitors with a linker group to facilitate coupling to a matrix support. Comparing the patterns of extract proteins bound to the active compound versus an inactive derivative allows identification of candidate target proteins, which can then be functionally characterized. This biochemical approach has been used previously to characterize inhibitors of cyclin-dependent kinases (CDKs), MAP kinases, GSK3 β , and tubulin, demonstrating that it can be used to identify diverse targets [e.g., 2–6].

Mitosis is initiated upon activation of cyclin-dependent kinase 1 (Cdk1), which directly phosphorylates several mitotic substrates and activates additional kinases, leading to a largely uncharacterized cascade of events resulting in the reorganization of microtubules into a bipolar spindle that mediates chromosome segregation. Small molecule inhibitors of spindle assembly could be valuable tools for identifying novel proteins involved in spindle or microtubule function, and the compounds could also prove useful as antiproliferative agents. Our screening efforts started with a combinatorial library of substituted purines, as these compounds could in theory bind a wide variety of nucleotide-dependent enzymes involved in mitotic spindle morphogenesis [7]. The screening library consisted of 1561 compounds, most of which were derived from a combinatorial library of 2,6,9-trisubstituted purines designed to display diverse side chains, and 128 purified compounds derived from kinase-directed medicinal chemistry efforts. From this library, we identified 15 compounds that inhibited spindle assembly in *Xenopus* egg extracts by shrinking microtubules without targeting tubulin directly. Affinity

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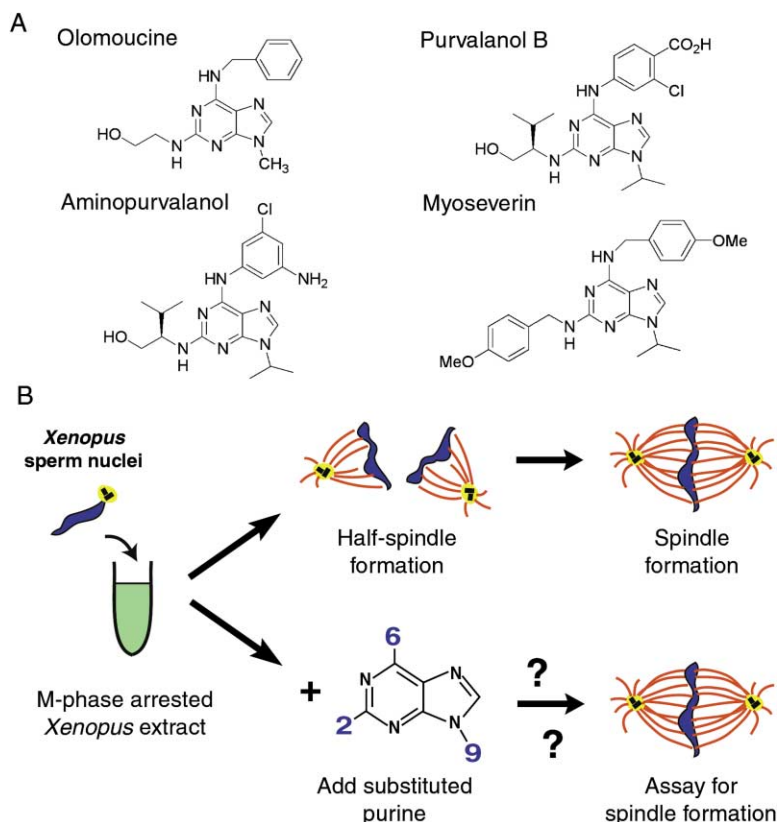


Figure 1. Screening of Purine Compounds in *Xenopus* Egg Extracts

(A) Various 2,6,9-trisubstituted purines. Olomoucine, purvalanol B, and aminopurvalanol are inhibitors of CDKs [2, 7]. Myoseverin has been shown to inhibit tubulin [3, 8].

(B) In vitro assay used for library screening. Addition of demembrated sperm nuclei to CSF-arrested *Xenopus* egg extract induces microtubule polymerization. By 30 min, half spindles can be visualized. By 45 min, bipolar spindles form [11]. Compounds were added to the spindle reactions at 100 μ M, and the reactions were visually screened by fluorescence microscopy to identify purine derivatives that interfere with normal spindle formation.

chromatography was successfully applied to identify a target of one of these inhibitors as NADP-dependent quinone oxidoreductase (NQO1). A role for NQO1 in influencing microtubule dynamics was confirmed through inhibition studies using known inhibitors and immunodepletion. Therefore, this approach provides an entry point for discovering and characterizing the roles of novel proteins required for microtubule morphogenesis and cell division.

Results

2,6,9-Trisubstituted Purines Are Potential Kinase Inhibitors

In order to identify novel mitotic inhibitors, we chose to screen a combinatorial library of 2,6,9-trisubstituted purine compounds. This strategy was based on the observation that a purine, olomoucine (Figure 1A), inhibits a family of CDKs with good selectivity (IC_{50} [50% kinase inhibition] = 7 μ M) [7]. The purine ring mimics the purine ring of ATP, and the three substituents make specific interactions with the nucleotide binding pocket. Interestingly, olomoucine was shown to bind to kinases in a novel way, with the purine ring rotated 160° compared to the purine ring of ATP. Modification of the three substituents led to a greatly improved Cdk2 inhibitor, purvalanol B (IC_{50} = 6 nM) (Figure 1A) [7]. Therefore, this study and others demonstrated that purines could be potent and selective inhibitors of kinases [2, 7, 8].

Using these compounds as a model, two libraries of 2,6,9-trisubstituted purines were synthesized. The first

group consisted of 128 compounds, each individually purified following synthesis (compounds labeled as NG or YC). In addition, a combinatorial library of 1433 compounds was synthesized (NSG001 library; described in [9] and in the Supplemental Data online at <http://www.chembiol.com/cgi/content/full/11/1/135/DC1/>). A few compounds in these libraries have been previously shown to inhibit other CDKs, including Cdk1 and Cdk2 [7, 10]. However, many compounds did not inhibit CDKs in vitro, and we therefore chose to screen both libraries to identify inhibitors of novel kinases or other factors involved in spindle or microtubule function.

