

2-(m-Azidobenzoyl)taxol binds differentially to distinct β -tubulin isotypes

Chia-Ping Huang Yang^{a,b,1}, Eng-Hui Yap^c, Hui Xiao^d, Andras Fiser^c, and Susan Band Horwitz^{a,1}

^aDepartment of Molecular Pharmacology, Albert Einstein College of Medicine, Bronx, NY 10461; ^bDepartment of Obstetrics and Gynecology and Women's Health, Division of Gynecologic Oncology, Albert Einstein College of Medicine, Bronx, NY 10461; ^cDepartment of System and Computational Biology, Albert Einstein College of Medicine, Bronx, NY 10461; and ^dLaboratory of Macromolecular Analysis and Proteomics, Albert Einstein College of Medicine, Bronx, NY 10461

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There are seven β -tubulin isotypes present in distinct quantities in mammalian cells of different origin. Altered expression of β -tubulin isotypes has been reported in cancer cell lines resistant to microtubule stabilizing agents (MSAs) and in human tumors resistant to Taxol. To study the relative binding affinities of MSAs, tubulin from different sources, with distinct β -tubulin isotype content, were specifically photolabeled with a tritium-labeled Taxol analog, 2-(m-azidobenzoyl)taxol, alone or in the presence of MSAs. The inhibitory effects elicited by these MSAs on photolabeling were distinct for β -tubulin from different sources. To determine the exact amount of drug that binds to different β -tubulin isotypes, bovine brain tubulin was photolabeled and the isotypes resolved by high-resolution isoelectrofocusing. All bands were analyzed by mass spectrometry following cyanogen bromide digestion, and the identity and relative quantity of each β -tubulin isotype determined. It was found that compared with other β -tubulin isotypes, β III-tubulin bound the least amount of 2-(m-azidobenzoyl)taxol. Analysis of the sequences of β -tubulin near the Taxol binding site indicated that, in addition to the M-loop that is known to be involved in drug binding, the leucine cluster region of β III-tubulin contains a unique residue, alanine, at 218, compared with other isotypes that contain threonine. Molecular dynamic simulations indicated that the frequency of Taxol-accommodating conformations decreased dramatically in the T218A variant, compared with other β -tubulins. Our results indicate that the difference in residue 218 in β III-tubulin may be responsible for inhibition of drug binding to this isotype, which could influence downstream cellular events.

Taxol | microtubule stabilizing agents | tubulin isotypes | drug binding | photolabeling

The tubulin/microtubule system is a validated target for a number of important antitumor drugs; examples are the vinca alkaloids, the taxanes, and the epothilones, all of natural product origin. Our laboratory has studied extensively the microtubule stabilizing agent (MSA) Taxol (paclitaxel), which has been approved for treatment of a variety of malignancies, including ovarian, breast, and lung carcinomas, and Ixempria (ixabepilone), approved for metastatic breast cancer. MSAs abrogate normal microtubule function by stabilizing the polymer, leading to inhibition of cell division and cell death. By altering the dynamicity of microtubules, Taxol also exerts a variety of effects in interphase cells, such as interfering with microtubule trafficking (1, 2).

Microtubules are made up of α - and β -tubulin, the latter containing the binding site for Taxol (3, 4). There are seven β - and eight α -tubulin isotypes present in distinct quantities in mammalian cells of different origin (5). Each isotype is the product of an individual gene. Expression of different tubulin isotypes, as well as altered posttranslational modifications (PTMs) of tubulin, have been found in cancers (6, 7). It has been reported that in several cancers, overexpression of β III-tubulin is associated with resistance to Taxol and other classes of antitumor drugs (8, 9). β III-Tubulin-induced Taxol resistance has been associated with reduced effects on microtubule dynamic instability (10).

