Published OnlineFirst May 4, 2010; DOI: 10.1158/1535-7163.MCT-09-0679

Research Article

Molecular Cancer Therapeutics

Microtubule Dynamics, Mitotic Arrest, and Apoptosis: Drug-Induced Differential Effects of βIII-Tubulin

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Abstract

Overexpression of βIII-tubulin is associated with resistance to tubulin-binding agents (TBA) in a range of tumor types. We previously showed that small interfering RNA silencing of βIII-tubulin expression hypersensitized non–small cell lung cancer cells to TBAs. To determine whether βIII-tubulin mediates its effect on drug-induced mitotic arrest and cell death by differentially regulating microtubule behavior, the effects of βIII-tubulin knockdown on microtubule dynamics were analyzed in H460 non–small cell lung cancer cells stably expressing green fluorescent protein-βI-tubulin. Interphase cells were examined at three vincristine and paclitaxel concentrations that (*a*) inhibited cell proliferation, (*b*) induced 5% to 10% mitotic arrest, and (*c*) induced 30% to 40% mitotic arrest. In the absence of either drug, βIII-tubulin knockdown caused no significant change in microtubule dynamic instability. At 2 nmol/L vincristine (IC_{50}), overall microtubule dynamicity was significantly suppressed in βIII-tubulin knockdowns (−31.2%) compared with controls (−6.5%). Similar results were obtained with paclitaxel, suggesting that knockdown of βIII-tubulin induces hypersensitivity by enhancing stabilization of microtubule dynamics at low drug concentrations. At higher drug concentrations (≥40 nmol/L vincristine; ≥20 nmol/L paclitaxel), βIII-tubulin knockdown resulted in significantly reduced suppressive effects on microtubule dynamicity with little or no further increase in mitotic arrest, compared with control cells. Importantly, apoptosis was markedly increased by βIII-tubulin knockdown independent of further suppression of microtubule dynamics and mitotic arrest. These results show that βIII-tubulin knockdown enhances the effectiveness of TBAs through two mechanisms: suppression of microtubule dynamics at low drug concentrations and a mitosis-independent mechanism of cell death at higher drug concentrations. *Mol Cancer Ther; 9(5); 1339–48. ©2010 AACR.*

Introduction

Microtubules are multifunctional cytoskeletal proteins involved in many essential cell functions including maintenance of cell shape, intracellular transport, and in mitosis, functioning as part of the spindle to ensure proper chromosome segregation and cell division. The ability of cells to organize the different arrays of microtubules used in different cellular processes is due in part to the dynamic behavior of microtubules. One such behavior, called dynamic instability, is characterized by switching at microtubule ends between phases of slow growth and rapid shortening. Both microtubule ends display dynamic instability behavior, with the β-tubulin plus end being more dynamic than the minus end that has α-tubulin exposed (1).

Vinca alkaloids and taxanes are two major groups of tubulin-binding agents (TBA) that are widely used in the treatment of human cancers. These agents target distinct sites on the β-tubulin subunit of the α/β-tubulin heterodimers that assemble to form the microtubules. A key antitumor action of the TBAs is kinetic stabilization of spindle microtubule dynamics, leading to mitotic arrest and subsequent cell death (2). Development of resistance to TBAs, either intrinsic or acquired, can be a significant clinical obstacle. Overexpression of the neuronal-specific βIII-tubulin has been implicated in resistance to TBAs in both preclinical and clinical models (reviewed in refs. 3, 4). Given the crucial role of microtubule dynamics in the pharmacologic action of these drugs, one mechanism by which βIII-tubulin has been thought to mediate resistance to TBAs such as the taxanes is to constitutively increase microtubule dynamics (5–7). Previous *in vitro* studies have shown that depletion of βIII-tubulin from unfractionated tubulin results in increased microtubule assembly both in the absence and presence of paclitaxel (8). Furthermore, microtubules composed of purified βIII-tubulin were more dynamic than microtubules assembled from unfractionated tubulin (5, 6).

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Note: Supplementary material for this article is available at Molecular Cancer Therapeutics Online (http://mct.aacrjournals.org/).

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doi: 10.1158/1535-7163.MCT-09-0679

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However, functional data assessing the abilities of βIII-tubulin to modulate dynamics in complex cellular environments are limited. Exogenous overexpression of HA-tagged βIII-tubulin in Chinese hamster ovary cells did not significantly alter any parameters of microtubule dynamic stability but reduced the ability of paclitaxel to suppress microtubule dynamics and resulted in resistance to paclitaxel (9). Moreover, to date, there has been no study addressing the functional significance of βIII-tubulin on the ability of *Vinca* alkaloids to suppress microtubule dynamics.

