Indibulin, a Novel Microtubule Inhibitor, Discriminates between Mature Neuronal and Nonneuronal Tubulin

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Abstract

Microtubule inhibitors interfere with microtubule dynamics, causing cell cycle arrest and apoptosis. These effects are responsible for the chemotherapeutic activities of members of the taxane and Vinca alkaloid families in oncology. Unfortunately, a major side effect of the taxanes and Vinca alkaloids is the development of peripheral neuropathies. Indibulin (N-[pyridin-4-yl]-[1-(4-chlorbenzyl)-indol-3-yl]-glyoxylic acid amid; D-24851; ZIO-301), a novel synthetic small molecule microtubule inhibitor, destabilizes microtubules and has antitumor activity but does not exhibit neurotoxicity in preclinical animal studies. In the present study, it has been found that indibulin is able to discriminate between highly posttranslationally modified tubulin present in mature neuronal microtubules, and less-modified tubulin present in immature neuronal or nonneuronal microtubules. Vinblastine and colchicine act on either tubulin equally well. The binding site of indibulin on mature neuronal microtubules seems to be inaccessible due to the posttranslational modifications, a theory that is supported by the observation that indibulin did not disrupt the integrity of highly modified microtubules present in neurites of pheochromocytoma (PC12) cells. The specificity of indibulin for unmodified microtubules seems to be dependent on the pyridyl moiety of indibulin because derivatives that have the pyridyl moiety replaced are not able to discriminate between highly and less-modified tubulins. The observed broad antitumor activity of indibulin and the lack of central and peripheral nervous system toxicity in preclinical studies make it a promising candidate for development as a cancer treatment. Indibulin is currently in phase I clinical trials. [Cancer Res 2009;69(1):171–7]

Introduction

The involvement of microtubules in a wide variety of cellular structures and processes led to the multitubulin hypothesis of tubulin diversity and distinct microtubule structures within a cell. Most eukaryotic cells express multiple isotypes of α- and β-tubulins, and this diversity is further enhanced by posttranslational modifications of tubulins (1–3). In addition to α- and β-tubulin isotypes, the microheterogeneity includes a variety of γ, δ, ε, ζ, etc., and FtsZ family members (4). The posttranslational modifications of the different isotypes include acetylation, phosphorylation, polyglutamylation, detyrosination, and polyglycylcycylation (1, 2). This tubulin microheterogeneity is much higher in neuronal cells than in other cells (5–9).

The degree of posttranslational modifications of tubulin is dependent on the developmental stage of the tissue (10–13). Posttranslational modifications of mouse embryo brain tubulins are low and comparable with nonneuronal HeLa cell tubulin modifications. However, during mouse development, the degree of brain tubulin modification strongly increases; in particular, the expression of β-tubulin isotypes, which changes qualitatively and quantitatively (10). Similarly, isolated neurons from fetal mouse brain in primary cultures undergo neuronal development and reveal increased posttranslational tubulin modifications, including polyglutamylation, acetylation, and detyrosination (14). These effects can also be mimicked in nerve growth factor (NGF)-induced outgrowth of rat pheochromocytoma PC12 neurites in vivo (15–17).

Microtubule polymerization and depolymerization are required for mitosis and neuronal functions. The dynamics of microtubules in mitotic spindles and the subsequent chromosome segregation are affected by all microtubule inhibitors inducing cell cycle arrest (G2/M arrest; ref. 18). Microtubules accumulate in axons in response to paclitaxel, whereas Vinca alkaloids induce spiralization of axon microtubules, and it is thought that the role of microtubules in axon transport and neurite elongation contribute to the toxicity of microtubule inhibitors (19). Disruption of axonal microtubules results in peripheral sensory-motor neuropathy, which is one of the most common side effects associated with Vinca alkaloids (e.g., vincristine) or taxane (e.g., paclitaxel) treatment and is often a limiting factor in their utility as a treatment of neoplastic diseases (20–24).

