Microtubule-Disrupting Chemotherapeutics Result in Enhanced Proteasome-Mediated Degradation and Disappearance of Tubulin in Neural Cells

Lyn M. Huff, Dan L. Sackett, Marianne S. Poruchynsky, and Tito Fojo

Abstract

We sought to examine the effects of microtubule-targeting agents (MTA) on neural cells to better understand the problem of neurotoxicity, their principal side effect, and to possibly develop a model of clinical toxicity. Studies showed that microtubule-depolymerizing agents (MDA) not only disassembled microtubules in neural HCN2 cells but also led to rapid disappearance of tubulin, and that this was specific for MDAs. Tubulin levels decreased to 20% as early as 8 hours after adding vincristine, and to 1% to 30% (mean, 9.8 ± 7.6%; median of 7%) after 100 nmol/L vincristine for 24 hours. This disappearance was reversible. An increase in both glut-terminated and acetylated tubulin, markers of stable tubulin, preceded reaccumulation of soluble tubulin, suggesting a priority for stabilizing tubulin first as microtubules before replenishing the soluble pool. Similar results were shown with other MDAs. Furthermore, microtubule reassembly did not arise from a central focus but instead appeared to involve dispersed nucleation, as evidenced by the appearance of small, stable microtubule stubs throughout the cytoplasm. In contrast, experiments with four nonneural "normal" cell lines and four cancer cell lines resulted in microtubule destabilization but only modest tubulin degradation. Evidence for proteasome-mediated degradation was obtained by demonstrating that adding a proteasome inhibitor before vincristine prevented tubulin disappearance. In summary, MDAs lead to rapid disappearance of tubulin in neural but not in other normal or cancer cells. These results underscore the fine control that occurs in neural cells and may further our understanding of neurotoxicity following MDAs.

Cancer Res; 70(14); OF1–10. ©2010 AACR.

Introduction

Microtubules are filamentous polymers that, along with actin and intermediate filaments (vimentin, lamin, and keratin), comprise the cytoskeleton. Microtubules are composed of α/β-tubulin heterodimers that form linear protofilaments and then arrange in parallel to create cylindrical "tubules." The continuous equilibrium of microtubule assembly (growth) and disassembly (shortening) make the microtubules dynamic structures that maintain cell shape, polarity, and motility; provide a scaffold for cellular trafficking of proteins and organelles; and play an integral role in mitosis.

Because microtubules and their dynamics are required for mitotic spindle formation and chromosome separation during mitosis, they are an important target for a chemically diverse group of anticancer drugs that induce mitotic arrest and cell death in vitro.

Microtubule-targeting agents (MTA) have been used clinically since the 1960s as single agents or in combinatorial regimens for the effective treatment of leukemia, lymphoma, and various solid tumors. MTAs inhibit cell proliferation by disrupting the dynamics of microtubule polymerization (1, 2). These mitotic inhibitors include the Vinca alkaloids (vincristine, vinblastine, vinorelbine, vinflunine) that destabilize or depolymerize microtubules, and the taxanes (paclitaxel, docetaxel) and epothilones (ixabepilone) that polymerize microtubules, thus causing mitotic arrest. Although it is often stated that MTAs kill proliferating cancer cells by causing mitotic arrest that in turn leads to apoptosis (3–6), such a mechanism is unlikely to be important in patients whose tumors divide on average every 30 to 60 days (7, 8). Evidence that such agents can bring about cell death without affecting mitotic arrest includes the primary toxicity of these agents; that is, the neurotoxicity that inflicts damage on somatic nerve cells that rarely divide (9).

Although neurotoxicity is the principal toxic effect observed clinically with MTAs, how and why this happens is not well understood (10), although it is thought to be due,
at least in part, to the inhibition of axonal microtubules that are necessary for axonal transport in neurons (11). Other than the obvious explanation that neural cells have large amounts of tubulin, studies addressing this phenomenon have been hampered by the lack of suitable in vitro or animal models.

