When C6 cells in culture were exposed at 37 °C to 1 μM colchicine or to 1 μM colcemid, a tubulin-binding antimitotic alkaloid, levels of αB crystallin in cells began to increase after about 10 h, reaching a maximum of more than 1 μg/mg protein after 24 h. The level of αB crystallin returned to near the control level within two subsequent days of culture in the normal medium. Northern blot analysis showed that the accumulation of αB crystallin was preceded by an increase in the level of the mRNA for αB crystallin. Nuclear run-off transcription assays showed that colchicine induced new synthesis of mRNA for αB crystallin. Immunofluorescence staining revealed that αB crystallin accumulated in the peripheral areas of cells, as did the depolymerized tubulin, after several hours of treatment with colcemid, and then it gradually became more conspicuous in the cytoplasm. Vinblastine and nocodazole, which also promote the disassembly of microtubules by binding to tubulins, also induced the synthesis of αB crystallin. Furthermore, induction of αB crystallin by these drugs was observed in quiescent cells that had been cultured in serum-free medium. However, taxol, a microtubule-stabilizing antimitotic agent, did not stimulate the synthesis of αB crystallin, but rather, it suppressed the induction of synthesis of αB crystallin by the microtubule-disrupting drugs. Induction of αB crystallin by colchicine or by other drugs that promote the disassembly of microtubules was sensitive to staurosporine, an inhibitor of protein kinases, and the induction was completely suppressed in the presence of 10 nM staurosporine. These results suggest that the expression of αB crystallin is stimulated, via phosphorylation reactions that are sensitive to staurosporine, when the depolymerization of microtubules is enhanced.

αB crystallin, a major structural protein of vertebrate lenses, is also expressed in various nonlenticular tissues such as central nervous tissues (1–6), and it is a member of the family of small stress or heat shock proteins. The expression of αB crystallin has also been observed under various stressful conditions, as is that of HSP27 (7–10). However, the expression of stress proteins is under the control of the cell cycle and that the transcription of genes for HSP70 (18) and HSP90 (19) increases during the synthetic (S) phase of the cell cycle.

We report here that agents that promote the depolymerization of microtubules also induce the synthesis and accumulation of αB crystallin, but not of HSP27 and HSP70, in rat C6 glioma cells in culture and that this process is sensitive to staurosporine, an inhibitor of protein kinases.

EXPERIMENTAL PROCEDURES

Reagents—Colchicine, colcemid, vinblastine sulfate, taxol, aphidicolin, hydroxyurea, staurosporine, and phorbol 12-myristate 13-acetate were obtained from Wako Pure Chemicals Co., Osaka, Japan. Nocodazole and forskolin were obtained from Sigma. Affinity-purified Texas red-labeled goat antibodies against rabbit IgG and affinity-purified fluorescein isothiocyanate-labeled goat antibodies against mouse IgG that had been absorbed with rat serum were purchased from Southern Biotechnology Associates, Inc. (Birmingham, AL) and Protos Immunoresearch (San Francisco, CA), respectively. The mouse monoclonal antibody against β-tubulin from physearum polypephalum myxamoebae was obtained from Boehringer Mannheim (Tokyo, Japan).

Culture and Treatment of Cells—C6 cells (obtained from the Jap- nese Cancer Research Resource Bank, Tokyo) were grown in Dulbecco’s modified Eagle’s medium (Nissui Pharmaceutical Co., Tokyo), supplemented with 10% fetal calf serum (Life Technologies, Inc., Tokyo) at 37 °C in a humidified atmosphere of 95% air and 5% CO₂. The cells were seeded in 35- or 90-mm dishes for large scale culture, and the medium was changed every 2 or 3 days. Cells that had grown to about 80% confluency (1.5–2 × 10⁶ cells/35-mm dish) were treated as follows.

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To whom correspondence should be addressed. Tel.: 81-568-88-0811 (ext. 3582); Fax: 81-568-88-0829.

The abbreviations used are: HSP27, mammalian small stress protein of 27–28 kDa; HSP70, inducible mammalian stress protein of 70 kDa; HSP90, mammalian stress protein of 90 kDa; PBS, phosphate-buffered saline; S, synthetic; M, mitotic.

