Proc. Natl. Acad. Sci. USA
Vol. 77, No. 3, pp. 1561–1565, March 1980
Cell Biology

Taxol stabilizes microtubules in mouse fibroblast cells

(cell cycle/cytoskeleton/cell migration/antimitotic agents)

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Communicated by Harry Eagle, December 18, 1979

ABSTRACT **Taxol, a potent inhibitor of human HeLa and mouse fibroblast cell replication, blocked cells in the G_2 and M phase of the cell cycle and stabilized cytoplasmic microtubules. The cytoplasmic microtubules of taxol-treated cells were visualized by transmission electron microscopy and indirect immunofluorescence microscopy. More than 90% of the cells treated with 10 μM taxol for 22 hr at 37°C displayed bundles of microtubules that appeared to radiate from a common site (or sites), in addition to their cytoplasmic microtubules. Untreated cells that were kept in the cold (4°C) for 16 hr lost their microtubules, whereas cells that were pretreated with taxol for 22 hr at 37°C continued to display their microtubules and bundles of microtubules in the cold. Taxol inhibited the migration behavior of fibroblast cells, but these cells did not lose their ability to produce mobile surface projections such as lamellipodia and filopodia.**

Taxol was isolated from the plant *Taxus brevifolia* and characterized as an experimental antitumor drug by Wani *et al.* (1). Our work has shown that taxol enhances *in vitro* the rate, extent, and nucleation phase of microtubule polymerization and stabilizes microtubules. Microtubules assembled *in vitro* in the presence of taxol are resistant to depolymerization by cold (4°C) or 4 mM $CaCl_2$. The optimal effects of the drug on *in vitro* polymerization and stabilization of microtubules are observed near stoichiometric equivalence with tubulin dimers (2).

We now report that taxol is a potent inhibitor of the replication of HeLa and BALB/c fibroblast cells. HeLa cells treated with taxol accumulate in the G_2 and M phase of the cell cycle. These cells contain microtubules plus bundles of microtubules all of which appear to have the structure of normal microtubules by transmission electron microscopy. Cytoplasmic microtubules in BALB/c fibroblasts treated with a concentration of taxol that completely inhibits the replication of these cells are resistant to depolymerization by cold (4°C) or by 10 μM steganacin. This is consistent with our observation that microtubules treated with taxol *in vitro* become resistant to depolymerization.

It has been reported that colchicine and other drugs that inhibit the polymerization of microtubules also inhibit fibroblast and macrophage cell migration but do not alter the ability of these cells to produce mobile surface projections (3–5). We find that taxol, a promoter and stabilizer of microtubules, completely inhibits fibroblast migration. However, the taxol-treated fibroblast cells are able to produce mobile lamellipodia and filopodia.

MATERIALS AND METHODS

Materials. Taxol was obtained from the National Cancer Institute. Steganacin was kindly provided by the late S. Morris Kupchan. Both drugs were dissolved in dimethyl sulfoxide at a concentration of 10 mM and stored at −20°C. The final concentration of dimethyl sulfoxide in each experiment was 0.5% or less, a concentration that had no effect on control reactions.

Cells. HeLa (human) cells, strain S_3, were grown in suspension culture in Joklik's modified Eagle's minimal essential medium supplemented with 5% fetal calf serum and 1% glutamine. A primary cell line of male BALB/c mouse fibroblasts was provided by Susie Chen. These fibroblasts and Swiss 3T3 mouse fibroblasts were grown as monolayers in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum. Fibroblast cells used in experiments were no older than 20 passages.

Flow Microfluorometry. Flow microfluorometric analysis of the DNA content per cell by propidium iodide (50 μg ml^{-1}) staining in 0.1% sodium citrate has been described (6). Suspensions of stained cells were analyzed in a Cytofluorograph (model 4802A, Ortho Diagnostics Instruments, Westwood, MA), using an argon ion laser at 488 nm.

Transmission Electron Microscopy. HeLa cells were sedimented (200 × *g* for 5 min at room temperature) after incubation with taxol at 37°C for 20 hr, resuspended in Joklik's modified Eagle's medium without serum, and sedimented again. The pellet of cells was fixed with 2% (wt/vol) glutaraldehyde buffered with Joklik's modified Eagle's medium for 1 hr at room temperature. Cells were postfixed in 1% osmium tetroxide, dehydrated, and embedded in Epon 812. Thin sections of cells were stained with 4% uranyl acetate in 40% (vol/vol) ethanol, then with 0.1% lead citrate, and viewed with a Siemens Elmiskop 1A electron microscope.

