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Microtubule Disassembly and Inhibition of Mitosis by a Novel Synthetic Pharmacophore

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Abstract Microtubule drugs, which block cell cycle progression through mitosis, have seen widespread use in cancer chemotherapies. Although microtubules are subject to regulation by signal transduction mechanisms, their pharmacological modulation has so far relied on compounds that bind to the tubulin subunit. A new microtubule pharmacophore, diphenyleneiodonium, causing disassembly of the microtubule cytoskeleton is described here. Although this synthetic compound does not affect the assembly state of purified microtubules, it profoundly suppresses microtubule assembly in vivo, causes paclitaxel-stabilized microtubules to cluster around the centrosomes, and selectively disassembles dynamic microtubules. Similar to other microtubule drugs, this new pharmacophore blocks mitotic spindle assembly and mitotic cell division. J. Cell. Biochem. 98: 102–114, 2006. © 2005 Wiley-Liss, Inc.

Key words: microtubule; centrosome; mitosis; cell cycle

Cell proliferation is under stringent cell cycle-dependent control of mitotic cell division. In particular, cell division is dependent on the precise segregation of chromatin and centrosomes during mitosis [Scholey et al., 2003]. This requires the assembly of a mitotic spindle, which is composed primarily of microtubule fibers. Following attachment of the chromosomes to these microtubule fibers, and their subsequent alignment in the equatorial plane between the spindle poles, the chromosomes are segregated by microtubule-dependent transport to the spindle poles. Disruption of the mitotic spindle therefore blocks mitotic cell division [Jordan and Wilson, 2004]. Assembly of a functional mitotic spindle can be affected by several mechanisms, including inhibition of centrosome disjunction [Mayer et al., 1999]. In particular, pharmacological modulation of microtubule assembly has proven highly effective in blocking mitosis [Jordan and Wilson, 2004]. Many anti-mitotic microtubule drugs bind to microtubule ends, thereby blocking incorporation of additional tubulin subunits [Jordan and Wilson, 2004].

While microtubules are the principal constituent of stable cellular components such as centrioles, flagella, and neurites, they are generally highly dynamic structures, constantly undergoing addition and loss of tubulin subunits [Howard and Hyman, 2003]. This is known as "dynamic instability," and is required for functioning of the mitotic spindle. Although a mitotic spindle can still form following stabilization of microtubules by taxanes, the loss of microtubule dynamic-instability, precludes mitotic progression of the cells beyond metaphase [Jordan and Wilson, 2004]. Indeed, even at very low microtubule drug concentrations, that do not significantly affect microtubule or spindle assembly, perturbation of microtubule dynamic instability can block the metaphase to anaphase transition required for normal exit from mitosis [Jordan and Wilson, 2004].

In addition to pharmacological modulation of their assembly dynamics by compounds that bind directly to tubulin, microtubules are also

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subject to signal transduction-dependent regulation [Watanabe et al., 2005; Wordeman, 2005]. While the signal transduction-mediated regulation of microtubule dynamics has yet to be fully characterized, profound modulation of microtubule assembly by pharmacological alteration of signaling mechanisms has recently been reported [Scaife et al., 2003b]. Here, a novel microtubule pharmacophore is described which similarly appears to act indirectly on microtubules, possible by affecting signal transduction mechanisms. This novel microtubule pharmacophore, previously described as a flavoprotein inhibitor [Hancock and Jones, 1987], causes rapid, pronounced, and reversible disassembly of the microtubule cytoskeleton. While its molecular mode of action remains undetermined, this compound, which selectively disassembles dynamic microtubules, also affects the organization, or intra-cellular distribution, of paclitaxel-stabilized microtubules.

Importantly, in light of its microtubule disassembling activity, this novel pharmacophore blocks mitotic cells in pro-metaphase. This compound therefore inhibits mitotic cell division in a manner that is similar to the action of other microtubule drugs.

MATERIALS AND METHODS

Cell Culture and Transfection

Rat1 fibroblasts were obtained from ATCC and cultured in DMEM (Gibco/BRL, Mount Waverley, VIC, Australia) containing 10% FCS (Gibco/BRL) and 2 mM L-glutamine (Gibco/BRL) at 37°C and 5% CO2. Cells were transiently transfected with Qiagen (Qiagen Pty Ltd., Clifton Hill, VIC, Australia) column-purified plasmids, as described by the manufacturer. Plasmids coding C3-transferase endotoxin and H2B-GFP were cotransfected using Lipofectamine-PLUS reagents (InVitrogen, Mount Waverley, VIC, Australia), while pZeoSV2/CAT plasmid coding Catalase [Bai and Cederbaum, 2003] and nuclear localization signal-tagged GFP [Scaife et al., 2003a] were transfected using Fugene (Roche, Indianapolis, IN).

Immunofluorescence Microscopy

For immunofluorescence microscopy, cells were seeded onto coverslips coated with polylysine (0.1 mg/ml). Fluorescence staining of microfilaments was achieved by fixation of the cells, in 4% paraformaldehyde in phosphate-buffered saline (PBS), permeabilization using 0.2% Tx-100 in PBS containing 2.5 mg/ml bovine serum albumin (BSA), and incubation for 30 min with 0.5 μg/ml Tritc-phalloidin (Sigma). All other immunofluorescence imaging was achieved by fixation in −20°C methanol, followed by a 4% paraformaldehyde post-fix. Coverslips were rinsed with PBS and incubated with either anti-β-tubulin (7.5 μg/ml Sigma mAb, # T-4026) or anti-α-tubulin antibodies (1/1,000 YL 1/2 rat mAb), anti-γ-tubulin antibodies (3.5 μg/ml Sigma mAb, # T-6557), anti-phospho-Aurora 2 (Thr288) antibodies (1/200 diluted Cell Signaling #3091), or anti-phospho-histone H3 (1/200 Cell Signaling, Beverley, MA, mAb 6G3 #9706) at 37°C for 60 min in PBS containing 2.5 mg/ml BSA. Following a PBS wash, the coverslips were incubated with 5 μg/ml biotin-SP conjugated goat anti-mouse (Jackson Laboratories, Bar Harbor, MI, # 115-065-003) or 7.5 μg/ml biotin conjugated goat anti-rabbit (Vector, Burlingame, CA, # BA-1000) at 37°C for 60 mins in PBS containing 2.5 mg/ml BSA. Biotin-labeled antigen–antibody complexes were then visualized by incubation for 60 min with PBS containing 2.5 mg/ml BSA and 2 μg/ml Alexa 488 conjugated streptavidin (Molecular Probes, Eugene, OR) and nuclear DNA was stained with Hoescht 33342. For double labeling with rabbit and mouse primary antibodies, the rabbit antibody was detected as described above, while the mouse antibody was detected using highly species cross-absorbed Alexa 546 conjugated anti-mouse (Molecular probes). For α- and γ-tubulin double labeling, α-tubulin was stained first using rat mAb YL1/2, 5 μg/ml biotin-SP conjugated goat anti-rat (Jackson Laboratories, Bar Harbor, MI, #112-065-003), and Alexa 546 conjugated streptavidin (Molecular probes). This was then followed by anti-γ-tubulin mAb and highly species cross-absorbed anti-mouse antibody conjugated to Alexafluor 488 (Molecular Probes).

