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J Clin Invest. 1993;92(1):431-440. https://doi.org/10.1172/JCI116585.

Research Article

We have previously shown that forskolin and 3-isobutyl-1-methylxanthine (IBMX) increased accumulation of cisplatin (DDP) in DDP-sensitive 2008 human ovarian carcinoma cells in proportion to their ability to increase cAMP. Since the major function of cAMP is to activate protein kinase A, it was conjectured that the stimulation of DDP accumulation was mediated by a protein kinase A substrate. We now show that exposure of 2008 cells to forskolin resulted in phosphorylation of a prominent 52-kD membrane protein. Microsequencing of the band demonstrated it to be human beta-tubulin. Similarly, pretreatment of 2008 cells with the microtubule stabilizing drug taxol increased platinum accumulation in a dose-dependent manner. In 11-fold DDP-resistant 2008/C13*5.25 cells, decreased DDP accumulation was associated with enhanced spontaneous formation of microtubule bundles and decreased expression of beta-tubulin and the tubulin-associated p53 antioncogene relative to 2008 cells. 2008/C13*5.25 cells had altered sensitivity to tubulin-binding drugs, being hypersensitive to taxol and cross-resistant to colchicine. We conclude that pharmacologic alterations of tubulin enhance accumulation of DDP, and that the DDP-resistant phenotype in 2008/C13*5.25 cells is associated with tubulin abnormalities.

In Vitro Modulation of Cisplatin Accumulation in Human Ovarian Carcinoma Cells by Pharmacologic Alteration of Microtubules

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Abstract

We have previously shown that forskolin and 3-isobutyl-1-methylxanthine (IBMX) increased accumulation of cisplatin (DDP) in DDP-sensitive 2008 human ovarian carcinoma cells in proportion to their ability to increase cAMP. Since the major function of cAMP is to activate protein kinase A, it was conjectured that the stimulation of DDP accumulation was mediated by a protein kinase A substrate. We now show that exposure of 2008 cells to forskolin resulted in phosphorylation of a prominent 52-kD membrane protein. Microsequencing of the band demonstrated it to be human β-tubulin. Similarly, pretreatment of 2008 cells with the microtubule stabilizing drug taxol increased platinum accumulation in a dose-dependent manner. In 11-fold DDP-resistant 2008/C13*5.25 cells, decreased DDP accumulation was associated with enhanced spontaneous formation of microtubule bundles and decreased expression of β-tubulin and the tubulin-associated p53 antioncogene relative to 2008 cells. 2008/C13*5.25 cells had altered sensitivity to tubulin-binding drugs, being hypersensitive to taxol and cross-resistant to colchicine. We conclude that pharmacologic alterations of tubulin enhance accumulation of DDP, and that the DDP-resistant phenotype in 2008/C13*5.25 cells is associated with tubulin abnormalities. (*J. Clin. Invest.* 1993. 92:431–440.) Key words: taxol • colchicine • protein kinase A • p53 antioncogene • drug resistance

Introduction

Cisplatin (DDP)[1] has been used successfully to treat several types of cancer. However, both intrinsic and acquired resistance to DDP occurs frequently. A variety of cell types with acquired resistance to DDP exhibit decreased accumulation of DDP as part of their resistant phenotype (reviewed in reference 1). Decreased DDP accumulation occurs at an early stage in the development of resistance and accounts for the low levels of resistance found after in vivo selection with DDP (2). It is not yet known how DDP enters cells. Several lines of evidence indicate that transport is not carrier mediated (i.e., accumulation cannot be saturated or completely inhibited by structural analogues). On the other hand, DDP accumulation can be modulated by a variety of different agents indicating that DDP transport is more complex than simple passive diffusion (1). Several recent reports have described a link between DDP and the cAMP signal transduction pathway (3, 4). On one hand, DDP has been shown to inhibit the production of cAMP in human thyrocytes (3). On the other hand, forskolin, an adenyl cyclase agonist, and 3-isobutyl-1-methylxanthine (IBMX), a phosphodiesterase inhibitor, caused 2.1- and 2.3-fold increases, respectively, in the short-term accumulation of DDP relative to untreated cells (4). In contrast, the inactive analogue, 1,9-dideoxyforskolin, decreased DDP accumulation. Stimulation of uptake did not occur in the DDP-resistant 2008/C13*5.25 subline. The effects were detectable as early as 1 min and persisted at 60 min. Forskolin and IBMX caused marked increases in cAMP levels in 2008 cells. cAMP-dependent protein kinase activity was intact in both 2008 and 2008/C13*5.25 cells. These results indicate that a target downstream of cyclic AMP-dependent protein kinase (PKA) is an important modulator of DDP accumulation, and that there was a defect in this pathway distal to PKA in the 2008/C13*5.25 cells.

We have now identified one such target in 2008 cells as membrane-associated tubulin on the basis of differential phosphorylation in response to treatment with forskolin or IBMX. In further support of the identification of tubulin as an intermediate in the induction of DDP uptake, we found that dipyridamole, another drug that increased DDP uptake, also induces phosphorylation of tubulin, and that altering the state of polymerization with the tubulin-binding drug taxol also increased platinum accumulation in 2008 cells. Thus, alteration of tubulin function by agents which act on tubulin through different mechanisms caused increased platinum accumulation in 2008 cells. Because decreased accumulation of DDP is an important determinant of DDP resistance in 2008/C13*5.25 cells (1), we asked whether in this cell line the DDP-resistant phenotype was associated with alterations in tubulin function. We report here that 2008/C13*5.25 cells exhibit an enhanced spontaneous formation of microtubule bundles and decreased levels of β-tubulin and the tubulin-associated p53 antioncogene protein relative to 2008 cells. Furthermore, 2008/C13*5.25 cells are cross-resistant to colchicine and hypersensitive to taxol relative to 2008 cells, indicating that during the selection process for resistance to DDP, 2008/C13*5.25 cells have acquired an alteration in tubulin function.

Portions of this work were presented at the 83rd Annual Meeting of the American Association of Cancer Research, May 20–23, 1992, San Diego, CA, and published in abstract form (1992. *Proc. Am. Assoc. Cancer Res.* 33:535).

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Received for publication 2 September 1992 and in revised form 6 January 1993.

1. *Abbreviations used in this paper:* DEP, *cis*-dichloro(ethylenediamine)plantinum(II); DDP, cisplatin; IBMX, 3-isobutyl-1-methylxanthine; MAP, microtubule-associated protein; PKA, cyclic AMP-dependent protein kinase; PVDF, polyvinylidene difluoride; RIPA buffer, 20 mM sodium phosphate, pH 7.2, 150 mM NaCl, 2 mM EDTA, 1% (wt/vol) deoxycholic acid, 1% (vol/vol) Nonidet P-40, 0.1% (wt/vol) SDS.