Phenotypic Screen to Identify Inhibitors of Spindle Assembly

To identify inhibitors of spindle formation, the purine libraries were screened using an in vitro spindle assembly assay (Figure 1B). Reactions consisting of metaphase-arrested *Xenopus* egg extracts and sperm nuclei were incubated with individual purine compounds at a final concentration of 100 μ M. An equal amount of the compound solvent DMSO was used in control reactions, and in all cases X-rhodamine-labeled tubulin was added to allow visualization of microtubules by fluorescence microscopy. After a 45 min incubation at 20°C to allow microtubule polymerization and organization around sperm chromosomes, samples of each reaction were squashed between a slide and coverslip and visually screened to identify compounds that caused defects in spindle morphology compared to the control. This in vitro assay has been used to study the roles of many

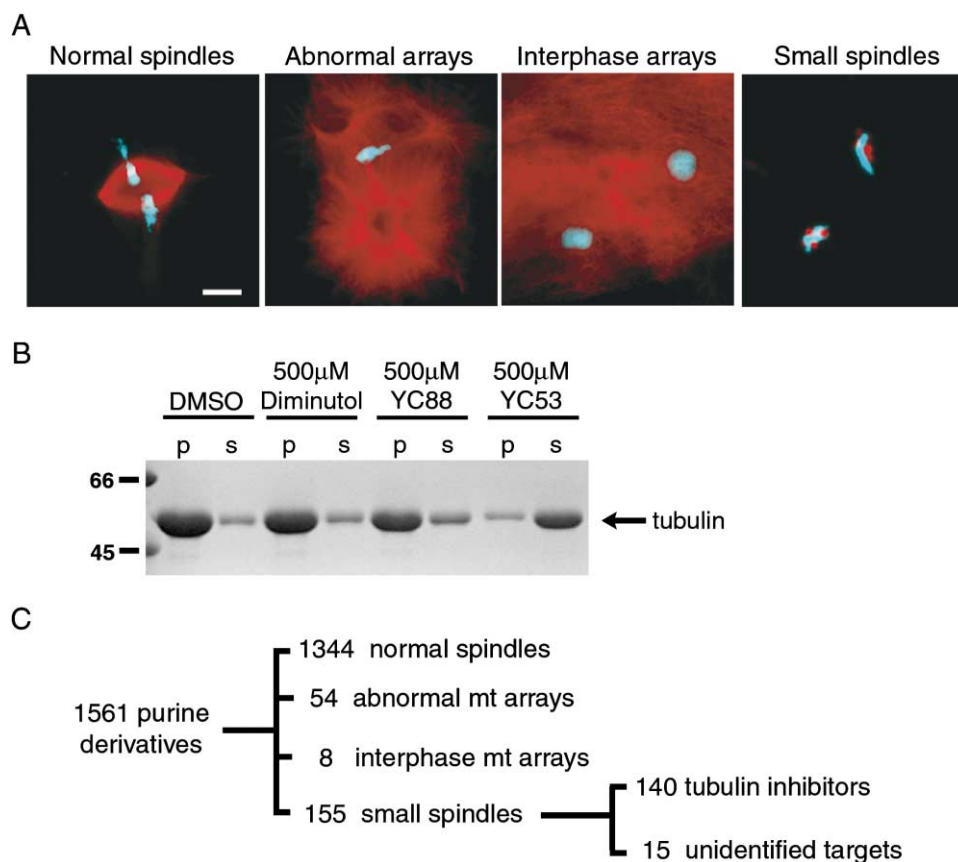


Figure 2. Summary of Purine Library Screen

(A) Examples of the four categories of microtubule structures observed: normal spindles, abnormal microtubule arrays, interphase microtubule arrays, and small spindles. In all fluorescence images, microtubules appear red due to incorporation of X-rhodamine-labeled tubulin, and chromosomes are stained blue with Hoechst 33258 (bar = 10 μ m).

(B) Tubulin pelleting assay. Microtubules were assembled *in vitro* by combining purified bovine brain tubulin with 5% DMSO and incubating at 37°C. Polymerized microtubules were then pelleted through a sucrose cushion and samples of the supernatant and pellet were run on a 10% gel and Coomassie stained. To determine if individual purine compounds inhibit microtubule polymerization, compounds were added to these reactions at 500 μ M. Shown are examples of compounds, such as diminutol and YC88, which had no effect. In contrast, YC53 depolymerized microtubules, shifting the tubulin to the supernatant fraction.

(C) Summary of the purine library screen. 1561 compounds were screened and grouped into the categories shown in (A).

proteins implicated in spindle assembly and mitosis [11] and is ideal for compound screening since it is fast and simple [12]. Although it does not fully mimic *in vivo* spindle assembly since the chromosomes do not replicate, the same essential proteins for spindle assembly are utilized. The assay protocol is schematized in Figure 1B.

Phenotypes Visualized in Purine Library Screen

A variety of microtubule structures formed in the presence of different purine compounds. These phenotypes were divided into four general classes, examples of which are shown in Figure 2A: normal spindles, abnormal microtubule structures, interphase microtubule arrays, and small spindles. A summary of this screen is shown in Figure 2C.

Eight compounds resulted in the formation of interphase microtubule arrays and decondensed chromosomes, indicating that the extract had exited mitosis. One of these compounds, NG97, (also called aminopur-

valanol, Figure 1A) was previously characterized in *Xenopus* egg extracts and was shown to inhibit the master mitotic kinase Cdk1 [2]. To determine if the other seven purine compounds also inhibited Cdk1, histone H1 kinase assays were performed on extracts treated with each compound. Each of the compounds lowered histone H1 kinase activity to interphase levels (data not shown), suggesting that this class of compounds targets Cdk1.

Another class of 54 compounds caused defects in microtubule organization, resulting in abnormal microtubule arrays. The most common structures observed in this category were large astral microtubule arrays. To determine if these compounds altered the cell cycle state of the extract, histone H1 kinase assays were performed for the most potent compounds. Interestingly, the extracts with astral microtubule arrays also had lowered histone H1 kinase activity, but the levels were intermediate between mitotic and interphasic extract (data not shown). Since microtubules are more stable in in-