Immunofluorescence. Cytoplasmic microtubules were visualized by indirect immunofluorescence microscopy (7). Fibroblast cells were grown on glass coverslips in tissue culture dishes (Falcon) and were allowed to attach for 24 hr prior to the addition of drug. After the cells were incubated with drug for the desired time, the coverslips were washed once in phosphate-buffered saline and fixed in 3.7% (wt/vol) formaldehyde for 8 min at room temperature. The coverslips were washed with phosphate-buffered saline, immersed in cold methanol (−20°C) for 4 min, immersed in cold acetone (−20°C) for 3 min, and then allowed to air dry. The dry coverslips were covered with 10 μl of rabbit antiserum to tubulin, provided by Marc Kirschner (8), diluted 1:30 in phosphate-buffered saline. After 50 min at 37°C the coverslips were washed extensively and covered with 10 μl of rhodamine-conjugated goat antiserum to rabbit IgG (Cappel Laboratories, Cochranville, PA), diluted 1:10 in phosphate-buffered saline. After 50 min at 37°C they were again washed and mounted with Aqua-mount (Lerner Laboratories, Stamford, CT) on microscope slides. A Zeiss Photomicroscope III equipped with epifluorescent optics and a ×63 oil immersion objective lens was used to view the cells. Photographs were taken on Kodak Tri-X 35-mm film and developed in Diafine.

Migration Assay. A method for the visualization of phagokinetic paths of individual cultured cells moving on a gold particle-coated substrate has been described by Albrecht-Buehler (9).

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Dark-Field and Phase-Contrast Microscopy. The phagokinetic tracks (9) of the 3T3 cells were observed in dark-field illumination with a Zeiss Photomicroscope II, using a ×2.5 objective lens. A ×40 objective was used for the phase-contrast micrographs. Photographs were exposed and developed as described above.

RESULTS

Flow microfluorometry was used to examine the effect of taxol on the distribution of DNA in HeLa cells as a function of time. Cells in exponential growth (3.2×10^5 cells ml^{-1}) were incubated with 0.25 μM taxol. Untreated cell cultures had a doubling time of 19 hr. DNA content of individual cells in taxol-treated cultures was observed 3, 6, 9, 18, and 27 hr after addition of the drug (Fig. 1). At 18 and 27 hr, essentially all of the drug-treated cells had a tetraploid DNA content. Approximately 70% of the taxol-treated cells were in mitosis at 20 hr, as determined by transmission electron microscopy; such cells contained condensed chromosomes and had lost their nuclear membranes.

When these cells were examined at higher magnification, it was noted that, in addition to normal microtubules, they contained bundles of microtubules (Fig. 2 *A* and *B*). These results were observed when HeLa cells were incubated with either 0.25 or 10 μM taxol for 20 hr. The microtubules had an average diameter of 250 Å, the same value obtained for control cells.