J. Clin. Invest.

0021-9738/93/07/0431/10 $2.00
Volume 92, July 1993, 431–440

Methods

Materials. The following compounds were obtained from the indicated sources: bromodecane, Aldrich Chemical Co. (Milwaukee, WI); 1,9-dideoxyforskolin, bromododecane, forskolin, IBMX, and 3-(cyclohexylamino)-1-propanesulphonic acid from Sigma Chemical Co. (St. Louis, MO). Concentrated stock solutions of forskolin, 1,9-dideoxyforskolin, and IBMX were maintained at −20°C in ethanol. The following monoclonal mouse IgG antibodies were purchased from Sigma Chemical Co.: anti-microtubule-associated protein 1 (MAP1) (5), anti-MAP2 (5), anti-tau (6), anti-α-tubulin (7), and anti-β-tubulin (8). The anti-β-tubulin antibody was known to react with the carboxy-terminal part of β-tubulin on immunoblots (9). A mouse monoclonal IgG antibody specific for mammalian wild type and mutant p53 (10) was obtained from Oncogene Science Inc. (Manhasset, NY). A rabbit polyclonal antitubulin antibody was purchased from Sigma Chemical Co. A donkey anti-rabbit peroxidase conjugated secondary antibody (absorbed against mouse IgG) and a rhodamine-stained goat anti-mouse antibody were purchased from Jackson ImmunoResearch Laboratories, Inc. (West Grove, PA). FITC-labeled goat anti-mouse IgG/IgM antibody was obtained from Caltag Laboratories (San Francisco, CA), and polyvinylidene difluoride (PVDF) protein sequencing membrane and Coomassie brilliant blue from Bio-Rad Laboratories (Richmond, CA). [^{3}H] *cis*-dichloro-(ethylenediamine)-platinum(II) ([^{3}H]DEP) was synthesized as previously described (11). Taxol and [^{3}H]taxol were kind gifts from Dr. Karl Flora, National Institutes of Health (Bethesda, MD) and Susan B. Horwitz, Albert Einstein College of Medicine (New York, NY), respectively.

Cell lines. The parental 2008 human ovarian carcinoma cell line (12) and a 11-fold DDP-resistant cell line designated 2008/C13*5.25 were used in these studies. 2008/C13*5.25 cells were generated by 13 monthly selections with 1 μM DDP, followed by chronic exposure to stepwise increasing concentrations of DDP ranging from 0.25 to 5.25 μM (13). The major mechanisms mediating resistance in 2008/C13*5.25 cells include impairment of drug uptake and enhanced DNA repair (1, 14). 2008 and 2008/C13*5.25 cells were grown on tissue culture dishes in a humidified incubator at 37°C and 5% CO_2 atmosphere. Cells were maintained in complete medium consisting of RPMI 1640 supplemented with 5% heat-inactivated fetal calf serum, 2 mM L-glutamine, and 50 μg/ml gentamicin sulfate.

Biosynthetic labeling of phosphoproteins. Subconfluent cultures of 2008 cells were grown in 60-mm dishes until the day of treatment. The cells were then incubated in serum- and phosphate-reduced Krebs-Ringer bicarbonate-buffered medium. After 2 h, 25 μCi [^{32}P]-orthophosphoric acid was added for 1 h. During the last 2 min of the labeling period, modulators were added including 50 μM forskolin, 20 μM IBMX, or 25 μM dipyridamole.

Digitonin fractionation of membrane-associated proteins. Fractionation of cells with digitonin was performed according to the method described by Jansky, Cornell, and Garrison (15, 16). Cells were recovered in 200 μl PBS and the suspension was injected into fractionation medium consisting of 4 mg/ml digitonin, 10 mM *N*-tris(hydroxymethyl)-methyl-2-aminoethane-sulfonic acid, pH 7.4, 50 mM NaF, 10 mM EDTA, 5 mM EGTA, and 200 mM sucrose. A hydrocarbon with a specific density of 1.05 g/ml was layered under the fractionation medium (90:110 bromodecane/bromododecane). The fractionation in digitonin was allowed to proceed for 6–8 s to ensure release of 90–95% of the cytoplasmic proteins, then the rest of the cell structures were removed by rapid centrifugation at 13,000 *g* through the hydrocarbon layer.

Protein fractionation. Analytical SDS-PAGE was performed with 1-mm-thick slab gels, with 4% stacking and 10% resolving gels. The gels were calibrated with a standard molecular mass kit from Bethesda Research Laboratories (Gaithersburg, MD). The gels were run at room temperature with a constant current of 15 mA until samples had focused in the stacking gel and thereafter at 35 mA constant current for the rest of the separation time. The gels were removed and stained with 1% (wt/vol) Coomassie brilliant blue in 10% acetic acid and 45% methanol for 4 h and destained with 10% acetic acid and 45% methanol in water overnight. Gels were then dried on filter paper in a slab gel dryer (model 543; Bio-Rad Laboratories) under heat and vacuum. The dried gels were subjected to autoradiography for 3–4 d (X-Omat XAR 5; Kodak, Rochester, NY) with intensifying screens (Quanta III; Dupont.

Immunoprecipitation. For immunoprecipitations, cells were lysed in RIPA buffer (20 mM sodium phosphate, pH 7.2, 150 mM NaCl, 2 mM EDTA, 1% [wt/vol] deoxycholic acid, 1% [vol/vol] Nonidet P-40, 0.1% [wt/vol] SDS). A volume of 120 μl of cellular extract was incubated with a monoclonal mouse anti-p53 antibody at 4°C. After 30 min, 60 μl of protein A–Sepharose slurry (50% packed beads in RIPA buffer) was added to each tube. After another 10 min at 0°C, the samples were centrifuged at 13,000 *g* for 1 min at room temperature. The pellet was washed three times with 0.5 ml of RIPA buffer.

Immunodetection. Immunoprecipitated proteins and proteins from lysed whole cells were separated by SDS-PAGE as described above. Proteins were transferred to PVDF membranes overnight at 14 V and blocked at room temperature for 2 h with 5% nonfat dry milk in PBS. After the blocking step, the membrane was incubated with 10 μg/ml monoclonal mouse anti-β-tubulin antibody in 5% nonfat dry milk for 2 h. The membrane was washed six times for 5 min each in PBS, and incubated with goat anti-mouse peroxidase conjugated antibody. β-tubulin was visualized by developing the membrane in a solution containing diaminobenzidine at 6 g/liter and H_2O_2 at 0.03% in 50 mM Tris (pH 7.6).

Protein purification and automated microsequence analysis. Cellular proteins of broken 2008 cells were separated by SDS-PAGE with 1 mM thioglycolic acid added to the upper reservoir to help prevent amino-terminal blocking of amino acid side chains. The proteins were blotted onto PVDF membranes at 90 V for 30 min in 3-(cyclohexylamino)-1-propanesulphonic acid buffer (100 mM, pH 11). The forskolin-responsive 52-kD protein band was cut out of the membrane and subjected to microsequence analysis. Automated Edman degradations were performed on a gas phase sequencer (model 470A; Applied Biosystems, Inc., Foster City, CA) using the "03RPTH" program as supplied by the manufacturer. Phenol-thiol-hydantoin amino acid derivatives were identified using an on-line HPLC system (model 120; Applied Biosystems, Inc.). Data reduction was accomplished by computer (model 7500; Perkin Elmer Corp., Norwalk, CT) using software (Chrom 3; Perkin Elmer Corp.) (17).

Quantitation of tubulin subunits and MAPs. Monoclonal antibodies directed against α-tubulin, β-tubulin, MAP1, MAP2, tau, and p53 antioncogene were used to quantitate tubulin subunits and MAPs in 2008 and 2008/C13*5.25 cells. Cells were harvested by trypsinization, and incubated with 100 nM of the respective monoclonal antibody in 3% BSA/PBS. After washing, the cells were stained with a fluorescent goat anti-mouse IgG/IgM antibody for 45 min in the dark. The cells were analyzed (CytoFluorograf; Ortho Diagnostics Systems, Inc., Raritan, NJ) with excitation and emission settings of 488 and 530 nm, respectively.

Histochemical staining of tubulin. 2008 and 2008/C13*5.25 cells were grown on plastic dishes until ~ 80% confluence was reached, fixed in 3.7% (vol/vol) formaldehyde in PBS (pH 7.4) for 10 min at room temperature. Cells were pretreated with 20% normal goat globulin, 0.1% saponin, and 1% EGTA in PBS for 5 min. The cells were washed with PBS and treated with 10 nM monoclonal antibody specific for β-tubulin in 3% BSA/PBS for 15 min. The slides were washed with PBS and then treated with rhodamine-conjugated goat anti-mouse IgG in 3% BSA/PBS. The slides were viewed and photographed with an immunofluorescence light microscope (Diastar®; Cambridge Instruments, Buffalo, NY) and on a confocal laser scanning microscope (model 600; Bio-Rad Laboratories).