We have demonstrated that the expression level of β -tubulin isotypes is altered in cancer cell lines resistant to MSAs, such as

Taxol, epothilone B (EpoB), and ixabepilone (Ixab), all of which bind in the taxane binding site in β -tubulin (11, 12). For example, the level of β III-tubulin is very low in the human ovarian cancer cell line, Hey, but increased 8- to 15-fold in drug-resistant daughter cell lines (12). It has been reported that β III-tubulin present in the cytoskeleton is phosphorylated and glycosylated, and associated with the resistant phenotype in ovarian cancer cells (13). Although many factors play a role in drug resistance, binding of drugs to their primary cellular target is of fundamental importance. Therefore, it is worthwhile to study the binding of MSAs to different β -tubulin isotypes, particularly to β III-tubulin, and determine if there is a correlation between isotype expression, drug binding, and drug sensitivity. To study binding of MSAs to human tubulin, in particular to different human tubulin isotypes, sufficient quantities of soluble tubulin is necessary. However, the expression of significant levels of human tubulin in a cell-free system has been difficult to accomplish, although recently recombinant $\alpha\beta$ III-tubulin has been expressed in a baculovirus system (14). As a first step, we have performed binding studies using tubulin from three eukaryotic sources, bovine brain (BBT), porcine brain (PBT), and chicken erythrocytes (CET). BBT and PBT contain mainly β I-, β II-, β III-, and β IV isotypes, whereas CET contains only β VI-tubulin.

We previously demonstrated that [³H]Taxol binding to BBT was almost completely inhibited by equal molar discodermolide, another MSA, but its binding to CET was not inhibited (15). These results suggested that the binding affinity of discodermolide (Disco) was different for tubulins with distinct β -tubulin isotype content. In this report, we investigated the relative binding affinities of MSAs to β -tubulin isolated from different eukaryotic sources, using a radiolabeled photoaffinity Taxol

Significance

β -Tubulin is a validated target for Taxol. There are seven β -tubulin isotypes present in distinct quantities in mammalian cells of different origin. Altered expression of β III-tubulin is found in cancer cell lines and in human tumors resistant to Taxol. A tritium-labeled Taxol analog was used to determine that β III-tubulin binds the least amount of drug, compared with other isotypes. Sequence analysis near the Taxol binding site revealed the presence of a unique Ala218 in β III-tubulin. Molecular dynamics simulations indicated that this residue could influence the ability of Taxol to interact with its binding site. These results link drug response with the β -tubulin isotypes present in a tumor and emphasize the importance of designing Taxol analogs that specifically interact with β III-tubulin.

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¹To whom correspondence may be addressed Email: chia-ping.h.yang@einstein.yu.edu or susan.horwitz@einstein.yu.edu.

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analog, [^3H]2-(m-azidobenzoyl)taxol (2-m-AzTax). We also determined the amount of [^3H]2-m-AzTax binding to different β -tubulin isotypes resolved by high-resolution isoelectrofocusing (IEF), followed by identification with mass spectrometry (16).

Our studies demonstrated that, compared with other β -tubulin isotypes, β III-tubulin binds the least amount of [^3H]2-m-AzTax. Analysis of sequences of β -tubulin near the Taxol binding site indicated that there is a unique alanine residue at 218 in the leucine cluster region (17) of β III-tubulin, whereas all other isotypes have a threonine at this position. Subsequent computational molecular simulation studies indicated that the frequency of Taxol-accommodating conformations decreased dramatically in the T218A variant compared with the other isotypes.

Results

MSAs Have Distinct Effects on Photolabeling of β -Tubulin from Different Eukaryotic Sources. To study the relative binding affinities of MSAs, tubulin from bovine brain, porcine brain, HeLa cells, and chicken erythrocytes was specifically photolabeled with [^3H]2-m-AzTax, in the presence and absence of a fourfold molar excess of either Taxol, EpoB, Disco, Ixab, laulimalide (LML), or peloruside A (PeLA). GTP (1 mM) was included in all experiments. β -Tubulin isotype content from these sources are different, particularly for chicken erythrocytes that contain only one β -tubulin isotype, β VI. The inhibitory effects elicited by Taxol, EpoB, Disco, and Ixab on photolabeling were distinct for β -tubulin from BBT and CET (Fig. 1A). Image quantification of each band indicated that a fourfold molar excess of unlabeled 2-m-AzTax inhibited the photolabeling of β -tubulin by 80–90%, demonstrating the specificity of this photolabeling. Taxol had a minimal inhibitory effect on BBT, but a strong inhibitory effect on CET. In contrast, EpoB and Disco exhibited strong inhibitory effects on BBT but moderate effects on CET (Fig. 1). This finding is most likely a result of the presence of distinct tubulin isotypes in BBT and CET (Table S1) (18–21). Ixab had moderate effects on both systems. It is to be noted that fourfold molar excess of LML or PeLA had a stimulatory effect (~15–30% increase) on photolabeling of tubulin from these two sources (Fig. 1). When BBT, PBT, and HeLa cell tubulin (HeLaT) were compared, all MSAs exhibited similar effects on photolabeling, except that Ixab had a stronger inhibitory effect on HeLaT, compared with BBT and PBT (Fig. S1). Although Taxol's effects on BBT and PBT are minimal, Taxotere (docetaxel) appeared to have a stronger inhibitory effect than Taxol on photolabeling of β -tubulin, indicating that structural modifications of Taxol can alter its interaction with β -tubulin (Fig. S2).

