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Bistratene A Induces a Microtubule-Dependent Block in Cytokinesis and Altered Stathmin Expression in HL60 Cells

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Bistratene A is a cyclic polyether which affects cell cycle progression and can induce phosphorylation of cellular proteins. Treatment of HL60 cells with 100 ng/ml bistratene A was found to inhibit cytokinesis but had no effect on DNA synthesis and nuclear division. Consequently, bistratene A-treated cells became polyploid and multinucleate. In association with the development of this phenotype, the cytoplasmic protein stathmin was biphasically phosphorylated and levels of expression were doubled. Immunostaining of binucleate cells (bistratene A for 24 h) revealed increased α-tubulin localization where the cleavage furrow might be expected to form, i.e., along the equatorial plane. Treatment of these binucleate cells with the microtubule depolymerizing agent nocadazole promoted cleavage furrow formation and partially ameliorated the bistratene A-induced block in cell division. These findings implicate the polymerization status of microtubules and stathmin function in the regulation of cytokinesis.

Progression through the cell cycle is coordinated via changes in levels of protein expression, protein phosphorylation and direct protein:protein interactions [1–3]. These mechanisms affect the function of key regulatory protein kinases and phosphatases, but also act on cytoskeletal proteins which provide the framework enabling cell division to proceed [4–7].

Bistratene A is a cyclic polyether derived from the marine ascidian *Lissoclinum bistratum* [8]. Depending on cell type and dosage, bistratene A has been shown to induce cell differentiation [9, 10], alter cell morphology [9, 11] and inhibit cell proliferation [12, 13]. In addition, bistratene A treatment leads to activation of protein kinase Cδ [12] and results in increased protein phosphorylation [12, 14]. Bistratene A may also affect cytoskeletal structures [9, 15]. HL60 promyeloid leukemic cells treated with bistratene A growth arrest in G2/M phases of the cell cycle [12, 16] and are unable to undergo cytokinesis [16]. This inhibition of cytokinesis does not prevent further DNA synthesis or nuclear division and results in an accumulation of polyploid and multinucleate cells [16]. Human lymphocytes treated with bistratene A undergo DNA damage and become polyploid [15]. Furthermore, whilst bistratene A treatment induces morphological changes and melanin synthesis in MM96E melanoma cells, a proportion of treated cells become multinucleate [9]. It is clear, therefore, that although bistratene A induces a variety of responses in different cells, a consistent effect is inhibition of cytokinesis, suggesting impingement on a common regulatory pathway. However, the mechanisms which result in this block in cell division are currently unknown. The studies described here were undertaken to determine how bistratene A induces inhibition of HL60 cell cytokinesis.

MATERIALS AND METHODS

Cell culture. Stock cultures of HL60 cells [17] were maintained in RPMI 1640 tissue medium (Gibco, Paisley, UK) supplemented with 10% fetal calf serum (Gibco) and in exponential growth at a density of between 2.5×10^5 cells/ml and 10×10^5 cells/ml. In experiments, HL60 cells were seeded into 6 well plates (Nunc, Gibco) at a density of 3×10^5 cells/ml and treated with bistratene A at a variety of doses. Bistratene A was isolated from *L. bistratum* as previously described [8]. A stock solution of 1 mg/ml bistratene A was prepared in dimethyl sulfoxide (Fisons Scientific Equipment, Loughborough, UK)

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and kept at −20°C. This stock was freshly diluted into tissue culture medium for each treatment. Control HL60 cell cultures were established in medium containing carrier alone.

HL60 cell growth, differentiation and morphology. The growth kinetics and viability of bistratene A-treated and control HL60 cell cultures were assessed by phase-contrast microscopy. A minimum of 400 cells/sample were enumerated as viable or non-viable. The differentiation status of HL60 cells was determined by staining cytocentrifuge preparations (cytospins) for α-napthyl acetate esterase (ANAE) activity [18] and analysis of the ability of cells to phagocytose complement-coated yeasts [19]. Cellular morphology was examined by staining cytospins with Jenner and Giemsa stain.

The DNA content of cells was determined as follows: One million cells were harvested from cultures and washed twice with phosphate buffered saline (PBS). These cells were then suspended in 200 μl of PBS prior to the vigorous addition of 2 ml of an ice-cold mixture of 70% ethanol and 30% PBS (v/v). The resultant cell suspension was stored at 4°C for 30 min. Cells were then re-harvested and resuspended in 800 μl of PBS and checked microscopically to ensure there was no cell clumping. One hundred μl of RNase (at 1 mg/ml, Sigma, Poole, UK) and 100 μl of propidium iodide (PI at 400 μg/ml, Sigma) were added to the suspension for 30 min at 37°C. PI stained cells were analyzed using a FACS IV flow cytometer (at a wavelength of 488 nm). The resultant histogram of DNA content was gated and analyzed using the Lacel single parameter system to determine the proportions of cells which were 2, 4, 8, and 16N.

Gel electrophoresis and Western blotting. To compare levels of stathmin protein expressed by control and bistratene A-treated HL60 cells, cell extracts were separated by one dimensional sodium dodecyl sulfate–polyacrylamide gel electrophoresis (1D SDS–PAGE). Twelve percent separating gels were used prior to Western blotting and immunodetection with antibody to stathmin and ^{125}I-labeled protein A, as described previously [20]. Gel lanes were loaded with an equivalent amount of total cellular protein. The degree to which stathmin was phosphorylated within HL60 cells was assessed by performing two dimensional gel electrophoresis prior to Western blotting and immunodetection [20]. Scanning densitometry of Western blots was performed to determine relative levels of overall stathmin expression and of the nonphosphorylated and phosphorylated forms of stathmin.