Recently, a novel microtubule inhibitor, indibulin, was identified in a cell-based screening assay. Indibulin has shown potent antitumor activity in preclinical models but seems not to affect neuronal microtubules. Neurotoxicity that is normally seen with other microtubule inhibitors was not observed with indibulin (25). In the present study, the mechanism of action and the absence of neurotoxicity of indibulin were investigated in more detail.

Materials and Methods

Materials and Cell Lines

Indibulin (N-[pyridin-4-yl]-[1-(4-chlorbenzyl)-indol-3-yl]-glyoxylic acid amid; Baxter Oncology GmbH) was synthesized as described (25). Other chemicals including vincristine, vinblastine, colchicine, and tubulin antibodies anti–α-tubulin (Clone B-5-1-2), anti–acetylated-α-tubulin (Clone 6-11B-1), anti–tyrosinated-α-tubulin (Clone TUB-1A2), anti–β-tubulin I, II isotypes (Clone JDR.3B8), and anti–β-tubulin III isotype (Clone SDL3D10)?were purchased from Sigma. Cy3-conjugated and peroxidase-conjugated goat anti-mouse antibodies were obtained from Dianova. Rat pheochromocytoma (PC12) cells were purchased from American Type Culture Collection (CRL-1721) and Chlamydomonas reinhardtii (CW15) was a gift from D. Sultemeyer (University of Kaiserslautern, Kaiserslautern, Germany).
Purification of Tubulin from Bovine Brain

Tubulin was purified from bovine brain as previously described (26). Bovine brain (ages 12–18 mo) was obtained directly after slaughter and stored in ice-cold PEM-buffer [100 mmol/L PIPES-NaOH, 1 mmol/L EGTA, 1 mmol/L MgSO4 (pH 6.6)]. Large blood vessels and meninges were removed. Cerebral cortex (600 g) was mixed in a commercial blender with PEM-buffer containing 0.1% 2-mercaptoethanol (900 mL) in 2 lots for 4 s each at low speed. The suspension was homogenized in a Teflon-in-glass homogenizer (2 passes at 1,700 rpm; Potter S, B. Braun Biotech International) the homogenate centrifuged [12,000 rpm (GSA rotor), 2°C, 90 min], and the supernatant incubated with 1 mmol/L GTP at 37°C for 30 min under gentle shaking. It was then transferred into 250 mL centrifuge bottles and carefully underlayered with 30 mL of a freshly prepared sucrese solution, warmed to 37°C (PEM buffer containing 10% sucrose and 1 mmol/L GTP). After centrifugation (11,000 rpm, 37°C, 45 min), the pellet was resuspended in ice-cold PEM buffer (2.5-fold pellet volume) and incubated on ice for 30 min while being homogenized in a Teflon-in-glass-homogenizer. After additional centrifugation (18,000 rpm, 2°C, 30 min), the supernatant was incubated with 1 mmol/L GTP (37°C, 20 min) and centrifuged again (18,000 rpm, 30 min, 37°C). The pellet was frozen in liquid nitrogen for storage. The frozen pellet was rapidly thawed, resuspended in 4 mL ice-cold PEM buffer, and transferred in a Teflon-in-glass-homogenizer followed by incubation on ice for 45 min. Every 15 min, the suspension was homogenized with 5 passes. The homogenate was then centrifuged in 1.5 mL tubes (15,000 rpm, 40 min, 2°C) and the supernatant used directly in the tubulin polymerization assay or stored at −80°C.

