In Vivo Measurement of Microtubule Dynamics Using Stable Isotope Labeling with Heavy Water

EFFECT OF TAXANES*

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In eukaryotic cells, microtubules exist in a state of dynamic instability. Tubulin dimers, consisting of $\alpha$ and $\beta$ monomers, are believed to cycle rapidly through polymerization as microtubules and depolymerization back to dimers. This dynamic instability is important for many functions of microtubules, particularly mitosis (1).

During interphase, many microtubules appear static, although others alternately extend and shorten (2, 3), and yet others may engage in treadmilling, i.e. simultaneous growth at plus ends and shrinkage at minus ends (4–6). During mitosis, the interphase microtubule array is disassembled, and a bipolar spindle is assembled, connecting the microtubule organizing centers to the kinetochore of chromosomes. Rearrangement of the microtubule network upon entry into mitosis involves a 10–100-fold increase in the number of microtubule ends undergoing growth and shrinkage (7, 8). Dynamic instability and treadmilling in mitotic spindles mediate their complex movements during chromosome segregation (9–12). Microtubule dynamics are regulated by endogenous cellular regulators during specific checkpoints that control mitotic progression and cell proliferation (13) and play a crucial role in passage through the metaphase/anaphase checkpoint (14–16).

Because microtubules are in dynamic equilibrium with their subunits, normal cell function requires the maintenance of a specific free tubulin dimer/microtubule polymer ratio, and changes in this ratio have been associated with alterations in several cellular activities (8, 10, 17, 18). Drugs that alter dynamic exchange of tubulin dimers with microtubules (microtubule-targeted tubulin-polymerizing agents (MTPAs)1) interfere with tumor cell proliferation and thus have come to occupy a crucial role in cancer chemotherapeutics. MTPAs either can promote the formation of alternate lattice contacts and polymers at microtubule ends (e.g. colchicines, vinca alkaloids, cryptophycin-52) or they can stabilize existing microtubule polymers and interfere with their disassembly (e.g. epothilone A and paclitaxel) (15, 19–22). Paclitaxel (Taxol®), the prototype drug of this class, is an important cancer chemotherapeutic agent that is effective in the treatment of many types of cancer, including carcinoma of the breast, ovary, lung, head and neck, bladder, and esophagus (23–25).

Paclitaxel binds microtubules with high affinity and with a stoichiometry of 1 mol of paclitaxel/mol of $\beta$-tubulin in microtubules (19, 26–29). Modest concentrations of the drug stabilize microtubules by blocking their dynamic exchange with free tubulin. Higher concentrations (~100 nm) additionally cause a net increase in polymer mass and promote the formation of bundled microtubules (14, 26). Paclitaxel treatment interferes with the function of the mitotic spindle, blocking the G2/M transition (30, 31). Mitotic arrest occurs at the metaphase/anaphase junction and is followed by apoptotic cell death (22, 32, 33). Paclitaxel strongly inhibits HeLa cell proliferation at low concentrations that suppress microtubule dynamics but do

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¶ The abbreviations used are: MTPAs, microtubule-targeted tubulin-polymerizing agents; $^2$H, deuterium; $^2$H2O, heavy water; GC/MS, gas chromatography/mass spectrometry; MAPs, microtubule-associated proteins; Me2SO, dimethyl sulfoxide; PBS, phosphate-buffered saline.
not promote microtubule growth or bundling (14, 27, 34). Thus, suppression of microtubule dynamics, rather than the stimulation of microtubule polymerization, appears to be the key antiproliferative mechanism.

Much of our understanding of intracellular microtubule dynamics and the effects of MTPAs is based on studies in vitro (35–37). These studies are based on measurements of assembly/dissassembly of purified tubulin, using 32P pulse-chase labeling and fluorescence microscopy of cultured cells to track the incorporation of labeled tubulin into microtubules, and on Western blot analysis to detect accumulation or loss of free tubulin and microtubules (5, 38–41). Extending these studies to intact animals has been more challenging. Whereas Western blots have been used to quantify changes in the ratio of free tubulin to microtubules in vivo (39, 41, 42), no method exists to measure in vivo rates and extents of tubulin/microtubule exchange. Changes in the tubulin to microtubule ratio underestimate the effect of MTPAs on microtubule dynamics, however, because drug-induced microtubule stabilization up-regulates de novo synthesis of free tubulin (43–46). To address these issues, we describe here a stable isotope/mass spectrometric method for direct measurement of the overall microtubule dynamics in living cells and tissue. This approach evaluates microtubule assembly/disassembly dynamics as the ratio of bio-synthetic label incorporation into tubulin dimers and polymers.