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phase but which stabilizes and prevents the depolymerization of microtubules (29). Aphidicolin and hydroxyurea also failed to increase levels of αB crystallin. Western blot analysis clearly revealed the presence of αB crystallin in the insoluble fraction of cells treated with colchicine but not in that of cells that had been treated with taxol, aphidicolin, or hydroxyurea (data not shown). Colchicine and other drugs that enhance the disassembly of microtubules did not induce the accumulation of HSP27, another small stress protein (Table 1), or of HSP70, which was quantitated by Western blot analysis (data not shown).

The time course of the colchicine-induced accumulation of αB crystallin in cells is shown in Fig. 1A. After cells had been cultured for 9–10 h with 1 μM colchicine, the level of αB crystallin in the soluble extracts began to increase, reaching a maximal level after 48–72 h of treatment. A similar time course for the accumulation of αB crystallin was observed in the presence of 1 μM colchicine. When cells that had been exposed to colchicine for 24 h were washed and then cultured in normal medium, the level of αB crystallin returned to close to the control level within 2 days (Fig. 1A). The maximal accumulation of αB crystallin in cells that had been exposed to colchicine (Fig. 1B) or colchicine was seen when these drugs were present at 1–3 μM.

Northern Blot Analysis and in Vitro Nuclear Run-off Transcription Assays—Levels of mRNA for αB crystallin were determined by Northern blot analysis of cells that had been treated with colchicine. The accumulation of αB crystallin was preceded by increases in the level of mRNA for αB crystallin, and the mRNA was clearly detected after 7 h of exposure to colchicine (Fig. 2A). By contrast, mRNAs for HSP27 and HSP70 were barely detectable in the same samples (not shown).

In order to determine whether the increase in the level of mRNA for αB crystallin was due to increased synthesis de novo or to the accumulation of mRNA by some other mechanism, run-off transcription assays were performed with nuclei from C6 cells. As shown in Fig. 2B, exposure of cells to colchicine as well as to arsenite, which was included as a positive control, stimulated new transcription of the gene for αB crystallin. These results indicate that at least part of the increase in the

### Table 1

Effects of exposure to inhibitors of the progression of the cell cycle on levels of αB crystallin and HSP27 in C6 cells

| Addition          | αB crystallin (ng/mg protein) | HSP27 (ng/mg protein) |
|-------------------|------------------------------|-----------------------|
| None              | 32.5 ± 3.0                   | 0.075 ± 0.008         |
| Aphidicolin (3 μM)| 38.5 ± 3.9                   | 0.092 ± 0.009         |
| Hydroxyurea (10 mM)| 21.6 ± 2.0                   | 0.123 ± 0.006         |
| Taxol (0.2 μM)    | 21.2 ± 0.7                   | 0.091 ± 0.003         |
| Colchicine (1 μM) | 932 ± 147                    | 0.105 ± 0.003         |
| Colcemid (1 μM)   | 862 ± 128                    | 0.100 ± 0.003         |
| Vinblastine (1 μM)| 1200 ± 80                    | 0.102 ± 0.003         |
| Nocodazole (1 μM) | 686 ± 57                     | 0.098 ± 0.004         |

Previously (20) using affinity-purified antibodies (0.05 μg/ml) raised in rabbits against the carboxyl-terminal decapeptide of αB crystallin (6) and peroxidase-labeled antibodies raised in goat against rabbit IgG as second antibodies. Peroxidase activity on nitrocellulose sheets was visualized on x-ray film by use of a Western blot chemiluminescence reagent (Reanal; DuPont NEN).

Northern Blot Analysis and in Vitro Nuclear Run-off Transcription Assay—Cells cultured in 90-mm dishes were treated in duplicate. Total RNA was isolated from cells with an RNaseasy total RNA kit (Qiagen Inc., Hilden, Germany). Twenty μg of total RNA were subjected to electrophoresis on a 1.0% agarose, 2.2 μl formaldehyde gel and blotted onto a nitrocellulose membrane. For Northern blots, membranes were allowed to hybridize as described by Wahl et al. (22) with cDNA probes that had been labeled with a Multiprime DNA labeling system (Amersham Corp., Buckinghamshire, U.K.). A PstI fragment of cDNA for bovine αB crystallin (23) was kindly provided by Dr. H. Bloemendal of the University of Nijmegen. Nuclear run-off analysis was performed by the published procedure of Ausubel et al. (24). Briefly, nuclei from the lysed cells were incubated with 0.1 ml of [α-32P]UTP, and then nuclear RNA was prepared by phenol/chloroform extraction. A linearized cDNA probe for bovine αB crystallin was prepared as a set of slot blots, and it was allowed to hybridize with samples of nuclear RNA that contained equal amounts of radioactivity (about 2 × 106 cpm).