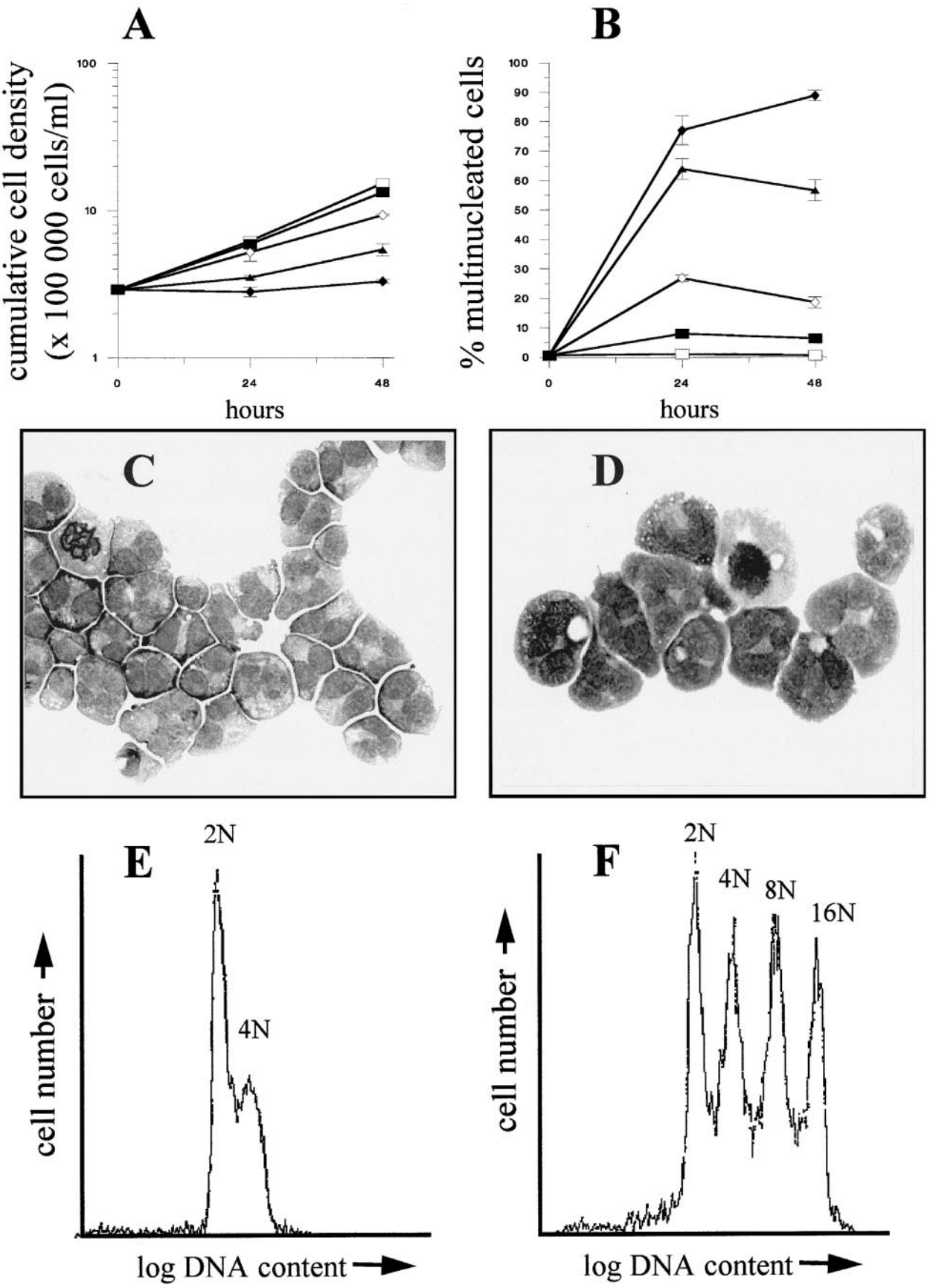
Immunostaining for α-tubulin. Cytospins were fixed in methanol and incubated for 1 h with a monoclonal antiserum specific for α-tubulin (B 1-5-2, Sigma) diluted 1/1000 in PBS containing 10% normal goat serum (Binding Site, Birmingham, UK). Following washes in PBS, a secondary FITC-labeled goat anti-mouse IgG (1/50, Bradsure Biologicals Ltd., Loughborough, UK) was applied to the cytospins for 1 h. Further washes in PBS were performed prior to counterstaining with Hoescht (bis-benzamide, No. 33342, Sigma). Alpha tubulin immunostaining was visualized by the use of a fluorescence microscope (Zeiss Axioplan microscope). An irrelevant and isotype-matched mouse monoclonal was used as a control primary antibody (Dako, Cambridge, UK; data not shown). Photographed and confocal microscope images (MRC Confocal Microscope, Bio-Rad, UK) of the α-tubulin immunostaining were subsequently digitized prior to lettering and printing.

Treatment with nocodazole and Taxol. Following treatment of HL60 cells for 24 h with 100 ng/ml bistratene A, cultures were treated with the microtubule depolymerizing agent nocadazole (Sigma) at a final concentration of 125 nM, or with the microtubule stabilizing agent paclitaxol (Taxol, Sigma) at a final concentration of 250 nM, or with carrier alone, for a period of 1 h at 37°C. These cultures were then photographed using an inverted microscope and time lapse images were collected using a video camera and computer package (SC Camera II, Fast Multimedia A.G., ImagoMicro, Manchester, UK). Photographs and captured images were subsequently digitized prior to lettering and printing. The morphology of treated cells was further determined by staining cytospins with Jenner and Giemsa.