Images of representative fields were obtained with Comos and Confocal Assistant software (Bio-Rad, Hercules, CA) following capture on a Nikon Diaphot 300 microscope equipped for UV laser scanning confocal microscopy (Bio-Rad MRC 1000/1024). The scale bar represents 25 μm.

Cell Lysis, Fractionation, and Western Blotting

Total cell lysates were obtained from PBS-washed cells by addition of ice-cold 50 mM

HEPES (pH 7.4) containing 150 mM NaCl, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, 10% glycerol, 1.5 mM MgCl2, 1 mM EDTA, 1 mM Na3VO4, 10 mM NaF, and protease inhibitors.

Cytoskeletal fractions were obtained after extraction of cytosolic proteins by a 1-min incubation of the cells with 80 mM PIPES (pH 6.7), 1 mM EGTA, 1 mM $MgCl_2$, 10% glycerol, 0.3% Triton X-100, 5% polyethylene glycol, and protease inhibitors at 37°C. After sonication, aliquots of total cell lysates and cytoskeletal fractions were subjected to SDS–PAGE and transferred onto nitrocellulose membranes (Amersham Biosciences, Piscataway, NJ). Membranes were blocked with 10% nonfat milk (Carnation, Las Vegas, NV) in TBS containing 0.5% Tween-20, and probed with antibodies directed against β-tubulin, nucleolin, Glu-tubulin (L4), and Tyr-tubulin (1/10,000 diluted YL1/2), followed by HRP conjugated secondary antibody (Silenus, Boronia, VIC, Australia). Antigens were then visualized by chemiluminescence (ECL, Amersham) using Hyperfilm MP (Amersham).

Microtubule In Vitro Assembly Assay

One milligram of purified microtubule protein (Cytoskeleton, Inc., Denver, CO, #ML113) was resuspended in 0.6 ml of general tubulin buffer (GTB) (Cytoskeleton, Inc. #BST01-001) supplemented with 0.1 mM GTP (Sigma) and centrifuged at 100,000*g* for 30 min at 4°C in a Beckman TL100 centrifuge. The supernatant fraction was placed at 37°C for 20 min following supplementation with 0.4 mM GTP. A 1/20 volume of GTB, or GTB containing either 20 μg/ml nocodazole or DPI, ranging from 0.12 to 4 mM, was added, and the microtubule samples were centrifuged at 100,000*g* for 30 min at 37°C. The resulting supernatant and pellet fractions were collected, subjected to SDS–PAGE, and the intensity of the Coomassie-stained tubulin band determined using a Bio-Rad Universal Hood II densitometer.

RESULTS

Selective Disassembly of the Microtubule Cytoskeleton by DPI

In order to examine the effect of diphenyleneiodonium (DPI) on the cytoskeleton, microfilaments, and microtubules were visualized by fluorescence microscopy. The actin cytoskeleton was observed following phalloidin staining of the cells. The phalloidin staining of Rat1 fibroblasts indicated that exposure to DPI did not substantially alter the assembly, or the organization, of the actin cytoskeleton. The actin cytoskeleton of untreated and DPI-treated cells appeared similar (Fig. 1A, panels 1 and 2), although a minor increase in actin stress-fibers was discernable for the DPI-treated cells. The microtubule cytoskeleton was observed following anti-α-tubulin staining of the cells. In contrast with its relatively minor effect on the actin cytoskeleton, DPI profoundly affected the microtubule cytoskeleton. While anti-α-tubulin immunofluorescence staining of untreated Rat1 fibroblasts revealed a dense radial network of cytoplasmic microtubules (Fig. 1B, panel 1), following a 30 min exposure of these cells to 10 μM DPI the cytoplasmic microtubules were largely disassembled (Fig. 1B, panel 2). This disassembly of microtubules by DPI appears to be fully reversible. Within 20 min of removal of DPI from the culture medium, a radial microtubule network resembling that of untreated cells reformed (Fig. 1B, panel 3).

In light of the disassembly of the microtubule cytoskeleton by DPI, its effect on in vitro microtubule assembly was examined. Purified microtubule protein (brain tubulin and MAPS, see Materials and Methods) was assembled at 37°C by addition of GTP, and then exposed to a range of DPI concentrations. Centrifugal fractionation of the samples into assembled (pellet) and unassembled (supernatant) fractions indicated that DPI did not cause disassembly of the microtubules. Unlike microtubule disassembly by 1 μg/ml nocodazole, which resulted in a recovery of most of the tubulin in the supernatant fraction, DPI at concentrations of up to 200 μM did not affect the amount of tubulin recovered in the supernatant and pelleted fractions (Fig. 1C).

Disassembly of the Microtubule Cytoskeleton is Independent of Rho

DPI is a well characterized flavoprotein inhibitor that, through its effect on NADPH oxidase, can increase intracellular Rho-GTP levels [Nimnual et al., 2003]. As Rho has been shown to influence the microtubule cytoskeleton [Watanabe et al., 2005], the effect of DPI on the microtubule cytoskeleton could reflect its ability to increase Rho-GTP levels by inhibition of NADPH-mediated generation of ROS. The