The starting point for this work was a motivation to further understand the effects of MTAs on neural cells to (a) comprehend what role, if any, they might have in the phenomenon of chemo-brain (chemotherapy-associated cognitive dysfunction presumably due to penetration of the blood-brain barrier by anticancer agents); (b) attempt to better understand the problem of neurotoxicity, often a treatment-limiting side effect of this class of drugs; and (c) possibly develop a model of some relevance to neurotoxicity. In the conduct of these studies, we noticed that the microtubule-destabilizing agents (MDA) not only led to the dissolution of microtubules when administered to neural cells but also to the rapid disappearance of tubulin—an observation we had never made in hundreds of similar experiments with cancer cell lines. Prior studies have described the tight regulation of tubulin in neural cells (12, 13). This regulation is not surprising given that (a) tubulin makes up >20% of the soluble protein in brain and (b) the precise polarity of microtubule can differ in different regions of a single neuron, and this polarity is essential for proper intracellular transport and hence for synaptic function. We sought to further investigate our observation of tubulin degradation in neural cells and compare the results with those obtained in cancer cells treated under similar conditions. We describe herein the results of these experiments.

Materials and Methods

Cell culture and reagents
HCN2 and HCN1A neural cells and CRL 2127 (skin fibroblasts) were obtained from American Type Culture Collection (ATCC) and were grown in DMEM supplemented with 10% fetal bovine serum, 2 mmol/L glutamine, 100 units/mL penicillin, and 100 μg/mL streptomycin. MCF-10A (breast epithelial) were grown in DMEM/F12 supplemented with 5% horse serum, 10 ng/mL epidermal growth factor, 500 ng/mL hydrocortisone, 10 μg/mL insulin, and penicillin and streptomycin as described above. Human umbilical vein endothelial cells (HUVEC) were obtained from Lonza and grown in endothelial cell growth medium (EGM BulletKit, Lonza). Cancer cell lines used include MCF-7 (breast), A549 (lung), SY5Y (neuroblastoma and obtained from ATCC), and A19 (ovarian, a A2780 subclone; ref. 14), and were grown in endothelial cell growth medium (EGM BulletKit, Lonza). Cancer cell lines used include MCF-7 (breast), A549 (lung), SY5Y (neuroblastoma and obtained from ATCC), and A19 (ovarian, a A2780 subclone; ref. 14), and were grown in RPMI and supplemented as described above. All cells obtained from cell repositories were grown in culture for less than 6 months.

The following chemicals were used in this study: vincristine, cycloheximide, doxorubicin, paclitaxel, 5-fluorouracil (5-FU), 3-isobutyl-1-methylxanthine, 12-0-tetradecanoylphorbol-13-acetate, nerve growth factor, and bafilomycin A (Baf; Sigma); ixabepilone (Bristol Myers Squibb); dolastatin and MG132 (Calbiochem); and ammonium chloride (NH₄Cl; Fisher Scientific). Reagents were reconstituted and stored according to the manufacturer's instructions.

MTT assays
Cells were plated in 96-well plates and treated with increasing concentrations of vincristine. Vincristine IC₅₀ values for each cell line described in Supplementary Table S1 were determined using the CellTiter 96 AQueous One Solution Cell Proliferation Assay (Promega) as per the manufacturer's protocol. Plates were read on a Bio-Rad Imager (Bio-Rad).

Immunoblots
Cells grown to 70% to 80% confluency in 12-well plates were harvested after drug treatment in 100 μL/well of protein lysis buffer containing 1 mmol/L MgCl₂, 2 mmol/L EGTA, 0.5% NP40, and 20 mmol/L Tris-HCl (pH 6.8) supplemented with protease inhibitors (protease inhibitor cocktail tablets, Roche) plus 200 units/mL aprotinin (Sigma-Aldrich) and 10 μmol/L trichostatin A (Cayman Chemical). Protein concentrations were determined using the Bio-Rad Protein Assay Reagent (BioRad), and 20 μg protein were loaded per well onto 10% or 4% to 15% gradient SDS-PAGE gels. After transfer onto nitrocellulose, blots were blocked in 0.5% milk and each blot was probed with an anti-actin antibody (C4 monoclonal, Chemicon) as an internal control. Other primary antibodies used were anti-p53 (Ab-6 monoclonal Do1, Oncogene Science), anti-acetylated tubulin (clone 6-11B-1, Sigma), and anti-α-tubulin (DM1A monoclonal, Sigma). Infrared species-specific secondary antibodies (LiCor) were used, and protein expression was quantitated using the Odyssey Infrared Imager (LiCor).