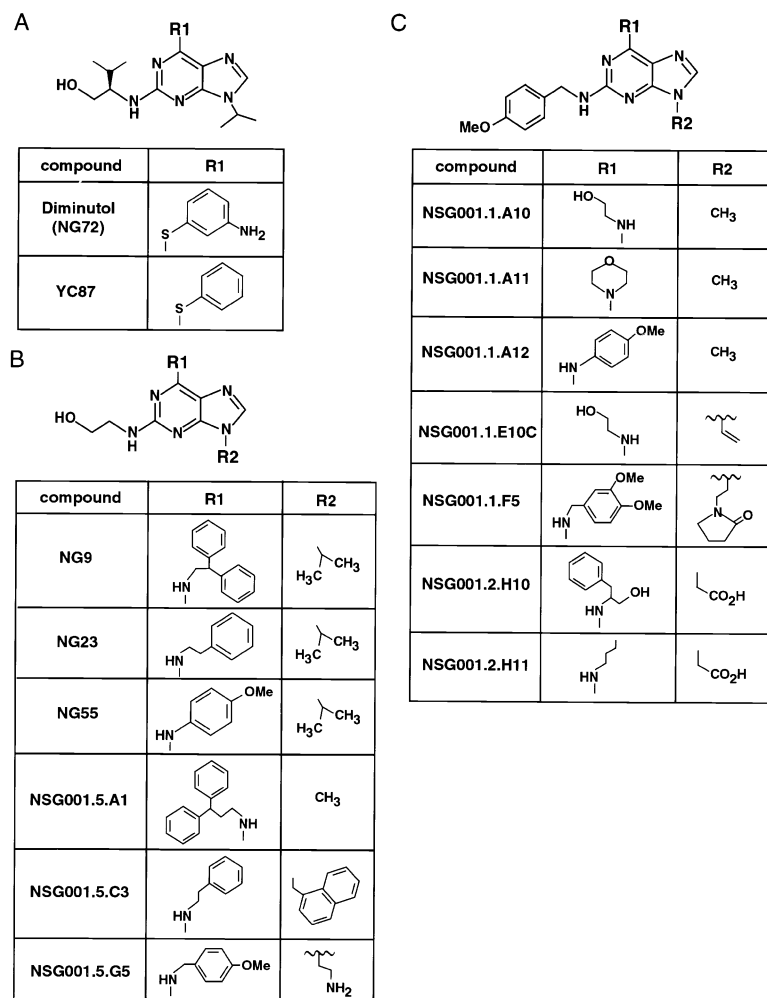


Figure 3. Three Structural Classes of Small Spindle Inhibitors Identified in Library Screen (A) Class 1: Two closely related compounds, diminutol and YC87. (B) Class 2: This cluster consists of six compounds that share a C2-hydroxyethylamino scaffold. (C) Class 3: These seven purines share a C2-4-methoxybenzylamino side chain.

terphase, this phenotype is likely to arise at least in part from incomplete inactivation of Cdk1, resulting in microtubule stabilization and ectopic aster formation. Since we were interested in identifying inhibitors of novel proteins involved in microtubule morphogenesis, we did not characterize these potential weak Cdk1 inhibitors further.

Finally, a class of 155 compounds caused microtubule depolymerization, shrinking microtubules and resulting in a small spindle phenotype. To determine if this phenotype was due to a direct effect on tubulin polymerization, each of the 155 compounds was incubated with pure tubulin, and microtubule assembly was monitored using a quantitative pelleting assay (Figure 2B). Most of the compounds tested (140/155) inhibited pure tubulin polymerization to some extent. Many of these tubulin inhibitors are structurally similar to compound 210 (also called mayoseverin, Figure 1A), a member of the purine library that has been shown to inhibit microtubule polymerization in vitro and in vivo [3,8]. However, 15 compounds did not affect microtubule polymerization directly, thereby potentially targeting extract proteins involved in regulating microtubule dynamics. These 15 compounds fall into three structural classes, based largely on the substituent found at the C2 position of the purine ring. Class 1

consists of two closely related compounds, which differ only slightly on the C6 substituent (Figure 3A). Class 2 consists of six compounds that all have a C2-hydroxyethylamino group (Figure 3B). Finally, the remaining seven compounds share a C2-4-methoxybenzylamino side chain (Figure 3C).

Characterization of Inhibitors in *Xenopus* Egg Extract

Next, we wanted to determine if these inhibitors targeted proteins that regulate microtubules throughout the cell cycle or whether they might be targeting mitotic-specific proteins. Therefore, the extract was cycled into interphase by adding calcium to trigger inactivation of Cdk1, and then the compounds were added at 100 μ M to this interphasic extract. All 15 compounds depolymerized microtubules in interphase, suggesting that they target proteins that act throughout the cell cycle (data not shown). In addition, we wanted to determine if the inhibitors target proteins specifically required for spindle formation around chromosomes. Therefore, microtubule asters were assembled in the absence of chromatin by adding the microtubule-stabilizing agent DMSO in the presence of various purine compounds. Each of the 15 compounds depolymerized microtubules in this

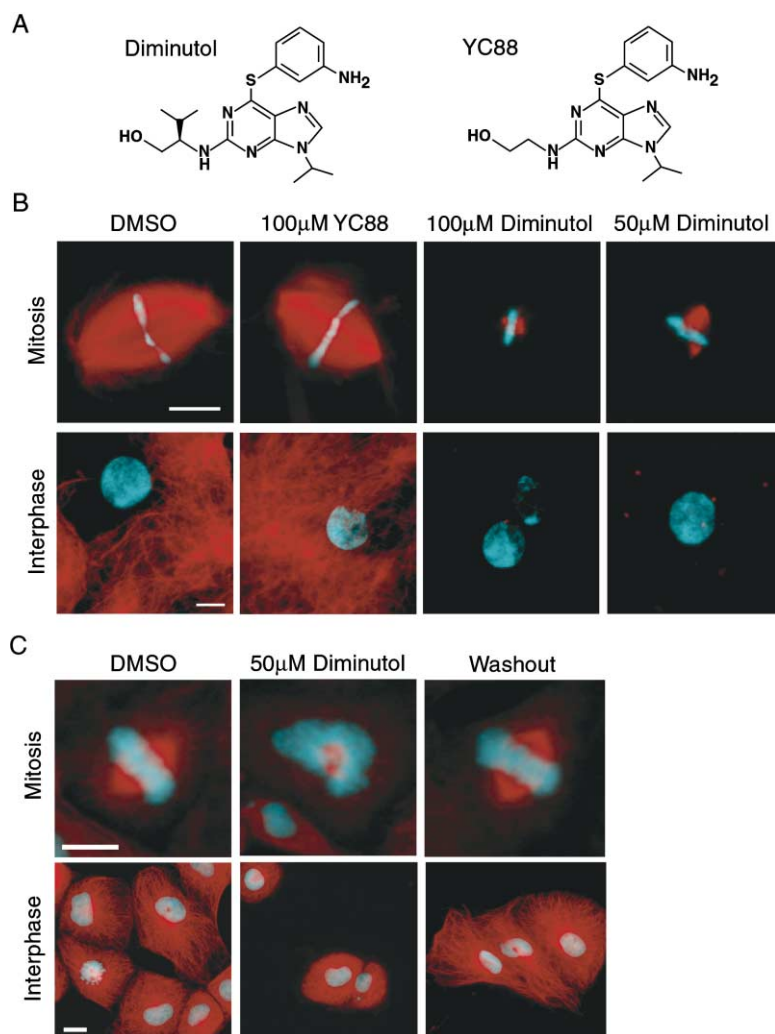


Figure 4. Diminutol Depolymerizes Microtubules in *Xenopus* Extracts and Cells

(A) Structure of diminutol and a control compound, YC88.

(B) Microtubule structures formed in mitotic or interphasic *Xenopus* egg extracts in the presence of control compound (YC88) or various concentrations of diminutol. As an additional control, an equivalent volume of the solvent DMSO was added (bar = 10 μ m).

(C) *Xenopus* XL177 cells treated with either 50 μ M diminutol or an equivalent volume of the solvent DMSO as a control. Diminutol depolymerizes microtubules in interphase and prevents normal spindle formation in mitosis. These effects are reversible, and normal interphase microtubule arrays and bipolar spindles reform 3 hr after diminutol was washed out of the cell culture media (Washout). Microtubules are shown in red and DNA in blue (bar = 10 μ m).

assay (data not shown), suggesting that they target proteins involved in the global regulation of microtubule dynamics.