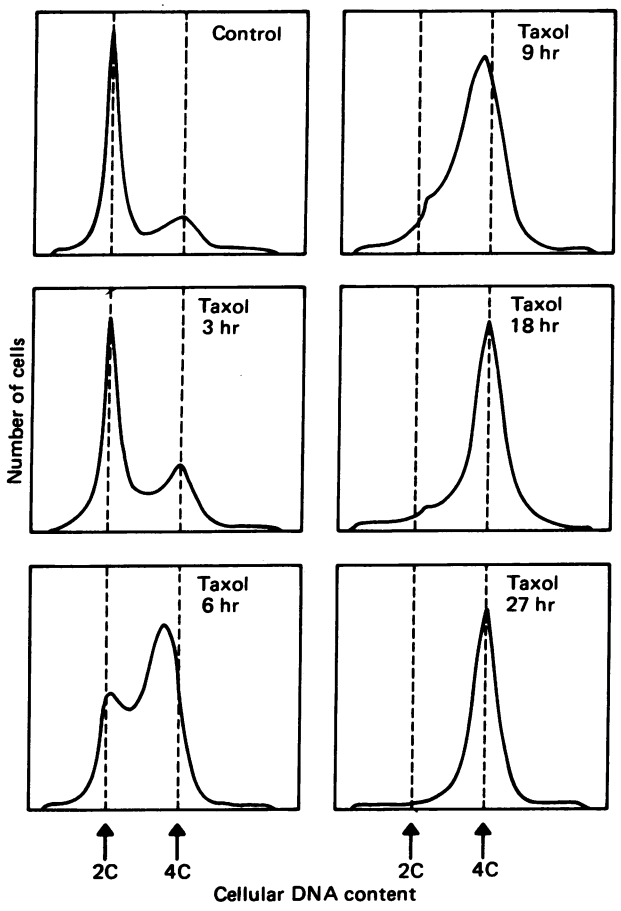

FIG. 1. Flow microfluorometry of the DNA content of HeLa cells in the absence and presence of taxol. Cells growing exponentially were diluted to 3.2×10^5 ml^{-1} at the start of the experiment. Approximately 6×10^4 cells were analyzed for DNA content at each time point. The histograms depict 100-channel analyses of cellular DNA content. The arrows indicate the modal positions of cells having diploid (2C) and tetraploid (4C) DNA contents. The full ordinate scale indicates a cell count of approximately 4×10^3 cells per channel. In control cultures, the proportion of cells with various DNA contents did not vary significantly during the time course of the experiment. The DNA distributions shown were determined in cultures exposed to 0.25 μM taxol for the indicated times.

Indirect immunofluorescence microscopy, with antibodies against tubulin, was used to examine the effect of taxol on cytoplasmic microtubules in BALB/c fibroblast cells. Interphase fibroblast cells presented a characteristic display of cytoplasmic microtubules (Fig. 3*A*). The microtubules in these cells are seen radiating outward from the perinuclear region to the plasma membrane. Cells that had been treated for 22 hr with either 1 or 10 μM taxol, concentrations of drug that completely inhibit fibroblast cell replication, displayed, in addition to their cytoplasmic microtubules, bundles of microtubules that appear to radiate from a common site (or sites) (Fig. 3*B*). Small bundles of microtubules could be seen as early as 20 min after the addition of 10 μM taxol to the cells. The taxol-treated cells also differed from normal cells in having a microtubule-free zone between the distal ends of their microtubules and the plasma membrane (Fig. 3*B*). More than 90% (132 out of 145) of the drug-treated cells viewed at 22 hr in a typical experiment had a characteristic morphology that included microtubule bundles and microtubule-free zones. The structure of the microtubules was verified by transmission electron microscopy and the morphology was observed to be the same as that described above for HeLa cells.

Indirect immunofluorescence has been used to demonstrate that the display of cytoplasmic microtubules in mammalian cultured cells is sensitive to cold and antimitotic drugs such as colchicine (10, 11) that inhibit microtubule polymerization *in vitro*. Normally, cells that have been in the cold (4°C) for 16 hr lose their microtubules (Fig. 3*C*); however, cells incubated with 10 μM taxol for 22 hr at 37°C and then shifted to 4°C for 16 hr still displayed their microtubules and bundles of microtubules (Fig. 3*D*). The same results were observed after a 1-hr incubation with taxol. Steganacin, an antimitotic agent that is a competitive inhibitor of the binding of [^{3}H]colchicine to purified tubulin, is a potent inhibitor of microtubule polymerization *in vitro* (12, 13). BALB/c fibroblasts that had been incubated with 10 μM steganacin for 2 hr at 37°C did not display their microtubules. However, fibroblasts that were pretreated with 10 μM taxol for 22 hr at 37°C and then treated with 10 μM steganacin for 2 hr continued to display their microtubules.

The effect of taxol and steganacin on 3T3 fibroblast cell migration behavior has been examined by using the phagokinetic track assay (9). When cells were viewed at low magnification with dark-field microscopy at 24 hr, both taxol and steganacin were found to completely inhibit cell migration at concentrations of 1 or 10 μM, whereas control cultures produced numerous phagokinetic tracks of individual cells removing and ingesting gold particles (Fig. 4 *A*, *C*, and *E*). Addition of taxol to a 24-hr culture of migrating 3T3 fibroblast cells completely inhibited further cell migration. When the drug-treated cells were viewed at higher magnification by phase-contrast microscopy, it was evident that the cells were able to clear and ingest the gold particles from the area of attachment during the 24-hr experiment (Fig. 4 *B*, *D*, and *F*). The steganacin-treated cells generally appeared to extend fewer and smaller lamellipodia than the taxol-treated cells.