**EXPERIMENTAL PROCEDURES**

**Cell Culture**—The human epithelial cell lines MCF-7 (derived from a mammary carcinoma) and SW1573 (from a lung carcinoma) were obtained from the ATCC (Manassas, VA). Cells were cultured as monolayers in Dulbecco’s modified Eagle’s medium (Sigma) supplemented with 10% fetal bovine serum (Omega Scientific, Tarzana, CA) in a humidified incubator, were adjusted to 4 mol % heavy water (2H2O) by adding 2H2O (99.9 mol %2H2O) containing 0.9% w/v NaCl (Cambridge Isotope Laboratories, Andover, MA), resulting in 4–5% body water2H enrichment, and were maintained on 8% 2H2O in drinking water (to allow for dilution of label by metabolic water) for 24 h prior to sacrifice (49, 50). 

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**EXPERIMENTAL PROCEDURES**

**General Principle of Stable Isotope Labeling Technique**—One way to measure microtubule dynamics and the degree of equilibrium between tubulin dimers and microtubule polymers (the

**RESULTS**

2H enrichment in body water and culture media was done by using a modification of procedures described previously (58, 60). Briefly, proton from plasma water were transferred to acetylene by reaction with calcium carbide. Acetylene samples were then analyzed using a Series 3000 cycloidal mass spectrometer (Monitor Instruments, Cheshwick, MA), which was modified to record ions at m/z 28 and 27 (M0 and M1) and calibrated against a standard curve prepared by mixing 99.9% 2H2O with unlabeled water. Body water 2H enrichments were not affected by paclitaxel treatment (data not shown).

**Isolation of Colonocytes and Bone Marrow Cells**—Bone marrow cells were flushed from tibiae and femurs with sterile PBS using a 1-ml syringe with an 18-gauge needle.

Colonocytes were isolated from mouse colon tissue by a protocol modified from Kim et al. (55). Briefly, colon was dissected and digested in PBS containing 75 units/ml of collagenase type 2 (Worthington) and 10 μg/ml DNase (Roche Applied Science) at 37 °C for 40 min. Dissociated cells were collected and centrifuged at 800 × g at room temperature for 5 min. Pellets were gently suspended in 45% Percoll, loaded on top of a 75–45% (v/v) Percoll, 1.6% JKMEM in PBS (Sigma) gradient, and centrifuged at 350 × g for 30 min at room temperature. Colonocytes were collected from the top of the gradient, washed with PBS, and filtered through a 35-μm sterile filter.

**Processing of DNA for GC/MS Analysis—Extraction, hydrolysis, and derivatization of DNA from tumor tissue, bone marrow, and colonocytes was performed as described previously (56–58).** GC/MS analysis in negative chemical ionization mode was performed as described (58). 2H enrichment was calculated as the percent increase, over natural abundance, in the percentage of purine deoxyribose derivative present as the (M + 1) mass isotope (58, 59).

**Processing of Tubulin for GC/MS Analysis**—Tubulin samples were hydrolyzed by treatment with 6 N HCl for 16 h at 110 °C. Protein-derived amino acids were derivatized to pentafluorobenzyl derivatives, and 2H incorporation into alanine was measured by GC/MS as described in detail elsewhere (49). 2H enrichment was calculated as the percent increase, over natural abundance, in the percentage of an alanine derivative present as the (M + 1) mass isotope. An index of microtubule dynamics was calculated as the ratio of 2H enrichment in polymer to 2H enrichment in dimer.

**Measurement of 2H2O Enrichment of in Body Water**—Measurement of 2H2O enrichment in body water and culture media was done by using a modification of procedures described previously (58, 60). Briefly, protons from plasma water were transferred to acetylene by reaction with calcium carbide. Acetylene samples were then analyzed using a Series 3000 cycloidal mass spectrometer (Monitor Instruments, Cheshwick, MA), which was modified to record ions at m/z 28 and 27 (M0 and M1) and calibrated against a standard curve prepared by mixing 99.9% 2H2O with unlabeled water. Body water 2H enrichments were not affected by paclitaxel treatment (data not shown).