Diminutol Inhibits Microtubule Polymerization in *Xenopus* Egg Extracts

We next characterized one of the most potent spindle inhibitors, NG72, in detail. First, both the enantiomers were prepared and tested in *Xenopus* egg extracts and in the tubulin pelleting assay. Although both compounds retained activity, the (R)-enantiomer was more potent, and we therefore named it diminutol since it causes microtubules to be diminished (Figure 4A). In both interphase and mitosis, addition of 100 μ M diminutol caused a dramatic reduction in microtubule polymerization; 50 μ M was sufficient to cause significant depolymerization (Figure 4B), and slight effects on microtubules were seen at concentrations as low as 10 μ M (data not shown). In order to determine what structural features of diminutol were important for activity, two focused libraries were prepared. Each library consisted of 96 diverse substituents introduced at either the C2 or C6 position of the diminutol scaffold (Figure S1). The C2 library was constructed by capturing a 2-fluoro-6-

thiophenyl-9-isopropylpurine scaffold onto resins containing 96-diverse amines. The desired C2 diverse library was then obtained after cleavage of the acid labile linker. The C6 library was constructed by nucleophilic displacement at C6 of a 2-(2-hydroxyethylamino)-6-(benzenesulfonyl)-9-isopropyl purine immobilized to a dihydropyran linker through the primary alcohol. Screening the C6 library at a concentration of 100 μ M in *Xenopus* extracts revealed that only compounds containing a C6-thioether retained the short microtubule phenotype (Figure S1A). Several differently substituted thiophenyl substituents were tolerated at C6, indicating that the 3-amino functionality of diminutol is not necessary for activity. Replacement of the C6 thioether with an oxygen or oxidation to the sulfone also resulted in a loss of activity. Several compounds from the C6 library appear to operate by a different mechanism and resulted in aster formation or conversion to an interphasic morphology characteristic of compounds that inhibit cyclin-dependent kinase activity. Screening the C2-focused library resulted in the identification of eight active compounds with different replacements to the R-valinol side chain of diminutol (Figure S1B). Comparison between the active and inactive members of the C2 library did not lead to

any clear conclusions about functionality required at the C2 position. As the active compounds were only characterized in the spindle assembly assay, it remains to be determined if their mechanism of action is similar to that of diminutol. In addition to compounds that retained inhibitory activity, we also identified compounds that were structurally similar to diminutol that no longer destabilized microtubules in *Xenopus* egg extracts. One of these derivatives, YC88, was used throughout this study as a control compound (Figure 4A).

Diminutol Reversibly Inhibits Microtubule Polymerization in Somatic Cells

To determine if diminutol is active in cells, it was added to *Xenopus* XL177 cells at a concentration of 50 μ M. Compound treatment caused microtubules to shorten in interphase and prevented proper spindle formation in mitosis (Figure 4C). Upon prolonged incubation, many diminutol-treated cells became multinucleate, indicating chromosome segregation defects. To determine if these effects were reversible, cells treated with diminutol for 18 hr were transferred to media lacking the drug. After 3 hr in fresh media, the cells regained normal microtubule arrays in interphase and were able to form bipolar spindles in mitosis (Figure 4C). This result suggests that diminutol does not permanently inhibit the ability of tubulin to polymerize. Diminutol caused similar microtubule and spindle defects in HeLa cells, demonstrating that these effects are not species specific. However, the compound was slightly more potent in *Xenopus* cells.

To determine if the effect of diminutol was specific to the microtubule cytoskeleton, various other assays were performed in *Xenopus* egg extracts. Diminutol (100 μ M) did not affect histone H1 kinase activity, incorporation of radioactively labeled dCTP into DNA, or the pelleting of F-actin, demonstrating that diminutol does not inhibit Cdk1 activity, DNA replication, or actin polymerization, respectively (data not shown). In addition, diminutol addition had no effect on chromosome or nuclear morphology (Figure 4B). These results suggest that the compound is not a general inhibitor of cellular processes but, instead, seems to have a more specific effect on the microtubule cytoskeleton. Since we had previously demonstrated that diminutol did not inhibit tubulin polymerization directly (Figure 2B), we next sought to identify its functional target.

Potential Diminutol Targets Identified through Affinity Chromatography

To identify the protein targets of diminutol, affinity matrices were constructed using the compound or one of the inactive derivatives coupled to agarose beads (Figure 5A). Diminutol was coupled through the C6-thiophenyl substituent to mimic the immobilization strategy that was successful for identifying the cellular targets of the structurally similar purine Cdk1,2,5 inhibitor purvalanol [2, 4, 5]. The linker-modified version of diminutol retained activity in *Xenopus* egg extracts, suggesting that the linker did not prevent association of diminutol with its protein target. The matrices were incubated in extracts, retrieved, and spindle assembly reactions set up using the depleted extracts. Extracts depleted with the dimi-

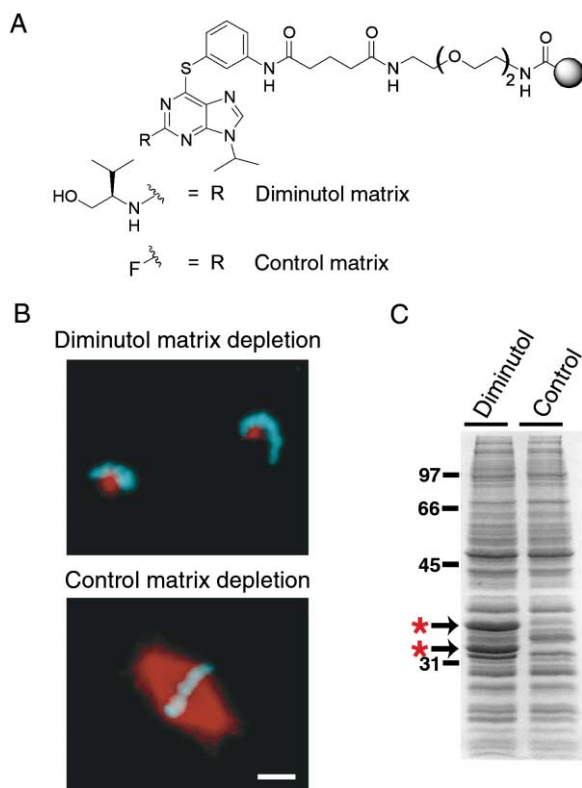


Figure 5. Affinity Chromatography to Identify Targets of Diminutol (A) Structures of the diminutol affinity matrix and a control matrix. (B) Spindles assembled in *Xenopus* extracts depleted with either the diminutol matrix or the control matrix. After removal of the diminutol matrix from extracts, microtubules were much shorter, suggesting that the matrix depleted a protein involved in regulating microtubules (bar = 10 μ m). (C) Coomassie-stained 15% SDS-PAGE gel showing proteins eluted from the diminutol matrix, compared to the control. Two proteins, marked with the red stars, are specifically enriched on the diminutol matrix. These bands are at 32 and 35 kDa. Other variations in band intensity were not reproducible.