Electron microscopy preparations. 2008 and 2008/C13*5.25 cells grown on tissue culture dishes were fixed in 2.5% (vol/vol) glutaraldehyde in 0.1 M sodium phosphate buffer (pH 7.4, total osmolarity = 0.520 osmol) and postfixed in 1% OsO_4. Sections of cells were cut parallel to the tissue culture dish on an ultramicrotome (model LKB-V; Pharmacia LKB Biotechnology, Piscataway, NJ). Thin sections of

cells were stained with saturated uranyl acetate in 50% (vol/vol) ethanol then with bismuth subnitrate (18). Cells were viewed with a transmission electron microscope (model H-600; Hitachi Instruments, Inc., Danbury, CT).

Stereological methods. Sections of 2008 and 2008/C13*5.25 cells taken parallel to the plane of the tissue culture dish were used to estimate the volume density of microtubule bundles by point counting (19, 20). Measurements were done at a final magnification of 16,200 by placing a transparent overlay of a 400-point grid on random micrographs of cell sections taken in the mid portion of the cells; i.e., including a section of the nucleus.

Colony-forming assay. Colony-forming assays were performed by seeding 300 cells per 60 mm tissue culture plastic dish, Corning Glass Works (Corning, NY). Cells were allowed to attach to the culture dishes and were then treated with taxol or colchicine for 20 h. The colonies that formed were counted after 10 d of incubation in humidified 5% CO_2 at 37°C. Cell clusters containing > 50 cells were scored as a colony.

Determination of [^{3}H]DEP accumulation. Parental 2008 and 2008/C13*5.25 cells were assayed in 60-mm dishes when subconfluent. Cells were pretreated at various concentrations of colchicine or taxol. After 19 h, 5 μM [^{3}H]DEP (2 μCi/ml) was added for 1 h. Control cells were exposed to 5 μM [^{3}H]DEP (2 μCi/ml) for 1 h. Immediately after completion of [^{3}H]DEP exposure, the cells were washed four times with 4°C PBS, lysed in 2 ml 1 N NaOH overnight, and an aliquot was removed for protein determination by the method of Bradford (21). Radioactivity was determined by liquid scintillation counting.

Determination of [^{3}H]taxol accumulation. Subconfluent 2008 and 2008/C13*5.25 cells were exposed to 27 nM [^{3}H]taxol (0.1 μCi/ml) for various periods of time. Taxol efflux was determined after loading of the cells by exposure to 27 nM taxol (0.1 μCi/ml) for 2 h. After [^{3}H]taxol exposure, the cells were washed in PBS, and protein content and radioactivity were determined as described above.

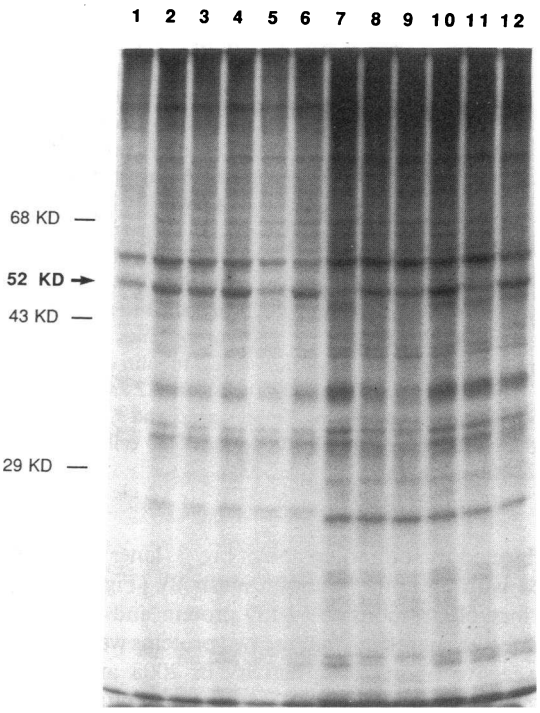

Figure 1. Effect of forskolin, IBMX, and dipyridamole on total cellular phosphoproteins. 2008 and 2008/C13*5.25 cells were labeled with ^{32}P for 1 h and exposed to modulators for 2 min. Cell lysates were fractionated by SDS-PAGE. Lanes *1–6*, 2008 cells; lanes *7–12*, 2008/C13*5.25 cells; lanes *1* and *7*, control cells; lanes *2* and *8*, 20 μM dipyridamole for 2 min; lanes *3* and *9*, 3 mM IBMX for 2 min; lanes *4* and *10*, 50 μM forskolin for 2 min; lanes *5* and *11*, 5 μM DDP for 1 h; lanes *6* and *12*, 50 μM forskolin for 2 min.

Results

Effect of modulators on phosphorylation of cellular proteins

2008 and 2008/C13*5.25 cells were labeled with ^{32}P for 1 h before treatment with forskolin, IBMX, and dipyridamole. Fig. 1 shows that treatment of 2008 cells with 50 μM forskolin for 2 min caused phosphorylation of a prominent 52-kD protein, as well as several smaller and less abundant phosphoproteins. A 2-min treatment with IBMX and dipyridamole, which are both inhibitors of phosphodiesterase, also induced phosphorylation of the 52-kD protein (Fig. 1, lanes *2, 3, 8,* and *9*), but the inactive analog of forskolin, 1,9-dideoxyforskolin, did not (data not shown). In 2008/C13*5.25 cells, forskolin, IBMX, and dipyridamole also caused increased phosphorylation of the 52-kD protein, however, the intensity was reduced compared to that observed in the 2008 cells.

To determine whether the cAMP-responsive 52-kD protein was membrane-associated, ^{32}P prelabeled cells were treated with forskolin for 2 min, and membrane-associated proteins of 2008 cells were recovered by digitonin fractionation and separated by SDS-PAGE. Digitonin fractionation showed that forskolin treatment resulted in a 1.5-fold increase in phosphorylation of a 52-kD membrane-associated protein, as quantitated by the integrated intensity (absorbance × surface area) of the 52-kD band by scanning densitometry.

Microsequence analysis

Cellular proteins from 2008 cells were separated by SDS-PAGE. The proteins were transferred to PVDF membranes and the forskolin-responsive 52-kD protein band was cut out of the membrane and subjected to automated Edman degradation. The sequence of the first 19 amino-terminal amino acids is shown in Fig. 2. This sequence is identical to that of the amino-terminal end of human β-tubulin (22). The amino-terminal sequence of the 52-kD protein was confirmed in a repeat experiment.