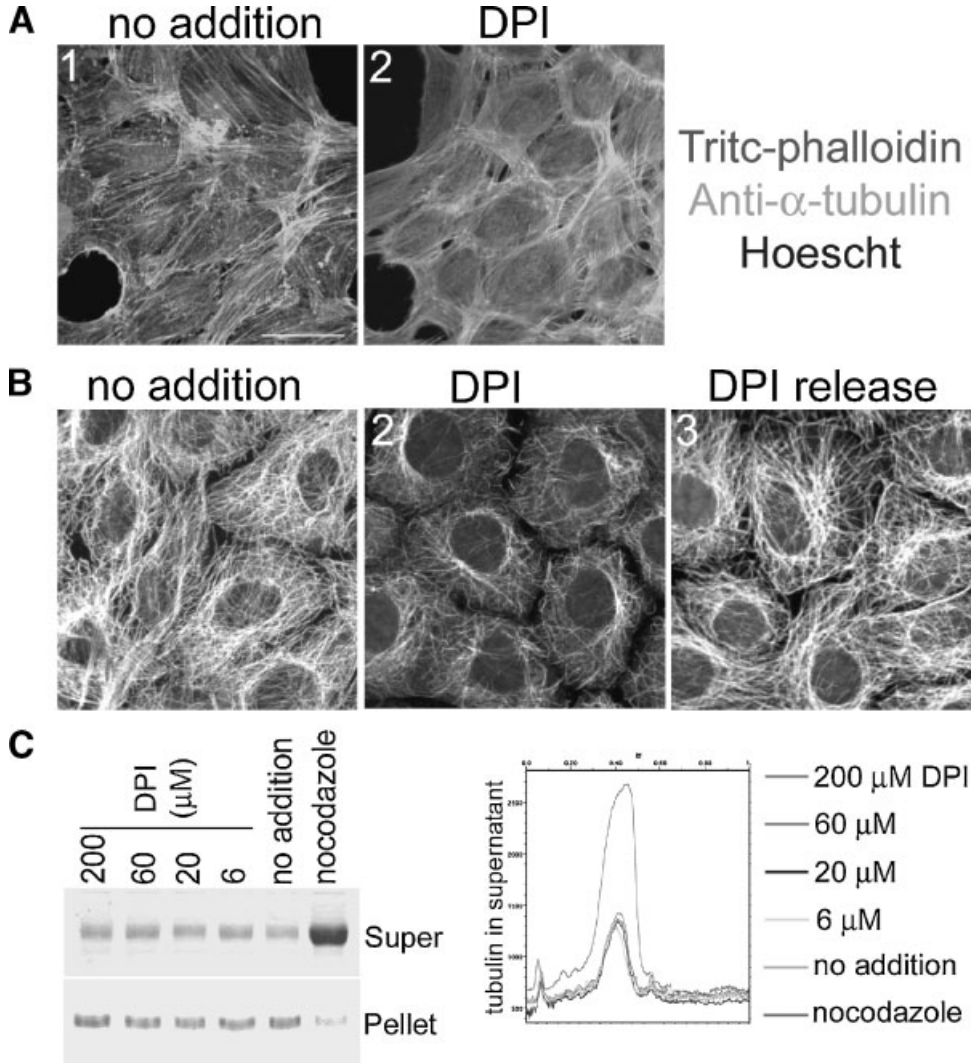

Fig. 1. Selective disassembly of the microtubule cytoskeleton by DPI. Rat 1 cells were left untreated (**panel 1**), exposed to 12 μM DPI for 60 min (**panel 2**) or exposed to 12 μM DPI for 60 min, followed by a switch to DPI-free culture medium for 20 min (**panel 3**). The cells were then fixed in 4% paraformaldehyde and stained with Tritc-phalloidin and Hoescht (**A**), or the cells were fixed in methanol and stained with anti-α-tubulin and Hoescht (**B**), and visualized by confocal fluorescence microscopy. **C**: Purified microtubule protein at 1.5 mg/ml was placed at 37°C for 20 min after which nocodazole (1 μg/ml) or DPI (6–200 μM) was added for 20 min. Following ultra-centrifugation, supernatant and pelleted fractions were subjected to SDS–PAGE. The tubulin component of the supernatant fraction was quantified by densitometric scanning following Coomassie staining of the gel. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

effect of DPI on the microtubule cytoskeleton was therefore examined in cells in which Rho had been inactivated. Rho was inactivated by transfection of cells with the ADP-ribosylating C3 transferase [Etienne-Manneville and Hall, 2002]. In order to identify cells expressing the C3 transferase, the plasmid coding for the C3-transferase was co-transfected with a plasmid that expresses a green-fluorescent marker [Scaife et al., 2003a]. Inhibition of Rho in Rat1 cells that were positive for the H2B-GFP marker was indicated by the absence Tritc-phalloidin-stained stress fibers (Fig. 2, panel 1). The effect of Rho inhibition on DPI-induced microtubule disassembly was then examined by anti-α-tubulin immunofluorescence microscopy. While expression of the C3 transferase did not perturb the cytoplasmic microtubule network (Fig. 2, panel 2), addition of DPI to these C3 transferase expressing cells caused a pronounced disassembly of cytoplasmic microtubules (Fig. 2, panel 3).

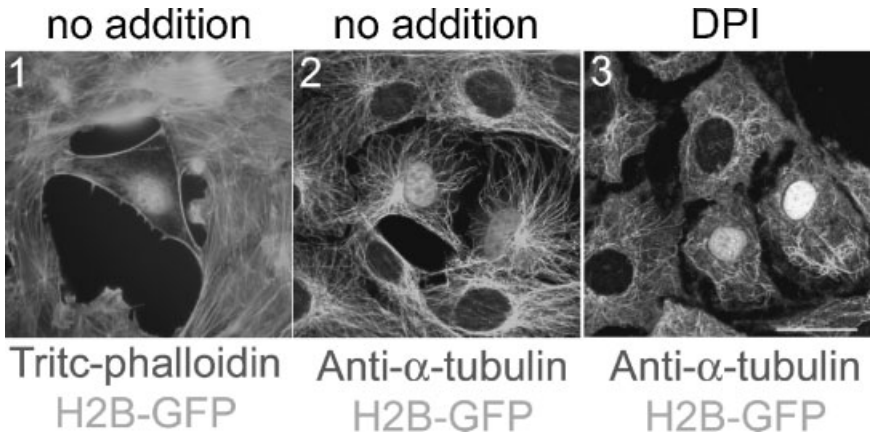

Fig. 2. Disassembly of the microtubule cytoskeleton is independent of Rho. Following cotransfection of Rat1 cells with C3-transferase and H2B-GFP, they were fixed in 4% paraformaldehyde for Tritc-phalloidin staining (**panel 1**) or the cells were fixed in methanol for anti-α-tubulin staining following a 60 min incubation without (**panel 2**) or with 12 μM DPI (**panel 3**). [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

DPI Represents a Novel Microtubule Pharmacophore

The ability of DPI to affect the microtubule cytoskeleton independently of Rho suggests that this effect may reflect a novel microtubule perturbing mechanism. This was examined further by evaluation of the effect of the closely-related flavoprotein inhibitor diphenyliodonium (Fig. 3A) [Chakraborty and Massey, 2002] on the microtubule cytoskeleton. As evidenced by anti-α-tubulin immunofluorescence microscopy, diphenyliodonium had no discernable effect on the microtubule cytoskeleton (Fig. 3B, panel 1).