In a separate experiment, photographic sequences were made of single 3T3 fibroblast cells incubated with 10 μM taxol at 37°C (Fig. 5). After 15 min in the presence of taxol, ruffling lamellipodia appeared in numerous places around the cell perimeter. At 25 min the cell extended a large lamellipodium. At 58 and 88 min the cell had retracted and, again, extended lamellipodia, respectively. At 130 min the cell had retracted its lamellipodia and seemed to have lost all its polarity; ruffling of the mem-

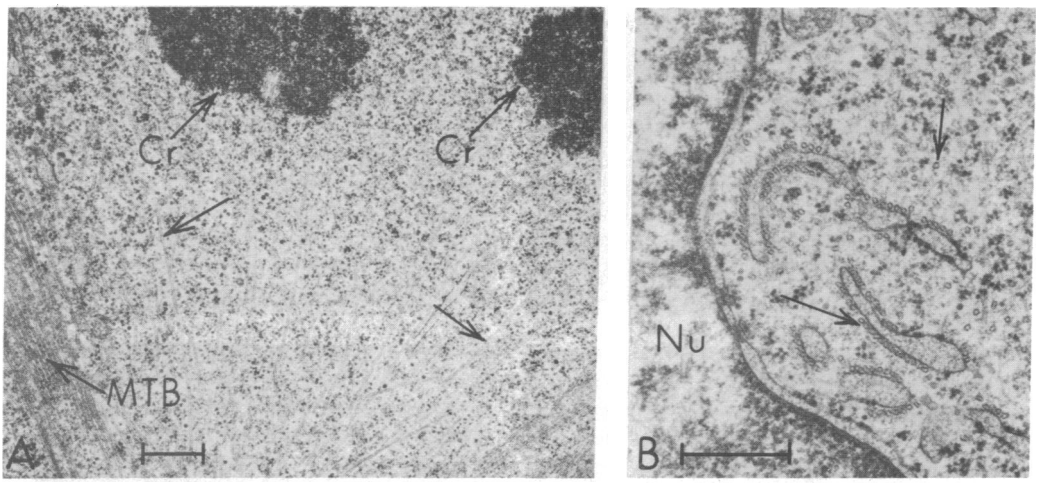

FIG. 2. Electron micrographs of thin sections of HeLa cells treated with 10 μM taxol for 20 hr. (*A*) Mitotic cell. MTB, microtubule bundle; Cr, chromosome; arrows point to individual microtubules in longitudinal profile (*B*) Interphase cell. Nu, nucleus; arrows point to microtubules in cross section. Scale bars: 0.5 μm.

brane was observed all around the perimeter of this fibroblast cell. The cell extended a lamellipodium again 223 min after the addition of taxol. Mobile filopodia were also observed during the photographic sequence. Untreated cells generally had ruffling only at the leading edge of the cell, as has been reported by other investigators (14, 15).

DISCUSSION

Flow microfluorometry was used to demonstrate that taxol does not inhibit the first round of DNA synthesis but blocks cells in the G_2 and M phases of the cell cycle. In addition to their individual cytoplasmic microtubules, the taxol-treated cells contain bundles of microtubules. Although approximately 70% of the taxol-treated cells are in mitosis after exposure to the drug for 20 hr, they do not make a normal mitotic apparatus even though they lose their nuclear membranes and condense their DNA. Using transmission electron microscopy, we have shown that the structure of the microtubules in these cells seems to be normal. Some of the microtubule bundles in the taxol-treated cells appear to be associated with the endoplasmic reticulum. It is not clear whether the association is a direct or indirect effect of taxol, but this observation does suggest that the endoplasmic reticulum may be involved in the organization of microtubules in cells.

Indirect immunofluorescence microscopy, using antibodies against tubulin, was used to examine the effect of taxol on cytoplasmic microtubules and to determine if taxol could convert "labile" cytoplasmic microtubules into "stable" microtubules like those found in cilia and flagella (16, 17). The "stable" microtubules of cilia and flagella are resistant to depolymerization by low temperatures and drugs that normally block polymerization of microtubules *in vitro*. More than 90% of the drug-treated cells viewed at 22 hr had a characteristic morphology consisting of microtubules, microtubule bundles, and microtubule-free zones between the distal ends of their microtubules and their plasma membranes. These microtubules were resistant to depolymerization by cold (4°C) and steganacin. The bundles appear to radiate from a common site (or