Real-time reverse transcriptase-PCR
HCN2 cells were treated with 100 nmol/L vincristine for 0, 8, 24, or 24 hours, followed by 24 hours incubation in a drug-free medium. RNA was isolated from HCN2 cells at these time points using the Qiagen RNaseasy method (Qiagen). One microgram of RNA was reverse transcribed. Real-time PCR was performed on cDNA template using Taqman Master UPL kit (Roche) and the following primers:

- α-Tub 5′: CTTTCGCTCTCTATCTCCTTA
- α-Tub 3′: AGCAGGCAATGACAATCT
- 28s 5′: AAATCGGTTGGAGCTCGT
- 28s 3′: CTATACAAAGTGCGCCTAC

Tubulin levels were normalized to 28S RNA and compared with untreated HCN2 control RNA.

Immunofluorescence and confocal microscopy
Cells plated on glass coverslips in 24-well dishes were incubated either with or without vincristine for 24 hours, rinsed in PBS twice, and either fixed immediately in 100% methanol at −20°C for 10 minutes (15) or incubated in medium without drug for washout (periods of 20 minutes, or 1, 4, 8, or 24 hours) before fixation. Following fixation, all coverslips were rinsed in PBS and incubated for 45 minutes in 20% goat serum in PBS, followed by incubation in either mouse monoclonal
anti-α-tubulin (DM1A) or mouse anti-acetylated α-tubulin (Sigma), and rabbit anti pericentrin (Abcam). Following rinses in PBS after each primary antibody incubation, the coverslips were incubated in either fluorescein-conjugated goat anti-mouse IgG or rhodamine-conjugated donkey anti-rabbit secondary IgG (both from Jackson Immuno Research Labs, Inc.). 4′,6-Diamidino-2-phenylindole (Sigma) was used to counterstain nuclei. Antibody control cells were incubated without any primary or secondary antibodies, with only nonspecific primary IgG, or with only secondary antibodies. Stained cells were visualized on a Zeiss Axiovert 100M microscope equipped with a Plan-NeoFluar 100×/1.3 oil immersion objective, and confocal images were generated using a Zeiss LSM510 META. Images in Results are shown as three-dimensional maximal projections reconstructed from Z-stacks.

Results

In an attempt to better understand how MTAs affect neural cells, we began exploratory studies using a neural cell line (HCN2) established from normal neural cells derived from the cerebral cortical tissue of a patient with Rasmussen's encephalitis (16). Although we recognize the limitations of such in vitro studies, we felt that given the lack of suitable models, these cells at a minimum presented us a starting point. Figure 1 shows results from one of the initial experiments performed to assess the response of the neural cell line HCN2 to several chemotherapeutic agents. An increase in p53 levels was seen in response to the DNA-damaging agent doxorubicin (400 ng/mL) when HCN2 cells were treated for 24 hours, an observation that was expected in these cells with wild-type p53. Consistent with our experience in other models, treatment with paclitaxel (50 nmol/L) or ixabepilone (50 nmol/L) for 24 hours stabilized microtubules, as shown by increased levels of the acetylated form of α-tubulin (acetyl tubulin), a marker of stable microtubules (similar results were observed with an antibody that detects the detyrosinated form of α-tubulin; not shown). We note here that the relative increases observed in these cells that under normal circumstances have a very stable microtubule network is not as pronounced as seen with cancer cell lines. As expected, overnight treatment of HCN2 cells with vincristine destabilized microtubules and lowered acetyl tubulin levels (and detyrosinated tubulin; not shown). Surprisingly, however, overnight treatment with vincristine (25, 50, or 100 nmol/L) also resulted to a marked reduction of tubulin levels as measured with several α-tubulin antibodies. The DNA-damaging agent, 5-FU (5 mmol/L) had no effect on tubulin levels or stability.