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**Measurement of 2H2O Enrichment of in Body Water**—Measurement of 2H2O enrichment in body water and culture media was done by using a modification of procedures described previously (58, 60). Briefly, protons from plasma water were transferred to acetylene by reaction with calcium carbide. Acetylene samples were then analyzed using a Series 3000 cycloidal mass spectrometer (Monitor Instruments, Cheshwick, MA), which was modified to record ions at m/z 28 and 27 (M0 and M1) and calibrated against a standard curve prepared by mixing 99.9% 2H2O with unlabeled water. Body water 2H enrichments were not affected by paclitaxel treatment (data not shown).

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fig. 1. Measuring microtubule dynamics using 2H2O labeling. A. 2H atoms from heavy water (2H2O) are incorporated into C–H bonds of nonessential amino acids (NEAAs) such as alanine, glycine, and glutamine. The 2H label is thus incorporated into newly synthesized tubulin dimers, which in turn are incorporated into microtubules. B. When microtubules are dynamically unstable, there is rapid exchange between dimers and polymers, and 2H label accumulates in polymers at nearly the same rate as it appears in dimers. In contrast, when microtubules are less dynamic or when dynamic exchange with tubulin dimers is disrupted by the action of MTPAs, 2H will enter the dimer pool at the biosynthetic rate, whereas incorporation into polymers is diminished.

putative target of MTPAs) is through isotopic labeling. If an isotopic label is introduced into the tubulin dimer pool via endogenous synthesis of new α/β-tubulin dimers (50, 62), it will rapidly distribute among both dimers and polymers and accumulate in both pools at the same rate, if dimers and polymers undergo rapid, dynamic exchange with each other (49, Fig. 1, A and B). If, however, microtubules exchange slowly, or if dynamic exchange is disrupted by the action of MTPAs, accumulation of label in microtubules should lag behind label incorporation into tubulin dimers. Moreover, trapping of tubulin in polymer form by agents such as MTPAs might stimulate new biosynthesis of tubulin dimers (22, 44, 46, 63); this would further amplify the isotopic label incorporation rate into dimers and the disequilibrium relative to polymers (Fig. 1B).

The development of a heavy water (2H2O) labeling technique for measuring protein synthesis provided an ideal means of testing these kinetic predictions regarding microtubules (49). 2H2O administration in vivo and in vitro results in a nearly immediate plateau in the 2H enrichment in body water, which remains relatively constant for days and weeks (64–67). A constant isotopic enrichment in the biosynthetic precursor pool for proteins greatly simplifies interpretation of the label incorporation relationship between dimer and polymer (Fig. 1). Moreover, 2H2O labeling is easily carried out for several days or longer, if necessary, and allows concurrent measurements of DNA replication and, thereby, cell proliferation (57–59). The relationship between microtubule dynamics and cell division can therefore be measured through a single labeling protocol.

Absence of 2H2O effects on Microtubules—To use 2H labeling as a probe of microtubule dynamics, we first had to establish that nontoxic doses of 2H2O (4–5% 2H2O in body water) routinely used for in vivo biosynthetic labeling (49, 50, 61) had no effect on tubulin polymerization. Previous studies have shown no phenotypic consequences of raising animals on chronic body 2H2O enrichment of 20–30% (67, 68), including no effect on highly proliferative tissues such as bone marrow. Nonetheless, direct examination of tubulin polymerization was considered important because at higher levels (≥50% 2H2O), which are acutely toxic or lethal in vivo, previous studies have reported alterations of microtubule polymerization (69–71). Thus, we cultured human lung cancer cells (SW1573 cell line) for 36 h in the presence or absence of 4% 2H2O in body water and isolated tubulin dimer and polymer fractions from post-nuclear supernatants. To assess the abundance of tubulin in the dimer and polymer fractions, we performed densitometry of immunoblots stained with antibodies to α-tubulin. The amount of tubulin in the dimeric and polymerized tubulin fractions was indistinguishable in the presence or absence of 4% 2H2O (Fig. 2A), indicating no discernible net effect on microtubule polymerization. In contrast, addition of 0.4 μM paclitaxel, as a positive control, caused a slight increase in total tubulin levels, attributable largely to an increase in the polymer fraction (Fig. 2A).