By inhibiting NADPH oxidase, DPI causes a decrease in intracellular ROS levels. Since ROS levels can similarly be decreased by exposure of cells to anti-oxidants such as *N*-acetylcysteine (NAC) [Nimnual et al., 2003; Scaife, 2004], the effect of NAC on the microtubule cytoskeleton was examined. Exposure of Rat1 cells to NAC did not appreciably affect the cytoplasmic microtubule network (Fig. 3B, panel 2). In addition to affecting NADPH oxidase, DPI can also inhibit the flavoprotein nitric oxide synthase (NOS) [Stuehr et al., 1991]. Treatment of the Rat1 cells with the NOS inhibitor *N*ω-L-Nitroarginine methyl ester (L-NAME), however, had no discernable effect on the microtubule network (Fig. 3B, panel 3).

Further indication that the action of DPI on microtubules does not involve ROS was obtained by examination of the microtubule network in cells that overexpress the ROS scavenging enzyme catalase. Anti-β-tubulin immunofluorescence microscopy of cells transfected with plasmid coding for catalase (evidenced by expression of the co-transfected GFP marker plasmid, Fig. 3C, panel 1), indicated that overexpression of catalase did not appear to affect the microtubule cytoskeleton (Fig. 3C, panel 2).

DPI Profoundly Suppresses Microtubule Reassembly

In order to further characterize the effect of DPI on microtubule assembly, its effect on microtubule reassembly was investigated. Microtubule reassembly was assayed by anti-α-tubulin immunofluorescence staining of Rat1 cells following release from the microtubule depolymerising drug nocodazole. Exposure of cells to 0.2 μg/ml nocodazole caused extensive microtubule depolymerisation, and microtubules rapidly reassembled following removal of the nocodazole from the culture medium (Fig. 4, panels 1–3). Within 15 min of removal of nocodazole from the culture medium, the cells had reassembled a radial cytoplasmic microtubule network, concomitant with the partial reassembly of mitotic spindles (Fig. 4, panel 2). At 45 min following removal of the nocodazole, both the cytoplasmic microtubules, as well as the mitotic spindle microtubules, appeared to be fully reassembled (Fig. 4, panel 3). This rapid reassembly of the microtubule cytoskeleton following nocodazole removal contrasts with

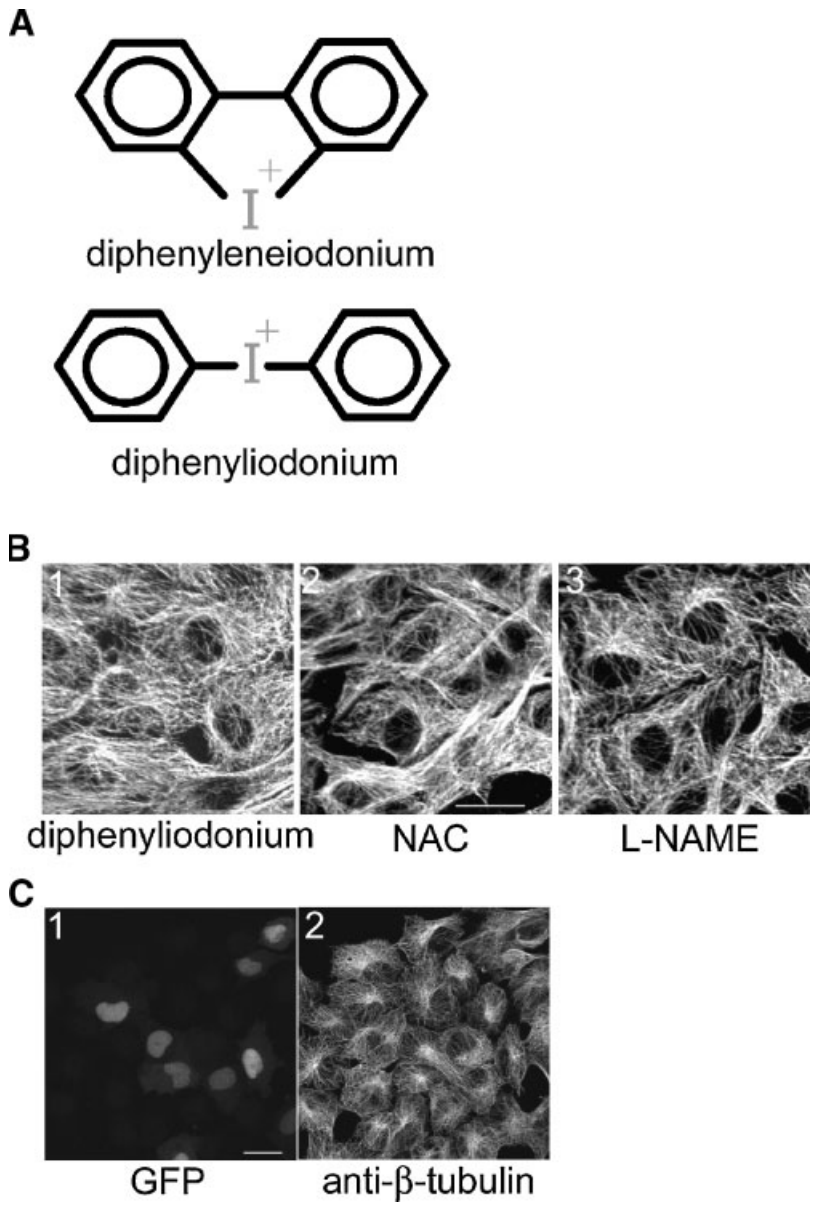

Fig. 3. DPI represents a novel microtubule pharmacophore. **A**: The chemical structures of diphenyleneiodonium and diphenyliodonium are indicated. **B**: Rat 1 cells were exposed to 20 μM diphenyliodonium for 30 min (**panel 1**), 20 mM NAC for 4 h (**panel 2**), or 20 μM L-NAME for 4 h (**panel 3**). The cells were then fixed in methanol and stained with anti-α-tubulin, and visualized by confocal fluorescence microscopy. **C**: Following co-transfection of Rat1 cells with pZeoSV/Catalase and NLS-GFP for 24 h, the cells were fixed in methanol and the GFP (panel 1) and anti-β-tubulin staining (panel 2) were observed by confocal microscopy. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

the greatly decreased reassembly following nocodazole release in the presence of DPI. In the presence of DPI there was essentially no microtubule formation 15 min after nocodazole removal (Fig. 4, panel 4). Even following 45 min of nocodazole release in the presence of DPI, there was only limited reassembly of microtubules (Fig. 4, panel 5). Double labeling with anti-α-tubulin and anti-γ-tubulin indicated that following 45 min of nocodazole release, the DPI-treated cells reassembled a cluster of relatively short microtubules emanating from the MTOC/centrosome (Fig. 4, panel 5). Further, in the presence of DPI, the reassembly of mitotic spindle microtubules failed to occur. Indeed, although a significant degree of cytoplasmic microtubule reassembly had occurred 120 min following nocodazole release in the presence of