Although we anticipated that treatment with vincristine would destabilize microtubules, the decrease in total tubulin levels was unexpected and not previously noted in our work with numerous cancer cell lines. Figure 2A shows a representative immunoblot of HCN2 cells treated with 100 nmol/L vincristine (IC₅₀ ≈ 11 μmol/L) from 8 to 48 hours. Tubulin levels decreased to 30% of their starting levels as early as 8 hours after the addition of vincristine to the culture medium, a decrease that was detectable after 4 hours in other experiments. A further decrease in tubulin levels occurred by 24 hours, and tubulin was undetectable by 48 hours. As the figure shows, vincristine-induced tubulin degradation was reversible. An increase in acetylated tubulin, a marker of stable microtubules, preceded the increase in total tubulin, suggesting a priority for incorporating any remaining tubulin as well as newly synthesized tubulin into stable microtubules, followed subsequently by a replenishment of the soluble tubulin pool. This is seen when vincristine was removed from the medium after 8 or 24 hours of drug treatment. In fact, the results of a separate experiment shown in the graph in Fig. 2A shows that acetyl tubulin levels increased as early as 4 hours after the removal of vincristine from the culture medium. We emphasize at this point that the marked reductions in tubulin levels observed in these experiments were detected with four different antibodies, making it unlikely these results could be explained by the loss or masking of an epitope rather than an actual reduction in tubulin levels. Although tubulin levels varied slightly, in 18 successive experiments, total tubulin levels were reduced to an average of 9.8 ± 7.6% that of the starting level (median of 7%) after treating HCN2 cells with 100 nmol/L vincristine for 24 hours.
That the effect seen with vincristine was not drug specific but rather a consequence of microtubule destabilization was confirmed by demonstrating similar results with the MDA dolastatin. To ensure that the tubulin recovery seen when vincristine was withdrawn from the culture medium was not due to a washout effect of vincristine, dolastatin, a drug that has sustained intracellular retention due to its high affinity for tubulin, was chosen, and these results are shown in Fig. 2B. HCN2 cells were treated for 24 hours with 1 nmol/L dolastatin (IC$_{50}$ ≈ 0.5 nmol/L), and the effect on total and acetyl tubulin
was assessed at various time points after drug removal. A representative immunoblot shown in Fig. 2B shows the near-total disappearance of total tubulin after 24 hours of dolastatin treatment (0 hour off drug). Similar to the results observed after treatment with vincristine, tubulin loss was reversible, and again the increase in acetylated tubulin occurred sooner than the increase in total tubulin, consistent with a preferential incorporation of newly synthesized tubulin into stabilized microtubules. However, the results shown in Fig. 2B show an increase that far exceeded the starting level, an observation that became clear when the cells were examined by immunofluorescence (see below). Although dolastatin uptake is considered irreversible, the time course of the recovery was similar to that following vincristine treatment and was consistent with the synthesis of new tubulin, which in the absence of free dolastatin was then preferentially incorporated into microtubules. We also observed similar results with combretastatin, another depolymerizing agent that binds not the Vinca but the colchicine site of tubulin—establishing not drug binding but microtubule depolymerization as the essential event in tubulin degradation (data not shown). In addition, we observed the destabilization of microtubules and the disappearance of total tubulin with vincristine treatment in HCN1a cells, a second neural cell line (ref. 16; Fig. 2C). Like in the HCN2 cells, acetylated tubulin and total α-tubulin levels were reconstituted after drug removal in HCN1 cells. Finally, we examined tubulin gene expression in HCN2 cells in the presence and absence of vincristine. The graph in Fig. 2D shows that tubulin RNA levels normalized to the control gene 28S. RNA levels only decreased to 52% at 8 hours and to 39% by 24 hours with vincristine treatment, indicating that factors other than reduced transcription were responsible for the marked decrease in tubulin protein levels to less than 10% of control (Fig. 2A). As expected, mRNA levels then increased when drug is removed, likely as a result of reduced tubulin protein levels and the absence of a repressive feedback effect on transcription (17).

Although HCN2 cells were isolated from human cerebral cortex, to confirm that they had maintained their neural properties in culture, we differentiated them as previously described (16) and observed neurite extensions within 4 hours of drug exposure (data not shown). Supplementary Fig. S1A–B shows phase microscopy of neurite production and increased syaptophysin levels, respectively, in HCN2 cells after differentiation for 48 hours. However, total tubulin levels were still markedly decreased after 8 and 24 hours treatment with 100 nmol/L vincristine (Supplementary Fig. S1C).

To establish that the extent of tubulin degradation seen in HCN2 cells exceeded that in other “normal” cells, we compared the results in HCN2 cells with additional normal cell lines as shown in Fig. 3. Relative to HCN2 cells (panel A), when 100 nmol/L vincristine was added to MCF10A (breast), CRL2127 (fibroblast), and HUVEC (umbilical endothelium) cells (panels B–D), only minimal α-tubulin degradation occurred despite clear evidence of microtubule destabilization as shown by the marked decrease in acetylated tubulin.