The Stimulatory Effect of Taxol, EpoB, and Disco on Tubulin Polymerization Correlates with Their Inhibitory Effect on Photolabeling of β -Tubulin. Tubulin from BBT and CET was treated with Taxol, EpoB, or Disco and the polymerized microtubules isolated. The protein levels of microtubules after each treatment were compared with the photolabeling data, and it was found that there was an excellent correlation between the stimulatory effect of Taxol, EpoB, and Disco on tubulin polymerization and their inhibitory effect on photolabeling of β -tubulin ($r = 0.99$) (Fig. S3). These results suggest that binding affinities of MSAs correlate with their effects on tubulin polymerization.

It is noted that Taxol inhibits photolabeling of BBT only $\sim 8 \pm 5\%$, whereas 2-m-AzTax inhibits 80–90%. To determine if this difference is a result of different quantities of polymer formed with the two compounds, a polymerization experiment was performed using 4 μM BBT and 5 μM Taxol or 5 μM 2-m-AzTax (stoichiometry: $\sim 1:1$). It was found that 2-m-AzTax induced approximately twice as much polymer compared with Taxol (Fig. S4). The correlation between the effect of these two drugs on tubulin polymerization and on the logarithm of β -tubulin photolabeling was plotted, and a correlation coefficient of 0.843 was obtained.

Unlike the minimal effect on 2-m-AzTax photolabeling elicited by Taxol in BBT, both Taxol and 2-m-AzTax significantly inhibited photolabeling in CET. These two compounds do not induce a major difference in polymerization of CET (Fig. S5). Taxol actually causes a slightly higher level of polymerization, compared with 2-m-AzTax. These polymerization results correlated well with the CET photolabeling pattern (Fig. 1).

Taxol Analog 2-m-AzTax Binds Differentially to Distinct Tubulin Isotypes. To determine the amount of drug that binds to different β -tubulin isotypes, tubulin from different sources was photolabeled and the isotypes resolved by high-resolution IEF. Approximately 20 bands were seen with BBT (Fig. 2A), but only 3–5 bands were resolved with CET (Fig. S6). Tubulin is known to be extensively PTM, so many of the bands represent PTM tubulin (22). [^3H]2-m-AzTax bound essentially only to β -tubulin and not α -tubulin (Fig. 2B). Each band was isolated and analyzed by mass spectrometry following cyanogen bromide (CNBr) digestion, and the identity and relative quantity of each β -tubulin isotype determined (Fig. 2C). After examining the identity of each band, it was found that some bands contained more than one isotype. Therefore, the exact amount of drug that binds to each individual tubulin isotype, except for β III- and PTM β II-tubulin, could not be determined. After measuring the radioactivity associated with each band and normalizing the values to the relative protein level of the corresponding band, it was found that