Cancer cells can display multiple genetic alterations, and increased expression of βIII-tubulin has been associated with aggressive and drug refractory disease in several cancer types (4). The functional role of βIII-tubulin expression and TBA drug resistance is poorly understood. Our laboratory recently showed that small interfering RNA (siRNA)–mediated knockdown of βIII-tubulin expression hypersensitized non–small cell lung cancer (NSCLC) cells to TBAs and DNA-damaging agents, suggesting that this protein may have multiple functions within the cell (10). In contrast, siRNA-mediated knockdown of βII- or βIVb-tubulin hypersensitized NSCLC cells to *Vinca* alkaloids but not paclitaxel (10). To date, the functional significance of βIII-tubulin suppression on microtubule dynamics, mitotic arrest, and cell death in the presence or absence of TBAs in cancer cells has not been addressed. Herein, we examined the role of βIII-tubulin on microtubule dynamics and the link between TBA-induced suppression of microtubule dynamics, mitotic arrest, and cell death associated with this tubulin isotype.

Materials and Methods

Cell culture. Human NSCLC H460 cells or H460 cells stably transfected with green fluorescent protein (GFP)-βI-tubulin were cultured in RPMI supplemented with 10% FCS (growth media) at 37°C in 5% CO_2. To visualize microtubule dynamic instability, H460 cells expressing GFP-βI-tubulin were generated by transfecting H460 cells with the pd2EGFP-N1 vector (Clontech) containing the full-length HM40 (βI-tubulin) using Lipofectamine 2000 transfection reagent according to the manufacturer's protocol (Invitrogen). This vector was constructed by cloning the full-length HM40 β-tubulin cDNA into the *EcoR I* and *Sac II* site of the pd2EGFP-N1 using standard procedures. Cells stably expressing GFP-βI-tubulin were selected in G418 (1 mg/mL). Pooled cell populations were screened for GFP expression by fluorescence microscopy before cell sorting by flow cytometry. Sorted GFP-positive cells were then maintained in growth media for further experiments. The presence or absence of GFP-βI-tubulin did not affect the degree of sensitivity to either paclitaxel or vincristine induced by transfection with βIII-tubulin siRNA (data not shown). H460 cells stably expressing GFP-βI-tubulin are denoted as H460-GFP-βI-tubulin in the remainder of this article and were used for all experiments unless otherwise specified.

Cytotoxic drugs. Vincristine (Sigma-Aldrich) was prepared at a stock concentration of 2 mmol/L in ethanol. Paclitaxel (Sigma-Aldrich) was prepared at a stock concentration of 10 mmol/L in DMSO and was further diluted to 10 μmol/L in ethanol before a final dilution in media for each experiment.

Western blotting. Western blot analyses using antibodies directed against different β-tubulin isotypes were done as previously described (10). Glyceraldehyde-3-phosphate dehydrogenase was used as a loading control (10).

Analyses of mitotic progression and apoptosis. Mitotic index was determined by nuclear staining with 4,6 diaminophenylindole as previously described with slight modifications (11). Briefly, H460-GFP-βI-tubulin cells were plated at 1.2×10^5/2 mL into six-well plates and transfected with nonfluorescent labeled βIII-tubulin SMARTpool siRNA and a corresponding nonfluorescent labeled nonsilencing control siRNA as described (10). The SMARTpool βIII-tubulin siRNA reagent is a pool of four siRNA duplexes targeting separate regions of the βIII-tubulin gene to suppress expression. After 72 hours of transfection, cells were incubated in the absence or presence of drug at a range of concentrations (1.6–60 nmol/L for paclitaxel and 2–60 nmol/L for vincristine) for 20 hours. Cells were harvested (both adherent and detached cells) and fixed with 10% formalin for 30 minutes, permeabilized in 0.1% Triton X-100 for 5 minutes, and stained with 4,6 diaminophenylindole (Prolong Gold antifade, Invitrogen) to visualize nuclei. Mitotic indices and apoptotic cells were quantitated by microscopy. Apoptotic cells were identified based on chromatin condensation and fragmentation, morphologic features characteristic for apoptosis (12). Results are the means and SEM of three experiments and 500 cells were counted per drug concentration.

Preparation of cells for analysis of microtubule dynamics. To monitor siRNA uptake by individual cells, H460-GFP-βI-tubulin cells were transfected with tetramethyl-6-carboxyrhodamine (TAMRA)–labeled SMARTpool βIII-tubulin siRNA (Dharmacon Research, Inc.) at a final concentration of 100 nmol/L using Lipofectamine 2000 following the manufacturer's instructions. Control experiments were done in parallel by transfecting the cells with a rhodamine-labeled nonsilencing control siRNA (Qiagen) at equivalent concentrations to the target siRNA. To promote cell spreading, transfected cells were seeded onto glass coverslips that had been pretreated with poly-L-lysine (0.5 mg/mL; Sigma-Aldrich) for 2 hours, followed by laminin and fibronectin (Invitrogen) coating as previously described (11). The transfection mix was removed after 24 hours of incubation and replaced with medium containing a reduced concentration of FCS (2%) to promote cell flattening for an additional 48 hours. Cells were incubated with drugs for an additional 4 hours before imaging and transferred to RPMI-recording