RESULTS

100 ng/ml Bistratene A specifically inhibits HL60 cell cytokinesis. Treatment of HL60 cells with Bistratene A resulted in a dose dependent decrease in cell proliferation (Fig. 1A). This was associated with an increase in the proportion of multinucleate cells (Fig. 1B). Control cultures of HL60 cells grew from a seeding density of 3×10^5 cells/ml to a cumulative total of $15.3 \pm 0.8 \times 10^5$ cells/ml after 48 h. The proportion of multinucleate control cells, at any time point, did not exceed $1 \pm 0.5\%$ (all values are the mean $\pm$ standard error, $n \geq 3$ independent experiments). By contrast, the cell density in cultures treated with 100 and 200 ng/ml bistratene A for 48 h were $5.4 \pm 0.5 \times 10^5$ cells/ml and $3.3 \pm 0.1 \times 10^5$ cells/ml, respectively. Furthermore, after 24 h of treatment with 100 and 200 ng/ml bistratene A, the proportions of multinucleate cells were 64 ± 4 and $77 \pm 5\%$, respectively. Slight decreases in the frequency of multinucleate cells were observed in cultures treated with bistratene A for 48 h at all doses except 200 ng/ml (Fig. 1B). Bistratene A treatment resulted in only a slight decrease in cell viability. Following 48 h of treatment with 100 or 200 ng/ml bistratene A, the proportions of viable cells were 92 ± 0.4 and $91 \pm 0.4\%$, respectively, compared to $98 \pm 1.2\%$ in control cultures. Bistratene A did not result in expression of ANAE activity at any dose tested. There was an increase in the phagocytic activity of HL60 cells treated with 200 ng/ml of bistratene A; at 48 h, $7.9 \pm 1.9\%$ of treated cells were phagocytic compared with $0.3 \pm 0.2\%$ of control cells. But other doses of bistratene A did not induce HL60 cells to become phagocytic.

The appearance of HL60 cells treated with 100 ng/ml bistratene A for 24 and 48 h shows that the number of nuclei per multinucleate cell increased over this time period (Figs. 1C and 1D). In these treated cultures, the proportion of multinucleate cells containing greater than 2 nuclei per cell increased from $5 \pm 0.8\%$ after 24 h to $58 \pm 2.5\%$ after 48 h. This observation indicates that although bistratene A-treated cells were unable to complete cytokinesis, DNA synthesis and nuclear division were not affected. To confirm that DNA synthesis was unaffected, the ploidies of control and 100 ng/ml bistratene A-treated cells were determined by propidium iodide staining and flow cytometry (Figs. 1E and 1F). The proportions of control cells which contained 2N and 4N of DNA at 48 h in culture were $49 \pm 2.4\%$ and $17.6 \pm 1.6\%$, respectively. At no time did control cells contain greater than 4N DNA. By contrast, the DNA content of bistratene A-treated cells increased such that after 24 h $15 \pm 0.4\%$ of cells contained 2N, $24 \pm 0.4\%$ contained 4N, and $40 \pm 2.3\%$ contained 8N DNA. After 48 h of treatment, $21 \pm 2.2\%$ of cells contained 2N, $19 \pm 0.8\%$ contained 4N, $14 \pm 1.8\%$ contained 8N and $7 \pm 2.3\%$ contained 16N DNA.


A
cumulative cell density
(x 100 000 cells/ml)
100
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B
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8N
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It is clear, therefore, that 100 ng/ml bistratene A-treated HL60 cells did not undergo cytokinesis, but otherwise remained in cell cycle and underwent successive rounds of endoreduplication.

Stathmin is phosphorylated and upregulated in response to bistratene A. Stathmin was biphasically phosphorylated following treatment of HL60 cells with bistratene A (Fig. 2A). In control cells, stathmin was present predominantly in a non-phosphorylated form, termed n (82 ± 5% of total stathmin), and to a lesser extent as a phosphoform, termed p1 (18 ± 5% of total stathmin). This pattern of stathmin phosphorylation was unchanged throughout the observed 48 h time course of control cultures and has been reported previously in exponentially growing HL60 cells [21]. By contrast, stathmin was rapidly phosphorylated following treatment with 100 ng/ml bistratene A. After 30 minutes, the proportion of stathmin in the p1 phosphoform had increased to 35 ± 2% of total stathmin. A more acidic phosphoform, termed p2, was also detected at this time and constituted 5 ± 1% of total stathmin. Subsequently, there was a transient decrease in the proportion of phosphorylated stathmin in treated cells such that the p2 phosphoform was no longer detected and the proportion of the p1 form was comparable to control values. This decrease was followed by a second period during which elevated levels of phosphorylated stathmin were observed. After 24 h of 100 ng/ml bistratene A treatment, the p1 and p2 phosphoforms constituted 42 ± 5% of total stathmin, and following 48 h of treatment, although the p2 phosphoform was not detected, 35 ± 7% of the protein was in the p1 phosphoform.

One dimensional SDS–PAGE and Western blotting were performed to compare levels of total stathmin expressed (whether phosphorylated or non-phosphorylated) in bistratene A-treated and control HL60 cells. Increased levels of stathmin were observed within 12 h of the addition of bistratene A to HL60 cultures, when the proportion of multinucleate cells was 5 ± 1%. After 24 h, stathmin expression in treated cells had increased to levels which were 205 ± 25% of that expressed in control cells, and at 48 h, stathmin levels in treated cells remained approximately double control levels. Within control cell cultures, stathmin levels did not vary over the 48 h time course by greater than 18% of time zero values.