Comigration of the forskolin-responsive 52-kD protein and β-tubulin

To confirm that the forskolin-responsive 52-kD protein was β-tubulin, proteins of ^{32}P-labeled, forskolin-treated 2008 and 2008/C13*5.25 cells were separated by SDS-PAGE and transferred to PVDF membrane (Fig. 3). The PVDF membrane

```
          1                                          10
p52       Met  x  Glu Ile Val  x  Leu Gln Ala Gly Gln Cyst
tubulin   Met Arg Glu Ile Val His Leu Gln Ala Gly Gln Cyst

          13                          19
p52       Gln Asn Gln Ile Gly Ala Lys
tubulin   Gln Asn Gln Ile Gly Ala Lys
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Figure 2. Purification and amino acid sequencing of the 52-kD protein which is overphosphorylated in response to forskolin. Proteins of 2008 cells were fractionated by SDS-PAGE and blotted onto PVDF membranes. Membrane strips containing the blotted 52-kD protein were subjected to microsequence analysis. The amino-terminal sequences of the forskolin-responsive 52-kD protein and β-tubulin (22) are shown.

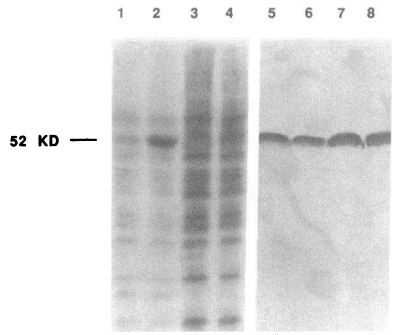

Figure 3. Comigration of the forskolin-responsive 52-kD protein and β-tubulin. Proteins of ^{32}P labeled control and forskolin-treated 2008 cells were separated by SDS-PAGE. Proteins were transferred to PVDF membrane. The membrane was first subjected to autoradiography (lanes *1–4*). The same membrane was then stained with an anti-β tubulin antibody (lanes *5–8*). Lanes *1* and *5*, control 2008 cells; lanes *2* and *6*, 2008 cells treated with 50 μM forskolin for 2 min; lanes *3* and *7*, control 2008/C13*5.25 cells; lanes *4* and *8*, 2008/C13*5.25 cells treated with 50 μM forskolin for 2 min.

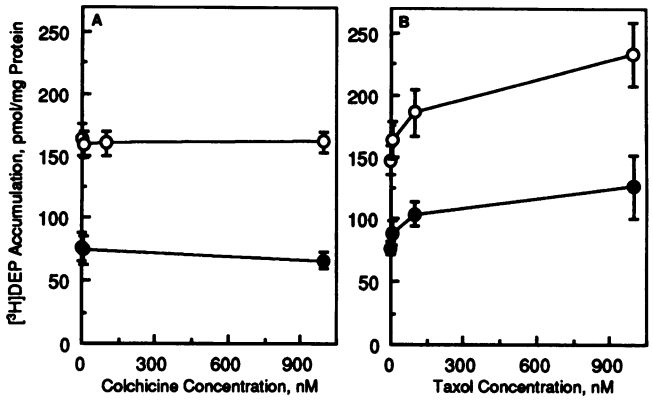

Figure 4. Effect of colchicine and taxol on [^{3}H]DEP accumulation. (*A*) 2008 and 2008/C13*5.25 cells were treated with increasing concentrations of colchicine for 19 h and then with both colchicine and [^{3}H]DEP concurrently for another hour. (*B*) 2008 and 2008/C13*5.25 cells were treated with increasing concentrations of taxol for 19 h and then with taxol and [^{3}H]DEP concurrently for another hour. *Open circles*, 2008 cells; *closed circles*, 2008/C13*5.25 cells. Data points indicate mean±SD of three different experiments. At concentrations of 100 nM and 1 μM, taxol induced a statistically significant increase in [^{3}H]DEP accumulation ($P < 0.05$ by two-sided unpaired *t* test).

was first subjected to autoradiography (Fig. 3, lanes *1–4*) and then stained with an anti–β–tubulin antibody (Fig. 3, lanes *5–8*). The forskolin-responsive 52-kD protein and β-tubulin matched perfectly, suggesting that the two proteins were identical. The excessive phosphatase activity in 2008 and 2008/C13*5.25 cells prevented proof by immunoprecipitation that the forskolin-responsive 52-kD protein was identical with β-tubulin. No phosphorylation could be demonstrated after immunoprecipitation of ^{32}P-labeled control and forskolin-treated cells with an anti–β-tubulin antibody. In these cell lines, phosphatase activity was resistant to treatment with phosphatase inhibitors such as okadaic acid, microcystin-LR, and sodium vanadate (data not shown).

Effect of other tubulin binding drugs on [^{3}H]DEP accumulation

To further strengthen the argument that microtubule function has an impact on [^{3}H]DEP accumulation, we determined the effect of colchicine and taxol, two drugs that bind to and alter microtubule function, on [^{3}H]DEP accumulation in 2008 and 2008/C13*5.25 cells. Subconfluent cells were treated with colchicine or taxol at increasing concentrations for 19 h and then with colchicine or taxol concurrently with [^{3}H]DEP for another hour. Pretreatment with colchicine, a drug that prevents tubulin polymerization, had no effect on [^{3}H]DEP accumulation (Fig. 4 *A*). In contrast, pretreatment of 2008 and 2008/C13*5.25 cells with taxol caused a dose-dependent increase in [^{3}H]DEP accumulation (Fig. 4 *B*). At concentrations of 100 and 1,000 nM taxol induced a statistically significant increase in [^{3}H]DEP accumulation in 2008 and 2008/C13*5.25 cells ($P < 0.05$ by two-sided unpaired *t* test). After a 20-h treatment with colchicine and taxol at the indicated concentrations, trypan blue was excluded from cells, indicating that the observed changes in [^{3}H]DEP accumulation were not caused by generalized permeabilization of the plasma membrane.

Tubulin abnormalities in DDP-resistant cells

11-fold DDP-resistant 2008/C13*5.25 cells exhibit decreased DDP accumulation relative to parental 2008 cells (23). We thus asked whether in 2008/C13*5.25 cells decreased DDP accumulation was associated with alterations in tubulin function and expression.

