G2 cell cycle arrest, down-regulation of cyclin B, and induction of mitotic catastrophe by the flavoprotein inhibitor diphenyleneiodonium

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Abstract

Because proliferation of eukaryotic cells requires cell cycle–regulated chromatid separation by the mitotic spindle, it is subject to regulation by mitotic checkpoints. To determine the mechanism of the antiproliferative activity of the flavoprotein-specific inhibitor diphenyleneiodonium (DPI), I have examined its effect on the cell cycle and mitosis. Similar to paclitaxel, exposure to DPI causes an accumulation of cells with a 4N DNA content. However, unlike the paclitaxel-mediated mitotic block, DPI-treated cells are arrested in the cell cycle prior to mitosis. Although DPI-treated cells can arrest with fully separated centrosomes at opposite sides of the nucleus, these centrosomes fail to assemble mitotic spindle microtubules and they do not accumulate the Thr288 phosphorylated Aurora-A kinase marker of centrosome maturation. In contrast with paclitaxel-arrested cells, DPI impairs cyclin B1 accumulation. Release from DPI permits an accumulation of cyclin B1 and progression of the cells into mitosis. Conversely, exposure of paclitaxel-arrested cells, DPI impairs mitotic cells to DPI causes a precipitous drop in cyclin B and Thr288 phosphorylated Aurora-A levels and leads to mitotic catastrophe in a range of cancerous and noncancerous cells. Hence, the antiproliferative activity of DPI reflects a novel inhibitory mechanism of cell cycle progression that can reverse spindle checkpoint-mediated cell cycle arrest. [Mol Cancer Ther 2004;3(10):1229–37]

Introduction

Cell proliferation requires a multitude of events to translate a mitogenic signal into cell division. Mitotic division

is a key event in cell proliferation, whereby cytokinetic fission, following segregation of the condensed chromatin, results in a doubling of cell numbers. Both the chromatin segregation and the cytokinetic fission require assembly of specialized cytoskeletal structures composed of microtubules, microfilaments, and centrosomes (1, 2). Pharmacologic perturbation of the microtubule cytoskeleton has hence been used to target cell proliferation in human disease, and the microtubule stabilizing taxanes (such as paclitaxel) have proven effective in the treatment of cancers (3).

In addition to mitosis, cell proliferation also requires mitogenic signaling to achieve DNA replication during S phase of the cell cycle. Among the broad range of mitogenic signaling components, reactive oxygen species (ROS) are emerging as important factors in cell proliferation (4). By targeting the catalytic sites of phosphatases, ROS can influence a range of intracellular signaling events, including mitogenic signaling by mitogen-activated protein kinases (MAPK; ref. 5). Indeed, inhibition of ROS production has been shown to inhibit cell proliferation (6, 7).

Intracellular ROS levels can be lowered by antioxidants such as N-acetyl-l-cysteine (NAC) or through inhibition of the flavoprotein complex NAD(P)H oxidase by the flavoprotein-specific inhibitor diphenyleneiodonium (DPI; ref. 8). As reported previously (7), I have found that NAC and DPI profoundly inhibit cell proliferation. My results indicate that the effects of NAC and DPI on the cell cycle are fundamentally different. Indeed, I have found that, whereas NAC delays cell cycle progression through G1, DPI can prevent mitotic cell division by blocking the cell cycle progression in G2. This antiproliferative effect of DPI correlates with its ability to impair cyclin B1 and Thr288 phosphorylated Aurora-A accumulation. Further, in mitotically arrested cells, DPI can induce down-regulation of cyclin B and Thr288 phosphorylated Aurora-A levels and a forced mitotic exit. I have therefore identified a novel antiproliferative pharmacophore that seems to target mitotic cell cycle signaling components. In light of the inability of p53-compromised and retinoblastoma protein–compromised cells to undergo tetraploidy-mediated cell cycle arrest following mitotic catastrophe (9), it is possible that iodonium-based pharmacophores (such as DPI) could increase the efficacy of spindle checkpoint-mediated anticancer strategies.