**Figure 3.** Compared with HCN2 cells, a decrease in total α-tubulin levels in normal cells was less pronounced despite microtubule destabilization. Immunoblots of tubulin protein levels in HCN2 cells and three normal epithelial cell lines (A): MCF10A, breast (B); CRL 2127, fibroblast (C); and HUVEC, endothelial cells (D) after treatment with 100 nmol/L vincristine.
Microtubule destabilization was reversible in the absence of vincristine in the nonneural normal cell lines, although recovery occurred at a later time point (24–48 hours) than in the neural cells (8 hours).

We considered the effect on the HCN2 cells unique, having never observed such results when working with numerous cancer cell lines in the laboratory. However, to exclude the possibility that we had missed such an effect, we performed similar experiments with cancer cell lines representing four different tissues of origin: A549 non–small cell lung cancer cells, A2780 (1A9) ovarian carcinoma cells, MCF7 breast cancer cells, and SY5Y neuroblastoma cells. As shown in Supplementary Table S1, in a 4-day cytotoxicity experiment, the IC50 for HCN2 neural cells was found to be 11 μmol/L—far in excess of the 100 nmol/L maximum used in these experiments—whereas that of the cancer cell lines was 1.6 to 40 nmol/L. When the stability of tubulin was examined using vincristine concentrations equal to the IC50 values, large differences were noted between the extent of tubulin disappearance in the cancer cell lines compared with HCN2 cells (Fig. 4A). Degradation was observed in the cancer cell lines only at higher concentrations that exceeded the IC50; however, at these concentrations, this was accompanied by cell death and the associated protein degradation. A comparison at 100 nmol/L vincristine was only possible at 8 hours before the onset of widespread cell death, and as shown in the graph, demonstrated preferential degradation in HCN2 neural cells (Fig. 4B).

Because in HCN2 cells tubulin degradation occurred in the absence of cytotoxicity and was reversible, we assumed that new protein synthesis must have occurred. Support for this assertion is found in Fig. 5. As seen in Fig. 5A, even after 48-hour inhibition of protein synthesis with cycloheximide (10 μg/ml), very little turnover of tubulin had occurred. This underscores active tubulin degradation as the process behind the decrease in tubulin levels following exposure to a MDA. In addition, following vincristine withdrawal in the presence of the protein synthesis inhibitor cycloheximide, total tubulin levels did not recover, but a recovery of “stable” tubulin levels occurred, as seen by the increase in acetylated tubulin shown in Fig. 6B, demonstrating stabilization of the remaining tubulin.

Degradation through the lysosomal or proteasomal pathways seemed logical starting points as possible mechanisms of active tubulin degradation. To that end, we sought to determine the extent to which inhibition of these pathways would affect the disappearance of tubulin. We investigated the lysosomal pathway by pretreating HCN2 cells with the lysosome inhibitors bafilomycin (10 nmol/L) and ammonium chloride (10 mmol/L) for 24 hours followed by 100 nmol/L vincristine for 24 hours in the presence of the inhibitors. The results in Fig. 5C show that the inhibitors did not prevent the vincristine-induced α-tubulin degradation. We concluded that degradation through the lysosome was not a major contributor to the rapid disappearance of α-tubulin.

However, pretreatment of cells with the proteasome inhibitor MG 132 (50 μmol/L) for 2 hours before the addition of 100 nmol/L vincristine, as shown in Fig. 5D, prevented the disappearance of tubulin at 8 hours (MG 132 + vincristine), and to a lesser extent after 24 hours when compared with 8 or 24 hours vincristine alone. Although this is consistent with proteasome-mediated degradation of tubulin, we cannot conclude that proteasome degradation is the only mechanism for the MG132 effect because, as we have shown previously, and confirm here, inhibition of the proteasome stabilizes microtubules (18). This is shown clearly by the increase in stabilized (acetylated) tubulin seen with the addition of the proteasome inhibitor alone. However, this also shows that vincristine alone works in the presence of MG132. Because of this, one cannot exclude the possibility that, to some extent, the stabilization that occurs as a result of proteasome inhibition is in part mediated by the stabilization effect preventing tubulin degradation. That the protection seen after 24 hours is not as marked as that seen at 8 hours in part reflects cellular toxicity. Nonetheless, the loss of acetylated tubulin when cells were treated with vincristine + MG132 indicates that the microtubules still depolymerized but the tubulin was not degraded, consistent with MG132 inhibition of the proteasome.