Expression of tubulin and MAPs. Expression of tubulin subunits and MAPs in 2008 and 2008/C13*5.25 cells was quantitated by flow cytometry after immunostaining with appropriate antibodies. In 2008/C13*5.25 cells, β-tubulin expression was 55±8% (mean±SD, $n = 4$, $P = 0.0014$ by two-sided unpaired *t* test) of that in the 2008 cells, as quantitated by the ratio of the mean intensity of fluorescence. Decreased β-tubulin expression in 2008/C13*5.25 cells was confirmed by using Coomassie stains of whole cell lysates. Proteins of 2008 and 2008/C13*5.25 cells were separated by SDS-PAGE and stained with Coomassie brilliant blue. In 2008/C13*5.25 cells, the 52-kD band representing β-tubulin was decreased in intensity to ~ 50% relative to 2008 cells (Fig. 5). In contrast to β-tubulin, α-tubulin and MAP1, MAP2, and tau were expressed at the same level in 2008 and 2008/C13*5.25 cells.

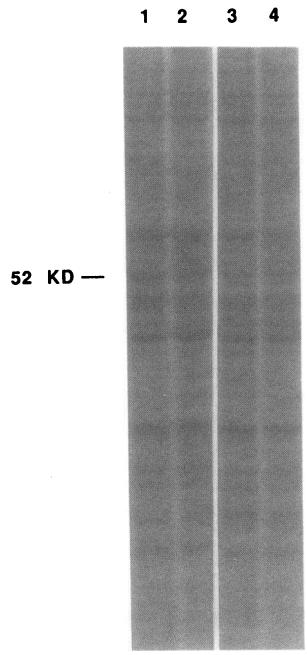

Figure 5. Expression of β-tubulin in 2008 vs 2008/C13*5.25 cells. Proteins of 2008 and 2008/C13*5.25 cells were separated by SDS-PAGE and stained with Coomassie brilliant blue. 100 μg protein was loaded in each lane. Lanes *1* and *2*, 2008 cells; lanes *3* and *4*, 2008/C13*5.25 cells. In 2008/C13*5.25 cells, the 52-kD band representing β-tubulin was decreased in intensity to ~ 50% relative to 2008 cells.

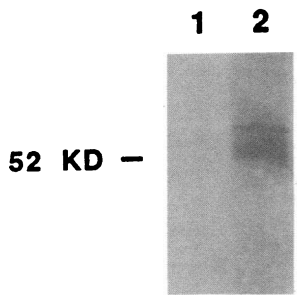

Figure 6. Coimmunoprecipitation of β-tubulin and p53 antioncogene. 2008 cells were lysed in RIPA buffer and immunoprecipitated with a non-specific mouse antibody (lane *1*) and with a monoclonal mouse anti-p53 antibody (lane *2*). The immunoprecipitates were separated by SDS-PAGE and probed with a rabbit anti-β-tubulin antibody and a peroxidase-conjugated donkey anti-rabbit antibody.

Interestingly, expression of the p53 antioncogene, which was associated with β-tubulin in 2008 cells (see below), was also decreased in 2008/C13*5.25 cells to 60±15% of that in the 2008 cells (mean±SD, $n = 5$, $P = 0.0053$ by two-sided unpaired t test).

Coprecipitation of β-tubulin and p53. In the SV40-transformed BALB/c mouse cell line mKSA, p53 was shown to coprecipitate with β-tubulin (24). Since in 2008 cells the DDP-resistant phenotype was associated with a similar decrease in both β-tubulin and p53, we sought to determine whether p53 was also associated with β-tubulin in 2008 cells. Whole cell lysates of 2008 cells were precipitated with a monoclonal mouse anti-p53 antibody. Blots of the precipitate were stained with a polyclonal rabbit anti-β-tubulin antibody and a secondary donkey anti-rabbit antibody conjugated to peroxidase. As shown in Fig. 6, β-tubulin coprecipitated with p53 in 2008 cells.

Microtubule morphology. Fixed preparations of 2008 and 2008/C13*5.25 cells were examined by indirect immunofluorescence on a light microscope and on a laser microscope using a β-tubulin-specific monoclonal antibody. Figs. 7 and 8 show that in 2008 cells, the microtubules were long and arranged in a typical perinuclear network and systems of fibers throughout the cell with most microtubules radiating from the perinuclear area towards the plasma membrane. In 2008/C13*5.25 cells, the microtubules were shorter and more randomly arranged.

Effect of taxol on microtubule morphology. 2008 and 2008/C13*5.25 cells were treated with 100 nM taxol for 24 h before fixation and indirect immunofluorescence staining with an anti-β-tubulin antibody. In 2008 cells, taxol caused microtubules to stain more intensely and to form clusters (Figs. 7 and 8). In contrast, in 2008/C13*5.25 cells, taxol treatment induced a marked loss of microtubule structure and the formation of intensively staining, prominent amorphous clumps. Al-

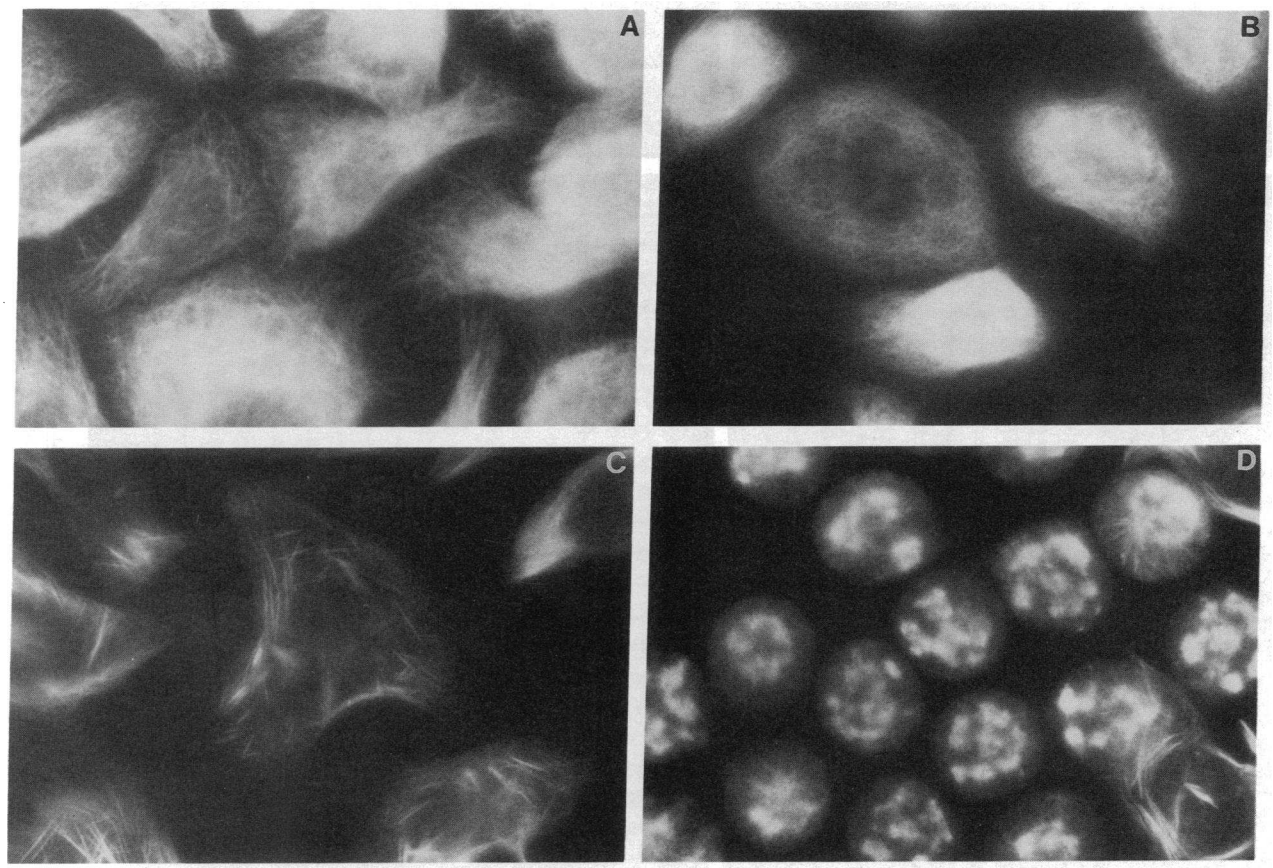

Figure 7. Microtubule morphology in 2008 and 2008/C13*5.25 cells. Fixed, detergent-extracted preparations of 2008 and 2008/C13*5.25 cells were examined under a light microscope by indirect immunofluorescence using a monoclonal anti-body specific for β-tubulin and a rhodamine-conjugated goat anti-mouse IgG antibody. (*A*) 2008 cells; (*B*) 2008/C13*5.25 cells. Note that the staining of microtubules is denser and that the microtubules are arranged more randomly in 2008/C13*5.25 cells as compared to 2008 cells. (*C* and *D*) Effect of taxol on tubulin assembly. 2008 and 2008/C13*5.25 cells were treated with 100 nM taxol for 24 h before fixation. In 2008 cells, taxol induced microtubules to aggregate and to form bundles (*C*). In 2008/C13*5.25 cells, the response to taxol was much more pronounced relative to that observed in 2008 cells with widespread clumping of microtubules (*D*).

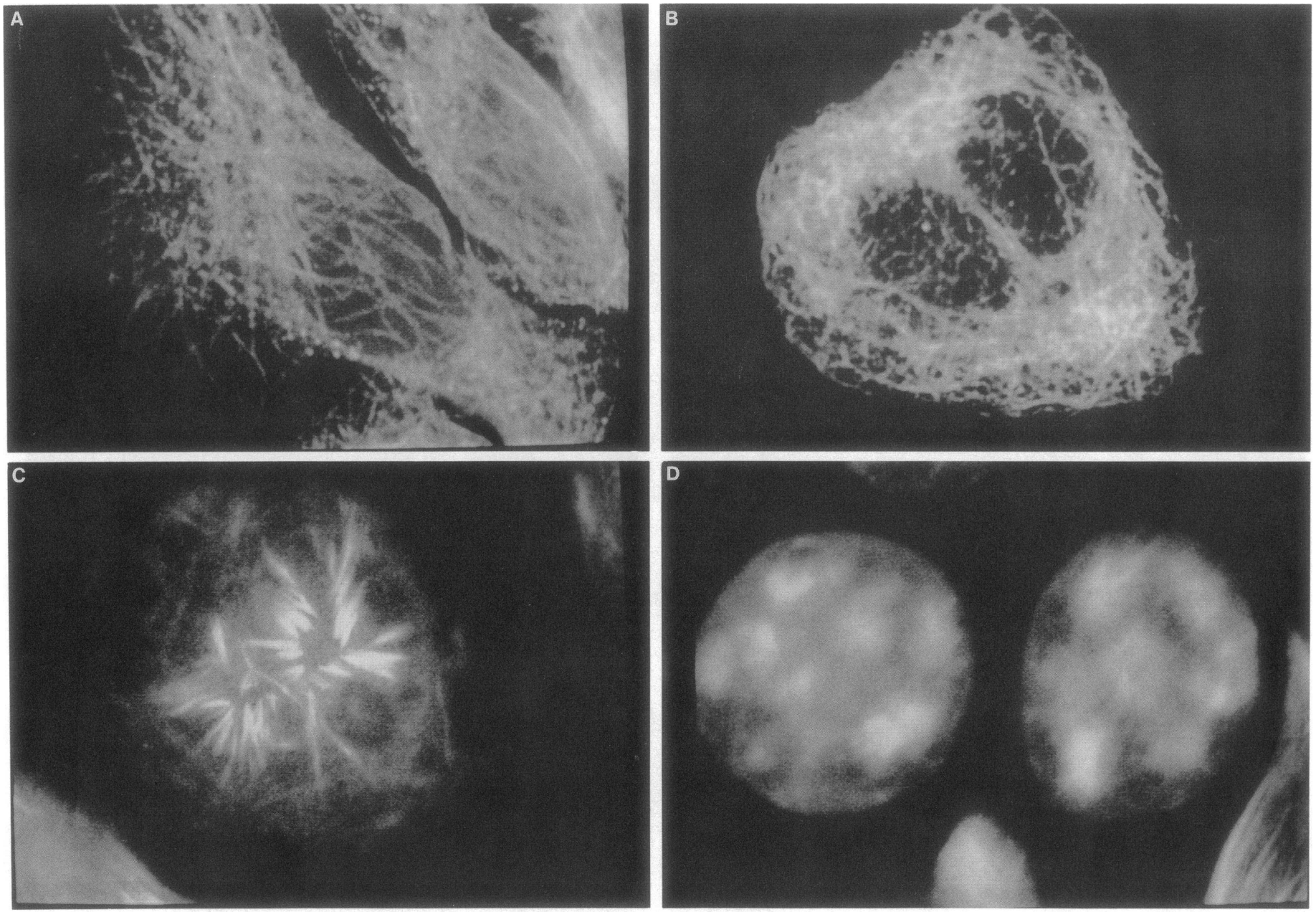

Figure 8. Microtubule morphology in 2008 and 2008/C13*5.25 cells assessed by confocal laser scanning microscopy. Cells were prepared as described in the legend to Fig. 7. *A*, control 2008 cells; *B*, control 2008/C13*5.25 cells; *C*, taxol-treated 2008 cells; *D*, taxol-treated 2008/C13*5.25 cells.

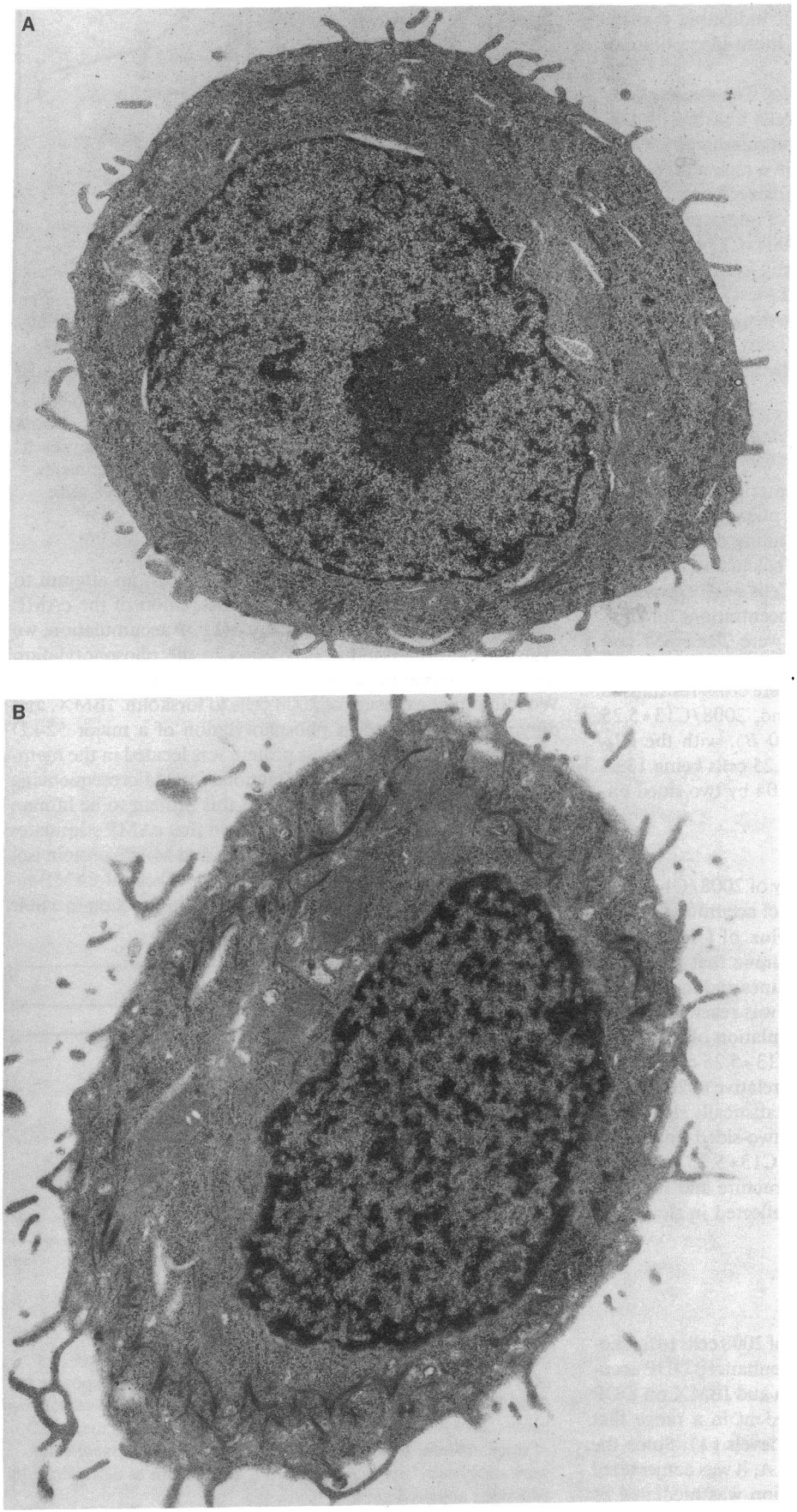