Materials and Methods

Cell Culture

NIH 3T3, Rat1, HepG2 and MCF-7 cells were cultured in DMEM (Trace Biochemicals, Sydney, New South Wales,
Australia) containing 10% FCS (Life Technologies) and 2 mmol/L L-glutamine (Trace Biochemicals) at 37°C and 5% CO₂. Human mammary epithelial cells were obtained from Clonetics (Walkersville, MD) and cultured in fully supplemented MEGM (Clonetics). Cells were grown to ~50% confluency prior to overnight serum starvation.

**Fluorescence and Phase-Contrast Microscopy**

For immunofluorescence microscopy, cells were seeded onto coverslips coated with polylysine (0.1 mg/mL) prior to fixation in 4% paraformaldehyde-PBS. The fixed cells were then permeabilized for 1 minute with 0.2% Triton X-100 in PBS containing 2.5 mg/mL bovine serum albumin. Coverslips were rinsed with PBS and incubated with 0.5 μg/mL tetramethylrhodamine isothiocyanate-phalloidin (Sigma Chemical Co., St. Louis, MO), anti-β-tubulin antibodies (10 μg/mL, monoclonal antibody T-4026, Sigma Chemical), anti-γ-tubulin antibodies (3.5 μg/mL, monoclonal antibody T-6557, Sigma Chemical Co.), or anti–Thr288 phosphorylated Aurora A antibodies (1:200 dilution, 3091, Cell Signaling, Beverly, MA) at 37°C for 60 minutes in PBS containing 2.5 mg/mL bovine serum albumin. Following a PBS wash, the coverslips were incubated with 5 μg/mL biotin-SP-conjugated goat anti-mouse (115-065-003, The Jackson Laboratory, Bar Harbor, ME) or 7.5 μg/mL biotin-conjugated goat anti-rabbit (BA-1000, Vector Laboratories, Burlingame, CA) at 37°C for 60 minutes in PBS containing 2.5 mg/mL bovine serum albumin. Biotin-labeled antigen-antibody complexes were then visualized by incubation for 60 minutes with PBS containing 2.5 mg/mL bovine serum albumin and 2 μg/mL Alexa 488–conjugated streptavidin (Molecular Probes, Eugene, OR) and nuclei were stained with Hoechst 33342. For antibody double labeling, rabbit secondary antibody was employed as described above, whereas mouse antigens were detected using Cy3-conjugated donkey anti-mouse serum (715-165-150, The Jackson Laboratory). Following a PBS rinse, coverslips were mounted with SlowFade Light Antifade reagent (Molecular Probes). Images of representative fields were obtained with Comos and Confocal Assistant software (Bio-Rad, Hercules, CA) following capture on a Diaphot 300 microscope (Nikon, Tokyo, Japan) equipped for UV laser scanning confocal microscopy (Bio-Rad MRC 1000/1024). Confocal microscopy images of anti-γ-tubulin staining were used to determine intercentrosomal distances by Optimas software–mediated measurement of triplicate samples of at least 100 cells. Optimas software was similarly used to capture phase-contrast images of cells using a 63× objective lens. Binding of Annexin V-Fluos (Roche, Indianapolis, IN) to cells was determined by fluorescence microscopy.

**Flow Cytometry**

The DNA content of cells was determined following fixation of the cells in 90% methanol-PBS at -20°C after trypsin-mediated detachment from the culture substrate. After permeabilization with 0.2% Triton X-100 and addition of 2 μg/mL DNase-free RNase, the cells were stained with 2 μg/mL propidium iodide (PI). The intracellular level of ROS was determined following incubation of the cells with 2′,7′-dichlorodihydrofluorescein diacetate (Sigma Chemical) for 30 minutes. The 2′,7′-dichlorofluorescein and the PI signal were collected using CellQuest software on a FACSCalibur machine (Becton Dickinson, Mountain View, CA). Histograms of the 2′,7′-dichlorofluorescein or PI signal were obtained using FlowJo software (Tree Star, Inc., Ashland, OR).