Stable Isotope Incorporation into Tubulin in the Presence or Absence of Paclitaxel in Cultured SW1573 Cells—To determine whether 2H2O labeling revealed effects of paclitaxel on microtubule dynamics in asynchronous cell populations, cultured SW1573 cells were labeled during exponential growth with culture media containing 4% 2H2O for various times. Tubulin dimer and polymer fractions were isolated from post-nuclear supernatants, and tubulin was purified from each fraction to 90% purity as determined by SDS-PAGE (Fig. 2B). After acid hydrolysis, amino acids were derivatized for GC/MS analysis, and 2H label incorporation into the alanine derivative was quantified (Fig. 3). Label incorporation in the tubulin dimer fraction steadily increased throughout the labeling period (Fig. 3A), indicating [2H]alanine incorporation into newly synthesized tubulin dimers. Isotopic enrichments in the tubulin polymer fractions were almost superimposable on those from the tubulin dimers (Fig. 3). The nearly identical rates of [2H]tubulin labeling in both fractions demonstrates the presence of a rapid equilibrium between tubulin dimers and microtubules (Fig. 1B), i.e. the flux of dimers into polymers occurred on a time scale that was fast compared with the overall rate of label incorporation into the pool of dimers. In actively dividing cells, tubulin dimers and polymers are therefore in a kinetic equilibrium on a time scale of hours.

In contrast, in the presence of 0.4 μM paclitaxel, the rate of 2H incorporation into polymers was reduced by 75%, compared with the rate of label incorporation into the dimer (Fig. 3B). This isotopic gradient indicates a substantial inhibition of [2H]tubulin dimer flux into the polymer, reflecting stabilization of microtubule polymers and, thus, prevention of dynamic exchange (22, 72, 73). Of note, the failure to incorporate newly synthesized [2H]tubulin into microtubules did not result in a net loss of microtubules (Fig. 2A), suggesting that paclitaxel may prevent microtubules from being proteolytically degraded as well. Finally, the labeling rate of dimeric tubulin was slightly but consistently higher in the paclitaxel-treated cells; this likely reflected up-regulation of α/β-tubulin biosynthesis by paclitaxel, which has been reported previously (44, 46, 74–76). This is a well described autoregulatory phenomenon that is sensitive to the level of free tubulin in the cell and acts to maintain a steady supply of tubulin for microtubule assembly (45).

Stable Isotope Incorporation Reveals Effects of Paclitaxel on Microtubule Dynamics on Implanted Tumors in Vivo—To demonstrate the feasibility of applying this stable isotope assay of microtubule dynamics in vivo, we implanted SW1573 lung cancer cells and breast cancer cells (MCF-7) into nude mice. Xenografted tumors were allowed to grow to ∼1000-mm3 volume, mice were then injected intraperitoneally with increasing doses of paclitaxel. We administered 2H2O (8%) in drinking water for a 24-h period, resulting in about 5% 2H enrichment in body water (49, 50). Animals were sacrificed 24 h after drug treatment, and tumor tissue was removed for analysis of 2H label incorporation into tubulin dimers and polymers (Fig. 4). In
SW1573 xenografts from untreated control animals, tubulin synthesis was somewhat lower than during in vitro culture (1.4% ^2H enrichment, Fig. 4A, versus 2% at 24 h in culture cells, Fig. 3), but importantly, label incorporation into tubulin dimers and microtubule polymers was again indistinguishable, demonstrating rapid exchange between the two pools (dynamic equilibrium). In contrast, paclitaxel treatment decreased incorporation of [2H]tubulin into microtubule polymers in a dose-dependent manner (Fig. 4A), indicating disruption of rapid exchange between free tubulin and microtubule. The fractional synthesis of tubulin dimers increased, similar to what was measured in culture. Similar effects of paclitaxel were also seen in implanted MCF-7 tumors, although the amount of ^2H label incorporation was lower than in SW1573 tumors (Fig. 4B). Paclitaxel-dependent reduction of ^2H label in polymers was less pronounced, but statistically significant at the 5 and 10 mg/kg doses. There was no significant paclitaxel induced up-regulation of tubulin dimer fractional synthesis over base line in this tumor. We concluded that the modulation of the dynamic exchange of tubulin dimers with microtubules by paclitaxel was detectable in vivo.