Immunofluorescence—Cells cultured on coverslips were fixed with 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.4, for 30 min at room temperature. Fixed cells were preincubated in 10% normal goat serum for 30 min to block nonspecific staining. For the staining of microtubules or αB crystallin, the cells were incubated with mouse monoclonal antibody against β-tubulin or affinity-purified rabbit antibodies against carboxyl-terminal peptide of rat αB crystallin for 30 min at room temperature. After washing with PBS, they were stained with fluorescein isothiocyanate-labeled antibodies against mouse IgG or Texas red-labeled antibodies against rabbit IgG. All specimens were observed under a confocal laser scanning microscope equipped with a krypton-argon laser (MRC1024; Bio-Rad, Watford, UK).

Other Methods—Concentrations of soluble protein in extracts were determined with a protein assay kit (Bio-Rad) with bovine serum albumin as the standard. Rat HSP27 and αB crystallin, which were used as the standards for immunoassays and electrophoresis, were purified from skeletal muscle (20, 25).

### Results

Effects of Exposure to Inhibitors of Progression of the Cell Cycle on the Levels of αB crystallin and HSP27 in C6 Cells—Proliferating C6 rat glioma cells in culture were exposed for 20 h at 37 °C to aphidicolin or hydroxyurea, inhibitors that act at the S phase of the cell cycle (26), or to colchicine, colcemid, vinblastine (27), nocodazole (28), or taxol (29), which are inhibitors that act at the mitotic (M) phase of the cell cycle. As shown in Table 1, levels of αB crystallin in soluble extracts of cells increased markedly in the case of cells that had been treated with colchicine, colcemid, vinblastine, or nocodazole, all of which interrupt the cell cycle at the early M phase by promoting the depolymerization of microtubules. In contrast, the levels of αB crystallin did not increase in cells that had been treated with taxol, which also interrupts the cell cycle at the M phase but which stabilizes and prevents the depolymerization of microtubules (29). Aphidicolin and hydroxyurea also failed to increase levels of αB crystallin. Western blot analysis clearly revealed the presence of αB crystallin in the insoluble fraction of cells treated with colchicine but not in that of cells that had been treated with taxol, aphidicolin, or hydroxyurea (data not shown). Colchicine and other drugs that enhance the disassembly of microtubules did not induce the accumulation of HSP27, another small stress protein (Table 1), or of HSP70, which was quantitated by Western blot analysis (data not shown).

The time course of the colchicine-induced accumulation of αB crystallin in cells is shown in Fig. 1A. After cells had been cultured for 9–10 h with 1 μM colchicine, the level of αB crystallin in the soluble extracts began to increase, reaching a maximal level after 48–72 h of treatment. A similar time course for the accumulation of αB crystallin was observed in the presence of 1 μM colchicine. When cells that had been exposed to colchicine for 24 h were washed and then cultured in normal medium, the level of αB crystallin returned to close to the control level within 2 days (Fig. 1A). The maximal accumulation of αB crystallin in cells that had been exposed to colchicine (Fig. 1B) or colchicine was seen when these drugs were present at 1–3 μM.

Northern Blot Analysis and in Vitro Nuclear Run-off Transcription Assays—Levels of mRNA for αB crystallin were determined by Northern blot analysis of cells that had been treated with colchicine. The accumulation of αB crystallin was preceded by increases in the level of mRNA for αB crystallin, and the mRNA was clearly detected after 7 h of exposure to colchicine (Fig. 2A). By contrast, mRNAs for HSP27 and HSP70 were barely detectable in the same samples (not shown).

In order to determine whether the increase in the level of mRNA for αB crystallin was due to increased synthesis de novo or to the accumulation of mRNA by some other mechanism, run-off transcription assays were performed with nuclei from C6 cells. As shown in Fig. 2B, exposure of cells to colchicine as well as to arsenite, which was included as a positive control, stimulated new transcription of the gene for αB crystallin. These results indicate that at least part of the increase in the
level of mRNA for \( \alpha B \) crystallin was due to the production of new mRNA.