**Cell Lysis and Western Blotting**

Cells were washed with PBS and lysed in ice-cold 50 mmol/L HEPES (pH 7.4) containing 150 mmol/L NaCl, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, 10% glycerol, 1.5 mmol/L MgCl₂, 1 mmol/L EDTA, 1 mmol/L Na₃VO₄, 10 mmol/L NaF, and protease inhibitors. Following centrifugation at 10,000 x g for 5 minutes, aliquots of cell lysates were subjected to SDS-PAGE and transferred onto nitrocellulose membranes (Amersham, Piscataway, NJ). Membranes were blocked with 10% nonfat milk (Carnation, Las Vegas, NV)-5% bovine serum albumin (Boehringer, Indianapolis, IN) in TBS containing 0.5% Tween 20 and probed with antibodies directed against either phospho–extracellular signal-regulated kinase (ERK) MAPK (monoclonal antibody 7833, Santa Cruz Biotechnology, Santa Cruz, CA), ERK MAPK (094, Santa Cruz Biotechnology), Thr288 phosphorylated Aurora 2 (3091, Cell Signaling), cyclin B1 (monoclonal antibody GNS-1, 554176, BD Biosciences, San Jose, CA), or cyclin A (571, Santa Cruz Biotechnology) followed by horseradish peroxidase–conjugated secondary antibody (Silenus, Boronia, Victoria, Australia). Antigens were then visualized by enhanced chemiluminescence (Amersham) using Hyperfilm MP (Amersham).

**MAPK Assays**

Following lysis of Rat1 cells into 20 mmol/L HEPES (pH 7.7), 0.1 mmol/L EDTA, 100 mmol/L NaCl, 20 mmol/L β-glycerophosphate, 100 μmol/L orthovanadate, 0.5 mmol/L DTT, and 0.2% (w/v) Triton X-100 containing protease inhibitors, lysates were clarified by centrifugation at 10,000 x g for 5 minutes and incubated for 3 hours with Sepharose-bound GST-Elk. The washed Sepharose beads were resuspended in 20 mmol/L HEPES (pH 7.6), 20 mmol/L MgCl₂, 20 mmol/L β-glycerophosphate, 100 μmol/L orthovanadate, and 0.5 mmol/L DTT, and after incubation with 10 μmol/L γ-[³²P]ATP for 30 minutes at 30°C, the samples were subjected to SDS-PAGE and autoradiography.

**Results**

**The Antioxidant NAC Slows Cell Cycle Progression in G₀ but Does Not Fully Block Mitotic Cell Division**

Because exposure of cells to NAC can decrease intracellular ROS levels, the effect of NAC on the cell cycle was probed. Incubation of Rat1 fibroblasts for increasing periods with NAC led to a progressive accumulation of cells with 2N DNA content (Fig. 1A, red flow cytometry profiles). Exposure of these cells to NAC therefore seems to
inhibit cell cycle progression, resulting in an accumulation of cells in the G_1 phase of the cell cycle. Inhibition of cell cycle progression by NAC was confirmed by treating the cells with paclitaxel (Fig. 1A, blue flow cytometry profiles). By arresting cells in mitosis, paclitaxel leads to an accumulation of cells with 4N DNA content (Fig. 1A, top). Exposure of the cells for increasing periods to NAC prior to the paclitaxel treatment led to a progressive reduction in the accumulation of cells with a 4N DNA content (Fig. 1A, middle and bottom). However, NAC did not lead to a complete arrest of cell cycle progression. Indeed, anti-β-tubulin and Hoescht staining revealed significant numbers of mitotic cells in the presence of both NAC and paclitaxel (Fig. 1B). A quantitative analysis of the anti-β-tubulin and Hoescht-stained mitotic cells confirmed that NAC reduced but did not fully block the paclitaxel-mediated accumulation of cells in mitosis (Fig. 1C).

**Inhibition of Cell Proliferation by Decreased ROS**

Levels Does Not Correlate with Apoptosis

ROS levels can be lowered by antioxidants such as NAC as well as by inhibitors of the flavoprotein NAD(P)H oxidases such as DPI. Intracellular levels of ROS can be assayed by measuring the intracellular oxidation of non-fluorescent 2′,7′-dichlorodihydrofluoresceindiacetate to the fluorescent 2′,7′-dichlorofluorescein (6). Flow cytometry of 2′,7′-dichlorodihydrofluoresceindiacetate–treated Rat1 cells revealed that exposure of these cells to 25 mmol/L NAC or 5 μmol/L DPI led to a comparable decrease in 2′,7′-dichlorofluorescein signal relative to cells incubated with 2′,7′-dichlorodihydrofluoresceindiacetate alone (Fig. 2A).