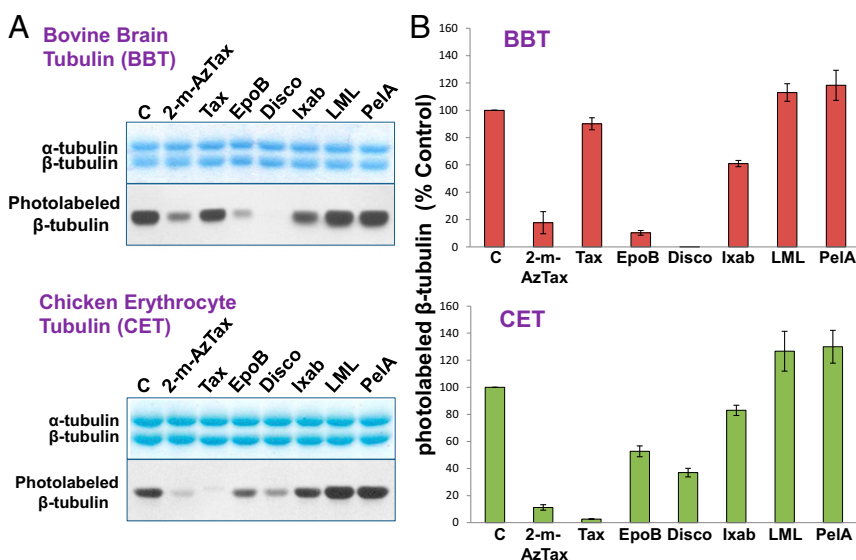


Fig. 1. MSAs exhibit distinct effects on 2-m-AzTax photoaffinity labeling of β -tubulin from bovine brain and chicken erythrocytes. Four micromolar soluble BBT or CET were incubated with 5 μM [^3H]2-m-AzTax and 1 mM GTP, in the absence or presence of a fourfold molar excess of MSAs, followed by UV irradiation as described in *Materials and Methods*. (A) Analysis by 9% SDS/PAGE and fluorography. Tax: Taxol. (B) Each band in A was image quantified by ImageJ. Results from three to four experiments are presented as mean \pm SE. C, control.

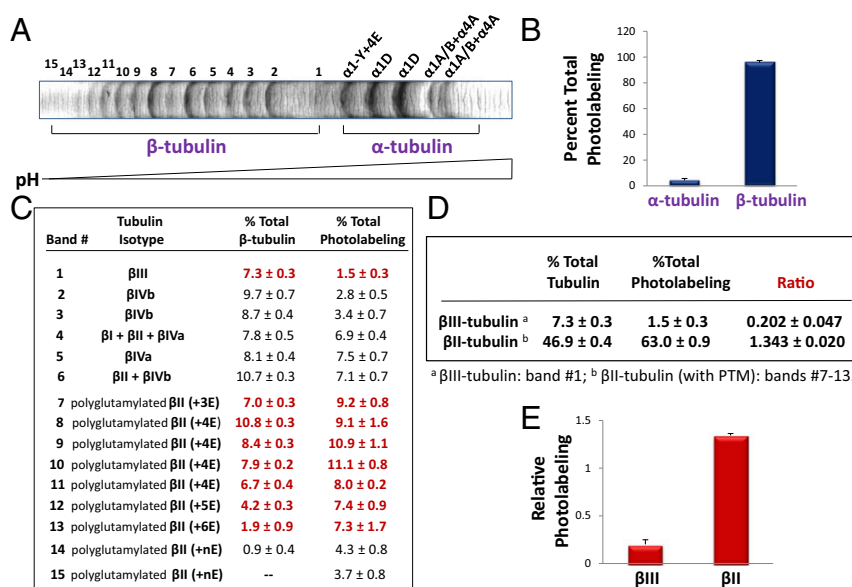


Fig. 2. Taxol analog 2-m-AzTax differentially photoaffinity labels distinct tubulin isotypes. Five micromolar BBT was incubated with 6 μ M [3 H]2-m-AzTax, in the presence of 1 mM GTP, at 37 $^{\circ}$ C for 30 min, followed by UV irradiation and centrifugation as described in *Materials and Methods*. (A) Separation of BBT by high-resolution IEF. Tubulin isotypes were identified by mass spectrometry. (B) Photolabeling of total α -tubulin and β -tubulin. (C) β -Tubulin isotypes were identified by CNBr digestion and mass spectrometry. Level of each isotype was determined by ImageJ and the amount of radioactivity associated with each band was determined by liquid scintillation counting as described in *Materials and Methods*. (D and E) Amount of radioactivity associated with β II- and β III-tubulin (red bolded values in C) was normalized to their protein levels, and relative photolabeling of β II- and β III-tubulin was plotted.

all β II-tubulin containing different PTMs bind approximately sixfold more drug than β III-tubulin (Fig. 2 C–E). β III-Tubulin bound the lowest amount of 2-m-AzTax, compared with other β -tubulin isotypes. The data also indicate that β IVb-tubulin binds a relatively low level of the drug. IEF patterns for BBT and PBT were very similar (Fig. S7A) and similar results were obtained when photolabeling and IEF experiments were performed with PBT (Fig. S7 B–D).