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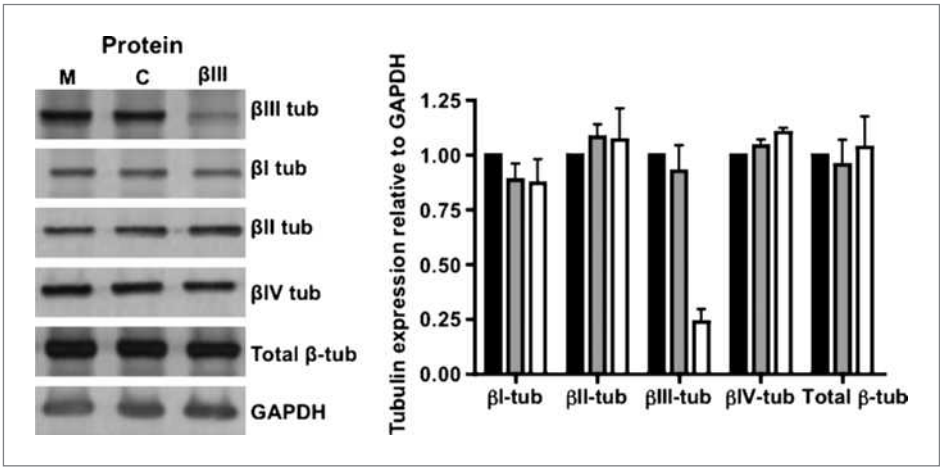

Figure 1. Expression of β-tubulin isotypes in siRNA-transfected H460 GFP-βI-tubulin cells. Cells were transfected with 100 nmol/L TAMRA-labeled SMARTpool βIII-tubulin (βIII-tub) siRNA and harvested 72 h after transfection. The lysates were probed with antibodies to β-tubulin isotypes and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as a loading control. M, mock; C, rhodamine-labeled negative control siRNA; βII, TAMRA-labeled SMART pool βIII-tubulin siRNA. Specificity of βIII-tubulin siRNA. No significant changes were observed for class I, II, and IV β-tubulin isotypes and total β-tubulin at the protein level.

medium lacking paclitaxel or vincristine during imaging (15 min–1 h; ref. 11).

Time lapse microscopy, image acquisition, and analysis of dynamic instability. Microscopy and analysis of dynamic instability were conducted as described (11, 13). Briefly, cells were placed in RPMI culture medium lacking sodium bicarbonate and phenol red, supplemented with 25 mmol/L HEPES, 3.5 g/l glucose, and 30 μL Oxyrase/mL (Oxyrase, Inc.) to reduce photodamage, in a double coverslip chamber. siRNA-transfected cells were identified by the presence of red fluorescence. Images were captured with a fluorescence microscope (Nikon Eclipse E800) maintained at 37 ± 1°C, with a Nikon plan apochromat 1.4 numerical aperture, ×100 objective lens. Thirty-one images per cell were acquired at 4-second intervals using a Hamamatsu Orca II digital camera driven by the Metamorph software (Universal Imaging). The positions of the plus ends of individual microtubules over time were tracked using the Metamorph software, converted to life history plots and analyzed using the Real Time Measurement software. Only changes of >0.5 μm were called growth or shortening events. Changes of <0.5 μm were considered to be pause or attenuation. The time-based catastrophe frequency was calculated by dividing the total number of catastrophes (transitions from growing or pause to shortening) by the total time spent in growth and pause. The rescue frequency was calculated by dividing the number of rescues (transitions from shortening to growth or pause) by the total time spent in shortening. The catastrophe and rescue frequencies based on length were also determined by dividing the number of transitions by the length of the microtubules that grew (for the catastrophe frequency) or shortened (for the rescue frequency). Dynamicity is the total length grown and shortened divided by the life span of the microtubules. Between 33 and 80 microtubules were analyzed for each condition from at least three independent experiments. Results are the means and SEM of at least three independent experiments.

Statistical analysis. All statistical tests of comparative data were done using two-sided, unpaired Student's *t* tests. Data were expressed as means of at least three independent experiments ± SEM, with $P < 0.05$ considered statistically significant.

Results

Stable overexpression of GFP-βI-tubulin does not alter the expression of other β-tubulin isotypes nor change the drug sensitivity profiles of H460 cells. The effects of silencing βIII-tubulin on microtubule dynamic instability in the absence and presence of TBAs were determined in H460 cells that stably expressed GFP-βI-tubulin. To assess the ability of these cells to take up siRNAs and to assess the efficacy of the fluorescent-labeled βIII-tubulin siRNAs, Western blot analysis was first done on siRNA-transfected H460-GFP-βI-tubulin cells to check the expression of different β-tubulin isotypes. As shown in Fig. 1, the TAMRA-labeled SMARTpool βIII-tubulin siRNA significantly knocked down βIII-tubulin expression 76% ± 0.03% without affecting the expression of other β-tubulin isotypes (Fig. 1), consistent with previous findings using nonfluorescent labeled SMARTpool βIII-tubulin siRNA in H460 cells (10).