As reported previously with both normal and transformed cells (6, 7), quantitative evaluation of Rat1 and NIH 3T3 cell proliferation with a range of DPI concentrations indicated that it has a potent antiproliferative activity. Proliferation of either Rat1 or NIH 3T3 cells was greatly reduced by 2.5 μmol/L DPI, whereas a concentration of 8 μmol/L DPI fully blocked proliferation (Fig. 2B).

To determine whether the DPI-mediated suppression of cell proliferation could be due to a loss of cells by DPI-mediated apoptosis, cells were stained with fluorescent Annexin V. Whereas exposure of the cells to UV light resulted in numerous Annexin V–stained cells (Fig. 2C, 3), the number of apoptotic cells arising from exposure to DPI was quite low (Fig. 2C, 2). Indeed, 9 hours after addition of DPI to the culture medium, there was essentially no sign of apoptosis. This contrasts with the high proportion of Annexin V binding cells that arise following exposure to UV light (Fig. 2D). As reported previously (6), prolonged exposure to DPI did however lead to a modest degree of apoptosis (Fig. 2D).

**Inhibition of Cell Proliferation by Decreased ROS**

Levels Does Not Correlate with MAPK Inhibition

Because ROS have been implicated in MAPK-mediated mitogenic signal transduction pathways (4, 10), the loss of cell proliferation in response to DPI or NAC treatment has been interpreted as being due to antioxidant-mediated lowering of intracellular ROS levels (6, 7, 11). The effect of NAC and DPI on mitogenic signaling was hence examined by Western blotting with phosphospecific MAPK antibodies (Fig. 3A) and by in vitro ERK kinase assays (Fig. 3B). Interestingly, whereas prolonged exposure of Rat1 cells to NAC significantly reduced ERK MAPK (p42 and p44 MAPK) phosphorylation (Fig. 3A, lane 6), exposure to DPI caused a pronounced increase in the levels of these phosphorylated MAPK as well as ERK activation (Fig. 3A and B, lane 3). Conversely, whereas NAC did not affect the levels of phosphorylated p38 MAPK, exposure to either DPI or the nitric oxide synthetase inhibitor l-NAME (which does not seem to appreciably affect Rat1 cell proliferation) substantially reduced the phosphorylation of this MAPK (data not shown).
Inhibition of Cell Cycle Progression by DPI

To further examine the antiproliferative activity of DPI, its effect on cell cycle progression was examined by flow cytometry of PI-stained cells. Exposure of Rat1 fibroblasts and NIH 3T3 cells to DPI led to a dose-sensitive increase in the number of cells with 4N DNA content (Fig. 4A; data not shown). In the absence of treatment, there were 62% 2N and 22% 4N Rat1 cells, whereas at 4 μmol/L DPI this distribution shifted to 36% 2N and 48% 4N DNA content (Fig. 4B). Accumulation of cells with 4N DNA content, as determined by PI flow cytometry, suggests that DPI affects cell cycle progression.

DPI Blocks Entry into Mitosis

Mitotic cells were clearly detectable by anti-β-tubulin and Hoescht fluorescence microscopy of untreated Rat1 cells (Fig. 5A, 1). However, essentially no mitotic cells were discernable following DPI treatment (Fig. 5A, 2). Similar to other microtubule perturbing compounds, such as paclitaxel, the antiproliferative activity of DPI also correlates with an accumulation of cells with 4N DNA content. The ability of DPI to block mitosis was therefore determined by treating cells with both DPI and paclitaxel. The ability of paclitaxel-treated cells to reach mitosis in the absence or presence of DPI was assayed by anti-β-tubulin and Hoescht fluorescence microscopy. Paclitaxel-treated cells were able to form partially assembled mitotic spindles (as evidenced by the anti-β-tubulin staining) and could condense their chromatin (as evidenced by the Hoescht staining; Fig. 5A, 3). Following 8 hours of exposure to paclitaxel, over 30% of the cells entered mitosis (Fig. 5B). By contrast, cells treated with paclitaxel and DPI failed to either condense their chromatin or rearrange their interphase microtubule array into a mitotic spindle alignment (Fig. 5A, 4). Paclitaxel-mediated mitotic arrest was reduced ~6-fold by exposure to 5 μmol/L DPI, whereas 10 μmol/L DPI fully abolished the entry into mitosis (Fig. 5B). This indicates that DPI can fully block mitotic cell division and that this occurs by a DPI-mediated cell cycle arrest prior to mitosis.