Unique Alanines at Residues 218 and 275 Are Present in the Leucine Cluster and the M-Loop Domains of β III-Tubulin. Sequence alignment of the leucine cluster region of bovine brain and human β -tubulin indicated that β III-tubulin harbors a unique alanine at residue 218 that is different from all other β -tubulin isotypes that contain a threonine at this residue (Fig. 3A). Murine β III-tubulin also has this unique Ala218. In the M-loop of human β -tubulin, β III- as well as β VI-tubulin each contains an alanine at residue 275, whereas other isotypes harbor serine at this position (Fig. 3B). Both of these domains have been shown to be important for the binding of taxanes to β -tubulin (4, 17, 23) in microtubules.

The Frequency of Taxol-Accommodating Conformations Is Reduced Dramatically in the T218A Variant. To gain insight into possible structural and functional differences between human β -tubulin isotypes I and III and their binding to Taxol, we built molecular models for these proteins in complex with Taxol and ran molecular dynamics simulations. To establish whether residues 218 and 275 are involved in drug binding and have contacts that could explain the differences observed in binding, comparative protein structure models were built for the β I-tubulin monomer with Taxol in the binding pocket and analyzed (Fig. 4). After running 20 independent 30-ps-long molecular dynamic simulations and analyzing 600 snapshots, no contacts between T218 and Taxol were found. S275 makes contacts with Taxol, but none are energetically favorable (hydrogen bond, hydrophobic, or aromatic). Hence, the lower binding affinity of β III-tubulin cannot be attributed to loss of direct interaction between Taxol and residues 218 and 275.

To establish if differences in binding pocket accessibility, if any, could explain the difference in binding, molecular dynamic

simulations were performed on the β I-tubulin monomer with GDP (Fig. 4). For each 1-ps snapshot, a representative set of six distances were monitored across the binding pocket (Fig. 4 and Table 1). Both the average distance across all snapshots and the percentage of snapshots that have a distance equal or greater than that in the corresponding β I-tubulin complex with Taxol inside the binding pocket are shown (Fig. 4).

Three sets of distances were significantly decreased in the variants (β I-tubulin containing T218A): the distances between residues 23 and 215, 23 and 279, and that between 279 and 360. Although the β I-tubulin maintains these pocket distances in greater than 90% of the time, the variants have a smaller fraction of time in which these pocket distances are large enough to accommodate Taxol. In particular, in the case of residues 279 and 360, the frequency of Taxol-accommodating conformations dropped from 90.7% in β I-tubulin to 36.3% and 38.9% in the variants T218A and T218A.S275A, respectively.

The same analysis was extended to a lateral dimer of β -tubulin with two molecules of GDP. The lateral interaction between the two β -tubulin subunits caused compaction around the M-loop. Indeed, a slight deformation in the binding pocket in our complex of the lateral dimer with Taxol bound was observed, resulting in different values of the six distances. The compaction also resulted in reduced flexibility around the binding pocket, leading to smaller differences in the distances between β I-tubulin and the variant. Nonetheless, the difference in distance between residues 279 and 360 persisted: the β I-tubulin maintained a distance equal or greater than the complex distance (13.05 \AA) in 23% of the time, whereas the variants rarely had this portion of the pocket opened (0.8% for T218A; 7.2% for T218A.S275A).

Discussion

Development of resistance to antitumor drugs is a complex process and it is for this reason that it has proven to be so difficult to overcome in patients. An increase in β III-tubulin has been associated with a reduced sensitivity to Taxol and other drugs, and there are a variety of interpretations for this in the literature (8, 9). In our study, we demonstrated that MSAs, such as Taxol, EpoB, Disco, and Ixab exhibited differential inhibitory effects on binding

Table 1. Distances across binding pocket in monomers of β I-tubulin and variants

Distance definition	Distance in complex	Average distance [Å] (% of snapshots with distance \geq distance in complex)		
		β I-Tubulin	T218A	T218A.S275A
23–215	18.39	20.1 \pm 0.9 (97.3)	18.9 \pm 1.4 (63.5)	19.3 \pm 1.4 (76.0)
23–279	23.34	26.5 \pm 1.3 (99.2)	23.1 \pm 2.6 (51.5)	23.8 \pm 3.4 (73.3)
23–360	11.34	12.4 \pm 1.6 (73.3)	12.0 \pm 1.4 (67.2)	13.2 \pm 1.4 (91.2)
215–279	9.8	11.3 \pm 1.0 (95.2)	12.8 \pm 2.5 (85.3)	12.6 \pm 1.5 (97.6)
215–360	16.43	17.6 \pm 1.4 (80.3)	17.5 \pm 1.3 (78.7)	17.8 \pm 1.8 (79.5)
279–360	16.06	18.5 \pm 1.7 (90.7)	14.7 \pm 2.7 (36.3)	14.9 \pm 3.4 (38.9)

Distance is measured between the C α atoms of the two residues in the definition.