nutol matrix had defects in microtubule formation and spindle assembly, whereas control-matrix-treated extracts were normal (Figure 5B). This result suggested that one or more proteins binding to diminutol were affecting microtubule polymerization. To identify these potential targets, beads retrieved from the extracts were washed under mild detergent conditions, and the bound proteins were eluted with sample buffer and analyzed by SDS-PAGE. Two major bands at approximately 32 and 35 kDa were found to be reproducibly enriched on the diminutol matrix (Figure 5C). Addition of excess free diminutol to the extract eliminated binding of both of these proteins to the diminutol matrix (data not shown), demonstrating that the interactions were specific and saturable. For protein identification, the bands were digested in-gel by trypsin overnight and the extracted peptides were analyzed by matrix-assisted laser desorption ionization time of flight mass spectrometry (MALDI-TOF MS). The most abundant peptides were then chosen for de novo sequencing. The sequence information obtained from this analysis was used in BLAST searches, which revealed the proteins to be two

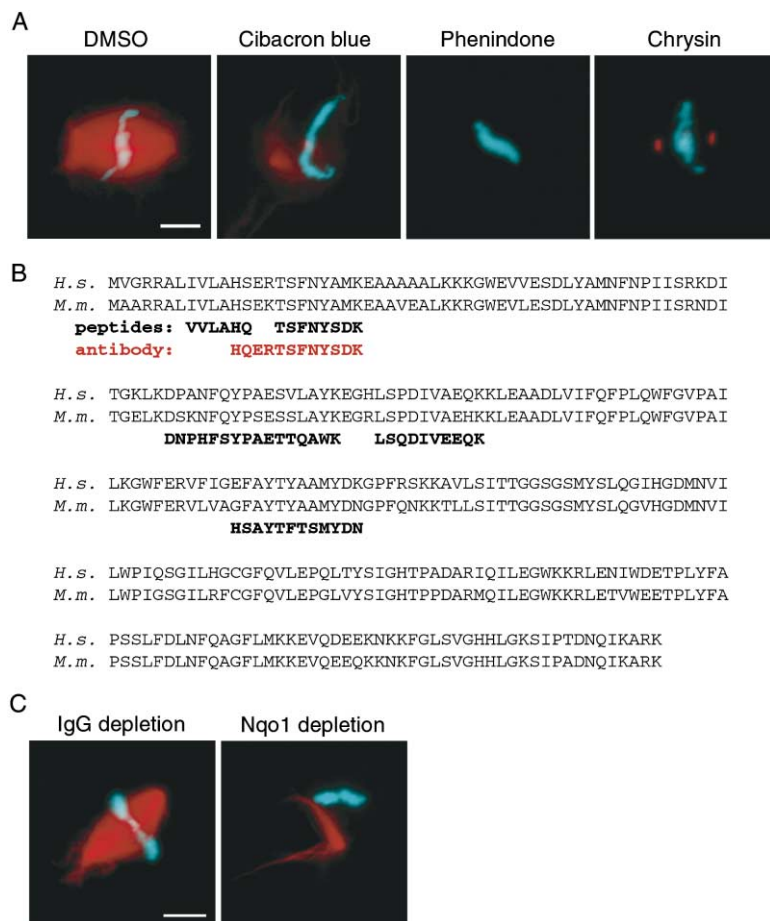


Figure 6. Inhibition of NQO1 Causes Microtubule Depolymerization

(A) Addition of the NQO1 inhibitors cibacron blue, phenindone, and chrysin depolymerize microtubules in *Xenopus* egg extracts. Each inhibitor was added to extract containing sperm nuclei to stimulate microtubule polymerization at 200 μ M, and the reactions were fixed at 60 min. As a control, an equivalent volume of the solvent DMSO was added (bar = 10 μ m).

(B) The amino acid sequences of human (*H. sapiens*) and mouse (*M. musculus*) NQO1. Aligned underneath are the *Xenopus laevis* peptide sequences identified in the mass spectrometry analysis, and the peptide sequence used to generate the NQO1 antibody (shown in red).

(C) Structures assembled in extracts depleted with control IgG or peptide antibodies raised against *Xenopus* NQO1. Microtubules are much sparser in NQO1-depleted extracts, suggesting that NQO1 is a regulator of microtubule stability (bar = 10 μ m).

NADP binding proteins, NADP-dependent quinone oxidoreductase (NQO1) (32 kDa band) and Leukotriene B4-12-hydroxydehydrogenase (35 kDa band).

To determine whether the proteins identified biochemically were indeed functional targets of diminutol, a separate approach was used to independently inhibit their functions. Addition of antibodies against Leukotriene B4-12-hydroxydehydrogenase or the purified recombinant protein to *Xenopus* egg extracts did not reveal any effect on microtubule dynamics. Though we cannot completely rule out a connection between Leukotriene B4-12-hydroxydehydrogenase and microtubules based on these data, we chose to focus on characterizing NQO1, a quinone oxidoreductase involved in redox regulation.

NQO1 Regulates Microtubule Polymerization

Diminutol was determined to be a competitive inhibitor of purified human NQO1 with respect to NAD(P)H, with an K_i of 1.72 μ M (S. Chen and K. Wu, personal communication). To determine if NQO1 inhibition was responsible for the effect of diminutol on the microtubule cytoskeleton, other methods were used to inhibit NQO1 activity in *Xenopus* egg extracts, and the effects on the microtubule cytoskeleton and spindle assembly were evaluated. The first approach was to test several compounds that have been previously shown to inhibit the activity of NQO1 in vitro. These include cibacron blue, chrysin,

and phenindone, which are competitive inhibitors with respect to NAD(P)H [13]. When added at various concentrations to *Xenopus* egg extract containing sperm nuclei to stimulate spindle formation, these inhibitors caused microtubule depolymerization, largely mimicking the phenotype induced by diminutol treatment (Figure 6A).

One caveat to the use of chemical inhibitors is that they may be binding to multiple cellular factors, making their specificity for NQO1 unclear. Therefore, we also used an immunodepletion approach to inhibit NQO1. Peptide antibodies were raised against *Xenopus* NQO1 using sequences obtained from mass spectrometry analysis (Figure 6B). The peptide chosen was well conserved in the human and mouse sequences, and the antibody recognized the 32 kDa band enriched on the diminutol matrix by immunoblot (data not shown). Although the antibody also crossreacted with a few other extract proteins, NQO1 was the only protein it recognized that was enriched on the diminutol matrix. Using this antibody, NQO1 was immunodepleted from *Xenopus* egg extracts. Microtubules were much sparser in the depleted extracts, and there were defects in spindle assembly consistent with the inhibitor phenotypes (Figure 6C). However, addition of recombinant human NQO1 did not rescue the depletion phenotype (data not shown). Although this failure to rescue might be explained by differences between the *Xenopus* and human proteins, we cannot be certain that NQO1 was the only

protein affecting microtubule stability depleted in this experiment until an add-back can be performed with *Xenopus* NQO1. In conclusion, inhibiting NQO1 function with chemical inhibitors or by immunodepletion mimics the effect of diminutol on microtubules, suggesting that NQO1 is a functional target of diminutol. However, it is possible that inhibition of other proteins could contribute to the phenotypes observed.