DPI Treatment Results in Centrosome Separation

Failure of DPI-treated cells to reach mitosis indicates that they have become arrested in the cell cycle prior to M phase.
To further characterize this cell cycle arrest, DPI-treated Rat1 and NIH 3T3 cells were stained with anti-g-tubulin to label centrosomes. During most of the cell cycle, centrosomes remain relatively near to each other and centrosome disjunction usually only occurs toward the end of G2 phase prior to the onset of mitosis (12). Anti-g-tubulin immunofluorescence staining of cells revealed that, whereas the centrosomes remained unseparated in the majority of untreated cells (Fig. 6A, 1), in most of the DPI-treated cells the centrosomes seemed to have separated (Fig. 6A, 2).

Indeed, in the presence of DPI, centrosomes of Rat1 cells, and NIH 3T3 cells in particular, were frequently aligned at opposite sides of the nucleus as evidenced by the frequent occurrence of intercentrosomal distances approximating the mean nuclear diameter (Fig. 6B; data not shown). This suggests that in the presence of DPI the cell cycle may continue up to late G2, at which point further cell cycle progression is blocked.

**Activation of the Centrosome-Associated Aurora-A Kinase Does Not Occur in DPI-Treated Cells**

Indicative of a G2-M arrest in the cell cycle, exposure to DPI leads to the accumulation of cells with 4N DNA content and fully separated centrosomes. Failure of these cells to progress into mitosis suggests that DPI may impair mitotic functions of the centrosomes. This was probed by assaying for mitotic activation of the centrosome through Thr288 phosphorylation of the Aurora-A kinase (13). Whereas Thr288 phosphorylated Aurora-A was localized to mitotic spindle poles from prophase to metaphase (Fig. 7A, 1-3), no Thr288 phosphorylated Aurora-A was detected, by immunofluorescence microscopy, on the fully separated centrosomes of DPI-treated cells (Fig. 7A, 4-6). Similarly, Western blots revealed that, whereas paclitaxel treatment caused an enrichment in Thr288 phosphorylated Aurora-A, exposure to DPI did not result in an increase in cellular levels of Thr288 phosphorylated Aurora-A (Fig. 7B). Failure of Thr288 phosphorylated Aurora-A to accumulate on the centrosomes of DPI-treated cells hence seems to be due to a DPI-mediated block in the cell cycle prior to Aurora-A Thr288 phosphorylation.

**DPI Reversibly Impairs Accumulation of Cyclin B1, Aurora-A Thr288 Phosphorylation, and Mitotic Entry**

Prior to mitotic centrosome activation at the G2-M transition, cell cycle progression through G2 to mitosis is accompanied by an accumulation of cyclin B1. Hence, anti-cyclin B1 Western blotting indicated that, essentially in parallel with mitotic arrest, paclitaxel treatment led to a pronounced increase in intracellular levels of cyclin B1.
To examine how DPI may block cell cycle progression from G2 to M phase, its effect on the accumulation of cyclin B1 was examined. Whereas DPI led to a progressive increase in the number of cells with 4N DNA content (see Fig. 4A and B), the accumulation of cyclin B1 was impaired (Fig. 8A).

Following removal of the DPI, cyclin B1 and Thr 288 phosphorylated Aurora-A could accumulate (Fig. 8B) and the cells could also enter into mitosis as determined by Hoescht staining of the condensed chromatin (Fig. 8C). The effects of DPI on cyclin B1 levels and cell cycle progression are hence not due to an irreversible nonspecific toxicity. Curiously, DPI did not appreciably affect the levels of cyclin A (Fig. 8B).

DPI Decreases the Levels of Cyclin B1 and Thr 288 Phosphorylated Aurora-A in Paclitaxel-Arrested Cells

To further probe the effect of DPI on mitotic events, cells were treated with paclitaxel prior to exposure to DPI. The paclitaxel caused mitotic arrest and a pronounced accumulation of cyclin B1 (Fig. 9A). Exposure of these paclitaxel-treated cells to DPI caused a substantial loss of cyclin B1, reaching levels comparable with (or less than) untreated cells 6 hours after the addition of DPI to the culture medium (Fig. 9A). Exposure of the paclitaxel-treated cells to DPI similarly led to a decrease in the levels of Thr 288 phosphorylated Aurora-A (Fig. 9B).