Recent reports have suggested that an important phosphodependent function of stathmin is to regulate the stability of microtubules [22, 30–34]. We therefore proceeded to investigate whether bistratene A treatment affected microtubule structures within HL60 cells.

Microtubule localization and stability within the equatorial plane of bistratene A-treated HL60 cells is associated with blocked cytokinesis. Following 24 h of 100 ng/ml bistratene A treatment, the majority of cells were binucleate (see above). Within these binucleate cells, intense α-tubulin immunopositivity was observed along the equatorial plane, i.e., between the two nuclei where the cleavage furrow might be expected to form (Fig. 3A). This localization of α-tubulin was confirmed by confocal microscopic analyses of sections of uniform depth through these cells (Fig. 3B). In cells containing greater than two nuclei, following 48 h of 100 ng/ml bistratene A treatment, intense α-tubulin immunostaining was observed at the center of cells (Fig. 3C). Mitotic spindles were occasionally observed in bistratene A-treated and control cell cultures, and multiple spindles were apparent in multinucleate cells (Fig. 3C). The frequency of cells containing mitotic figures, however, did not differ in control and bistratene A-treated cultures, indicating that cells were not arrested in mitosis (data not shown). The appearance of mitotic spindles nonetheless demonstrated that the α-tubulin monoclonal antibody immunostained microtubules, as has been reported previously [22]. Immunostaining of control cells for α-tubulin did not alter greatly over the 48 h time course. In some control cells, the immunopositivity was punctate and throughout the cytoplasm, whilst in other cells positivity was predominantly perinuclear to one side of the nucleus (Fig. 3D). This latter pattern of immunostaining may represent centrosome-associated microtubule structures.

Binucleate HL60 cells (100 ng/ml bistratene A for 24 h) were subsequently treated with nocodazole, which depolymerizes microtubules, or with Taxol, which stabilizes microtubules. Within an hour of treatment, nocodazole induced the formation of a cleavage furrow in the many of these cells (Figs. 4A and 4B). Furthermore, in a proportion of these nocodazole treated cells, cleavage furrow formation led to complete division into two daughter cells (Figs. 4C and 4D). Jenner and Giemsa staining of nocodazole treated cells further demonstrated that the microtubule depolymerizing agent partially restored the ability of binucleate cells to divide (Figs. 5A and 5B). Within 1 h of treat-

FIG. 1. Bistratene A specifically inhibits HL60 cell cytokinesis. (A) The growth kinetics of HL60 cells treated with carrier alone (control cells, □), 25 ng/ml (●), 50 ng/ml (♦), 100 ng/ml (▲) or 200 ng/ml (◆) bistratene A. Data are means ± standard errors of at least 3 independent experiments. (B) The proportion of HL60 cells which were multinucleate following treatment with bistratene A at these doses for the periods indicated. (C) 100 ng/ml bistratene A-treated HL60 cell morphology (Jenner and Giemsa staining, original magnification ×250) at 24 h. (D) 100 ng/ml bistratene A-treated HL60 cell morphology (Jenner and Giemsa staining, original magnification ×250) at 48 h. (E) Histogram of DNA content within control HL60 cells at 48 h. (F) Histogram of DNA content within bistratene A-treated HL60 cells at 48 h. Peaks corresponding to cells containing 2, 4, 8, and 16N DNA are indicated.