The immunoblots indicated that during the initial recovery of HCN2 cells from MDA exposure, most tubulin was incorporated into stable microtubules; however, we often saw what seemed to be an excessive amount of compensation, resulting in acetylated tubulin levels being higher than

![Figure 4](image-url). Vincristine treatment decreased tubulin levels in cancer cell lines but not to the levels seen in neural cells. A, cancer cells (A549, MCF-7, SY5Y, and 1A9) were treated with IC50 concentrations of vincristine (Supplementary Table S1) for 8, 24, and 48 h compared with 100 nmol/L vincristine treatment in HCN2 cells. Total α-tubulin levels in cancer cells compared with HCN2 cells are shown. B, total α-tubulin levels in cancer cells are plotted and compared with HCN2 cells when treated for 8 h with 100 nmol/L vincristine.

Assessment of active tubulin degradation in vitro. A, total α-tubulin levels in cancer cell lines treated with vincristine (VCR) at IC50 concentrations for 24 h were compared with those in HCN2 neural cells treated with 100 nmol/L vincristine. B, total α-tubulin levels in cancer cell lines compared with HCN2 cells treated with 100 nmol/L vincristine for 24 h.
drug-free controls. Thus, we sought to investigate this further by performing immunofluorescence experiments on HCN2 cells using acetylated tubulin (Fig. 6A–E) and total α-tubulin antibodies (Figs. 6F–J) in the presence or absence of 100 nmol/L vincristine. In Fig. 6A and F, untreated HCN2 cells displayed long microtubules that extend throughout the cell. After a 24-hour treatment with vincristine, the microtubules disappeared (Fig. 6B and G). Four hours after the drug is removed, short irregular microtubules appeared throughout the cell (Fig. 6C, C inset, and H). In untreated control cells, the microtubules were not organized in a radial array with a defined centrosomal focus. Following drug exposure, most tubulin staining was lost, although a few short Ac tubulin (+) stubs were seen (Fig. 6B). During recovery from drug, multiple short, Ac tubulin (+) microtubules formed throughout the cell. Microtubule formation did not arise from the centrosome because pericentrin staining showed two centrioles in the majority of cells (Supplementary Fig. S2). Eight hours after drug removal (Fig. 6D, I, and I inset), microtubules were found near the periphery of the cell, and by 24 hours (Fig. 6E and J) these short microtubules were replaced by a microtubule network that can be considered more normal in appearance. Thus, for a time of recovery after drug exposure, only short microtubules comprised the microtubule network.

Discussion

First introduced into clinical oncology in the 1960s, MTAs are essential components in the therapy of many cancers, including lymphoma as well as breast, ovarian, lung, and head and neck cancers (19). In cancer cells, the focus has often been on their ability to interfere with mitosis, a thesis developed with rapidly proliferating in vitro models that has never been proven in patients (20). However, because...
Somatic neurons do not divide, a different explanation must be considered to explain the most common side effect, neurotoxicity (21). The latter has largely centered on the likelihood that microtubule trafficking—especially crucial in neurons—is adversely affected. Clinical support for this thesis includes the fact that neuropathy usually begins in the hands and feet, consistent with a greater impact on longer neurons that are more likely to be affected by interference with trafficking (10). In the present study, we show that compared with cancer cells, neurons respond differently to microtubule disruption by rapidly degrading tubulin. Although we speculate on the importance this might have in terms of neurotoxicity, the results more directly highlight the delicate balance that exists in neurons with regard to handling tubulin.