Relationship between Microtubule Dynamics and Cell Proliferation in Response to Paclitaxel Administration—Various factors can interfere with the actions of paclitaxel in tumors, including alterations in tubulin isotype content, efflux pumps, and in vivo drug metabolism (17, 44, 77, 78). Regardless of these factors, if inhibition of microtubule dynamic is related to the antiproliferative activity of MTPAs in vivo, there should be a correlation between the two processes. To explore this relationship, we correlated the paclitaxel-induced inhibition of microtubule dynamics with the inhibition of DNA synthesis in tumor cell tissue (the latter serving as a quantitative, stable isotope-based measurement of cell proliferation) (50, 57). The results (Fig. 5, A and B) show a clear relationship, in both types of tumor, between inhibition of microtubule dynamics (expressed as fractional reduction, compared with controls, of label incorporation into polymerized microtubules) and inhibition of newly synthesized DNA in tumors. Thus, the inhibition of microtubule dynamics appeared to track in vivo with the antiproliferative action of MTPAs.

Most interestingly, the quantitative relationship between microtubule dynamics and cell proliferation appeared to be cell type-dependent (Fig. 5). In lung SW1573 cancer tumors, an 80% inhibition of microtubule dynamics was required to achieve 50% inhibition of DNA synthesis (Fig. 5A), whereas only 40% inhibition of microtubule dynamics was required to achieve the same result in MCF-7 tumors (Fig. 5B). The greater susceptibility of the latter tumor to inhibition of microtubule dynamics may contribute to its greater sensitivity to killing (IC_{50} = 1 μM, versus 3 μM for SW1573 cells in vitro, by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay; data not shown).
Despite the potent anti-tumor activity of paclitaxel, its clinical use is often hampered by dose-limiting side effects such as myelosuppression, diarrhea, and neuropathy (79–81). Accordingly, we evaluated potential toxic side effects of MTPAs in host tissues using heavy water (2H2O) labeling techniques.

First, we determined the effects of MTPAs on DNA replication in colonocytes and bone marrow cells. A trend toward dose-dependent reduction in DNA synthesis was observed in colon epithelia, but not in bone marrow, from tumor-bearing mice, at 24 h of 2H2O labeling following a single dose of paclitaxel (data not shown).

In contrast to the antiproliferative effects of MTPAs, their neurotoxicity cannot be evaluated by studying DNA replication, because neurons are differentiated, post-mitotic cells. To test whether modulation of microtubule dynamics by MTPAs could be detected in peripheral nerves, we measured 2H label incorporation into free tubulin and polymerized microtubules from sciatic nerve (Fig. 6). The results contrasted remarkably with those observed in tumor cells. Unlike the kinetic equilibrium between free and bound tubulin observed in tumor cells, sciatic nerve microtubules exhibited very low level of labeling (i.e., were largely static) at base line, and 2H label incorporation into polymer was only about 40% that measured in free tubulin (i.e., dynamic equilibrium) (82–85). Surprisingly, higher doses of paclitaxel increased the label incorporation into polymers, while slightly decreasing labeling of free tubulin, and reduced the labeling gradient between dimers and polymers. These results suggest a stimulation of microtubule polymer aggregation by paclitaxel. To explore this effect further, we assessed net changes in the abundance of unpolymerized tubulin and microtubule polymers by densitometric analysis (Fig. 6B). This experiment showed a dose-dependent increase in the content of microtubule polymers and a slight decrease in free tubulin dimers. It appears that, in sciatic nerve microtubules, paclitaxel-induced neurotoxicity is due to increased polymer aggregation.
nerves, paclitaxel increases the aggregation of microtubule polymers and promotes net incorporation of newly synthesized tubulin dimers into microtubule polymers.