Diminutol Depolymerizes Microtubules in High-Speed Extracts

Although NQO1 is a cytoplasmic protein, it is related to a family of mitochondrial oxidoreductases. These proteins are involved in reducing quinones in the respiratory chain and are important for proper cellular metabolism. Therefore, it is possible that diminutol inhibits these oxidoreductases in addition to NQO1 and that the phenotype of diminutol addition could be caused by disrupted mitochondrial function. In order to test this hypothesis, we assembled microtubule structures in high-speed *Xenopus* egg extract, which was centrifuged at $200,000 \times g$ for 2.5 hr to remove mitochondria. After addition of the microtubule-stabilizing agent DMSO, microtubules polymerize and are then organized into an aster by molecular motors (Figure 7A). Addition of diminutol to this reaction caused microtubule depolymerization, just as it had in low-speed extracts. Although we cannot rule out the possibility that residual mitochondria are present in the high-speed extract, it is likely that the effect of diminutol on the microtubule cytoskeleton is not related to mitochondrial function.

Diminutol Does Not Cause Oxidative Stress in *Xenopus* Egg Extract

We next sought to address the mechanism by which NQO1 affects microtubule polymerization. NQO1 catalyzes the two-electron reduction of quinones to hydroquinones using NAD(P)H as an electron donor. This reaction prevents other enzymes from catalyzing a one-electron reduction, which can result in the production of free radicals. In vivo, reactive quinones can alter cellular protein function by catalyzing the oxidation of sulfhydryls to disulfides, depleting intracellular thiols. Therefore, NQO1 is important to maintain redox balance and prevent oxidative stress [14]. Various studies have shown that microtubules are depolymerized in cells that are undergoing oxidative stress [15–17]. In agreement with these reports, induction of oxidative stress in *Xenopus* egg extracts by addition of 1 mM menadione caused microtubule depolymerization (data not shown). At this concentration, menadione also altered chromosomal morphology, suggesting that oxidative stress has pleiotropic effects on the extract.

To determine if the effect of NQO1 inhibition on microtubules is the result of sending the extract into an oxidatively stressed state, analogous to menadione addition, the abundance of free radicals in the extract was monitored using a chemical probe. Proxyl fluorescamine is a compound that increases in fluorescence in the presence of superoxide or hydroxyl radicals [18]. This probe was added to *Xenopus* egg extract at $50 \mu\text{M}$, and its fluorescence intensity was measured at regular intervals

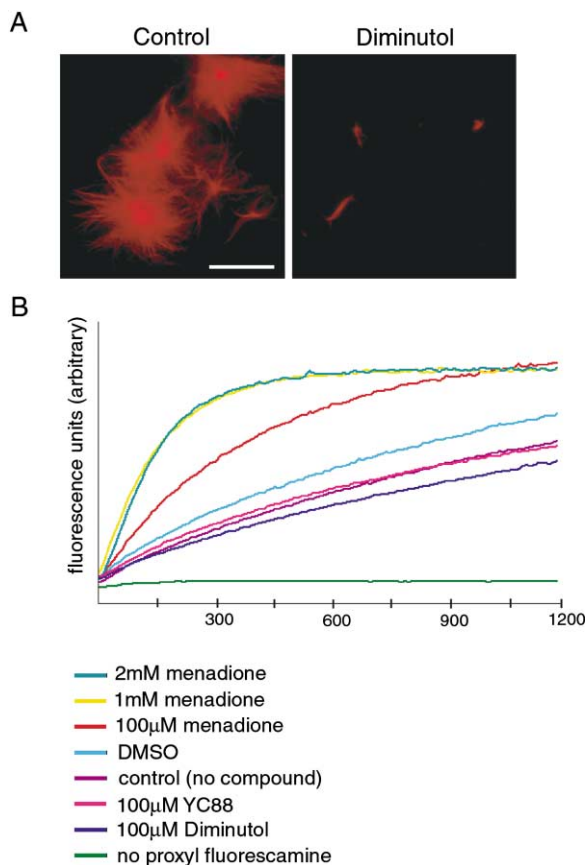


Figure 7. Diminutol Does Not Cause an Increase in Free Radical Production

(A) Microtubule asters assembled in high-speed extracts by addition of 5% DMSO. Microtubules are stained red due to incorporation of X-rhodamine-labeled tubulin. Addition of diminutol causes microtubule depolymerization, demonstrating that the compound retains activity in the absence of mitochondrial oxidoreductases (bar = $10 \mu\text{m}$).

(B) Proxyl fluorescamine increases in fluorescence in the presence of superoxide or hydroxyl radicals. Addition of this fluorophore to control *Xenopus* extracts (purple line) results in a slow, steady increase in fluorescence intensity. Menadione, a compound that generates free radicals, causes an increase in free radical production (red, yellow, and teal lines). In contrast, YC88 (pink) and diminutol (navy blue) resemble the control curve, demonstrating that diminutol does not cause oxidative stress in *Xenopus* egg extracts over a 20 min timecourse.

over a 20 min period at 20°C , an incubation period adequate to observe robust effects of diminutol on microtubule polymerization. In control extract, the fluorescence intensity increased steadily at a slow rate (Figure 7B). Addition of 1 mM menadione caused a marked increase in the fluorescence intensity, consistent with the fact that it is known to cause an increase in superoxide radicals. In contrast, addition of diminutol, YC88, or the other chemical inhibitors of NQO1 (cibacron blue, chrysin, phenindone) did not increase the fluorescence intensity above that of the control extract (Figure 7B and data not shown). Therefore, diminutol addition does not result in an increase in free radical production that is detectable by this assay during the time period in

which microtubules are depolymerized. Interestingly, addition of 100 μ M menadione increased the level of free radical production (Figure 7B), even though this concentration did not cause microtubule depolymerization in the extract (data not shown). Therefore, the level of superoxide radicals can be increased moderately without affecting microtubule integrity, further indicating that the effect of diminutol on the microtubule cytoskeleton is unrelated to free radical production. This experiment suggests that the effect of NQO1 inhibition on microtubules is independent of oxidative stress. We propose that in addition to preventing oxidative stress, NQO1 function is required to maintain the integrity of the microtubule cytoskeleton.