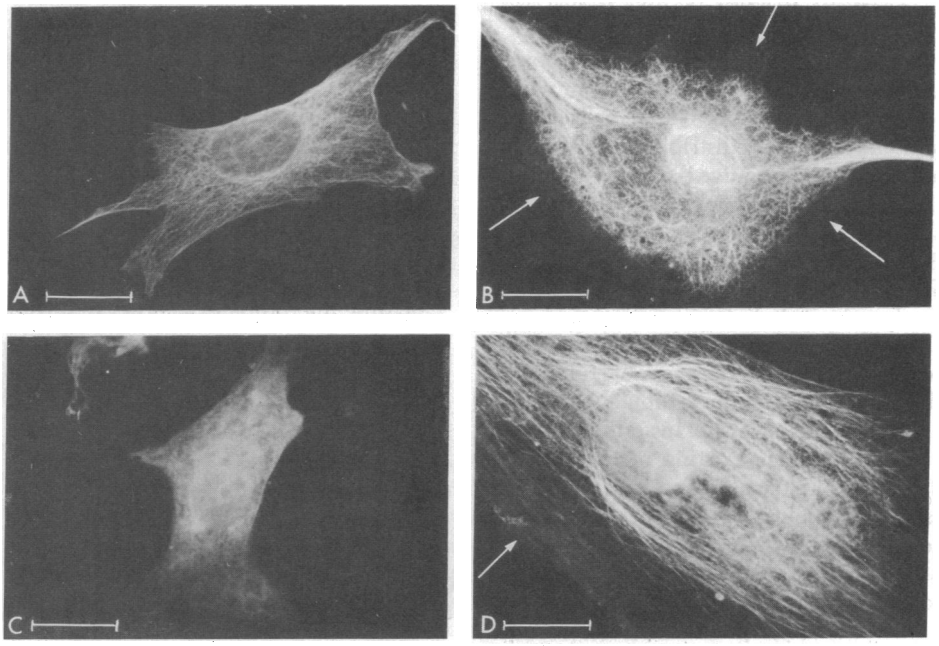

FIG. 3. Indirect immunofluorescence of BALB/c fibroblast cells, using antibodies against tubulin. (*A*) Control cell, (*B*) cell exposed to 10 μM taxol for 22 hr, (*C*) control cell kept at 4°C for 16 hr, (*D*) cell incubated with 10 μM taxol for 22 hr, then shifted to 4°C for 16 hr. Scale bars: 20 μm. Arrows indicate the edge of the plasma membrane in the plane of focus.