We recently showed that siRNA-mediated knockdown of βIII-tubulin increases sensitivity to both vincristine and paclitaxel in H460 cells (10). In the present study, we examined whether the same result would be obtained in H460 cells stably expressing GFP-βI-tubulin. The IC_{50} for vincristine and paclitaxel in H460 βIII-tubulin knockdown

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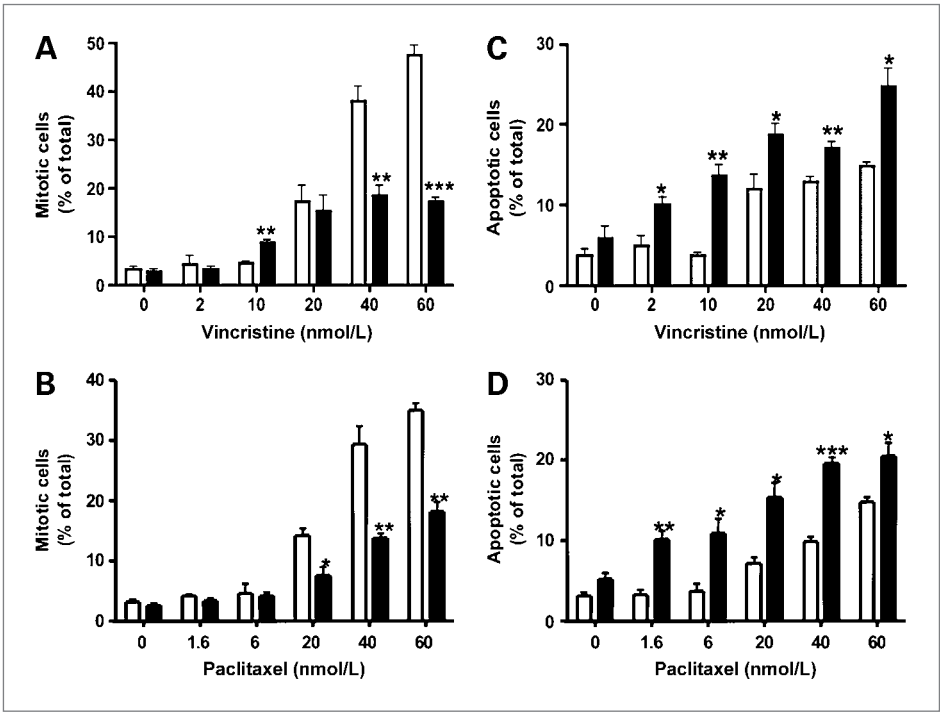

Figure 2. Quantitation of mitotic and apoptotic cell number. Percentage of siRNA-transfected H460 GFP-βI-tubulin cells in mitosis quantified after 20 h of incubation with (A) vincristine and (B) paclitaxel. Open columns, control siRNA–transfeted cells; filled columns, βIII-tubulin siRNA–transfected cells. Percentage of apoptotic cells for siRNA-transfected H460 GFP-βI-tubulin cells quantified after 20 h of incubation with (C) vincristine and (D) paclitaxel. Open columns, control siRNA–transfected cells; filled columns, βIII-tubulin siRNA–transfected cells. Cells were fixed and incubated with 4,6 diaminophenylindole to stain chromosomes and nuclei. Apoptotic cells were scored if nuclear staining was condensed and appeared as droplets of fragmented chromatin. In addition, refer to Supplementary Fig. S1 for morphologic features of interphase, mitotic, and apoptotic siRNA-transfected H460-GFP-βI-tubulin cells. Columns, mean; bars, SEM. Statistical analysis was done by Student's *t* test. Values significantly different from corresponding control siRNA cells are indicated as *, $P < 0.05$; **, $P < 0.01$; and ***, $P < 0.001$.

cells stably expressing GFP-βI-tubulin was determined using drug-treated clonogenic assays (10) and was found to be 2.3 ± 0.2 nmol/L for vincristine and 1.8 ± 0.2 nmol/L for paclitaxel. These IC_{50} values were very similar to our previously reported values of 1.7 ± 0.1 nmol/L for vincristine and 1.7 ± 0.04 nmol/L for paclitaxel in the βIII-tubulin knockdown H460 cells in the absence of GFP-βI-tubulin expression (10). These results show that the knockdown of βIII-tubulin induced similar sensitivity profiles to TBAs in the presence or absence of stable expression of fluorescent βI-tubulin.