Loss of these mitotic protein markers by exposure to both paclitaxel and DPI was not due to progression of the cells from M to G1 phase because flow cytometric analysis of PI-stained cells revealed that they continued to have a mostly 4N DNA content in the presence of paclitaxel alone or in combination with DPI (Fig. 9C).

DPI Causes Mitotic Catastrophe in Paclitaxel-Arrested Cells

Phase-contrast microscopy of cells exposed to either paclitaxel alone (Fig. 10A, 1 and 5) or paclitaxel followed by the addition of DPI (Fig. 10A, 2 and 6) indicated that, in addition to the loss of cyclin B1 and Thr 288 phosphorylated Aurora-A, exposure to DPI also caused a striking reversal of the rounded and translucent mitotic phenotype. By fluorescence microscopy of Hoescht staining, it was...
determined that, characteristic of mitotic catastrophe, the loss of the rounded and translucent mitotic phenotype was accompanied by chromatin decondensation and micronucleation (Fig. 10A, 4 and 8). By counting the number of paclitaxel-arrested cells with condensed chromatin prior to and following exposure to DPI, the DPI-induced mitotic exit seems to be very pronounced (Fig. 10B).

Discussion
The results presented here show that the flavoprotein inhibitor DPI can inhibit G2 cell cycle progression. A range of antioxidants, including DPI, have been reported to inhibit proliferation of both untransformed and cancerous cells (6, 7), and this inhibition is thought to occur by reduction in intracellular ROS levels. By targeting catalytic cysteine residues, ROS can inactivate phosphatases and thereby affect numerous cell signaling pathways (4). In accordance with previous reports (14, 15), the results presented here show that the antioxidant NAC may inhibit mitotic cell division by decreasing mitogenic signaling by MAPK. This leads to an accumulation of cells in the G1 phase (2N DNA content) of the cell cycle and a slowing of cell cycle progression. By contrast, although DPI has a comparable antioxidative potential, it leads to an accumulation of cells with 4N DNA content. Additional differences between NAC and DPI are apparent as NAC, but not DPI, suppresses MAPK activity. The antiproliferative effects of NAC and DPI therefore seem to be mechanistically distinct and may not necessarily be due to effects on MAPK.

Figure 7. Activation of the centrosome-associated Aurora-A kinase does not occur in DPI-treated cells. A, Rat1 fibroblasts were either left untreated (1-3) or exposed for 8 hours to 10 μmol/L DPI (4-6) and processed for fluorescence microscopy of anti-γ-tubulin (1 and 4), Thr288 phosphorylated Aurora-A (2 and 5), and Hoescht (3 and 6). Bar, 20 μm. B, Rat1 fibroblasts were left untreated (lane 1), serum starved (lane 8), or exposed to either 10 μmol/L DPI (lanes 2-4) or 1 μmol/L paclitaxel (Tx; lanes 5-7) for 2, 4, and 8 hours. The cells were then lysed and subjected to SDS-PAGE/Western blot using anti-Thr288 phosphorylated Aurora-A and anti-MAPK antibodies (as a loading control).

Figure 8. DPI reversibly impairs accumulation of cyclin B1, Aurora-A Thr288 phosphorylation, and mitotic entry. A, Rat1 fibroblasts were either left untreated (lane 1), serum starved (lane 8), or exposed to either 10 μmol/L DPI (lanes 2-4) or 1 μmol/L paclitaxel (Rx; lanes 5-7) for 2, 4, and 8 hours. The cells were then lysed and subjected to SDS-PAGE/Western blot using anti-Thr288 phosphorylated Aurora-A and anti-MAPK antibodies (as a loading control). B, Rat1 fibroblasts were left untreated (lane 1) or were exposed to either 10 μmol/L DPI for 8 hours (lane 2) followed by release from the DPI for 3 and 6 hours (lanes 3 and 4) or 1 μmol/L paclitaxel 4 and 8 hours (lanes 5 and 6). The cells were then lysed and subjected to SDS-PAGE/Western blot using anti-cyclin B1 and anti-MAPK antibodies (as a loading control). C, Rat1 fibroblasts were exposed for 8 hours to 10 μmol/L DPI and processed for fluorescence microscopy of Hoescht staining prior to (1) and following (2) a 6-hour release from the DPI. Bar, 50 μm.
Interestingly, unlike cells arrested by microtubule drugs, the DPI-treated cells fail to enter mitosis. Indeed, even in the presence of paclitaxel, DPI can fully block cell cycle progression to mitotic prophase. The block of DPI-mediated cell cycle progression hence seems to be due to failure of the cells to undergo the transition from G2 to M phase.