**Indirect Immunofluorescence Localization of \( \alpha B \) Crystallin**—
When C6 glioma cells were treated with 1 \( \mu \)M colcemid, the profiles of the cells changed from spindle shaped to polygonal. Depolymerization of microtubules in the colcemid-treated cells was confirmed by indirect immunofluorescence staining of \( \beta \)-tubulin. The depolymerized tubulin was concentrated in the peripheral areas of the cytoplasm (Fig. 3B), in contrast to the typical appearance of the microtubules in nontreated cells (Fig. 3A). In nontreated C6 cells, \( \alpha B \) crystallin was detected only in the nucleoli (Fig. 3C). When the cells were treated with colcemid for 6 h, \( \alpha B \) crystallin accumulated in the peripheral areas of some but not all cells (Fig. 3D). During incubation for 10 h, the amount of \( \alpha B \) crystallin increased throughout the cytoplasm of most cells although it was still abundant in the peripheral areas (Fig. 3E). \( \alpha B \) crystallin in C6 cells that had been treated with colcemid for 24 h was distributed in the cytoplasm of most cells (Fig. 3F).

**Induction of \( \alpha B \) Crystallin by Colchicine in Serum-starved or Aphidicolin-treated Cells**—In order to clarify whether or not the induction of \( \alpha B \) crystallin by the tubulin-binding agents was linked to progression of the cell cycle, cells that had been cultured for 24 h in serum-free medium or in normal medium that contained 6 \( \mu \)M aphidicolin were exposed to 1 \( \mu \)M colchicine in normal or serum-free medium. As shown in Fig. 4, the colchicine-induced synthesis of \( \alpha B \) crystallin was observed in serum-starved cells and in aphidicolin-treated cells with a similar time course in each case although the final level in the serum-starved culture (Fig. 4A) was significantly lower than that in the culture that included serum (Fig. 4, B and C).

Control cultures of synchronized cells that were not exposed to colchicine did not produce \( \alpha B \) crystallin in normal medium (Fig. 4, B and C). Colcemid, vincristine and nocodazole also induced the accumulation of \( \alpha B \) crystallin in cells in serum-depleted quiescent cultures (data not shown).

These results indicate that the expression of \( \alpha B \) crystallin was not enhanced at a specific stage (G2 or the early stage of the M phase) of the cell cycle and, moreover, that the synthesis of \( \alpha B \) crystallin induced by colchicine or by drugs that promote the disassembly of microtubules was due to a process that was not directly linked to a specific stage of the cell cycle.

**Suppression by Taxol and Staurosporine of the Synthesis of \( \alpha B \) Crystallin That Was Induced by Tubulin-binding Antimitotic Drugs**—When cells were exposed to 1 \( \mu \)M colchicine, 1 \( \mu \)M colcemid, 1 \( \mu \)M nocodazole, or 1 \( \mu \)M vincristine, each one together with 4 \( \mu \)M taxol that had been added 3 h before the
Induction of αB Crystallin during Microtubule Disassembly

Fig. 5. Suppression by taxol of the synthesis of αB crystallin induced by drugs that promote the disassembly of microtubules. A, cells that had been cultured for 3 h in the presence (+) or absence (−) of 4 μM taxol were exposed to 1 μM colchicine (Col), 1 μM colcemid (Ccd), 1 μM nocodazole (Noc), or 1 μM vinblastine (Vbt) with or without taxol, and cultures were continued for 20 h. Cells that had been preincubated with or without taxol were also exposed to 100 μM sodium arsenite (As) for 1 h and then cultured for 19 h in normal medium in the presence or absence of 4 μM taxol. None refers to control culture without treatment. Concentrations of αB crystallin in the soluble extracts of cells were determined as described in the legend to Fig. 1. B, aliquots, containing 20 μg of protein, of the same extracts as those that had been analyzed by immunoprecipitation were subjected to SDS-polyacrylamide gel electrophoresis and Western blot analysis with antibodies against αB crystallin (αB cry), total RNA was isolated from control cells (C) and from cells that had been exposed for 13 h to 1 μM colcemid (Ccd), nocodazole (Noc), colchicine (Col), or vinblastine (Vbt) in the presence (+) or absence (−) of taxol, as described above. Northern blot analysis of mRNA was performed as described in the legend to Fig. 2A. Bands of 28 S RNA are shown for reference.