Knockdown of βIII-tubulin reduces TBA-induced mitotic arrest. We examined the effects of βIII-tubulin knockdown on TBA-induced mitotic arrest by determining the mitotic index after a 20-hour incubation with a range of concentrations of vincristine or paclitaxel (see Materials and Methods). The control siRNA cells with unaltered levels of βIII-tubulin (Fig. 2A and B, open columns) showed a TBA dose-dependent increase in mitotic index, in contrast to the βIII-tubulin knockdown cells in which the mitotic index was clearly plateaued at higher concentrations of vincristine and tended to increase at a reduced concentration-dependent rate with paclitaxel (Fig. 2A and B, filled columns). Control cells accumulated in mitosis at ≥20 nmol/L for both vincristine and paclitaxel. Interestingly, βIII-tubulin knockdown cells had a slightly higher mitotic index at 10 nmol/L vincristine compared with the control cells ($P < 0.01$; Fig. 2A), but plateaued at higher concentrations. In contrast, the extent of mitotic block induced by paclitaxel was markedly reduced after βIII-tubulin knockdown at ≥20 nmol/L when compared with control cells ($P < 0.05$; Fig. 2B). At concentrations ≥40 nmol/L of both drugs, mitotic arrest was significantly lower in cells with silenced βIII-tubulin expression, ($P < 0.01$; Fig. 2A and B). Rather, there was a significant increase in the percentage of βIII-tubulin knockdown cells undergoing apoptosis, following exposure to either vincristine or paclitaxel when compared with the relevant control siRNA cells treated at the same drug concentrations (Fig. 2C and D). As illustrated in Supplementary Fig. S1, the βIII-tubulin knockdown cells showed chromosome condensation and nuclear fragmentation, morphologic features characteristic of apoptosis, following exposure to paclitaxel. We also sought to determine the time course of the cell cycle phases using cell cycle analysis at a low and high concentration of each drug. Consistent with the mitotic index data presented in Fig. 2A and B, there

Published OnlineFirst May 4, 2010; DOI: 10.1158/1535-7163.MCT-09-0679

was no significant change in the number of cells arresting at the G_2-M phase of the cell cycle with either low-dose vincristine (2 nmol/L) or low-dose paclitaxel (1.6 nmol/L) at 4, 8, and 20-hour treatments (Supplementary Figs. S2 and S3, respectively). Moreover, there was no major increase in cyclin B1 expression, a marker of mitosis, or cyclin D (Supplementary Fig. S4). At a high concentration of vincristine (40 nmol/L), G_2-M accumulation (mitotic block) is seen in both βIII-tubulin knockdown and control siRNA cells at 4 hours. Following 8 hours of treatment with high-dose vincristine, βIII-tubulin knockdown cells and control siRNA cells displayed G_2-M accumulation, but only the βIII-tubulin knockdown cells displayed an increase in sub- G_1 (cell death) content. Consistent with the cell cycle data, there was an increase in cyclin B1 expression (Supplementary Fig. S4). At 20 hours, the high-dose treatment caused a marked increase in sub-G_1 levels and a decrease in G_2-M (compared with 8 h), indicative of cell death in βIII-tubulin knockdown cells. The cyclin B1 levels were lower at 20 hours in high-dose vincristine–treated βIII-tubulin knockdown cells compared with 8 hours, most likely due to either degradation of cyclin B1, which occurs at the end of mitosis, or reflecting the increased cell death observed in these cells. Either way, the cyclin B1 levels correlate with the lower percentage of cells at G_2-M (Supplementary Fig. S4). The control cells treated with high-dose vincristine on the other hand had a marked increase in G_2-M (Supplementary Fig. S3) and increased cyclin B1 expression (Supplementary Fig. S4). Taken together, these data further support the mitotic index and apoptosis data presented in Fig. 2. High-dose paclitaxel treatment (20 nmol/L) caused the control siRNA cells to accumulate at G_2-M at 8 hours. The βIII-tubulin knockdown cells also display G_2-M accumulation at 8 hours with paclitaxel, but have much higher sub-G_1 content than the control cells at 20 hours (Supplementary Fig. S3). These results indicate that the most potent and durable effects of vincristine and paclitaxel on cell proliferation and mitosis in the H460-GFP-βI-tubulin cells occurred between 1.6 to 40 nmol/L. Thus, we chose to determine microtubule dynamics within this range, at three concentrations of vincristine (2, 10, and 40 nmol/L) and paclitaxel (1.6, 6, and 20 nmol/L).