Figure 9. Microtubule bundle formation in 2008 and 2008/C13*5.25 cells. Electron micrographs of thin sections of 2008 (*A*) and 2008/C13*5.25 cells (*B*) were viewed at ×16,200. Both cell lines exhibited spontaneous formation of microtubule bundles. In 2008/C13*5.25 cells, bundle formation was more marked relative to 2008 cells as quantitated by volume density determination ($P < 0.01$ by two-sided t test).

terations in taxol accumulation could not account for the enhanced morphological response to taxol in 2008/C13*5.25 cells compared to 2008 cells (see below), indicating that in 2008/C13*5.25 cells, microtubules were more susceptible to taxol-induced clumping.

Structural characteristics of microtubules. The microtubule structure of 2008 and 2008/C13*5.25 cells attached to the tissue culture dish was assessed by electron microscopy. Cells were cut parallel to the plane of the dish in a region including the nucleus. In both cell lines, discrete bundles of microtubules were found (Fig. 9). The volume density of microtubule bundles was 6±2% and 13±4% in 2008 and 2008/C13*5.25 cells, respectively (n = six pictures taken randomly from six different cells in each group, $P < 0.01$ by two-sided t test). Thus 2008/C13*5.25 cells contained a higher volume density of microtubule bundles compared to parental 2008 cells, indicating that 2008/C13*5.25 cells have acquired an alteration in microtubule function.

Cross-resistance pattern between DDP and antimitotic drugs

Because DDP has the potential to depolymerize microtubules (25), we asked whether the DDP-resistant phenotype was associated with cross-resistance to tubulin-binding drugs. Fig. 10 summarizes the dose response curves of colchicine and taxol for the 2008 and 2008/C13*5.25 cells. Cells were exposed to colchicine and taxol for 20 h. The IC_{50} concentrations for 2008 and 2008/C13*5.25 cells to colchicine were 20±5 nM and 52±8 nM, respectively ($P = 0.004$ by two-sided unpaired t test) indicating that the 2008/C13*5.25 cells were cross-resistant to colchicine (Fig. 10 *A*). On the other hand, 2008/C13*5.25 cells were hypersensitive to taxol (Fig. 10 *B*), with the IC_{50} concentration for 2008 and 2008/C13*5.25 cells being 13±4 nM and 2.5±0.7 nM, respectively ($P = 0.04$ by two-sided unpaired t test).

[^{3}H]Taxol influx and efflux.

To determine whether the hypersensitivity of 2008/C13*5.25 cells to taxol was caused by increased taxol accumulation, we determined the initial influx and the efflux of [^{3}H]taxol in 2008 and 2008/C13*5.25 cells. Fig. 11 shows that the initial influx of [^{3}H]taxol was rapid in both cell lines and that steady-state cellular accumulation of [^{3}H]taxol was reached by 2 h. The initial influx and steady-state accumulation of [^{3}H]taxol was almost identical in 2008 and 2008/C13*5.25 cells. Taxol efflux was slower in 2008/C13*5.25 cells relative to 2008 cells. The differences in [^{3}H]taxol efflux were statistically significant at 15, 30, and 90 min as determined by two-sided unpaired t test ($P < 0.05$). This suggests that 2008/C13*5.25 cells have acquired an alteration in microtubule structure and function resulting in enhanced binding of taxol reflected in slower release of taxol.

Discussion

We have previously shown that exposure of 2008 cells to forskolin, IBMX, and dipyridamole resulted in enhanced DDP accumulation (4, 26). The effects of forskolin and IBMX on DDP accumulation were concentration-dependent in a range that corresponded to their effects on cAMP levels (4). Since the major function of cAMP is to activate PKA, it was conjectured that the stimulation of DDP accumulation was mediated by

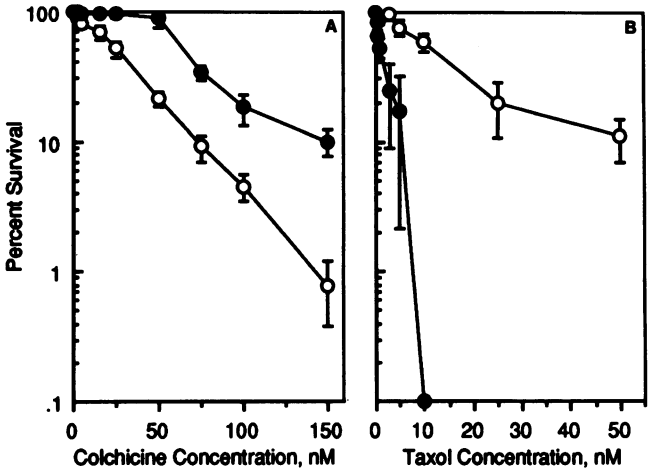

Figure 10. Pattern of cross-resistance to antimitotic drugs associated with the DDP-resistant phenotype. 2008 and 2008/C13*5.25 cells were treated with increasing concentrations of colchicine (*A*) or taxol (*B*) for 20 h. Survival was determined by colony-forming assay. Each data point represents the mean±SD of three different experiments, each performed with triplicate cultures. *Open circles,* 2008 cells; *closed circles,* 2008/C13*5.25 cells.

the action of PKA on one of its substrates. In an attempt to determine the mechanism by which activation of the cAMP signal transduction pathway enhances DDP accumulation, we assessed the cAMP-induced changes in the phosphorylation pattern of cellular proteins in 2008 and 2008/C13*5.25 cells. We found that exposure of 2008 cells to forskolin, IBMX, and dipyridamole resulted in phosphorylation of a major 52-kD phosphoprotein, and that this protein was located in the membrane fraction after digitonin fractionation. Microsequencing of the amino terminal end revealed this protein to be human β-tubulin. It has previously been shown that cAMP stimulates the phosphorylation of purified tubulin and MAP2 protein isolated from mammalian brain (27). The effects of cAMP-induced phosphorylation of tubulin on tubulin function in whole