Tubulin Polymerization Assay

The assay was basically performed according to Bollag and colleagues (27). Tubulin heterodimers (10 μmol/L) from calf brain (Sigma or tebu-bio GmbH) or bovine brain (purified as described above) were incubated with compounds as indicated in PEM buffer [100 mmol/L PIPES-NaOH, 1 mmol/L EGTA, 1 mmol/L MgSO4, and 0.05% Triton-X-100 (pH 6.6)] containing 1 mmol/L GTP at 37°C for 1 h. Samples (75 μL) were transferred to a 96-well Millipore Multiscreen Durapore hydrophilic 0.22-μm pore size filtration plate that had been previously washed with 200 μL PEM buffer [100 mmol/L PIPES-NaOH, 1 mmol/L EGTA, 1 mmol/L MgSO4, and 0.05% Triton-X-100 (pH 6.6)] under vacuum. Microtubules recovered on the filters were stained with 50 μL of Amido Black solution [0.1% naphthol blue black (Sigma), 45% methanol, and 10% acetic acid] for 2 min. Unbound dye was removed by vacuum wash 2 additions of 200 μL of destaining solution (90% methanol and 10% acetic acid). The microtubule-bound dye was then eluted by incubation with elution solution (25 mmol/L NaOH, 0.05 mmol/L EDTA, and 50% ethanol) for 10 min. The elution solution was then transferred to a 96-well plate and the absorbance measured at 600 nm.

Preparation of Flagella and Cell Body Extract from Chlamydomonas Reinhardtii

Cell body extract from Chlamydomonas reinhardtii (CW15) was prepared as previously described (28, 29). Cells were centrifuged for 5 min at 2,000 rpm and resuspended in ice-cold 10 mmol/L Tris-HCl (pH 7.0) containing 5% sucrose and adjusted to 5 to 6 × 104 cells/mL. While stirring the suspension pH was decreased from 7.0 to 4.5 by adding acetic acid (0.5 N). Within 30 s flagella became detached from cell bodies. After detachment, pH was immediately adjusted to 7.0 by adding KOH (0.5 N). Aliquots of 10 mL of the suspension were transferred into 15 mL tubes and underlayered with 10 mmol/L Tris-HCl (pH 7.0), containing 25% sucrose. Cell bodies were separated from flagella by centrifugation twice through 25% sucrose (10 min at 3,000 rpm). Interphase and 5% sucrose containing flagella were pooled and centrifuged at 31,000 g for 20 min. The pellet was resuspended in 50 mL HM-buffer (pH 6.8; 10 mmol/L Heps, 1 mmol/L MgSO4, 1 mmol/L DTT, 0.1 mmol/L GTP, 0.5 mmol/L phenylmethylsulfonyl fluoride (PMSF), 0.24 mol/L Sucrose, 0.2% Nonidet-P40) and incubated on ice for 15 min to disrupt the membranes. After centrifugation, a pellet containing the axonema was resuspended in 50 mL HM buffer (pH 6.8) containing 0.66 mol/L NaCl and incubated on ice for 30 min. After centrifugation at 20,000 g for 15 min, supernatant (Flagella extract) was dialysed against TDSA buffer (pH 7.5; 10 mmol/L Heps, 5 mmol/L MgSO4, 1 mmol/L DTT, 4% Sucrose). Deflagellated cell bodies were washed with 2 pellet volumes of HMDS-buffer [10 mmol/L Heps, 5 mmol/L MgSO4, 1 mmol/L DTT, 4% sucrose (pH 7)] and centrifugation (2,000 rpm, 5 min). After resuspension in HM-buffer cells [10 mmol/L Heps, 1 mmol/L MgSO4, 1 mmol/L DTT, 100 mmol/L GTP, 500 μmol/L PMSF, 240 mmol/L sucrose, 0.2% Nonidet-P40 (pH 6.8)], bodies were lysed by sonification (intensity 70%, 10 cycles, 1 min each). Cell debris was separated by centrifugation (20,000 g, 30 min). Treatment of tubulin with flagella extract from Chlamydomonas reinhardtii (CW15). Calf brain tubulin (1.8 mg) was polymerized in the presence of GTP (1 mmol/L) for 20 min at 37°C. Subsequently, 1 mmol/L Acetyl-CoA and 0.25 mL flagella extract was added and mixture was incubated for 1 to 1.5 h at 37°C. Samples were put on ice for 1 h and then centrifuged at 20,000 g at 4°C for 30 min. Supernatant was analyzed by SDS-PAGE and Western blotting.