βIII-tubulin knockdown does not affect microtubule dynamics in the absence of TBAs. The effects of βIII-tubulin levels on dynamic instability were determined in living interphase H460 cells expressing GFP-βI-tubulin as a marker to visualize microtubules. Images of GFP-labeled microtubules were captured by time-lapse fluorescence microscopy, and the changes in length of individual microtubules were graphed as life history plots from which the variables of microtubule dynamics were determined (Materials and Methods). The dynamic instability behavior of microtubules after βIII-tubulin knockdown was similar to that in control siRNA cells in the absence of drug (Tables 1 and 2; also compare life history traces in Fig. 3A,

Table 1. Effect of vincristine on the dynamic instability of microtubules in βIII-tubulin knockdown cells and control siRNA–transfected cells

Parameter	Control siRNA				βIII-tubulin siRNA			
	Nontreated	2 nmol/L VCR	10 nmol/L VCR	40 nmol/L VCR	Nontreated	2 nmol/L VCR	10 nmol/L VCR	40 nmol/L VCR
Growth rate (μm/min)	10.4 ± 0.5	NS	NS	*−65.9**	10.6 ± 0.2	*−19.6**	*−27.9**	*−35.4**
Growth length (μm)	1.4 ± 0.1	NC	NS	*−67.0**	1.5 ± 0.1	*−19.1*†	*−32.5*‡	*−38.7**
Shortening rate (μm/min)	15.2 ± 0.4	NS	*−17.1*†	*−70.2**	16.4 ± 0.5	*−20.7*‡	*−35.5**	*−43.2**
Shortening length (μm)	1.7 ± 0.1	NS	NS	*−68.8**	1.8 ± 0.1	*−21.4*†	*−34.2*‡	*−44.0**
Catastrophe/min	3.9 ± 0.2	NS	*−17.4*†	*−79.0**	3.7 ± 0.1	NS	*−31.5*‡	*−37.3**
Rescue/min	6.5 ± 0.3	NS	NS	NS	6.4 ± 0.4	NS	NS	NS
Catastrophe/μm	0.6 ± 0.03	NS	*51.3*†	*133.3**	0.7 ± 0.04	*32.0*†	*58.7*‡	*72.3**
Rescue/μm	0.5 ± 0.03	NS	*32.3*‡	*149.2**	0.5 ± 0.03	*52.2*‡	*102.0**	130.3*
% Growing	48.1 ± 1.5	NS	*−12.7*†	*−76.4**	46.1 ± 1.7	NS	*−30.3*‡	*−28.8*‡
% Shortening	31.4 ± 1.2	NS	NS	*−67.8**	30.6 ± 1.3	NS	*−21.9*†	*−25.1*‡
% Paused	20.5 ± 0.7	*19.7*‡	*47.3**	*286.3**	23.2 ± 1.6	*32.7*†	*90.0**	*91.3**
Dynamicity (μm/min)	9.6 ± 0.4	NS	*−27.9*‡	*−89.4**	10.1 ± 0.3	*−31.2**	*−50.3**	*−59.1**

NOTE: The values for nontreated cells are given in absolute units. The effects of vincristine are indicated as percentage change from nontreated cells, when statistically significant. Measurements are from 82 to 205 microtubules from between 18 and 50 cells for each condition. Values are means ± SEM. Tests of significance were done on all parameters of dynamics.

Abbreviations: NS, not significant; VCR, vincristine.

* $P < 0.001$.

† $P < 0.05$.

‡ $P < 0.01$.

Published OnlineFirst May 4, 2010; DOI: 10.1158/1535-7163.MCT-09-0679

Table 2. Effect of paclitaxel on the dynamic instability of microtubules in βIII-tubulin knockdown cells and control siRNA–transfected cells

Parameter	Control siRNA				βIII-tubulin siRNA			
	Nontreated	1.6 nmol/L PXL	6 nmol/L PXL	20 nmol/L PXL	Nontreated	1.6 nmol/L PXL	6 nmol/L PXL	20 nmol/L PXL
Growth rate (μm/min)	10.4 ± 0.5	NS	*–21.7**	*–38.9*†	10.6 ± 0.2	*–11.7*†	*–19.0*‡	*–23.9*‡
Growth length (μm)	1.4 ± 0.1	NS	NS	*–36.3*†	1.5 ± 0.1	NS	NS	NS
Shortening rate (μm/min)	15.2 ± 0.4	*13.4**	*–22.6*†	*–54.7*‡	16.4 ± 0.5	*–13.9**	*–15.7**	*–24.9*†
Shortening length (μm)	1.7 ± 0.1	NS	*–22.0**	*–49.4*‡	1.8 ± 0.1	NS	NS	*–19.9**
Catastrophe/min	3.9 ± 0.2	NS	*–23.0**	*–43.4*‡	3.7 ± 0.1	NS	NS	NS
Rescue/min	6.5 ± 0.3	NS	NS	NS	6.4 ± 0.4	NS	NS	NS
Catastrophe/μm	0.6 ± 0.03	NS	NS	*89.8*‡	0.7 ± 0.04	*24.7**	NS	NS
Rescue/μm	0.5 ± 0.03	NS	*44.8*†	*125.2*‡	0.5 ± 0.03	NS	NS	*37.9**
% Growing	48.1 ± 1.5	NS	NS	*–44.2*‡	46.1 ± 1.7	NS	NS	NS
% Shortening	31.4 ± 1.2	NS	NS	NS	30.6 ± 1.3	NS	NS	NS
% Paused	20.5 ± 0.7	NS	*34.0**	*126.4*‡	23.2 ± 1.6	NS	NS	NS
Dynamicity (μm/min)	9.6 ± 0.4	NS	*–30.5*†	*–64.9*‡	10.1 ± 0.3	*–20.9*†	*–16.7**	*–24.4*†