H6–H7 loop region, affecting Leu-215, Leu-217, and Leu-228 (17); (ii) expression of the transfected mutant genes (L215H, L217R, or L228F) conferred Taxol resistance (17, 35); (iii) the Taxol analog 2-m-AzTax photolabels a peptide, amino acids 217–231 of β -tubulin (23); and (iv) hydrogen/deuterium exchange mass spectrometry (HDX-MS) studies have demonstrated that peptide 212–230 of β -tubulin is very strongly protected by Taxol (15), in fact, more so than the M-loop. β III-Tubulin has a residue, Ala218, in this region that is unique to this isotype and appears to influence its interaction with Taxol.

It is also known that the M-loop of tubulin has a substantial role in interacting with the H1–S2 loop of an adjacent β -tubulin molecule. In addition, it has been reported that the M-loop of β -tubulin has a role in drug resistance (33, 34). β III-Tubulin of BBT differs from other major isotypes (β II- and β IV-tubulin) in the M-loop, with a difference at residue 275 (S275A). β I-Tubulin of BBT also harbors S275A, R276Q, as well as P287A alterations in the M-loop, but because β I-tubulin accounts for only 3% of total BBT (18), this isotype is not included in our comparison. The M-loop sequences of human β -tubulins are identical to those of BBT (Fig. 3). It was proposed that, because of the presence of S275A, the M-loop of β III-tubulin becomes much more flexible than other isotypes, leading to a weaker interaction with Taxol (36). However, our molecular dynamics results indicate that S275A appears not to have a significant influence on drug entrance into the binding pocket (Table 1). Unique alterations are found in the M-loop of human β VI-tubulin: R276Q, T285S, and P287A. These alterations may explain why the photolabeling competition results were so different between BBT/PBT and CET, the latter containing only one β -tubulin isotype, β VI. It is noted that neither T218A nor S275A make direct contact with the bound drug; however, our computational simulation studies suggest that the presence of T218A has a strong influence on the shape of the binding pocket, down-modulating drug accessibility to the binding pocket.

We have demonstrated previously that some MSA-resistant cell lines express increased levels of β III-tubulin (12). In intact cells, binding of microtubule interacting agents to the polymer is the primary event occurring when cells are challenged with such drugs, resulting in microtubule disarray that in turn influences downstream signaling cascades. Therefore, a difference in the extent of drug binding could easily lead to alterations in cellular events. It may also alter the expression of distinct tubulin isotypes and influence drug sensitivity in resistant cells. In addition, our laboratory has demonstrated, using HDX analysis, that Taxol binding to microtubules can influence the interaction of the endogenous dynamics regulator MAP4 with microtubules (37), suggesting that the presence of less Taxol in β III-tubulin could alter a variety of signaling processes.

Several investigators have considered β III-tubulin as a drug target for overcoming drug resistance and have designed drugs based on computational modeling of the Taxol binding site of β III-tubulin, with the focus on the M-loop. Many secondary taxanes were synthesized and have demonstrated cytotoxicity in β III-tubulin-overexpressing ovarian cell lines (36, 38, 39). In the future, the T218A variant should be considered when designing drugs for the binding site in β III-tubulin.

In addition to the finding that suppression of β III-tubulin increased drug sensitivity to tubulin binding agents in nonsmall cell lung cancer (NSCLC) cell lines (40), many observations have been reported concerning the effects of β III-tubulin in cells. For example, maspin, an adhesion-associated tumor suppressor protein, is directly regulated by β III-tubulin in NSCLC (41, 42). Suppression of β III-tubulin caused a reduction of tumor spheroid outgrowth and an increase in sensitivity to anoikis. Phosphatase and tensin homolog (PTEN)/AKT signaling has been demonstrated to be regulated by β III-tubulin in NSCLC (42). In addition, a high percentage of breast cancer patients develop brain metastasis and β III-tubulin overexpression is associated with brain metastasis (43). Recently, it was demonstrated that β III-tubulin is involved in a complex, pro-survival molecular pathway induced by hypoxia (44). Roles for β III-tubulin in stress-response signaling and glucose metabolism have been proposed (45). Therefore, β III-tubulin is not only involved in drug sensitivity, but also in a variety of other cellular events. Increased expression of β III-tubulin in drug-resistant ovarian cancer cells is likely to be the consequence of a combination of drug binding to tubulin and subsequent cellular processes.