Discussion

Phenotype-based screens are powerful tools to identify proteins involved in complex cellular processes. In classic model organism genetics, screens are performed to identify mutants in a process of interest and then the mutated genes are identified using well-established cloning techniques. In the analogous chemical approach, libraries of chemical compounds are screened to find inhibitors of a process, and the subsequent identification of the targets of these compounds can lead to the discovery of novel proteins.

We have applied a biochemical method to identify the target of a compound that destabilizes microtubules in *Xenopus* egg extracts. By comparing the proteins that bound to an active and inactive version of the inhibitor, we identified two candidate target proteins. Subsequent characterization of these potential targets led to the discovery of a novel regulator of microtubule morphogenesis, NQO1.

2,6,9-Trisubstituted Purines Can Inhibit NADP Binding Proteins

The identification of NQO1 as a target of diminutol was initially a surprise. Previously, various 2,6,9-trisubstituted purines had been characterized as cyclin-dependent kinase (CDK) inhibitors [7], since the purine ring mimics the purine ring of ATP. Therefore, we expected to find inhibitors of other kinases involved in microtubule function. Consistent with previous studies, we identified CDK inhibitors in the purine libraries; eight compounds inhibited Cdk1, releasing cytosolic factor (CSF)-arrested extracts from their mitotic arrest. In addition, another class of compounds inhibited histone H1 kinase activity to some extent, suggesting that they could be less potent inhibitors of Cdk1. However, biochemical analysis of diminutol revealed that the two proteins enriched on the compound matrix were not kinases but, instead, NADPH binding proteins. We subsequently demonstrated that diminutol was a competitive inhibitor of human NQO1 with respect to NADPH, suggesting that the compound interacts with the NADP binding site. Therefore, in addition to kinases, 2,6,9-trisubstituted purines can inhibit other classes of nucleotide binding proteins.

NQO1 and Microtubule Function

NQO1 is a 31 kDa cytoplasmic oxidoreductase that uses NAD(P)H as an electron donor to catalyze the two-electron reduction of quinone compounds. One *in vivo* role for NQO1 is to prevent oxidative stress by blocking the conversion of oxidized quinones into free radicals [14]. In this study, we have shown that another role for NQO1 is to maintain the integrity of the microtubule cytoskeleton. The mechanism by which NQO1 exerts its effects on microtubules is unknown, although previous studies suggested several possibilities. First, NQO1 inhibition could cause the extract to accumulate free radicals and enter an oxidatively stressed state, resulting in microtubule depolymerization. We have shown that diminutol does not cause an accumulation of free radicals over the timecourse of our experiments, thereby ruling out oxidative stress as the cause of microtubule depolymerization (Figure 7B). In agreement with this result, we have also shown that diminutol addition does not affect nuclear morphology or actin polymerization (Figure 4B and data not shown), which are both sensitive to oxidative stress [15]. Although we cannot rule out the possibility that there may be a slight increase in free radicals, undetectable in our assay, it is unlikely that such a small increase could cause the dramatic effect on the microtubule cytoskeleton that we observe with diminutol addition. This result suggests that the connection between NQO1 and microtubules may be more direct.

Another explanation for the effect of NQO1 inhibition on microtubules could be that NQO1 is required to reduce one or more specific quinones important to maintain the cytoskeleton. Such quinones could function directly or as cofactor for a required enzyme. Alternatively, it is possible that reduction of a specific quinone (or multiple quinones) is required to prevent chemical toxicity. For example, if a particular quinone was capable of causing microtubule depolymerization in its oxidized form, reduction of the compound by NQO1 might be required to protect the microtubules from destabilization.

Lastly, the effect of NQO1 on microtubules could be direct. NQO1 could physically associate with microtubules or with regulatory proteins to modulate microtubule function. For example, it is possible that tubulin could be a direct substrate of NQO1. It has been shown in a number of studies that tubulin contains disulfide bonds and that altering the number of bonds with reducing or oxidizing agents affects the ability of tubulin to polymerize *in vitro* [19, 20]. One recent study demonstrated that oxidation of tubulin sulfhydryls inhibits tubulin polymerization [20]. If this observation is correct, NQO1 could be required to reduce disulfides so that tubulin is able to polymerize. However, Chaudhuri and colleagues reduced tubulin disulfides using DTT and found that reduction inhibited microtubule assembly, contradicting the other study [19]. Therefore, further work will be needed to test this model. Measuring the disulfide bond content of tubulin retrieved from extracts in the presence and absence of diminutol could shed light on this controversy.

In support of the hypothesis that NQO1 directly affects tubulin polymerization, our preliminary experiments indicate that NQO1 binds to pure tubulin *in vitro* (data not

shown). However, we were not able to detect a biochemical interaction between NQO1 and microtubules in *Xenopus* egg extracts, and therefore further investigation will be required to reveal the connection between NQO1 function and microtubule regulation.

Antimitotic Drugs as Research Tools

Small molecules have proven to be very useful in the study of mitosis. In fact, tubulin itself was initially purified from cell extracts through its ability to bind to a radioactively tagged version of the antiproliferative drug colchicine [21, 22]. Like colchicine, the antimitotic drugs nocodazole, vinblastine, and taxol bind to tubulin directly. These drugs have allowed detailed analysis of microtubule dynamic properties in various organisms [23]. Recently, there has been much interest in identifying drugs targeting other components of the mitotic machinery. A high throughput screen in cells led to the identification of a specific drug targeting Eg5, a kinesin-like protein involved in spindle organization [24]. This inhibitor, monastrol, has been used to dissect the function of Eg5 both in vitro and in vivo [25]. In addition, a nonmuscle myosin inhibitor, blebbistatin, has been used to probe mechanisms of cytokinesis [26]. Diminutol does not target a protein functioning specifically in mitosis but identifies a pathway involved in general microtubule regulation different from the tubulin-specific drugs. As we have identified two other classes of inhibitors with similar effects (Figure 3), identification of the protein targets of these drugs will further elucidate microtubule regulation and could provide new antimitotic agents that can be used as research tools.

Significance

In this study, we have applied a chemical approach to the *Xenopus* egg extract system and have identified a novel regulator of microtubule dynamics, NQO1. Our approach is advantageous because it allows an unbiased screening approach to be applied to a powerful biochemical system. This methodology can now be applied to identify other regulators of microtubule dynamics and spindle assembly and could prove useful to generate new small molecule reagents. In the future, diminutol and the other compounds identified in this study can be used as research tools to elucidate novel pathways regulating microtubule function and cell division.

Experimental Procedures

Preparation of *Xenopus* Egg Extracts

Crude cytosolic factor (CSF) extracts were prepared from *Xenopus laevis* eggs arrested in metaphase of meiosis II as described [27, 28], except the crushing spin was performed at 10,200 rpm in an HB-4 rotor for 15 min. To prepare high-speed extracts, CSF extract was centrifuged an additional 150 min at 55,000 rpm in a TLS-55 rotor. Sperm nuclei and X-rhodamine-labeled tubulin were prepared as described [27, 29].