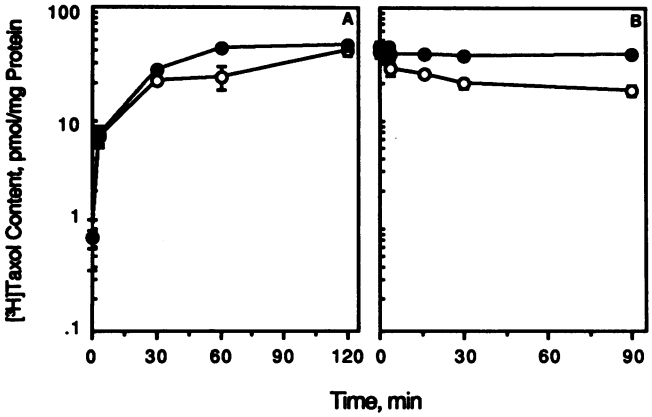

Figure 11. [^{3}H]taxol influx and efflux in 2008 and 2008/C13*5.25 cells. (*A*) Taxol influx during exposure to 27 nM [^{3}H]taxol (0.1 μCi/ml). (*B*) Taxol efflux after loading of the cells by exposure to 27 nM [^{3}H]taxol (0.1 μCi/ml) for 2 h. *Open circles,* 2008 cells; *closed circles,* 2008/C13*5.25 cells. Data points represent the mean±SD of three different experiments. The differences in [^{3}H]taxol efflux were statistically significant at 15, 30, and 90 min as determined by two-sided unpaired t test.

cells have not been well defined. The following observations link cAMP mediated phosphorylation of tubulin with alterations in membrane function that result in enhanced DDP accumulation. (*a*) Both forskolin, an adenyl cyclase agonist, and IBMX, an inhibitor of phosphodiesterase, caused enhanced DDP accumulation and induced phosphorylation of tubulin in 2008 cells. (*b*) Exposure of 2008 cells to dipyridamole, another drug that increases DDP uptake, also caused phosphorylation of tubulin. (*c*) The inactive analog of forskolin, 1,9-dideoxyforskolin, which does not activate adenyl cyclase (28), did not enhance DDP accumulation and failed to induce phosphorylation of membrane-associated tubulin.

To further investigate the hypothesis that tubulin is involved in regulating DDP accumulation, we determined the effect of tubulin-binding drugs such as taxol and colchicine on DDP accumulation. Pretreatment of 2008 cells with taxol caused a dose-dependent increase in DDP accumulation. On the other hand, pretreatment of 2008 cells with colchicine at concentrations which are expected to result in depolymerization of tubulin did not alter DDP accumulation. Since stabilization of polymerized tubulin is the only known effect of taxol, we conclude that taxol-induced polymerization of tubulin caused the alterations in the cell membrane that resulted in increased DDP accumulation.

2008/C13*5.25 cells contained a significantly smaller amount of β-tubulin and of the microtubule-associated p53 antioncogene relative to 2008 cells. This contrasts with unchanged levels of α-tubulin and the tubulin-associated proteins MAP1, MAP2, and tau. DDP has been shown to bind to free sulfhydryl groups in tubulin and to cause partial depolymerization of microtubules at concentrations as low as 12.5 μM (25). In addition, DDP has been shown to alter microtubule assembly by direct tubulin modification (29) and to induce changes in the cytoskeletal pattern of tumor cells (30). DDP is also mutagenic (31, 32) and it is conceivable that during the process of selection for resistance to DDP, 2008/C13*5.25 cells have acquired alterations in tubulin structure and/or expression in response to DDP-induced mutations or tubulin damage. In several yeast models, it has been shown that the accumulation of β-tubulin in excess over α-tubulin is uniquely toxic because it interferes with normal microtubule assembly (33). Overexpression of β-tubulin leads to loss of normal microtubule structures, formation of abnormal structures, and loss of viability (33). In the same yeast model, the excess of α-tubulin over β-tubulin was not toxic (33). Similarly, the excess of α-tubulin over β-tubulin present in 2008/C13*5.25 cells did not cause toxicity, since these cells proliferate as well and have the same cloning efficiency as 2008 cells. Whether this imbalance can account for the altered microtubule morphology and hypersensitivity to taxol is unknown. In 2008/C13*5.25 cells, both β-tubulin and p53 antioncogene were downregulated to a similar extent, suggesting that these two proteins might be closely associated. Indeed, immunoprecipitation with an anti-p53 antibody precipitated both p53 and β-tubulin, confirming the association of these two proteins in 2008 cells. Similarly, in transformed mouse fibroblasts, the p53 antioncogene has been shown to coprecipitate specifically with β-tubulin (24). Interestingly, decreased expression of p53 has been associated with a poor response to chemotherapy in patients with lymphoma (34). In this study, patients with deletions or translocations of the short arm of chromosome 17, where the gene encoding p53 is located, had a poor outcome because of a very high degree of refractoriness to chemotherapy (34). At this juncture, it is not known by which mechanism decreased expression of p53 results in resistance to chemotherapeutic agents.