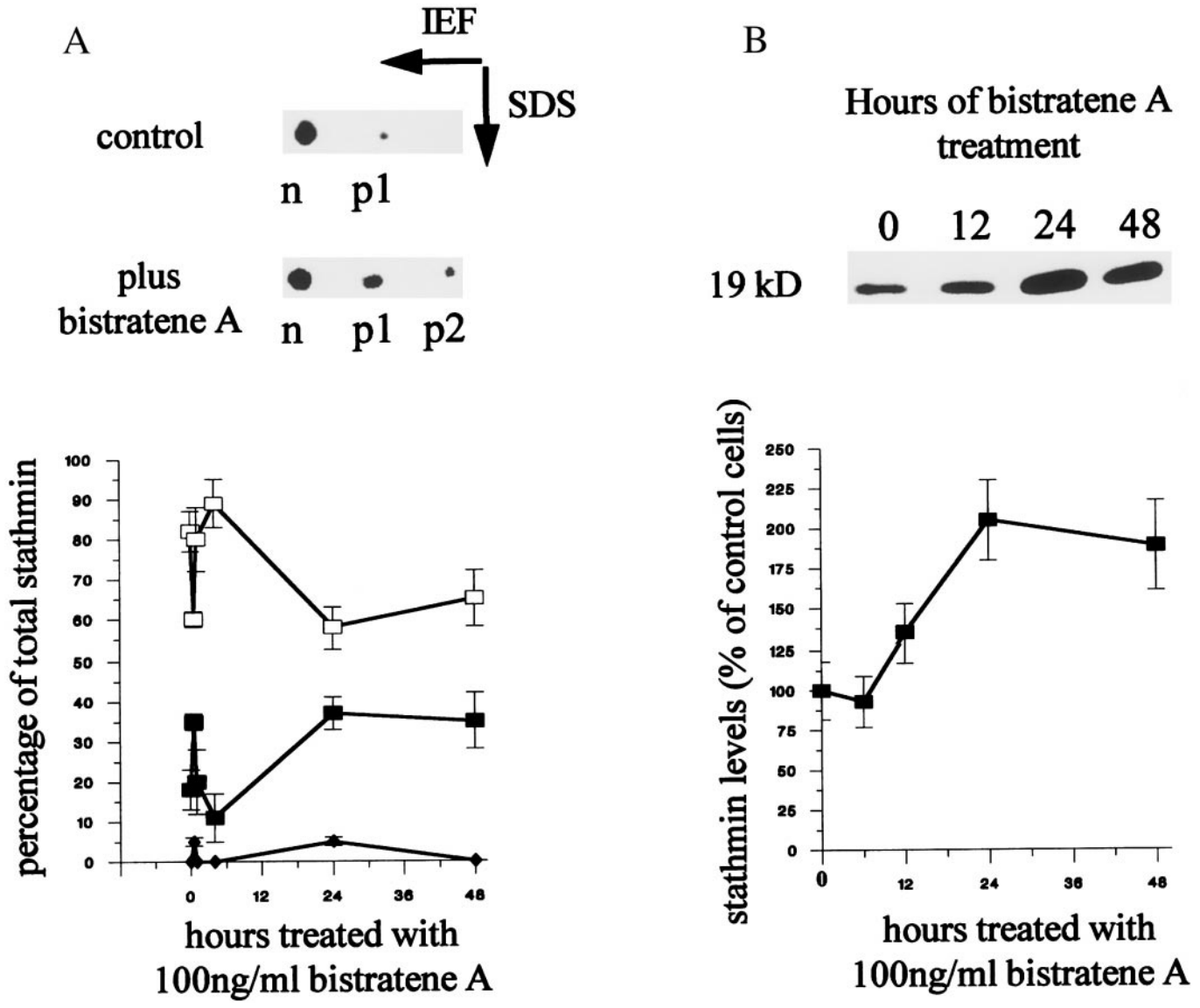

FIG. 2. Stathmin is biphasically phosphorylated and upregulated in response to bistratene A. (A) Top panel: representative autoradiograph of 2D gels immunoblotted for stathmin expression in control and 100 ng/ml bistratene A-treated HL60 cells at 24 h. n is nonphosphorylated, and p1 and p2 increasingly phosphorylated forms of stathmin. (B) Top panel: representative autoradiograph of 1D SDS gels immunoblotted for stathmin expression in 100 ng/ml bistratene A-treated HL60 cells at the times shown. Lanes were loaded for equal amounts of total cellular protein. Bottom panels show meaned data following scanning densitometry of 2D (A) and 1D (B) autoradiographs derived from at least 3 independent experiments. For the 2D data, □ represents the nonphosphorylated n form of stathmin, ■ the p1 phosphoform, and ◆ the p2 phosphoform.

ment, the proportion of nocodazole treated cells which were mononucleate increased by 15 ± 2%. The appearance of anucleate cytoplasmic parcels was also observed (Fig. 5B). Following 1 h of nocodazole treatment, anucleated cells represented 12 ± 3% of all cells, and their formation suggests that in some cells the cleavage furrow may have deviated from the equatorial plane. Binucleate cells treated with Taxol for 1 h did not form a cleavage furrow or divide (Fig. 5C). Furthermore, treatment of control cells with nocodazole for 1 h did not induce cleavage furrow formation, cytoplasmic division or the appearance of anucleate cytoplasmic parcels (Fig. 5D).

DISCUSSION

Treatment of HL60 cells with bistratene A has been shown to specifically inhibit the ability of the majority of cells to undergo cytokinesis. This inhibition led to a dose-dependent formation of polyploid multinucleate cells. Bistratene A treatment (100 ng/ml for 24 h) was