**DISCUSSION**

We have used $^2$H isotope labeling with heavy water ($^2$H$_2$O) as a universal metabolic tracer to study the effects of paclitaxel on microtubule dynamics and cell proliferation in malignant and normal tissues of tumor-bearing animals. Microtubules are dynamic polymers that are continuously built and degraded in living cells. To measure microtubule dynamics, two experimental approaches have been used (2, 9, 20). One approach measures the kinetics of fluorescence redistribution after photobleaching or photoactivation in assembled microtubule bundles harboring fluorescently labeled tubulin. Another approach is to visualize the dynamic behavior of individual microtubules. Our biosynthetic labeling approach measures overall microtubule dynamics in vivo as the fraction of total microtubules that is in rapid exchange with free tubulin. Microtubule dynamics in vivo is validated extensively and has been reported elsewhere (57, 63, 64). Details of the protein labeling method with $^2$H$_2$O will be reported elsewhere, but two features of our assay of microtubule dynamics are worth discussing here. First, all biosynthetic labeling methods for measuring protein turnover rates based on estimates of fractional synthesis (% new protein synthesized per unit time) rely on assumptions about the concentration of the metabolic tracer in the precursor pool. These assumptions can be difficult to test, as the aminoacyl-tRNA pool usually is not readily accessible to analysis of label incorporation. In the present application, however, the key metabolic precursor is not aminoacyl-tRNA; rather, the objective is to measure the flux of free tubulin dimer into microtubules (Fig. 1). $^2$H label incorporation into the dimer, which acts as the immediate metabolic precursor of polymerized tubulin, is readily measurable. This provides a simple way to calibrate the precursor-product relationship; the ratio of $^2$H enrichment in microtubules to that in free dimers is equal to the fraction of microtubules that have exchanged with free tubulin at any given labeling time. Second, even though stabilization of mi-

The use of $^2$H$_2$O as a source of stable isotope label to measure new DNA synthesis, and hence cell proliferation, in vivo is validated extensively and has been reported elsewhere (57, 63, 64). Details of the protein labeling method with $^2$H$_2$O will be reported elsewhere, but two features of our assay of microtubule dynamics are worth discussing here. First, all biosynthetic labeling methods for measuring protein turnover rates based on estimates of fractional synthesis (% new protein synthesized per unit time) rely on assumptions about the concentration of the metabolic tracer in the precursor pool. These assumptions can be difficult to test, as the aminoacyl-tRNA pool usually is not readily accessible to analysis of label incorporation. In the present application, however, the key metabolic precursor is not aminoacyl-tRNA; rather, the objective is to measure the flux of free tubulin dimer into microtubules (Fig. 1). $^2$H label incorporation into the dimer, which acts as the immediate metabolic precursor of polymerized tubulin, is readily measurable. This provides a simple way to calibrate the precursor-product relationship; the ratio of $^2$H enrichment in microtubules to that in free tubulin dimers is equal to the fraction of microtubules that have exchanged with free tubulin at any given labeling time. Second, even though stabilization of mi-

FIG. 5. Relationship between the effects of paclitaxel on microtubule dynamics and cell DNA turnover in tumor xenografts. SW1573 (A) and MCF-7 (B) tumor tissue from xenografted, 24-h $^2$H$_2$O-labeled, paclitaxel-treated mice was analyzed for $^2$H label incorporation into tubulin dimers and polymers (cf. Fig. 4), and an index of microtubule dynamics was calculated as the ratio of $^2$H enrichment in microtubules to that in free dimers from the same tissue sample (∼1 in untreated tissue). DNA also was extracted from aliquots of tumor tissue and used to quantify $^3$H incorporation into deoxyribonucleosides by GC/MS (a measure of de novo DNA synthesis). Inhibition of DNA synthesis was calculated as the percent decrease in $^2$H incorporation in paclitaxel-treated, compared with untreated tumors (x axis), and correlated with the index of microtubule dynamics (y axis). Both separate slopes are significantly different from zero.