A striking consequence of DPI treatment is the appearance of cells with fully separated centrosomes. Following their duplication during S phase, centrosomes usually remain tethered near to each other until the cells approach the G2-M transition prior to mitotic entry (12). Hence, usually only a relatively small fraction of the interphase cells have fully separated centrosomes because this is

Figure 9. DPI decreases the levels of cyclin B1 and Thr288 phosphorylated Aurora-A in paclitaxel-arrested cells. MCF-7 and HepG2 cells were either left untreated (lanes 1 and 4) or treated with 1 μmol/L paclitaxel for 8 hours followed by an additional incubation for 6 hours in the absence (lanes 2 and 5) or presence (lanes 3 and 6) of 10 μmol/L DPI. The cells were then lysed and subjected to SDS-PAGE/Western blot using anti-cyclin B1 (A) or Thr288 phosphorylated Aurora-A (B). Anti-MAPK antibodies are presented as loading controls. Following overnight treatment with 0.5 μmol/L paclitaxel, MCF-7 and human mammary epithelial cells (HMEC) were incubated for 6 more hours in the absence (blue) or presence (green) of 10 μmol/L DPI. The paclitaxel/DPI-treated cells and untreated cells (red) were then processed for PI flow cytometry (C).

Figure 10. DPI causes mitotic catastrophe in paclitaxel-arrested cells. A, MCF-7 (1-4) and human mammary epithelial cells (5-8) were treated overnight with 0.5 μmol/L paclitaxel and then incubated for an additional 6 hours in the absence (1, 3, 5, and 7) or presence (2, 4, 6, and 8) of 10 μmol/L DPI. The cells were then visualized by phase-contrast microscopy (1, 2, 5, and 6) and fluorescence microscopy of Hoechst staining (3, 4, 7, and 8). Bar, 50 μm for the phase-contrast images and 25 μm for the fluorescence images. B, percentage of mitotic paclitaxel-arrested Rat1, MCF-7, and human mammary epithelial cells was determined (by the number of cells with condensed chromatin) following a 6-hour incubation with and without 10 μmol/L DPI.
usually followed by assembly of the mitotic spindle and cell division. Centrosome separation is affected by a range of kinases (16, 17), and their mitotic activation is under the control of several cell cycle–regulated kinases (18). The NIMA kinase Nek1 is involved in centrosome separation (19), whereas Aurora-A and the polo-like kinase Plk1 regulate the centrosome-mediated G2-M transition and assembly of mitotic spindle microtubules, respectively (1). Binding of activated Aurora-A kinase to centrosomes permits subsequent recruitment and activation of cyclin B-cdk1 and entry into mitosis (13). Although the centrosomes of DPI-treated cells seem poised for entry into mitosis, their failure to assemble mitotic spindle microtubules suggests that DPI compromises their mitotic activation. This is further suggested by the finding reported here that DPI blocks the centrosomal accumulation of activated Aurora-A kinase.

The failure of the cells to initiate the G2-M transition seems related to the DPI-mediated down-regulation of cyclin B1 levels. The ability of DPI to selectively impair the accumulation of cyclin B1 could underlie its potent antiproliferative activity. Interestingly, DPI can cause an override of the metaphase checkpoint-mediated arrest. Although this is accompanied by down-regulation of cyclin B and Thr288 phosphorylated Aurora-A levels, it does not involve mitotic segregation of the chromatin. Exposure of paclitaxel-arrested cells to DPI can hence induce tetraploidy by mitotic catastrophe. Although much remains to be clarified regarding the action of DPI on the cell cycle, the ability of DPI to block mitotic cell division has exposed a potent and novel antiproliferative activity that may have significant implications for the targeting of cancerous cells by tetraploidization (9).

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