NOTE: The values for nontreated cells are given in absolute units. The effects of paclitaxel are indicated as percentage change from nontreated cells, when statistically significant. Measurements are from 82 to 205 microtubules from between 18 and 50 cells for each condition. Values are means ± SEM. Tests of significance were done on all parameters of dynamics.
Abbreviation: PXL, paclitaxel.
*$P < 0.05$.
†$P < 0.01$.
‡$P < 0.001$.

left and right). For example, the control microtubules grew and shortened at a mean rate of 10.4 ± 0.5 μm/min and 15.2 ± 0.4 μm/min, respectively, compared with βIII-tubulin–depleted microtubules that grew and shortened at a mean rate of 10.6 ± 0.2 μm/min and 16.4 ± 0.5 μm/min, respectively (Tables 1 and 2). The overall microtubule dynamicity was 9.6 ± 0.4 μm/min in control siRNA cells compared with 10.1 ± 0.3 μm/min after βIII-tubulin knockdown. Collectively, the results showed that knockdown of βIII-tubulin did not significantly affect any parameters of dynamic instability.

Vincristine and paclitaxel suppress microtubule dynamics in living βIII-tubulin knockdown cells at concentrations that inhibit cell proliferation. Representative life history plots of the changes in length of individual microtubules in the presence or absence of vincristine are shown in Fig. 3. At 2 nmol/L vincristine (the IC_{50} for βIII-tubulin knockdown cells, from clonogenic assay), the dynamic instability of microtubules in control cells with unaltered βIII-tubulin levels is virtually unchanged (compare Fig. 3A and B, left). In contrast, after βIII-tubulin knockdown, 2 nmol/L vincristine induced significant suppression of microtubule dynamic instability and the life history traces of length changes were nearly flat (compare Fig. 3A and B, right). As shown quantitatively in Table 1, 2 nmol/L vincristine induced almost no significant change in the parameters of dynamic instability of control cell microtubules. In strong contrast, several dynamic instability parameters were significantly altered after βIII-tubulin knockdown. For example, the growth and shortening rates, the growth and shortening lengths, and the dynamicity were reduced by 19.6%, 20.7%, 19.1%, 21.4%, and 31.2%, respectively by 2 nmol/L vincristine (Table 1). Hence, at the concentration that inhibited the proliferation of βIII-tubulin knockdown cells by 50% in a clonogenic assay but had no antiproliferative effect on control cells (10), microtubules grew and shortened more slowly and for shorter distances in βIII-tubulin–depleted microtubules compared with microtubules in the control siRNA cells treated at the same vincristine concentration (Table 1). Also at this concentration, apoptosis was significantly enhanced in the βIII-tubulin knockdown cells compared with control cells (Fig. 2C). Hence, at this low drug concentration, the increased sensitivity to vincristine in the βIII-tubulin knockdown cells can be explained by the increased suppression of dynamic instability and increased vincristine-induced cell death.

At 1.6 nmol/L paclitaxel (the IC_{50} for βIII-tubulin knockdown cells, from clonogenic assay), both the growth and shortening rates were significantly reduced by paclitaxel after βIII-tubulin knockdown, by 11.7% and 13.9%, respectively, compared with the control siRNA cells (Table 2). This led to an overall paclitaxel-induced 20.9% decrease in dynamicity after βIII-tubulin knockdown compared with only an insignificant 0.3% change in dynamicity of control cell microtubules (Table 2). In

Published OnlineFirst May 4, 2010; DOI: 10.1158/1535-7163.MCT-09-0679

addition, there was a significant increase in the percentage of apoptotic cells at this concentration when βIII-tubulin was knocked down (Fig. 2D). Thus, at this low drug concentration, the increased paclitaxel sensitivity after βIII-tubulin knockdown can be explained by the increased paclitaxel suppression of dynamic instability and also increased paclitaxel-induced cell death, similar to vincrisitine.

Higher vincristine and paclitaxel concentrations did not further suppress microtubule dynamicity after βIII-tubulin knockdown. The preceding section showed that vincristine suppressed both the growing and shortening rates of βIII-tubulin–depleted microtubules at concentration that inhibited cell proliferation (i.e., 2 nmol/L). Similarly, 10 nmol/L vincristine significantly decreased both the rates and lengths of growing and shortening, leading to >50% suppression in dynamicity in the βIII-tubulin knockdown cells. In contrast, only a few parameters of microtubule dynamic instability were significantly affected by 10 nmol/L vincristine in control siRNA cells and these parameters were affected to a significantly lesser extent than in βIII-tubulin knockdown cells (Supplementary Fig. S5; Table 1). Of note is that the mitotic index of βIII-tubulin knockdown cells was significantly increased at 10 nmol/L vincristine compared with control cells (Fig. 2A). Thus, at this particular vincristine concentration, there was a marked parallel between suppression of microtubule dynamic instability and degree of mitotic arrest.