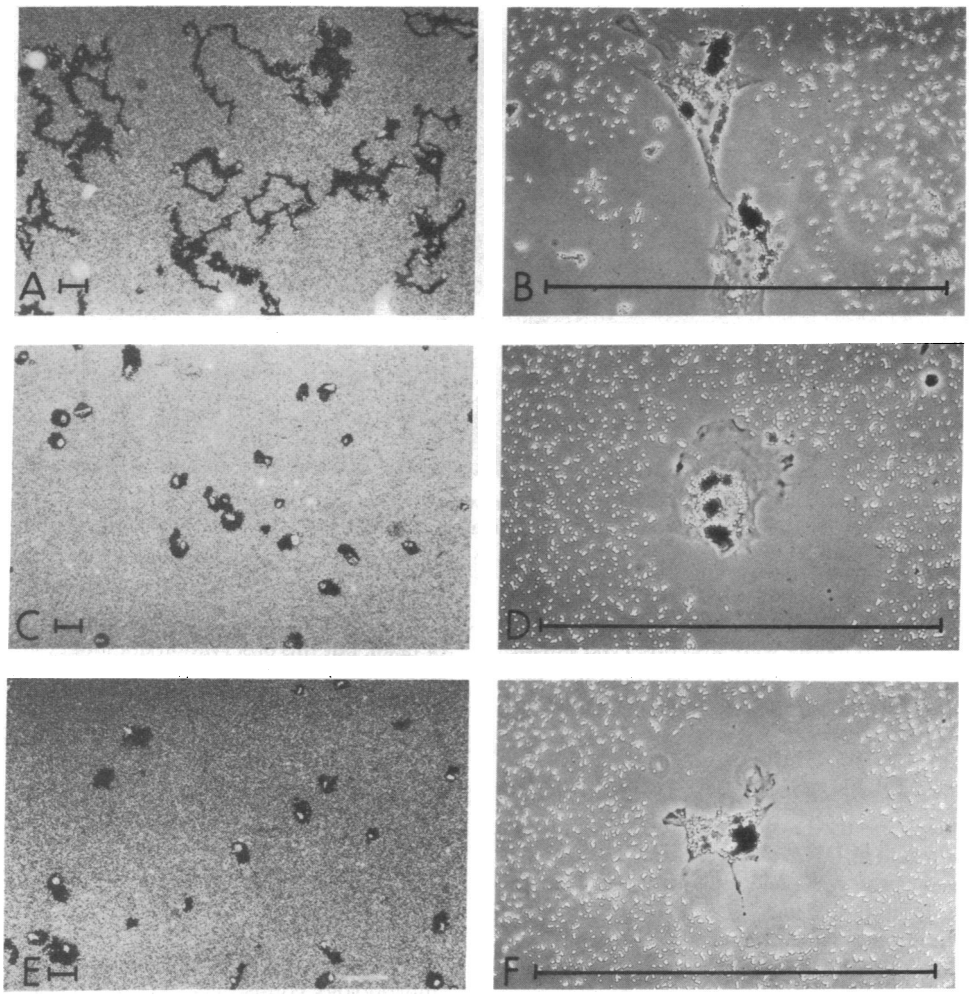

FIG. 4. Migration behavior of control (*A* and *B*), taxol-treated (*C* and *D*), and steganacin-treated (*E* and *F*) Swiss 3T3 fibroblast cells. Cells (1000–2000 per dish) were seeded into drug-containing medium in 35 × 10 mm tissue culture dishes. *A*, *C*, and *E* show the phagokinetic track patterns left by individual control, taxol-treated (10 μM), and steganacin-treated (10 μM) 3T3 cells, respectively. Particle-free dark areas result from the cells ingesting or removing the gold particles. Illumination is dark field. *B*, *D*, and *F* are phase-contrast micrographs of control, taxol-treated, and steganacin-treated cells, respectively, from the same experiment. Both drug-treated and control cells were able to make particle-free areas and ingest the gold particles. However, the drug-treated cells did not make tracks. Scale bars: 200 μm.

sites) within the cell, which may represent the microtubule-organizing centers. These may be the same sites from which microtubules have been reported to originate in cells (8, 11). Although it is difficult to rule out a redistribution of microtubules in the cells, an intriguing alternative is that these bundles represent new initiations that have occurred at microtubule-organizing centers. Mouse fibroblasts are known to contain a primary cilium (18); therefore, some of the bundles observed in taxol-treated cells may be elongated or newly formed cilium. One explanation for the microtubule-free zone is that the cell preferentially initiates formation of new microtubules at the microtubule-organizing centers with the available tubulin dimers, instead of elongating existing microtubules as the cell extends lamellipodia on the glass coverslip. In addition, the cell may not be able to depolymerize existing microtubules to provide additional dimers for elongation.

Little is known about the biological machinery involved in cell migration. The microtubule cytoskeleton may play a role in cell migration by determining the polarity of a migrating cell (19). A model based on intrinsic microtubule behavior has been proposed to explain the orderly separation of chromosomes during mitosis (20). Colcemid, colchicine, and vinblastine, drugs that inhibit mitosis, also inhibit fibroblast and macrophage cell migration (3–5). However, these drug-treated cells are not utterly immobile. They are still able to produce mobile lamellipodia and filopodia. Ruffling of the membrane becomes more evenly distributed around the cell perimeter of these drug-treated cells, instead of occurring mainly at the leading edge as is true of untreated cells (14, 15). The results of the time-lapse photographic sequences and the migration experiments demonstrate that taxol inhibits cell migration but does not affect cell motility. These experiments further suggest that migrating cells must be able both to polymerize and depolymerize their cytoplasmic microtubules in order to differentiate between their front and back ends.

The inhibition of HeLa and BALB/c fibroblast cell replication and the inability of 3T3 fibroblasts to migrate in the presence of low concentrations of taxol could derive from the cell being unable to depolymerize its microtubule cytoskeleton. This may represent a mechanism of action for a chemotherapeutic agent and explain the observed antitumor activity of taxol. The drug should be a useful tool in studying the regulation of microtubule assembly and cellular functions, such as cell migration, that may be mediated by microtubules. The drug could also provide a method for the isolation of intact cytoplasmic and spindle microtubules.