In summary, this study demonstrates that less 2-m-AzTax, a Taxol analog, binds to β III-tubulin than to other β -tubulin isotypes. We noted the presence of a unique alanine at residue 218 in the leucine cluster of β III-tubulin. To extend our understanding of this observation, we used molecular dynamic simulation that strongly suggested that the alanine residue 218 is likely responsible for the reduced binding of the drug to β III-tubulin. The low drug-binding characteristic of β III-tubulin may be compensated with an increased expression of this isotype that leads to reduced drug sensitivity.

Materials and Methods

Drugs and Tubulin. Taxol, EpoB, Disco, Ixab, LML, and PeLa were obtained as described previously (46). [3 H]2-m-AzTax was provided by GlaxoSmithKline. BBT, PBT, and HeLaT were purchased from Cytoskeleton. CET was isolated from the marginal bands of chicken erythrocytes as previously described (20). Reagents for IEF and IEF strips were from GE Healthcare.

Photoaffinity Labeling of Tubulin and Drug Competition Assays. Four micromolar of soluble tubulin in MEM buffer (0.1 M Mes, 1 mM EGTA, 1 mM MgCl₂, pH 6.7) containing 3 M glycerol and 1 mM GTP was incubated with 5 μ M [3 H]2-m-AzTax (specific activity: 5.5 Ci/mmol) in the absence or presence of a fourfold molar excess of competing MSAs (20 μ M) at 37 °C for 30 min (23). The samples were irradiated for 10 min at 4 °C with a UV lamp (254 nm) at a distance of 6 cm. The entire sample containing photolabeled microtubules was analyzed by SDS/PAGE on 9% gels, using a cathode buffer that was titrated to pH 9, and detected by fluorography.

Photolabeling of Tubulin and Resolution of Tubulin Isotypes. Five micromolar of BBT or PBT (200 μ g in 400 μ L MEM-glycerol buffer containing 1 mM GTP) was incubated with 6 μ M [3 H]2-m-AzTax at 37 °C for 30 min, followed by 10 min UV irradiation at 4 °C. The irradiated samples were layered over a 5% (wt/vol) sucrose pad in MEM-glycerol buffer and centrifuged at 120,000 \times g for 30 min at 37 °C. Pellets were resuspended in 450 μ L solubilization buffer [7 M urea, 2 M thiourea, 4% (wt/vol) CHAPS, 0.5% Triton X-100, 0.5% Pharmalyte pH 4.5–5.4

(GE), 20 mM DTT and Bromophenol blue], loaded onto 24-cm IPG strips [pH 3–5.6 NL (nonlinear)], and run on an IPGphor IEF system for a total of 75,000 V/h.

Identification of Tubulin Isotypes and Determination of Radioactivity Associated with Each Isotype. IPG strips were fixed in 20% (wt/vol) trichloroacetic acid, stained with GelCode Blue Stain Reagent (Thermo Scientific), and destained with water. The stained bands were excised, destained with 50% (vol/vol) acetonitrile and 200 mM ammonium bicarbonate, pH 8.9, treated with 100 mg/mL CNBr and 70% formic acid for 16 h, followed by mass spectrometry (MALDI-TOF). Parallel IPG strips were destained, scanned, and bands quantified by ImageJ. The stained bands were excised, treated with 90% hydroxide of Hyamine 10-X (PerkinElmer) and radioactivity associated with each band determined by liquid scintillation counting.

Tubulin Polymerization Assays. BBT or CET (2 μ M) in MEM-glycerol buffer containing 2 mM GTP was incubated with 10 μ M of the indicated MSAs at 37 °C for 30 min. Samples were centrifuged at 120,000 \times g at 37 °C for 1 h, and the pellet containing the polymerized tubulin was dissolved in SDS sample buffer and analyzed by SDS/PAGE.

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