FIG. 6. Effect of paclitaxel dose on microtubule dynamics in sciatic nerve of tumor-bearing mice. A, kinetic analysis. Sciatic nerves were dissected from SW1573 tumor-bearing mice that had been labeled with $^2$H$_2$O for 24 h following a single intraperitoneal injection of paclitaxel at the indicated dose. Tubulin dimer and polymer fractions were purified from sciatic nerve tissue, and $^2$H label incorporation into alanine was measured by GC/MS (mean ± S.D. for groups of three mice). At base line, $^2$H label incorporation into microtubule polymers was 40% that measured in the free dimeric tubulin fraction and increased with paclitaxel dose. A significant linear trend for the polymer was observed ($p < 0.003$ by analysis of variance with a contrast for linear trend over dose). B, tubulin abundance was measured by Western blotting for $^2$H enrichment (Fig. 2A) in total cytosolic extracts, as well as in dimer and polymer fractions, of sciatic nerves from the mice used in A. Data showed a paclitaxel dose-dependent increase in the content of microtubule polymer and a slight decrease of free tubulin dimers. The use of $^2$H$_2$O as a source of stable isotope label to measure new DNA synthesis, and hence cell proliferation, in vivo is validated extensively and has been reported elsewhere (57, 63, 64). Details of the protein labeling method with $^2$H$_2$O will be reported elsewhere, but two features of our assay of microtubule dynamics are worth discussing here. First, all biosynthetic labeling methods for measuring protein turnover rates based on estimates of fractional synthesis (% new protein synthesized per unit time) rely on assumptions about the concentration of the metabolic tracer in the precursor pool. These assumptions can be difficult to test, as the aminoacyl-tRNA pool usually is not readily accessible to analysis of label incorporation. In the present application, however, the key metabolic precursor is not aminoacyl-tRNA; rather, the objective is to measure the flux of free tubulin dimer into microtubules (Fig. 1). $^2$H label incorporation into the dimer, which acts as the immediate metabolic precursor of polymerized tubulin, is readily measurable. This provides a simple way to calibrate the precursor-product relationship; the ratio of $^2$H enrichment in microtubules to that in free tubulin dimers is equal to the fraction of microtubules that have exchanged with free tubulin at any given labeling time. Second, even though stabilization of mi-
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crotubules (69, 70), or rearrangement of microtubule arrays (71), can be promoted by high, toxic doses of \(^2\text{H}_2\text{O}\) (> 50%), our results showed no alteration of microtubules in the presence of the content of \(^3\text{H}_2\text{O}\) (4–5%) routinely used for in vivo labeling in our studies.

In vitro studies have highlighted the kinetic complexity of microtubule assembly and disassembly (2, 18, 20, 35, 37, 42). The kinetic resolution of biosynthetic labeling experiments with \(^2\text{H}_2\text{O}\) is intrinsically limited by the time scale on which \(^2\text{H}\) equilibrates in body water (15–30 min in mice and 30–120 min in humans) (86). Thus, many events involved in the dynamic exchange of tubulin dimers with microtubules on the second to minute time scale (e.g. nucleation versus elongation) are invisible to our method. Indeed, even in cultured log-phase tumor cells (Fig. 3), there is no detectable lag between label incorporation into tubulin dimers and polymers, even at the earliest labeling time points (30 min), and the time course of label incorporation is identical for both, suggesting that the entire microtubule network exchanges completely on this time scale. This highlights the dynamic nature of the microtubule network in actively (and asynchronously) proliferating cells.

In striking contrast, paclitaxel treatment prevents subsequent exchange of the majority of microtubules with free tubulin dimers as early as 30 min, and this effect lasts for at least 36 h. A constant, minor fraction of microtubules remains exchangeable throughout the time course, suggesting that a subpopulation of microtubules continues to exchange rapidly, whereas the remainder is inactivated by the drug. This dichotomous behavior is consistent with the presence of paclitaxel-resistant and -susceptible tubulin isoforms (29, 87, 88), and thus may serve as a marker of functional paclitaxel resistance of microtubules in vivo, although further studies will be required to address this issue. Overall, our results are consistent with previous studies in cell culture showing that paclitaxel induces formation of extremely stable and nonfunctional microtubules (22, 26, 35, 73, 89). Perhaps more intriguingly, sciatic nerve microtubules are only partially exchanged with free microtubules after 24 h of labeling. This suggests that neuronal MAPs are also capable of slowing exchange with free tubulin to a time scale of many hours.

Paclitaxel appeared to have similar effects on microtubule dynamics in cultured tumor cells and in intact tumor-bearing animals. In both settings, paclitaxel inhibited the flux of newly synthesized tubulin dimers into microtubules, although less so for MCF-7 than for SW1573 cells. This effect was dependent on paclitaxel dose. Inhibition of microtubule exchange with free tubulin dimers appeared to be relevant to the antitumor effect, because disruption of microtubule dynamic equilibrium correlated with the inhibition of new DNA synthesis in both types of tumor. This is consistent with the prevailing notion, based on cell culture studies, that interference with microtubule dynamics during the cell cycle is relevant to the antiproliferative activity of paclitaxel (14, 27, 29, 34). In contrast, the up-regulation of fractional tubulin synthesis, seen in SW1573, but not at the drug doses used in MCF-7 tumors, was clearly not required for inhibition of DNA synthesis. The stimulation of tubulin dimer biosynthesis likely represents a feedback mechanism that maintains a constant level of free tubulin dimer in the face of drug-induced stabilization of microtubules (44, 45, 74, 75).