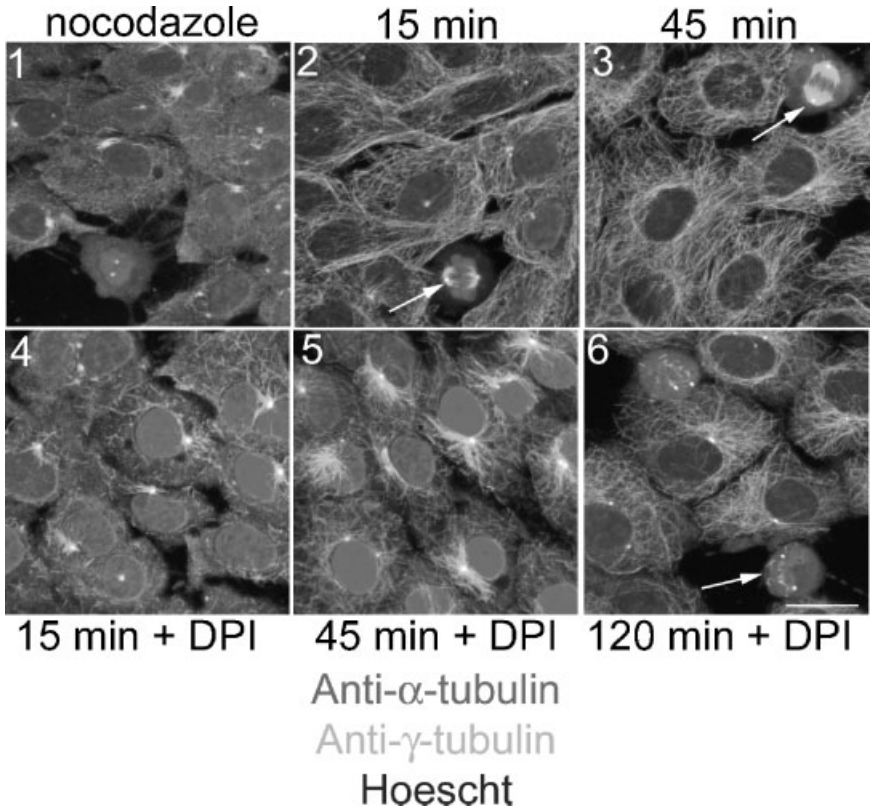

Fig. 4. DPI profoundly suppresses microtubule reassembly. Rat1 cells were fixed following exposure to 0.2 μg/ml nocodazole for 60 min (**panel 1**), and released into nocodazole-free medium for 15 min (**panel 2**) and 45 min (**panel 3**). Additionally, Rat1 cells were exposed to 0.2 μg/ml nocodazole for 45 min, at which point 12 μM DPI was added for a further 15 min prior to release into nocodazole-free medium in the presence of 12 μM DPI for 15 min (**panel 4**), 45 min (**panel 5**), and 120 min (**panel 6**). The cells were then stained with anti-α-tubulin, and visualized by confocal fluorescence microscopy. Mitotic cells are indicated by the arrows. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

DPI, nocodazole-released mitotic cells failed to reassemble a mitotic spindle. Double labeling with anti-α-tubulin and anti-γ-tubulin confirmed that the centrosomes of the mitotic cells did not nucleate any microtubules (Fig. 4, panel 6).

Paclitaxel Reverses Disassembly but not Microtubule Reorganization by DPI

Paclitaxel greatly promotes tubulin assembly into microtubule polymer. Since DPI profoundly suppressed microtubule assembly, the ability of paclitaxel to overcome this suppression of assembly was investigated. Firstly, the microtubule cytoskeleton and centrosomes were visualized by anti-α-tubulin and anti-γ-tubulin staining in the presence and absence of DPI. In the absence of DPI, the paclitaxel-treated cells formed numerous, relatively short, microtubules (Fig. 5A, panel 1). These paclitaxel-stabilized microtubules were dispersed evenly throughout the cytoplasm, and they had no clear physical association with the centrosomes (Fig. 5A, panel 1). Following exposure of paclitaxel-treated cells to DPI, there were similarly numerous, relatively short, microtubules (Fig. 5A, panel 2). In the presence of DPI, however, these paclitaxel-stabilized microtubules were often preferentially localized in the vicinity of the centrosomes (Fig. 5A, panel 2).

In order to assay whether DPI affects the polymerization of paclitaxel-stabilized microtubules, levels of assembled tubulin were assayed by anti-β-tubulin Western blotting of microtubule cytoskeletal fractions. This indicated that the pronounced increase in tubulin assembly by paclitaxel was not affected by the addition of DPI (Fig. 5B).

Since this data indicates that DPI does not cause disassembly of paclitaxel-stabilized microtubules, the ability of paclitaxel to reverse the DPI-induced microtubule assembly was investigated. Anti-β-tubulin Western blotting of microtubule cytoskeletal fractions indicated that addition of paclitaxel to DPI-treated cells