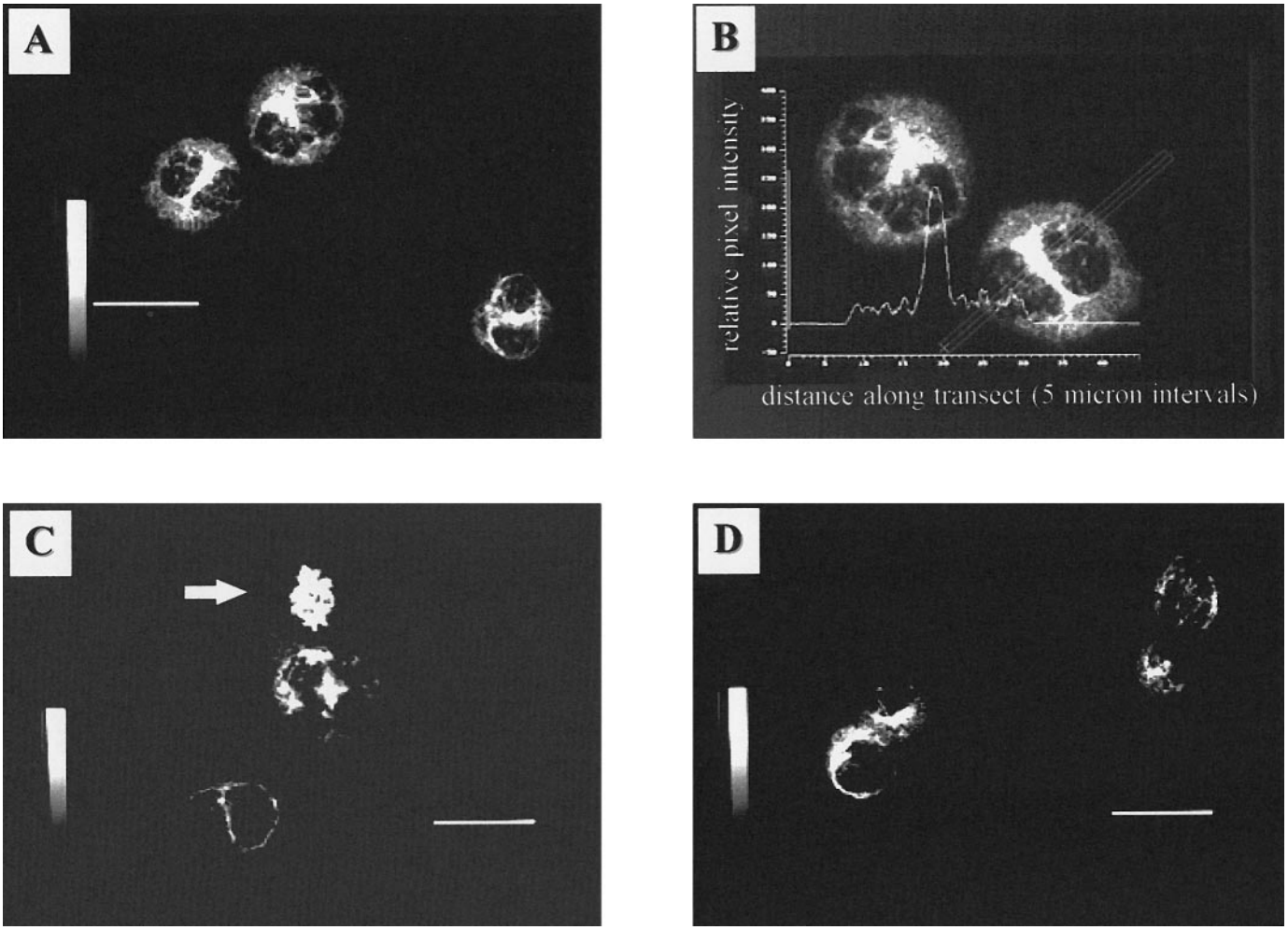

FIG. 3. Alpha-tubulin localizes within the cleavage furrow of binucleate HL60 cells. Immunostaining of HL60 cells for α-tubulin following treatment for 24 h (A) or 48 h (C) with 100 ng/ml bistratene A, and in control cells (D) (bar for all images = 25 μM). Arrow indicates mitotic spindles in a multinucleate cell (see main text). (B) Confocal microscopy analyses of the intensity of fluorescent immunostaining for α-tubulin (y axis, pixels) throughout a transverse section of uniform depth (x axis) in one of the binucleate HL60 cells shown in (A).

also associated with an accumulation of α-tubulin immunopositivity within the equatorial plane of binucleated cells. Subsequent treatment of these binucleated cells with a microtubule depolymerizing agent induced cleavage furrow formation and partially restored cell division. It has been concluded, therefore, that bistratene A treatment of HL60 cells causes a microtubule-dependent block in cytokinesis.

The function of microtubules in the regulation of cell cycle progression, particularly with regards to kinetochore and mitotic spindle activities during the transition of cells from G2 phase into mitosis, and from metaphase through anaphase, has been studied extensively [7, 23, 24]. However, the specific involvement of microtubules during cytokinesis is less well understood [25]. Prevention of sister chromatid separation and the formation of the spindle inter-zone has been shown to result in abnormal cleavage furrow formation and cytokinesis in rat kidney cells, leading to a suggested role for mid-zone microtubules in regulating cleavage activity [26]. The importance of mid-zone microtubules in regulating cytokinesis has also been implicated in *Caenorhabditis elegans* mutants null for the kinesin-like gene, *Zen*-4 [27]. Furthermore, in elegant studies using cultured epithelial cells, Cao and Wang [28] have shown that signals emanating from the mid-zone of the mitotic spindle during anaphase may regulate the formation of microtubules in the equatorial region of the cell and contractile activity. Interestingly, treatment of PtK1 cells during anaphase with the microtubule stabilizing agent, Taxol, has been found to retard cleavage furrow formation and cell division [29].

Bistratene A-induced cytokinetic arrest may provide a useful model for the study of microtubule function during this final act of cell division. The value of this model is that DNA synthesis and nuclear division apparently proceed as normal, allowing for analyses of the role that microtubules play in cell cleavage alone.