Culture and Differentiation of PC12 Cells

PC12 cells were seeded in Collagen-R–coated (Sigma) 6-well plates (7.5 × 104 cells per well). NGF-S7 (Sigma) was added in a final concentration of 100 ng/mL. Media (including NGF) were changed every second day. After the day 5 cultivation of PC12 cells, the compounds were added to the media for 24 h and the cells were subsequently subjected to indirect immunofluorescence staining.

Indirect immunofluorescence microscopy. Cells were incubated with different compounds as well as DMSO (control) for 24 h and then extracted for 3 min with ice-cold 0.5% Triton-X-100 in PHEM [60 mmol/L PIPES, 10 mmol/L Heps, 0.25 mmol/L MgCl2, and 0.5% sucrose (pH 6.8)] containing 10 μg/μL paclitaxel (this treatment removes unassembled tubulin while preserving microtubules). The cultures were then fixed by the addition of PHEM containing 8% paraformaldehyde and 0.3% glutaraldehyde at 4°C for 10 min. The cells were washed thrice with PBS. After incubation with blocking solution (PBS containing 2% FCS and 1% bovine serum albumin) for 30 min, microtubules were visualized using a mouse monoclonal antibody against acetylated α-tubulin (1:1,000; Sigma) and a Cy3-conjugated goat anti-mouse antibody (1:1,000), and a cooled AT200 charge-coupled device camera system (Photometrics Ltd.). Fluorescent images were further processed using Fluoro-Pro module for Image-ProPlus.

Generation of Indibulin-Resistant Cell Line

The indibulin-resistant subline of mouse leukemia L1210 cells (L1210/indibulin) was generated by long-term adaptation in a medium with stepwise increasing concentrations of indibulin. Expression of acetylated and tyrosinated tubulin was analyzed by Western blotting using the monoclonal antibodies, Clone 6-11B-1 and Clone TUB-1A2, respectively.

Results

Effect of indibulin on polymerization of bovine brain tubulin derived from different developmental stages. To test the effect of indibulin on the polymerization of neuronal tubulins from different developmental stages, tubulins derived from calf brain (ages 2 to 6 months; immature tubulins) or from bovine brain...
Indibulin Does Not Interact with Mature Neuronal Tubulin

Tubulins were allowed to polymerize in the absence or presence of indibulin, vincristine, vinblastine, or colchicine, and microtubule polymers were separated from tubulin heterodimers by filtration, as described (27). All compounds tested completely inhibited calf brain tubulin polymerization. In contrast, polymerization of mature tubulin from bovine brain was only partially inhibited by indibulin but was fully inhibited by Vinca alkaloids and colchicine (Fig. 1A). As shown in Fig. 1B, polymerization of purified calf brain tubulin was inhibited by indibulin in a dose-dependent manner, with an IC50 value of ~0.25 μmol/L and a maximal level of inhibition of 90%. In contrast, polymerization of mature tubulin from bovine brain was only inhibited up to a maximum of 25%. Vincristine or colchicine inhibited polymerization of tubulin by ~95% to 100% regardless of the origin of the tubulin (Fig. 1B). The IC50 values for vincristine and colchicine differed only slightly for bovine or calf brain tubulins, indicating that these compounds cannot discriminate between tubulins derived from the different sources.