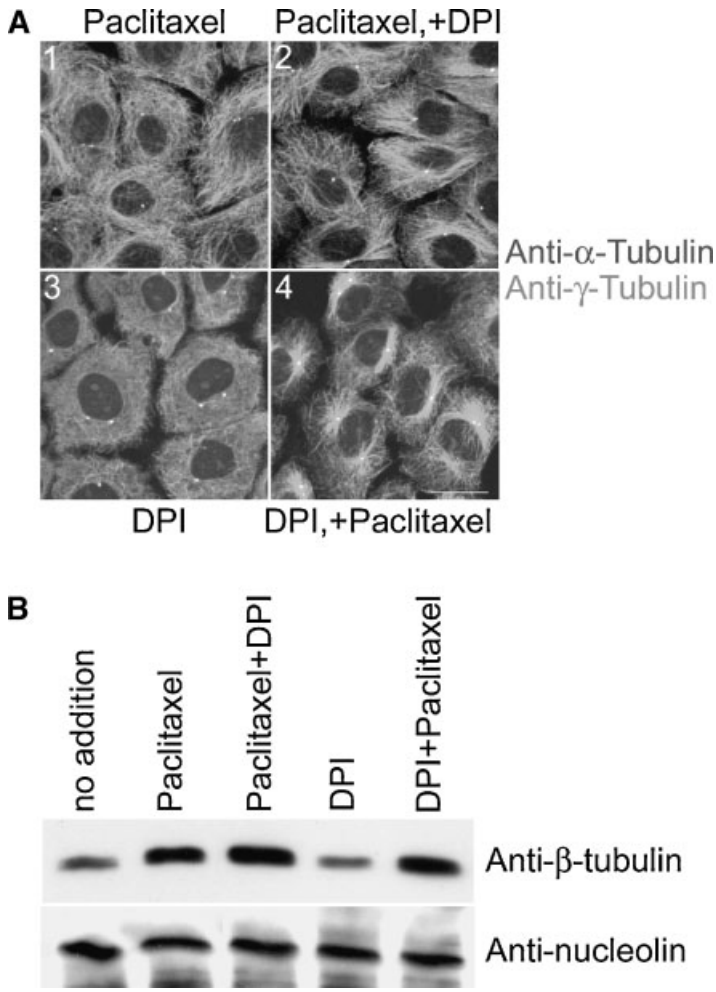

Fig. 5. Paclitaxel reverses disassembly but not microtubule reorganization by DPI. Rat1 cells were either treated with 0.4 μM paclitaxel for 60 min followed by a further 60 min incubation in the absence (**panel 1**) and presence (**panel 2**) of 6 μM DPI, or the cells were exposed to 6 μM DPI for 60 min followed by a further 60 min incubation in the absence (**panel 3**) and presence (**panel 4**) of 0.4 μM paclitaxel. The cells were then fixed in methanol and stained with anti-α-tubulin and anti-γ-tubulin, and subsequently visualized by confocal fluorescence microscopy (**A**). A parallel sample of paclitaxel- and DPI-treated Rat1 cells was lysed in microtubule extraction buffer and subjected to SDS–PAGE and anti-β-tubulin Western blotting (**B**). The anti-nucleolin Western blot is shown as a loading control. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

led to tubulin assembly levels comparable to those of cells treated with paclitaxel alone (Fig. 5B). However, anti-α-tubulin and anti-γ-tubulin immunofluorescence microscopy of microtubules and centrosomes, respectively, indicated that DPI greatly affected the intracellular organization of the paclitaxel-assembled microtubules. Unlike paclitaxel treatment of control cells, which led to the assembly of microtubules that were dispersed throughout the cytoplasm (Fig. 5A, panel 1), addition of paclitaxel to DPI-treated cells led to the assembly of microtubules that were clustered into dense bundles around the centrosomes (Fig. 5A, panel 4).

DPI Selectively Disassembles Dynamic Microtubules

As shown in Figure 4, DPI affected the highly dynamic microtubules of the mitotic spindle [Jordan and Wilson, 2004] much more than the microtubules in interphase cells. Since stable (or relatively non-dynamic) microtubules can undergo detyrosination of α-tubulin [Kreis, 1987], the levels of detyrosinated (Glu) and tyrosinated (Tyr) tubulin [Lafanechere et al., 1998] were determined in order to assay whether DPI selectively disassembles dynamic microtubules. Anti-Glu- and anti-Tyr-tubulin immunofluorescence microscopy staining of

untreated Rat1 fibroblasts revealed that these cells have very few Glu-microtubules, and that these short Glu-microtubules are usually clustered around the nucleus of the cell (Fig. 6A, panel 2). While exposure of these cells to DPI led to a pronounced disassembly of Tyr-microtubules (Fig. 6A, panel 3), this correlated with a marked increase in the number of Glu-microtubules (Fig. 6A, panel 4). The Glu-microtubules in DPI-treated cells were often dispersed throughout the cytoplasm. This DPI-induced increase in Glu-tubulin was further evidenced by Western blot analysis of cell lysates. While DPI treatment did not affect the total cellular levels of tubulin, it did significantly increase the amount of Glu-tubulin in the cells (Fig. 6B). This selective effect of DPI on the Glu-tubulin balance was particularly evident upon examination of microtubule cytoskeletal fractions. Somewhat analogous to the pronounced increase in Glu-tubulin in response to paclitaxel-mediated inhibition of microtubule dynamics, exposure of the cells to DPI similarly led to an increase in the levels of Glu-tubulin in the cytoskeletal fraction, relative to untreated cells (Fig. 6B). Since DPI causes substantial disassembly of the total microtubule population, the proportion of DPI-resistant Glu-microtubules, relative to the total, or β-tubulin, microtubule content of the cytoskeletal fraction was hence quite high (Fig. 6B).