Our observations are consistent with previous reports documenting precise regulation of tubulin metabolism in neural cells (13). In this regard, we note that consistent with the importance of microtubules in neural cells, the results show the priority given to assembling stable microtubules as recovery occurred, indicated by the increased acetylated tubulin staining, followed later by an increase in soluble tubulin. However, whereas previous reports highlighted centrosomal nucleation and release (22), we found that microtubule reassembly appeared to involve a mechanism of dispersed nucleation, as small, stable microtubule stubs appeared throughout the cytoplasm and not from a central focus. It should be noted that the microtubule arrays in the control HCN2 cells do not radiate from a clear center. Indeed, staining for pericentrin shows that the microtubule arrays are not organized around the centrosomes. Therefore, the apparently dispersed nucleation observed in drug washout is not surprising. Noncentrosomal microtubule arrays have been reported in a number of differentiated mammalian cell types (23). A number of different mechanisms have been reported for generation of these noncentrosomal microtubules, including nucleation and release from the centrosome as well as dispersed nucleation from membrane sites or microtubule oligomers. It has also been suggested that spontaneous dispersed nucleation can occur in cells in which the tubulin has all been depolymerized, resulting in a high concentration of

Figure 6. Confocal microscopy of acetylated tubulin (A–E) and total α-tubulin (F–J) levels in HCN2 cells treated for 24 h with 100 nmol/L vincristine and following drug removal. A and F, untreated control; B and G, 24 h vincristine; C and H, 24 h vincristine followed by 4 h washout of vincristine; D and I, 8 h washout; E and J, 24 h washout.
tubulin dimers. This is clearly not the case here because de-polymerized tubulin was degraded, resulting in a low level of total cellular tubulin. Hence, recovery from drug exposure starts with a low, not a high, concentration of dimeric tubulin.

Cellular control of the expression level of tubulin has been studied for many years; however, most research has been directed at transcriptional and especially translational control of synthesis, in particular autoregulation of tubulin mRNA stability by the level of unpolymerized dimers. Much less is known about regulation of the degradation of this very stable protein, whose half-life can exceed the doubling time of the cells in which it occurs (control of tubulin expression is reviewed in ref. 24). Some reports have addressed the degradation pathways of tubulin and documented that degradation can be enhanced by drugs, including traditional antimicrotubule agents in normal cells (25), isothiocyanates in some cancer cell lines (26, 27), and by peroxisome proliferator-activated receptor γ inhibitors (28, 29). Our study is the first report documenting this effect in neural cells, demonstrating that traditional MDAs that are in clinical use can produce significant and rapid loss of tubulin.

Although we cannot say with certainty that these results have clinical relevance, there is reason to suspect that they may. The concentrations used in the experiments with HCN2 cells (e.g., 100 nmol/L) are concentrations that can be achieved in patients receiving MTAs (30, 31). Moreover, although these concentrations are maintained transiently, and clinically one would expect a recovery of tubulin levels and microtubule stabilization as we observed in our in vitro models, it is not unreasonable to assume that recurrent events of microtubule depolymerization and tubulin degradation might take its toll and lead to a worsening of neurotoxicity over time (32). It is also not unreasonable to think that this might explain the often-noted observation of transient neurotoxicity in which symptoms occur after drug administration, but last only a few days and are followed by recovery (33). Previous reports have shown the neurotoxic effect of epothilone B on dorsal root ganglia and sciatic nerves in Wistar and Fischer rats, citing both neurophysiological and neuropathologic changes. In addition, increased polymerized tubulin levels were observed in sciatic nerve lysates after treatment with epothilone B compared with untreated control animals (34). This suggests that examination of the effects of MDAs in an animal model may be possible. Future studies will be directed toward the in vivo effects of vincristine in murine models to look for tubulin degradation following drug treatment and the in vitro effects of vincristine exposure in differentiated human neural stem cells to support the model of neurotoxicity proposed herein.

In summary, we report rapid degradation of tubulin in normal neural cells following the administration of a MDA. Tubulin levels were markedly reduced by 24 hours following drug treatment and by 48 hours had resulted in near-complete disappearance of tubulin. Tubulin degradation was also rapidly reversible when the MDA was withdrawn. This rapid degradation and recovery appears to be a property that is much more developed in normal neural cells. The latter is not unexpected given that, in neural cells, this protein is not only abundant but also crucial in neural trafficking.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Grant Support

Intramural Research Program, National Cancer Institute, NCI (Bethesda, MD).

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Received 12/01/2009; revised 05/06/2010; accepted 05/06/2010; published OnlineFirst 06/29/2010.

References

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*Published* Cancer Res Published OnlineFirst June 29, 2010.

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