Xenopus Egg Extract Assays

For half-spindle reactions, CSF extracts were supplemented with demembrated sperm (500 nuclei/ μ l final) and with X-rhodamine-labeled tubulin (50 μ g/ml final) on ice, and transferred to 20°C. To observe interphasic microtubule arrays, CSF-extracts were driven

into interphase by adding 1/10 extract volume of CaCl₂ buffer (4 mM CaCl₂, 10 mM Hepes (pH 7.7), 150 mM sucrose, 100 mM KCl, and 1 mM MgCl₂). To assemble microtubule asters, DMSO was added to extracts to 5%.

To visualize reactions, 1 μ l samples were spotted onto microscope slides, overlaid with 5 μ l of spindle fix (48% glycerol, 11% formaldehyde in MMR [5 mM Hepes (pH 7.8), 2 mM KCl, 1 mM MgSO₄, 100 mM NaCl, 2 mM CaCl₂, and 0.1 mM EGTA], 5 μ g/ml Hoechst 33258) and squashed beneath a coverslip.

Phenotypic Screen

Compounds were screened in extract using half-spindle reactions [12]. A large mix of CSF-extract, sperm nuclei, and rhodamine tubulin was set up on ice, and then 25 μ l was aliquotted into tubes containing 0.25 μ l of individual purine compounds. The compounds were dissolved in DMSO at 10 mM, making the final concentration in extract 100 μ M. As a control, DMSO alone was added. Reactions were squashed at 45–60 min and the phenotypes visually evaluated. Interesting compounds were rescreened to confirm their phenotypes.

Histone-H1 Kinase Assay

At different cell cycle states, 1 μ l extract samples were diluted in 19 μ l EB (80 mM β -glycerophosphate [pH 7.3], 20 mM EGTA, 15 mM MgCl₂, 10 μ g/ml LPC, and 1 mM DTT) and frozen in liquid nitrogen. The samples were then thawed and incubated with recombinant histone H1 and [γ -³²P]-ATP in a filter binding kinase assay as described [30].

Pure Tubulin Pelleting Assay

Bovine brain tubulin was purified according to Ashford [31]. 25 μ M of tubulin in 1 \times BRB80 (80 mM K-PIPES, 1 mM MgCl₂, and 1 mM EGTA [pH 6.8]) + 1 mM GTP was polymerized with 5% DMSO at 37°C for 30 min. These reactions were layered onto a 40% sucrose/BRB80 cushion and the microtubules pelleted at 40,000 rpm for 20 min at 37°C in a TL-100 rotor. A 10 μ l supernatant sample was taken and added to 10 μ l sample buffer. Half of the cushion was then aspirated, the interface was washed, and the pellet resuspended in 20 μ l sample buffer. The samples were run on a 10% polyacrylamide gel and Coomassie stained. For testing, compounds were added at 100 μ M at the start of the polymerization reaction. Compounds that did not affect tubulin polymerization were retested at 500 μ M, yielding 15 compounds that had no effect at either concentration. Pelleting assays were also performed for diminutol using *Xenopus laevis* tubulin purified from egg extracts [32] to rule out species-specificity differences.

Cell Culture

XL177 cells were grown at 27°C on sterile coverslips in 12-well plates. For immunofluorescence, these coverslips were fixed in -20°C methanol for 10 min, rehydrated in PBS for 10 min, and blocked with PBS/3% BSA for 30 min. Immunofluorescence was then performed using mouse monoclonal antibodies against tubulin and rhodamine-conjugated secondary antibodies. DNA was stained with 1 μ g/ml Hoechst 33258 in PBS.

Biochemical Target Identification

Compound affinity matrices were prepared as described ([2] and Supplemental Data). To analyze proteins binding to matrices, 250 μ l packed matrix (500 μ l slurry) was washed four times in CSF-XB (10 mM HEPES [pH 7.7], 2 mM MgCl₂, 0.1 mM CaCl₂, 100 mM KCl, 5 mM EGTA, and 50 mM sucrose) plus protease inhibitors and resuspended in 2 ml CSF-extract. This mixture was rotated at 4°C for 4 hr, and the matrix was retrieved by spinning at 4000 rpm for 5 min at 4°C. The matrix was washed four times in CSF-XB and two times in TBS + 0.1% Triton X-100 and resuspended in 100 μ l sample buffer. The samples were run on a 15% gel and stained with Coomassie. Bands were cut out of the gel, proteins were digested in-gel by Trypsin overnight, and the extracted peptides were analyzed by matrix-assisted laser desorption/ionization time of flight mass spectrometry. The most abundant peptides were then chosen for de novo sequencing.

For matrix depletion, two aliquots of 125 μ l packed diminutol or

control matrix (250 μ l slurry) were washed three times in CSF-XB plus protease inhibitors, and then one aliquot was resuspended in 200 μ l CSF-extract. This mixture was rotated for 1 hr at 4°C, and the matrix was retrieved by spinning for 5 min at 4000 rpm. The depleted extract was then added to the second matrix aliquot, incubated at 4°C for another hour, and retrieved as before. The extract was spun an additional time to remove residual matrix, and half spindle reactions were set up using this depleted extract. The matrices were washed four times in CSF-XB, two times in TBS + 0.1% Triton X-100, and resuspended in 100 μ l sample buffer for SDS-PAGE analysis.

Antibody Production

Peptide antibodies against *Xenopus* NQO1 were made using sequence obtained in the mass spectrometry analysis: (C)HQERTSF NYSDK. Synthetic peptide was generated and antibody production was carried out by Zymed Laboratories (South San Francisco, CA). Antibodies were affinity purified as described [33].

Inhibitor Studies and Immunodepletion

100 mM stocks of cibacron blue, chrysin, and phenindone were made in DMSO and stored at -20°C. For each experiment, fresh working stocks were made by diluting the stock in DMSO and adding to extracts at 1:100.

Extracts were depleted of NQO1 in two rounds. For each, 15 μ g of the NQO1 peptide antibody or 15 μ g rabbit IgG (control) were added to 25 μ l Protein A Dynabeads (Dyna), the volume brought to 200 μ l with TBS + 0.1% Triton X-100, and rotated at 4°C overnight. Following coupling, beads were washed with CSF-XB + protease inhibitors [28]. Beads were then used to deplete 70 μ l CSF extract in two 1 hr rounds. During each, extract was kept on ice and mixed frequently.

Oxidative Stress Assays

Proxyl fluorescamine was added to 60 μ l CSF extract at 50 μ M and then immediately transferred to a quartz cuvette and placed in a spectrophotometer. The fluorescence was then measured every 10 s over 20 min at 20°C (excitation wavelength = 393 nm; emission wavelength = 482 nm).

Microscopy and Data Analysis

Images were collected using a Nikon E600 fluorescence microscope equipped with an Orca II CCD camera (Hamamatsu), Lambda 10-2 (Sutter) shutter controller and Metamorph software (Universal Imaging). Images were imported into Adobe Photoshop for processing.

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