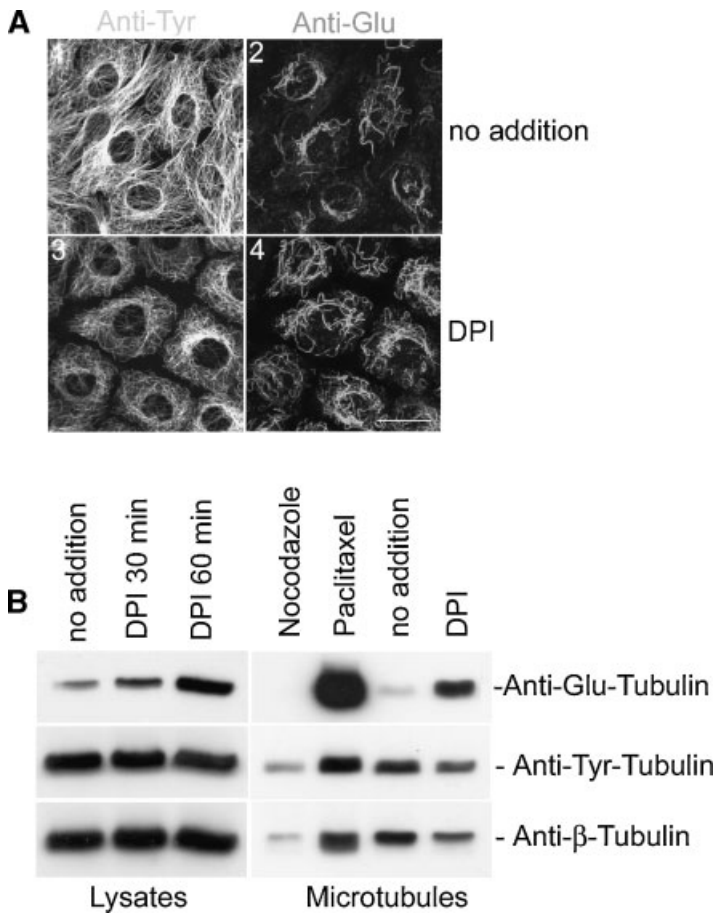

Fig. 6. DPI selectively disassembles dynamic microtubules. Rat1 cells that were untreated (**panels 1** and **2**) or exposed to 6 μM DPI for 60 min (**panels 3** and **4**) were fixed in methanol and stained with anti-Tyr and anti-Glu antibodies. The cells were then visualized by confocal fluorescence microscopy (**A**). Rat 1 cells were either left untreated or exposed to 6 μM DPI for 30 and 60 min. The cells were then lysed in SDS-containing buffer (Lysates). Additionally, Rat1 cells exposed to 0.1 μg/ml nocodazole, 0.4 μM paclitaxel, no treatment or 6 μM DPI were lysed in microtubule extraction buffer (Microtubules). The whole cell lysates (left hand panels), and the microtubule fractions (right hand panels), were then subjected to SDS–PAGE, followed by and anti-Glu-, anti-Tyr-, and anti-β-tubulin Western blotting (**B**). [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

DPI Causes Pro-Metaphase Mitotic Arrest

In light of the inhibitory effect of DPI on the assembly of mitotic spindle microtubules, the ability of DPI-treated cells to undergo mitotic cell division was examined. Mitotic cells in untreated and DPI-treated samples were identified by anti-phospho-histone H3 labeling (Fig. 7A). Immunofluorescence microscopy of cells co-stained with anti-α-tubulin and Hoescht revealed cells in various stages of mitosis in the untreated sample (Fig. 7A, panel 1). By contrast, the anti-α-tubulin immunofluorescence staining of DPI-treated mitotic cells indicated that they failed to assemble a mitotic spindle. (Fig. 7A, panel 2). Further, the Hoescht staining of the condensed chromatin in DPI-treated mitotic cells indicated that they were in the pro-metaphase stage of mitosis (Fig. 7A, panel 2). Examination of a large number of mitotic cells confirmed the failure of DPI-treated mitotic cells to progress past pro-metaphase. None of the DPI-treated mitotic cells were able to reach the latter stages (i.e., anaphase, telophase, or cytokinesis) of mitosis, and the condensed chromatin failed to align as a distinct metaphase plate (Fig. 7B).

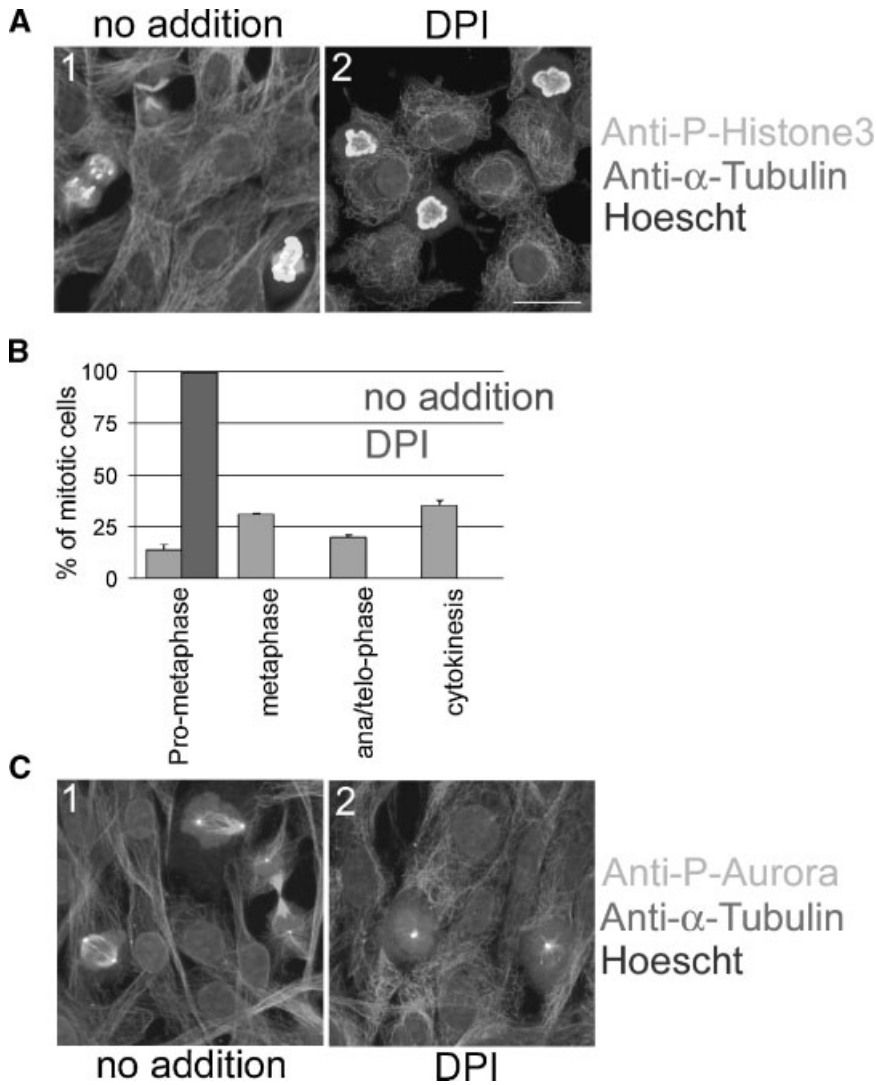

Fig. 7. DPI causes pro-metaphase mitotic arrest. Rat 1 cells were either left untreated (**panel 1**) or exposed to 6 μM DPI for 120 min (**panel 2**). The cells were then fixed in methanol and stained with Hoescht, anti-α-tubulin and either anti-phospho-histone H3 (**A**) or anti-phospho-Aurora (**C**). The cells were visualized by confocal fluorescence microscopy (A) and (C), and the percentage of mitotic cells in pro-metaphase, metaphase, ana/telophase, and cytokinesis is indicated in the bar graph (**B**). [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

In order to address whether the failure of DPI-treated mitotic cells to assemble a mitotic spindle, and progress through mitosis, might be due to perturbation of cell cycle signal transduction mechanisms [Scaife, 2004], rather than inhibition of microtubule assembly, the effect of DPI on signal transduction-mediated centrosome maturation was examined. Centrosome maturation was assayed by immunofluorescence staining with anti-phospho-Aurora [Hirota et al., 2003]. Similar to untreated mitotic cells, which had anti-phospho-Aurora labeled spindle poles (Fig. 7C, panel 1), the spindle poles of DPI-treated mitotic cells were also labeled with the phospho-Aurora antibody (Fig. 7C, panel 2).