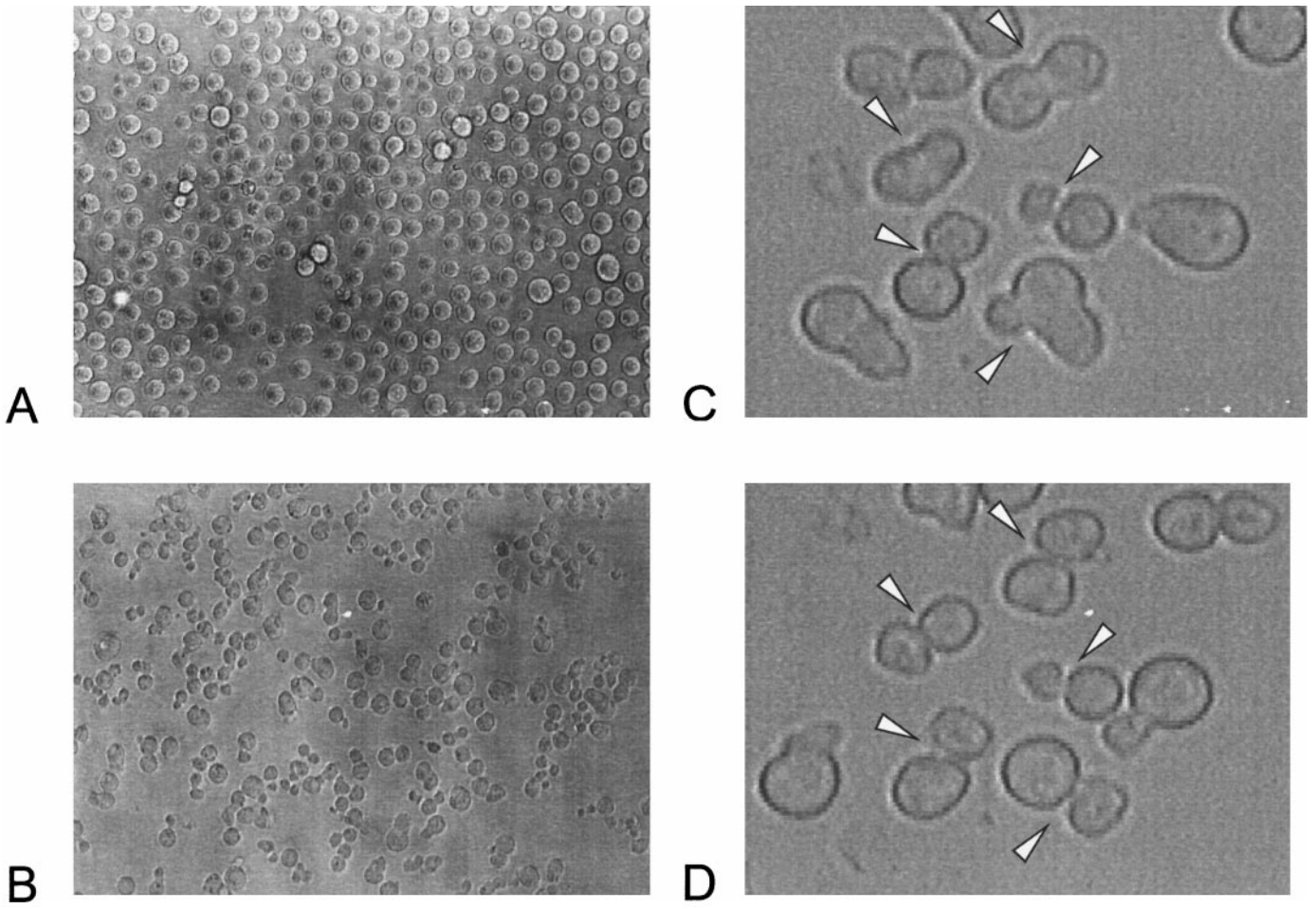

FIG. 4. The microtubule depolymerizing agent, nocodazole, induces cleavage furrow formation in bistratene A-treated HL60 cells. (A) 100 ng/ml bistratene A-treated HL60 cells (at 24 h) prior to the addition of nocodazole. (B) Bistratene A-treated HL60 cells, as in (A), following a subsequent treatment for 1 h with 125 nM nocodazole (original magnification for A and B, ×100). (C) and (D) Time lapsed images of cells, captured at 5 minute intervals, following nocodazole treatment (original magnification ×400). Arrows indicate cells in which the cleavage furrow formation is progressive, leading to division.

That the majority of the binucleate cells which arise following bistratene A treatment develop a cleavage furrow in response to nocodazole suggests not only that many of these cells are primed for cytokinesis, but also that dysfunctional microtubule stability largely accounts for the block in cell division.

The cause of microtubule localization and stabilization within the equatorial region of bistratene A treated HL60 cells is not known. But the observed changes in phosphorylation and expression of the cell cycle regulated protein stathmin are of particular interest. A number of recent studies have shown that non-phosphorylated stathmin binds directly with tubulin dimers thereby affecting microtubule stability [22, 30–34]. Both *in vitro* and *in vivo*, this interaction results in microtubules becoming destabilized, but is inhibited when stathmin is phosphorylated [22, 31, 32]. Ectopic expression of mutant forms of stathmin which cannot be phosphorylated results in a temporary block in the G2/M phase transition which has been associated with an inability to form mitotic spindles [22, 31]. It has therefore been suggested that phosphorylation of stathmin may be required early in metaphase in order to switch off its "microtubule-destabilizing" activity and thereby permit microtubule polymerization and formation of the spindle apparatus [31]. However, *in vitro* analyses of protein phosphatase activity in *Xenopus* egg extracts have demonstrated a role of protein phosphatase 2A-dependent stathmin dephosphorylation in maintaining the steady state of short microtubules during metaphase [35]. Furthermore, a recent study has shown that stathmin may also be dephosphorylated by protein phosphatases types 1, 2A, and 2B, as cells exit mitosis [36]. These findings suggest that the role of stathmin in regulating microtubule structures during mitosis is complex, but they allow for interpretation of the changes in stathmin detected in HL60 cells in response to bistratene A. The observed