Most interestingly, the two cell lines differed in the degree of inhibition of microtubule dynamics required to inhibit tumor growth (as measured by newly synthesized DNA). Clinically, tumor response to paclitaxel varies greatly, due to differences in systemic drug metabolism and various factors intrinsic to tumor cells. The latter include expression of multidrug resistance proteins which act as drug efflux pumps, up-regulation of intrinsically paclitaxel-resistant tubulin isoforms, and differences in the susceptibility to downstream consequences of drug-induced microtubule stabilization, such as cell cycle arrest and apoptosis (87, 88, 90–94). Whereas the two tumor cell lines could differ in any or all of these respects, many of these variables would be predicted to influence the overall efficacy of the drug but not the relationship between microtubule dynamics and cell proliferation. Drug efflux pumps can diminish the local concentration of paclitaxel in the tumor, and up-regulation of drug-resistant tubulin isoforms or mutants would also protect the tumor from the antiproliferative activity of the drug (22, 47, 76, 78, 95). Thus, although both factors could increase the IC\(_{50}\), neither should alter the relationship between drug-induced inhibition of microtubule dynamics and cell turnover. The fact that this relationship is cell type-dependent suggests that the combination of both assays provides information about the sensitivity of cells to actions of paclitaxel that are downstream of, or independent of, its antimicrotubule effect. It will be important to assess whether this type of variability is a major determinant of drug resistance of human tumors.

Mechanism-based toxicities of paclitaxel are well established and often dose-limiting, providing another constraint on the clinical application of the drug. This assay of microtubule dynamics was also able to detect the effects of paclitaxel in sciatic nerves, which are known targets of paclitaxel-induced neuropathy (96). Neuropathy cannot be due to antiproliferative effects, as neurons are postmitotic; rather, it can be attributed directly to the accumulation of aberrantly formed, bundled microtubules that are not part of the normal axonal cytoskeleton (97–99). In our studies of tubulin dynamics, we observed that paclitaxel caused a net increase in the amount of total and microtubule-associated tubulin, similar to that seen in cultured, drug-treated tumor cells. However, the effects of paclitaxel on kinetics were quite different in the two tissues. Whereas the drug diminished \(^3\text{H}\) label incorporation into the (nominally dynamic) tumor cell microtubules, labeling of the (nominally static) microtubules in sciatic nerves was increased. Thus, in tumor cells, paclitaxel appears to stabilize pre-existing microtubules and to prevent new label incorporation. In contrast, in peripheral nerves, axonal microtubules are already stabilized by MAPs, and paclitaxel appears to recruit newly synthesized tubulin into aberrant bundles (96–98, 100–103). How this happens remains unclear. One possibility is that paclitaxel promotes recruitment of newly made oligomeric “nuclei” into bundles by binding to, and thus stabilizing, otherwise transient oligomers that then polymerize into bundles. These effects are likely to contribute to neuropathy, as neuronal microtubules are normally key participants in axonal transport and thus in maintaining the functional integrity of axons.

In conclusion, we have established a simple, rapid assay of microtubule dynamics, based on stable isotope labeling technology with heavy water, that can be used in intact animals. The assay is readily adaptable for human use, as labeling with \(^2\text{H}_2\text{O}\) can be performed safely and easily in humans (50, 64, 86, 104). The technology reveals constitutive differences in microtubule dynamics between tissues and is exquisitely sensitive to the action of MTPAs, revealing significant in vivo effects at doses up to 30-fold lower than the maximum tolerated therapeutic dose of paclitaxel after only 24 h (105). In combination with simultaneous, stable isotope-based measurements of new DNA synthesis, this approach can reveal mechanism-based therapeutic and toxic actions of microtubule-stabilizing drugs. These tests can also provide information about variation among tumors with regard to susceptibility to downstream effects of such drugs. Insights about the mechanism of disruptive action