DISCUSSION

Previously described as a flavoprotein inhibitor [Hancock and Jones, 1987; Stuehr et al., 1991], diphenyleneiodonium has here been shown to have a novel biological activity that targets the microtubule cytoskeleton, and hence also mitotic cell division. As a flavoprotein inhibitor, DPI can inhibit NAD(P)H-oxidase-mediated generation of reactive oxygen species (ROS) and NOS-mediated formation of nitric oxide. While nitric oxide has not been reported to affect the cytoskeleton, reactive oxygen species, through their inhibitory effect on protein tyrosine phosphatases, affect Rho-activation, and hence also organization of the actin cytoskeleton [Nimnual et al., 2003]. Accordingly, an effect of DPI on the actin cytoskeleton was indeed observed here, although this modest effect on actin stress fibers was eclipsed by the pronounced DPI-mediated disassembly of microtubules. Importantly, the DPI-induced disassembly of microtubules appears to occur independently of the actin cytoskeleton, as the disassembling activity persists in C3-transferase expressing cells in which actin stress fibers are fully disassembled. The effect of DPI on the microtubule cytoskeleton is highly reversible, as the microtubules reassemble within minutes of its removal from the cell culture medium. This suggests that the microtubule depolymerising activity of DPI is due to its reversible binding to cellular targets, rather than a result of its chemically reactive nature that would result in a covalent linkage, and hence an essentially irreversible cellular response.

Unlike other microtubule pharmacophores, such as taxanes and vinca alkaloids, which exert their effect on microtubules in vitro by direct binding to the tubulin subunit [Jordan and Wilson, 2004], DPI did not cause disassembly of purified microtubules. The failure of DPI to cause disassembly of microtubules in vitro suggests that its effect on the microtubule cytoskeleton does not involve direct targeting of tubulin. Despite a degree of structural similarity with biphenyls (such as combretastatin), which cause microtubule disassembly in vitro [Young et al., 2001; Liou et al., 2004], the effect of DPI on the microtubule cytoskeleton therefore appears to represent a novel indirect action on the microtubule cytoskeleton.

The effect of DPI on the microtubule cytoskeleton may reflect an ability to perturb signal transduction-mediated regulation of the microtubule cytoskeleton. By affecting intracellular levels of reactive oxygen species, DPI can affect the activity of Rho. While Rho indeed also affects the microtubule cytoskeleton [Watanabe et al., 2005], the results presented here indicate that the disassembly of the microtubule cytoskeleton by DPI is independent of Rho. Similarly, failure of the NAD(P)H oxidase inhibitor diphenyliodonium, and the NOS inhibitor L-NAME, to cause disassembly of the microtubule cytoskeleton indicates that the activity of DPI on the microtubules is not due to effects on reactive oxygen, or nitric oxide levels. This is further substantiated by the failure of overexpression of the ROS scavenging enzyme catalase to cause microtubule disassembly.

As shown here, DPI greatly suppresses microtubule reassembly following release of cells from nocodazole-induced microtubule depolymerisation. While this could be a reflection of a direct effect of DPI on microtubule assembly by centrosomes, it could also be the result of a DPI-mediated increase in microtubule instability. Indeed, following nocodazole release in the presence of DPI, the cells are capable of assembling microtubules, provided they are anchored to the centrosomes. Similarly, at DPI concentrations that cause only a partial disassembly of the microtubule network, the remaining microtubules are generally anchored to the centrosomes. Centrosomes stabilize microtubules by capturing their kinetically unstable "minus" end. Therefore, a DPI-induced increase in "minus" end instability would result in a preferential loss of microtubules with free "minus" ends, relative to microtubules that have their "minus" ends tethered to

the centrosomes. By selectively disassembling unstable microtubules, exposure of cells to DPI could also lead to a relative increase in stable, or Glu-, microtubules. The data presented here indeed indicates that DPI selectively disassembles unstable, or Tyr-, microtubules. This effect of DPI on microtubule dynamics appears similar to the recently reported kinetic stabilization of microtubules by anti-proliferative organosulfides [Xiao et al., 2005], as exposure of cells to diallyl disulphide (DADS) also induces an accumulation of Glu-microtubules. The molecular mechanism of microtubule stabilization by DADS remains unclear however [Hosono et al., 2005]. Further efforts will similarly be required to establish how exposure to DPI changes microtubule organization and assembly.

In the presence of DPI, paclitaxel-stabilized microtubules redistribute into a striking clustered arrangement. In light of the very pronounced microtubule stabilization by paclitaxel [Watanabe et al., 2005], the preferential localization of paclitaxel microtubules in the vicinity of the centrosome is most likely not due to an additional kinetic stabilizing effect. Rather, it suggests that DPI also affects the centrosome, thereby increasing its microtubule-organizing function. DPI-induced inactivation of a "minus" end microtubule-associated protein could indeed lead to enhanced interaction of the microtubule "minus" end with the centrosomes. This would lead to kinetic stabilization and a consequent increase in Glu-tubulin content, as described here.

The data presented here unequivocally demonstrates that DPI can block mitotic cell cycle progression. Similar to other microtubule drugs, DPI prevents assembly of the mitotic spindle, thereby causing an arrest in pro-metaphase. Although this pro-metaphase block is very evident from the immunofluorescence microscopy analysis of mitotic cells, this effect of DPI on the cell cycle is difficult to discern due to the ability of DPI to profoundly affect other stages of the cell cycle [Scaife, 2004]. Indeed, exposure to DPI causes the majority of the cells to arrest prior to mitosis in either G1- or G2-phase [Scaife, 2005]. Only the small fraction of cells that are between the DPI-G2 arrest point and M-phase can be blocked in mitosis by DPI. Furthermore, this accumulation of pro-metaphase arrested cells by DPI is transient, as prolonged exposure to DPI forces the cells to exit mitosis [Scaife, 2004, 2005]. Hence, unlike other microtubule drugs, such as nocodazole and paclitaxel, which cause a progressive time-dependent accumulation of mitotic cells, exposure of cells to DPI entails only a minor and transient accumulation of mitotic cells. Interestingly, since mitosis is regulated by centrosome-associated signaling proteins [Nigg, 2001], it is possible that targeting of a critical centrosomal components could underlie the observed effects of DPI on microtubules (described here) and the mitotic check-point [Scaife, 2004, 2005].

The ability of DPI to profoundly affect multiple stages of the cell cycle, apparently by targeting of signal transduction to the microtubules cytoskeleton and other cell cycle components, indicates that this compound is ripe for more detailed structure-function analyses. Indeed, one can readily imagine how DPI analogs may exhibit more specific and more potent biological activities that would permit their application in anti-cancer strategies.

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