Figure 1. Effect of indibulin on polymerization of purified tubulin derived from different developmental stages of bovine brain. A, purified calf brain tubulin and purified bovine brain tubulin were incubated with GTP and DMSO or with GTP and 1 μmol/L of either Vincristine, Vinblastine or Indibulin, or 10 μmol/L Colchicine in DMSO. The concentrations of the compounds used in A were based on the minimal concentration that is sufficient to reach the plateau of the inhibition (maximum inhibition) of bovine brain tubulin polymerisation. See also B. Six independent experiments were performed and corresponding results were comparable. In total, six preparations of bovine brain tubulin, one preparation of calf brain tubulin, and six batches of commercially available calf brain tubulin from different providers were examined. B, purified tubulin was incubated with GTP with DMSO alone (100% polymerization) or with different concentrations of vincristine, colchicine, or indibulin. Polymerized microtubules were separated from heterodimeric tubulin by filtration and recovered on 0.22-μm pore-size filters in a 96-well plate. Subsequently, microtubules were stained with naphthol blue black, and bound dye was quantified. Inhibition of polymerisation and IC50 values were analyzed using GraphPadPrism software and were determined from two independent experiments for each compound. Results were comparable.

Figure 2. Effect of indibulin, D-51138, and D-69429 on polymerization of purified bovine brain tubulin. Purified tubulin was incubated with GTP with DMSO alone as a control (100% polymerization) or with different concentrations of D-51138, D-69429, or indibulin. Polymerized microtubules were separated as described in Fig. 1. The panel next to the graph shows chemical structures of indibulin, D-69429, and D-51138.
In general, inhibition of tubulin polymerization by indibulin ranged from 82% to 100% when using different preparations of tubulin from calf brains and ranged from 25% to 65% when using different preparations from bovine brains regardless of whether they were from different commercial providers or whether they were prepared in our own laboratory.

Closely related indibulin analogues in which the pyridyl residue of indibulin is substituted with 8-methyl-quinol-6-yl (D-69429) or with benzyl alcohol (D-51138) inhibited polymerization of bovine brain tubulin equally well, and similarly to vincristine and colchicine (Fig. 2). Both analogues showed slightly less antitumor activity compared with indibulin in vivo and in vitro (data not shown).

Characterization of calf and bovine derived tubulin preparations. Posttranslational modifications by acetylation or detyrosination of α-tubulins occur during murine brain development (10, 11). To investigate microheterogeneity of tubulin derived from calf and bovine brain in more detail, calf and bovine brain tubulin preparations were subjected to SDS-PAGE and analyzed by Western blotting, using antibodies to different tubulin isotypes and posttranslational modifications. As shown in Fig. 3, α-tubulin and all types of β-tubulin (β I/β II- and β III-tubulin) were present in nearly equal amounts in both calf and bovine brain preparations of tubulin. Acetylated α-tubulin, mostly of neuronal origin, was abundant in bovine brain but much less abundant in calf brain. Detyrosinated α-tubulin were higher in bovine than in calf brain. This indicates that posttranslational modifications such as acetylation and detyrosination of tubulins may influence the inhibitory activity of indibulin.

An attempt was made to test whether the ability of indibulin to inhibit polymerization of calf brain tubulins would be lost if calf brain tubulins were acetylated. Calf brain tubulins were treated with an extract containing α-tubulin–specific acetyl-transferase isolated from flagella of *Chlamydomonas reinhardtii* (30–32). The test could not be completed, however, because the high salt concentrations necessary for the acetylation reaction effectively inhibited polymerization in all preparations tested. Dissociation of microtubule-associated proteins from microtubules was also observed at the high salt concentrations present in the flagella extract (data not shown). The more effective approach, therefore, was to deacetylate bovine brain tubulin using a cell body extract of *Chlamydomonas reinhardtii* containing tubulin-specific deacetylase (TDA; ref. 30). Bovine brain tubulin was incubated with TDA for up to 4 hours, preparations were assayed for polymerization at different time points in the presence or absence of vincristine or indibulin. Vincristine blocked tubulin polymerization regardless of the length of treatment with the extract, whereas the extent of inhibition by indibulin increased with increasing deacetylation; after 4 h of TDA treatment, indibulin had completely blocked polymerization of the deacetylated tubulin derived from bovine brain (Fig. 4). These results indicate that removing the acetyl-group in α-tubulin restores the ability of indibulin to inhibit neuronal tubulin polymerization. However, it cannot be excluded that other tubulin modifications may also occur during the deacetylation process.