Fig. 6. Suppression by staurosporine of the synthesis of αB crystallin induced by drugs that promote the disassembly of microtubules. A, cells were exposed for 20 h to 1 μM colcemid together with staurosporine (STP) at the indicated concentrations. B, cells were exposed to 1 μM colchicine (Col), colcemid (Ccd), nocodazole (Noc), or vinblastine (Vbt) with (+) or without (−) 10 nM staurosporine. Concentrations of αB crystallin in the soluble extracts of cells were determined as described in the legend to Fig. 1. C, total RNA was isolated from control cells (C) and from cells that had been exposed for 13 h to 1 μM colchicine (Col), colcemid (Ccd), nocodazole (Noc), or vinblastine (Vbt) with (+) or without (−) 10 nM staurosporine. Northern blot analysis of mRNA was performed as described in the legend to Fig. 2A. Bands of 28 S RNA are shown for reference.

addition of tubulin-binding antimitotic drugs, the synthesis of αB crystallin was strongly suppressed. In the case of the colcemid- and nocodazole-induced accumulations, suppression was almost complete (Fig. 5A). Exposure of cells to taxol alone barely affected the control level of αB crystallin. Since the induction of αB crystallin by arsenite (10) was scarcely affected by taxol under the same conditions (Fig. 5, A and B), the suppressive effect of taxol on the colcemid- and nocodazole-inducible syntheses of αB crystallin seemed not to be due to a nonspecific effect of taxol on the synthesis of DNA or protein. Taxol also suppressed the colchicine-induced response of αB crystallin but at a lesser extent (about 50%) than the effect of taxol on the other drug-induced response. Northern blot analysis indicated that the suppressive effect of taxol was apparent at the level of the mRNA for αB crystallin (Fig. 5C). Taxol is a potent stabilizer of microtubules. Thus, these results suggest that the expression of αB crystallin might be stimulated under conditions that promote the disassembly of microtubules.

The induction of αB crystallin by tubulin-binding antimitotic drugs was very sensitive to staurosporine, an inhibitor of protein kinases (30). The presence of staurosporine inhibited the accumulation of αB crystallin in cells exposed to colcemid in a dose-dependent manner (Fig. 6A), and the presence of 10 nM staurosporine completely suppressed induction of the synthesis of αB crystallin by colchicine, colcemid, nocodazole, or vinblastine (Fig. 6B). This effect was also detected at the mRNA level (Fig. 6C).

DISCUSSION

The expression of HSP70 (18) and HSP90 (19) is regulated during the cell cycle, and the levels of mRNAs for HSP70 and HSP90 increase rapidly upon entry of cells into the S phase, declining by the late S and G2 phases. The levels of αB crystallin in C6 cells were significantly elevated during the growing phase (50–100 ng/mg of protein), as compared with those in confluent cultures (<10 ng/mg of protein). Therefore, the possible regulation of the expression of αB crystallin during the cell cycle was examined by the exposure of C6 cells to various inhibitors. Among the inhibitors tested, drugs that promote the disassembly of the microtubule by binding to tubulin and that interrupt the cell cycle at the early M phase induced the accumulation of αB crystallin but not that of HSP27 and HSP70.

However, the induction of αB crystallin by these drugs was also observed in quiescent cultures of cells without serum and, moreover, the level of αB crystallin barely increased in cells during synchronous culture in normal medium. These results suggest that the increased accumulation of αB crystallin was not due to the continuous expression at the early M phase but...
The addition of 1 μM colcemid (closed circles), and after 24 h, as indicated by an arrow, 1 μg/ml actinomycin D (+Acd), 5 μg/ml cycloheximide (+Chx), or 10 nM staurosporine (+STP) was added to the culture medium. Cells were also exposed to 1 μM colcemid together with 1 μg/ml actinomycin D (closed squares) or 5 μg/ml cycloheximide (closed triangles). After incubation for the indicated time in the CO2 incubator, cells were harvested for quantitation of αB crystallin as described in the legend to Fig. 1. Each point shows the mean ± S.D. of the results from 4 or 5 dishes. B, total RNA was isolated for Northern blot analysis of mRNA for αB crystallin from control cells (lane 1) or from cells that had been exposed to 1 μM colcemid for 24 h (lane 2), 48 h (lane 3), or 48 h with the addition of 1 μg/ml actinomycin D at 24 h of exposure (lane 4). Lane 5, cells exposed to 1 μM colcemid for 24 h and then cultured an additional 24 h in normal medium. Bands of 28 S RNA are shown for reference.