We are indebted to Danuta Hornig and Dr. Carl Schildkraut for advice concerning flow microfluorometry, Jane Fant for assistance with

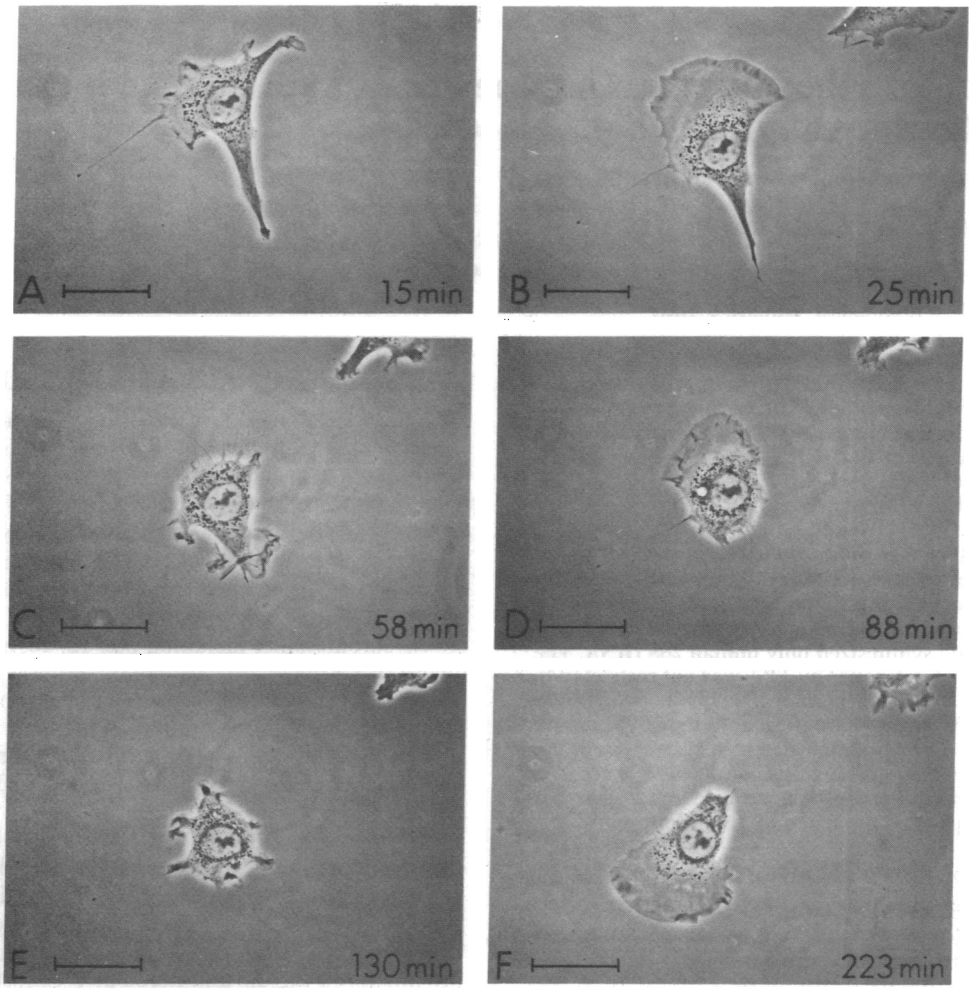

FIG. 5. Selected frames from a time-lapse phase-contrast study of a taxol-treated Swiss 3T3 fibroblast cell. Cells were allowed to attach to the glass coverslip 24 hr before the addition of 10 μM taxol at 37°C. After addition of the drug the coverslip was mounted on a slide and sealed with wax to form a chamber containing culture medium. The stage of the microscope and slide were kept at 37°C with an airstream incubator. Cells were exposed to light only during film exposure. Untreated cells behaved normally in these conditions. The photographic sequence begins 15 min after the addition of taxol to the cells. Scale bars: 40 μm.

the transmission electron microscopy, Dr. Robert Pollack for teaching us the methodology for immunofluorescence, and Dr. Richard Burger for his criticism of the manuscript. We are very grateful to Dr. Guenter Albrecht-Buehler for expert assistance with the cell migration experiments and valuable discussions. This work was supported in part by American Cancer Society Grant CH-86 and U.S. Public Health Service Grants CA 23187 and CA 15714. P.B.S. was supported by U.S. Public Health Service Training Grant in Pharmacological Sciences 6T 32 GM 07260. S.B.H. is a recipient of an Irma T. Hirschl Career Scientist Award.

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