The effect of different inhibitors on neurite outgrowth of pheochromocytoma (PC12) cells. The PC12 neurite-outgrowth assay is a suitable and reliable model for predicting the neurotoxicity of various compounds (15–17, 33). Microtubules in
neurites, axons, and dendrites of nerve cells have cell-specific posttranslational modifications that are distinct from nonneuronal microtubules (7). Particularly, \(\alpha\)-tubulins are highly acetylated in established neurites (15). Outgrowth of PC12 neurites was induced by neuronal growth factor for 5 to 6 d followed by treatment with indibulin, its derivatives, or with colchicine for 24 h (2 \times IC_{50} for each compound). Cells were fixed and visualized by phase contrast microscopy. As shown in Fig. 5A (control), neurites of PC12 cells form a network of axons of different diameters. When treated with colchicine, the axons lose their uniform shape and, instead, take on an irregular, dotted structure (Fig. 5B). Similar effects were seen in cells treated with the indibulin derivative D-69429 (Fig. 5D). In contrast, neurites of indibulin-treated PC12 cells retained their structure and integrity, similarly to control cells (Fig. 5C).

To directly visualize microtubules within neurites, cells were stained with an antibody that recognizes the acetylated \(\alpha\)-tubulins that are present in neurites but not in cell bodies (15). In control cells (DMSO treated), microtubules stained strongly and were highly organized (Fig. 5A). Cells treated with indibulin retained their highly organized microtubule structure in neurites similarly to untreated controls. This observation indicates that indibulin does not affect the stability of neurite microtubules (Fig. 5C). In contrast, treatment with either colchicine (Fig. 5B) or the indibulin analogue D-69429 (Fig. 5D) resulted in disruption of neuronal microtubules and strongly reduced the staining of acetylated tubulins, confirming that these compounds depolymerize microtubules within neurites. These data support observations made previously in rats where indibulin had no central nervous system toxicity and had no effect on peripheral nerve conductance velocity (25). D-69429 exerted neurotoxicity in the preclinical animal models similarly to other microtubule inhibitors (data not shown).

Characterization of indibulin-resistant cell line. L1210/indibulin is an indibulin-resistant cell line derived from the mouse leukemia L1210 cell line by long-term adaptation to increasing concentrations of indibulin. As cells adapted to higher concentrations of indibulin (80 nmol/L to 200 nmol/L), levels of acetylated \(\alpha\)-tubulins and detyrosinated tubulins increased (Fig. 6). The indibulin-sensitive L1210 leukemia cell line contained a low level of acetylated \(\alpha\)-tubulin and detyrosinated \(\alpha\)-tubulin (DMSO control; Fig. 6). These results are compatible with acetylation of tubulin being a major cause of the resistance of L1210/indibulin to indibulin. The only acetylation site of \(\alpha\)-tubulin is Lys40 (34); therefore, modification of Lys40 might prevent indibulin from accessing its binding site.

Discussion

Indibulin (D-24851) was previously shown to have broad antimitotic activity against a variety of human tumors (25). Indibulin seemed to cause G2/M arrest by destabilizing cellular tubulin, as shown in HeLa and SKOV-3 cells by indirect immunofluorescence. Previously, HeLa cells were shown to contain only a few tubulin isotypes and modifications compared with the...
high number found in tubulin isolated from mouse brain (10). Highly modified microtubules are primarily present in neuronal tissue, with the degree of modification dependent on the developmental stage of the tissue (8–13). For example, mouse neuronal tissue contains highly acetylated tubulin, whereas liver, heart, spleen, testis, and kidney tissues contain only low levels (34). Moreover, modifications of nonneuronal tubulin and tubulin derived from mouse embryos are relatively low, whereas the degree of modifications, including polyglutamylation, strongly increases during development of the animals (10, 14). Thus in general, it seems that nonneuronal tumors would be expected to contain less posttranslational modifications of tubulin than normal neuronal tissue. As described previously, indibulin does not seem to cause the neurotoxicity that normally occurs with other microtubule inhibitors (25). The present studies were, therefore, mainly focused on an investigation of the effects of indibulin on neuronal tubulins to elucidate the molecular basis for the lack of neurotoxicity of indibulin in vivo. We show here that indibulin can discriminate between highly posttranslationally modified and less-modified microtubules.