was due to an unknown mechanism that was not directly related to progression of the cell cycle. Stimulation of the synthesis of αB crystallin was also observed in rat BRL-3A and 3Y-1 cells that had been exposed to 1 μM colchicine (data not shown).

It has been reported (31) that the depolymerization of microtubules early in the cell cycle is sufficient to initiate DNA synthesis and that colchicine and other drugs that promote the disassembly of microtubules enhance the synthesis of DNA, as does thymbin, a growth factor. However, human thymbin (2 μg/ml), when added to the culture medium of C6 cells with or without serum, did not induce the synthesis of αB crystallin nor did it stimulate the induction by colchicine (data not shown). The induced synthesis of αB crystallin by colcemid or nocodazole was strongly suppressed by taxol, a drug that stabilizes cytoplasmic microtubules and prevents their depolymerization by tubulin-binding antimitotic drugs (32). The suppressive effect of taxol on the colchicine-inducible response was not as great as its effect on the colcemid- or nocodazole-induced response. This difference was probably due to the low reversibility of the binding of colchicine to tubulin, as compared with that of colcemid (33) or nocodazole. It is likely that exposure to colcemid or nocodazole increases the pool of free tubulin in cells, whereas taxol reduces the size of this pool by preventing depolymerization. Therefore, it is suggested that the increased depolymerization of microtubules might be a trigger for the increased synthesis of αB crystallin.

The results of nuclear run-off assays indicated that the increased synthesis of αB crystallin was a result of the increased rate of transcription of the gene for αB crystallin. The elevated level of αB crystallin protein in cells that had been exposed to colcemid for 24 h decreased rapidly from 24 to 48 h after the addition of cycloheximide or staurosporine (Fig. 7A) and in cultures in the normal medium (Fig. 1A). However, high levels of αB crystallin protein (Fig. 7A) and mRNA (Fig. 7B) were maintained for 24 h even after the addition of 1 μM actinomycin D, which completely blocked the synthesis of αB crystallin when added simultaneously with colcemid. These results suggest that the microtubule-disrupting drugs enhance not only the transcription of the gene for αB crystallin but also the stabilization of the mRNA for αB crystallin in cells.

During several hours of exposure of cells to colcemid, the αB crystallin in some cells was colocalized with the depolymerized tubulin in the peripheral areas of cells. However, it remains to be determined whether or not the two proteins are associated under these conditions.

The induction of the synthesis of αB crystallin by colchicine and by other microtubule-disrupting drugs was completely suppressed in the presence of 3–10 nM staurosporine. It has been reported (34) that the heat-induced synthesis of HSP70 and HSP27 is suppressed in the presence of staurosporine at 2–10 μM concentrations about 1,000-fold higher than the present effective concentrations. Staurosporine is a potent inhibitor of protein kinases, with IC50 values of 2.7 nM for protein kinase C, 8.2 nM for protein kinase A, and 6.4 nM for the tyrosine protein kinase p60c-src (30). The IC50 value for the colchicine-induced response of αB crystallin was about the same as that for protein kinase C (Fig. 4). However, exposure of cells for 20 h to 0.1 μM phorbol 12-myristate 13-acetate (an activator of protein kinase C), 20 μM forskolin (an activator of protein kinase A), or 30 μM sodium orthovanadate (an inhibitor of tyrosine phosphoprotein phosphatase) did not induce the accumulation of αB crystallin nor did these treatments stimulate the colchicine-induced accumulation of αB crystallin (data not shown).

It is not known which protein kinases are involved in the signal transduction cascade of the colchicine-induced response. However, the present results suggest that the synthesis and accumulation of αB crystallin are stimulated, via phosphorylation reactions that are sensitive to staurosporine, when the depolymerization of intracellular microtubules is enhanced.

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