Several lines of evidence indicate that 2008/C13*5.25 cells have altered tubulin function relative to 2008 cells. First, 2008/C13*5.25 cells have acquired an alteration in microtubule morphology as assessed by indirect immunofluorescence using an anti-β-tubulin antibody and by electron microscopy. In 2008/C13*5.25 cells, the microtubule staining was denser and microtubules were arranged more randomly than in 2008 cells. Furthermore, electron microscopic analysis revealed that 2008/C13*5.25 cells contained significantly more microtubule bundles than parental cells. Second, the morphologic response of microtubules to taxol was markedly enhanced in 2008/C13*5.25 cells as compared with 2008 cells. Increased taxol accumulation was ruled out as a cause of the enhanced morphologic response. Third, 2008/C13*5.25 cells were hypersensitive to taxol and cross-resistant to colchicine. This same pattern of resistance to antimitotic drugs has been found in CHO cells selected for resistance to drugs that inhibit microtubule assembly such as colcemid (35). Thus, the DDP-resistant 2008/C13*5.25 cells had many characteristics in common with the colcemid-resistant mutants described by Minotti et al. (35). These similarities in phenotype may, in part, be accounted for by a common target of the selecting agents DDP and colcemid. Both agents bind to tubulin and depolymerize microtubules (25, 36). The colcemid-resistant mutants were shown to have changed the conformation of tubulin in such a manner that hyperstable microtubules were formed (35). Similarly, the microtubules of 2008/C13*5.25 cells were more dense and compact as compared with 2008 cells, and exhibited an enhanced spontaneous formation of microtubule bundles, reflecting a shift in the microtubules to a state of higher intrinsic stability. Thus, DDP-resistant and colcemid-resistant cells recruit similar defense mechanism(s) to cope with damage produced by chronic selection with microtubule depolymerizing agents, and this may account for the similarities in phenotype in DDP and colcemid-resistant cells. In human leukemic cell lines, microtubule bundle formation has been shown to be related to the sensitivity of the cell line to taxol (37, 38). Thus in 2008/C13*5.25 cells the acquired shift in the microtubules to enhanced spontaneous formation of microtubule bundles may represent the basis for the hypersensitivity to taxol. The observation that DDP-resistant ovarian carcinoma cells were hypersensitive to taxol has important clinical ramifications with respect to the treatment of DDP-resistant ovarian cancer. In this situation, taxol should be particularly effective. Indeed, phase II clinical trials with taxol in patients with advanced refractory ovarian cancer indicate that a notable proportion of responses occurred in patients who were considered to be resistant to DDP (39).

The major mechanisms mediating resistance to DDP include impairment of drug uptake, elevated levels of glutathione or metallothioneins, and enhanced DNA repair (1, 14). Numerous laboratories have selected DDP-resistant sublines from cancer cells in vitro that accumulate less platinum, implicating a platinum accumulation deficit as an important mechanism of DDP resistance (1). We have now shown that in 2008 cells, pharmacologic alterations of microtubule function alter DDP accumulation, and that in 2008/C13*5.25 cells, decreased DDP accumulation is associated with alterations in tubulin function and expression relative to 2008 cells. The challenge is

now to unravel the interconnection between altered tubulin function and DDP accumulation in carcinoma cells.

Acknowledgments

The authors wish to thank Matthew Williamson, University of California San Diego (La Jolla, CA), for microsequencing of the forskolin-responsive 52-kD protein; Michael L. Costello, La Jolla Cancer Research Foundation (La Jolla, CA), for electron microscopy; Dr. Odile A. Mathieu-Costello, University of California San Diego, for expert advice on stereology and morphometric assessment of microtubule bundles; Dr. Lingna Li, AntiCancer Inc. (San Diego, CA), for analyzing cells on the confocal laser scanning microscope; Dennis Young, University of California San Diego, for analyzing cells on the flow cytometer; Dr. Karl Flora, National Institutes of Health (Bethesda, MD), and Dr. Susan B. Horwitz, Albert Einstein College of Medicine (Bronx, NY), for supplying taxol and [^{3}H]taxol, respectively.

Supported by grants CA 23,100 from the National Institutes of Health, CH 377 from the American Cancer Society, 100-R041 from Bristol-Myers Squibb, Co., and grants from the Finnish Academy, the Swiss Cancer League, the Swiss National Science Foundation, and the Swiss Society of Gynecology and Obstetrics. This work was conducted in part by the Clayton Foundation for Research, California Division. Drs. Howell, Jekunen, Thiebaut, and Christen are Clayton Foundation Investigators. Dr. Shalinsky was supported in part by NIH CA 09290, the CRCC and National Research Service Award CA 08993. Dr. Christen is a recipient of a Young Investigator Award and a Clinical Research Career Development Award from the American Society of Clinical Oncology.

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