As observed previously in mice, rats, and guinea pigs in which brain tubulins from older animals are more extensively modified posttranslationally than in young animals, the tubulins from calf brain (age 2–6 months; before sexual maturity) and bovine brain (age 12–18 month) also differ in their degree of acetylation and detyrosination of α-tubulins. α-Tubulins derived from bovine brain were shown to be more highly acetylated and detyrosinated than those from calf brain. The levels of modification seemed to correlate with sensitivity to indibulin: polymerization of highly acetylated and detyrosinated tubulins from mature bovine brain was not affected by indibulin, whereas polymerization of the less-modified tubulins from calf brain was inhibited by indibulin.

Consistent with the above findings, the integrity of microtubules in PC 12 neurites that contain highly acetylated α-tubulins (Fig. 5B) was not affected by indibulin (15). In contrast, colchicine and an indibulin derivative, neither of which discriminates between neuronal and nonneuronal cellular tubulins, disrupted the microtubule structure in these neurites. Thus, acetylation of α-tubulins in the neurites of both the PC12 cells and in mature bovine brain seems to make them resistant to the effect of indibulin. The only acetylation site of α-tubulin, Lys40, is acetylated (35), and it is possible that modification of this amino acid might prevent indibulin from accessing its binding site.

This hypothesis is supported by the observation that indibulin is able to inhibit polymerization of mature bovine brain tubulin as deacetylation progresses during incubation with an α-tubulin-specific deacetylase enzyme present in an extract from cell body Chlamydomonas reinhardttii (29, 30). However, in addition to its deacetylation activity, Chlamydomonas reinhardttii extract might also catalyze other tubulin modifications, for example, the addition of tyrosine to detyrosinated tubulin (29). Therefore, other posttranslational modifications of tubulin, in addition to acetylation, could play a role in determining its sensitivity to indibulin. In addition, acetylation and detyrosination of nonneuronal cellular α-tubulin can be induced through the adaptation of L1210 leukemia cells to high indibulin concentrations in vitro, suggesting that these tubulin modification are associated with indibulin resistance.

It is conceivable that the α-tubulin binding site of indibulin overlaps with the Lys40 position of α-tubulin. The amino group of Lys40 may interact directly with the pyridyl moiety of indibulin. Therefore, acetylation of this amino group could interfere with indibulin binding. When the pyridyl moiety was replaced by 8-methyl-quinol-6-yl (D-69429) or by benzyl alcohol (D-51138), the tubulin binding activity was not affected by acetylation of Lys40, and the indibulin derivatives were unable to discriminate between tubulins derived from nonneuronal tissue or immature neurons versus mature neurons. It seems that highly modified mature neuronal microtubules are protected from the destabilizing effect of indibulin but are sensitive to the indibulin derivatives as well as to colchicine and Vinca alkaloids. These findings correlate with previous findings in animal models where indibulin did not cause the neurotoxicity associated with the other tubulin binders such as D-69429 or Vinca alkaloids (25).

Indibulin is a novel tubulin binder with cytotoxic activity comparable with that of taxanes and Vinca alkaloids, but it lacks the neurotoxicity associated with other tubulin binders in preclinical models. Its inability to prevent tubulin polymerization in mature neuronal tubulin, possibly because acetylation of Lys40 of α-tubulin prevents binding, is unique. The resulting lack of neurotoxicity observed in preclinical models positions indibulin as a promising chemotherapeutic agent. Indibulin is orally available and is currently being tested for safety and clinical activity in Phase 1 studies.

**Disclosure of Potential Conflicts of Interest**

No potential conflicts of interest were disclosed.

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