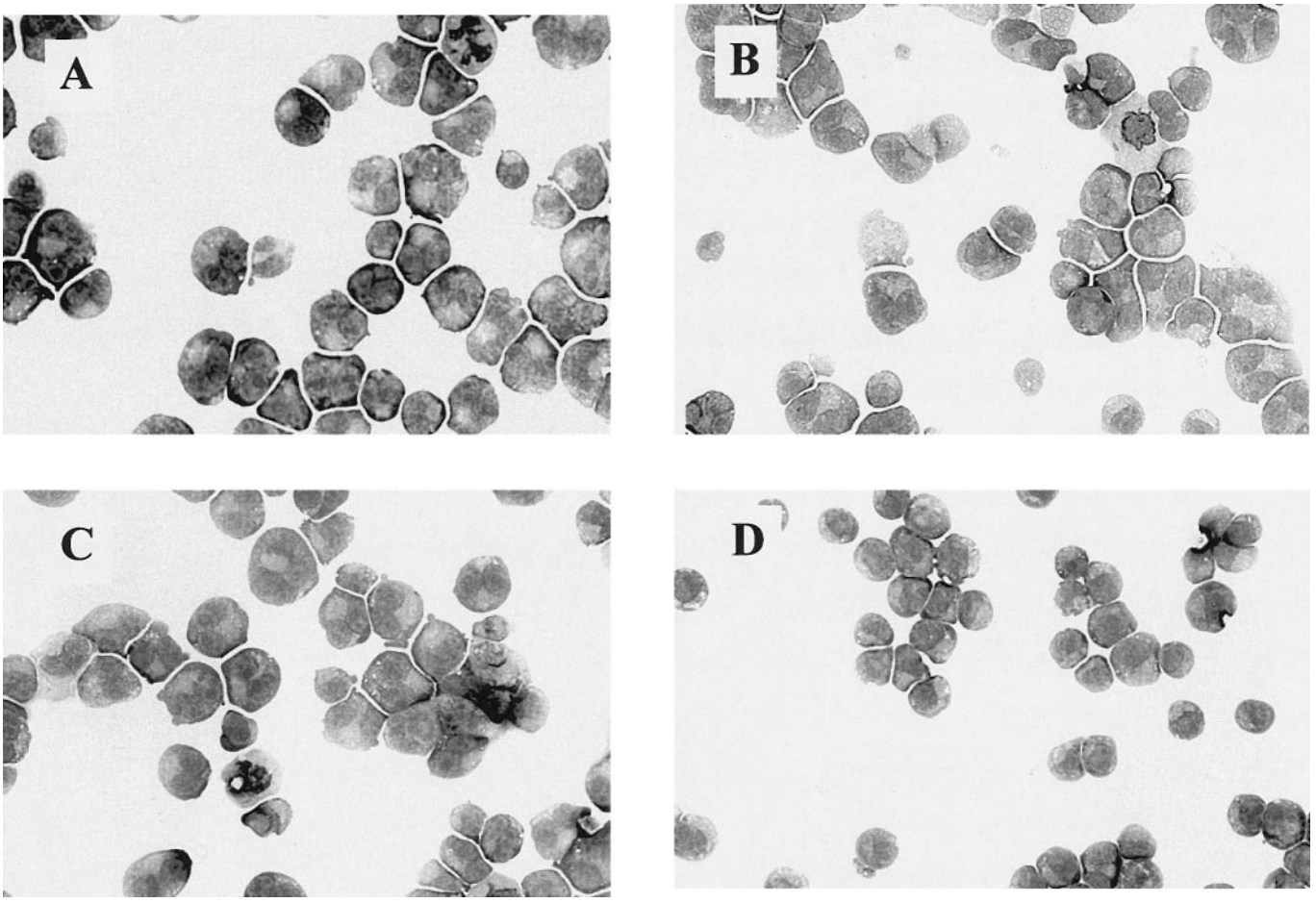

FIG. 5. Nocodazole induced cell division in binucleate bistratene A-treated HL60 cells. HL60 cells were stained with Jenner and Giemsa to reveal their morphology. (A) 24 h bistratene A-treated cells, subsequently treated with carrier alone for 1 h. (B) 24 h bistratene A-treated cells, subsequently treated with 125 nM nocodazole for 1 h. Note an increased incidence of mononucleate cells and the appearance of anucleate cytoplasmic parcels. (C) 24 h bistratene A-treated cells, subsequently treated with 250 nM Taxol for 1 h. (D) Control HL60 cells at 24 h, subsequently treated with 125 nM nocodazole for 1 h (original magnification ×250 for all images).

increase in stathmin phosphorylation following 24 h and 48 h of bistratene A treatment is consistent with the observed development of stable equatorial microtubules. Given the effects of nocodazole in promoting cell cleavage, stathmin dephosphorylation in binucleate cells would be predicted to result in increased microtubule destabilization and cell division. Such an interpretation allows us to form a putative model of stathmin function during normal cell cycle progression: wherein stathmin phosphorylation is required early in metaphase to permit microtubule stability and the subsequent formation of a functional mitotic spindle, but stathmin dephosphorylation is required during cytokinesis to permit microtubule destabilization, the consequent formation of a cleavage furrow and septation. Stathmin phosphorylation in response to bistratene A was, in fact, biphasic, with stathmin also becoming rapidly but transiently phosphorylated at 30 minutes posttreatment. It seems unlikely that this initial phosphorylation of stathmin could directly and mechanistically lead to an inability of cells to destabilize microtubulules during cell cleavage. It is also difficult to envisage how the elevation in stathmin expression which was observed at 24 h of bistratene A treatment could, in itself, contribute to increased microtubule stabilization and the inhibition in cell division. Indeed, this stathmin overexpression may represent a cellular response to the cytokinetic block, reflecting an attempt to raise the overall levels of non-phosphorylated stathmin and thus destabilize microtubules.

In conclusion, our study suggests that although during normal mitosis the dual processes of nuclear and cellular division are tightly orchestrated, their execution is at least in part separately controlled. Bistratene A treatment of HL60 cells allows the dissection of these processes and therefore will permit future studies of abnormal cytokinesis in the context of apparently normal chromosomal replication and nuclear division. In this regard the observed increase in α-tubulin staining seen in bistratene A treated cells and the subsequent

effects of nocadazole, indicate that the onset of cytokinesis is dependent on an appropriate depolymerization of equatorial microtubules. The mechanism by which microtubule destabilization is inhibited following bistratene A treatment is uncertain but the fact that increased stathmin phosphorylation is coincident with aberrant microtubule stability at this time suggests that this known modulator of microtubule function may have a role to play.

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