Surprisingly, the suppression changed very little with higher concentration of vincristine (40 nmol/L), as shown quantitatively in Table 1 and graphically in Supplementary Fig. S5 (filled columns). In the control siRNA cells, 40 nmol/L vincristine induced >40% mitotic arrest (Fig. 2A, open columns); the microtubule shortening and growing rates were reduced to such an extreme extent that they were barely measurable and the microtubules spent ~80% of the time in the attenuated state (Supplementary Fig. S2; Table 1). In contrast, with the cells in which βIII-tubulin was knocked down, 40 nmol/L vincristine induced <20% mitotic arrest (Fig. 2A, filled columns) and the parameters of dynamic instability were reduced to lesser extents than in the control cells (Supplementary Table S1; Table 1).

Similarly with paclitaxel, the degree of mitotic arrest reflected the degree of suppression of microtubule dynamicity in the control siRNA and βIII-tubulin knockdown cell lines at higher concentrations. The microtubules of control siRNA cells became increasingly stabilized in a paclitaxel concentration–dependent manner until their life history plots became almost flat at 20 nmol/L paclitaxel (data not shown) and the individual parameters of dynamics instability were reduced 2- to 3-fold (Supplementary Fig. S6; Table 2). The few dynamic microtubules grew and shortened more slowly and for shorter lengths, leading to an overall 64.9% decrease in dynamicity. Interestingly, at 20 nmol/L paclitaxel, only ~7% of the βIII-tubulin knockdown cells accumulated in mitosis compared with ~14% mitotic arrest in the control siRNA cells (Fig. 2B). Thus, the degree of suppression of microtubule dynamic instability by paclitaxel reflected the degree of mitotic arrest in the βIII-tubulin knockdown and control siRNA cells at this concentration.

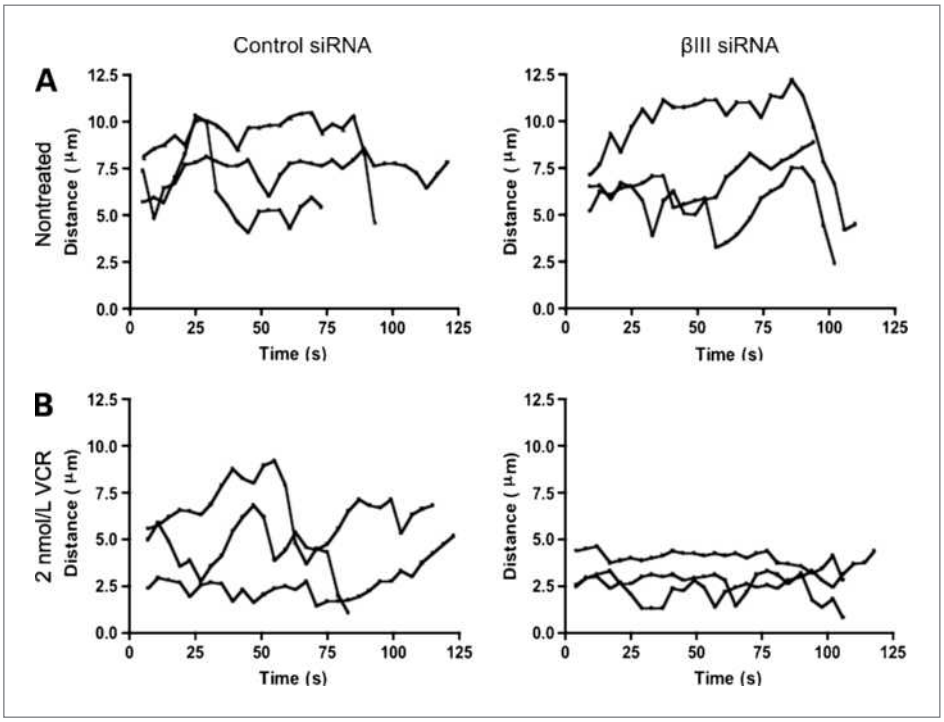

Figure 3. Dynamic life history plots of individual microtubules. Changes in length of the plus ends of individual microtubules over time were followed in control cells (left) and βIII-tubulin knockdown cells (right) in the absence and presence of vincristine. Each line represents a single microtubule. Changes in the length of the plus ends of microtubules were plotted versus time. A, nontreated; B, 2 nmol/L vincristine (VCR). From these life history plots, individual growth, shortening, and attenuation events were identified and rates and durations of each event were measured as described in Materials and Methods.

Published OnlineFirst May 4, 2010; DOI: 10.1158/1535-7163.MCT-09-0679

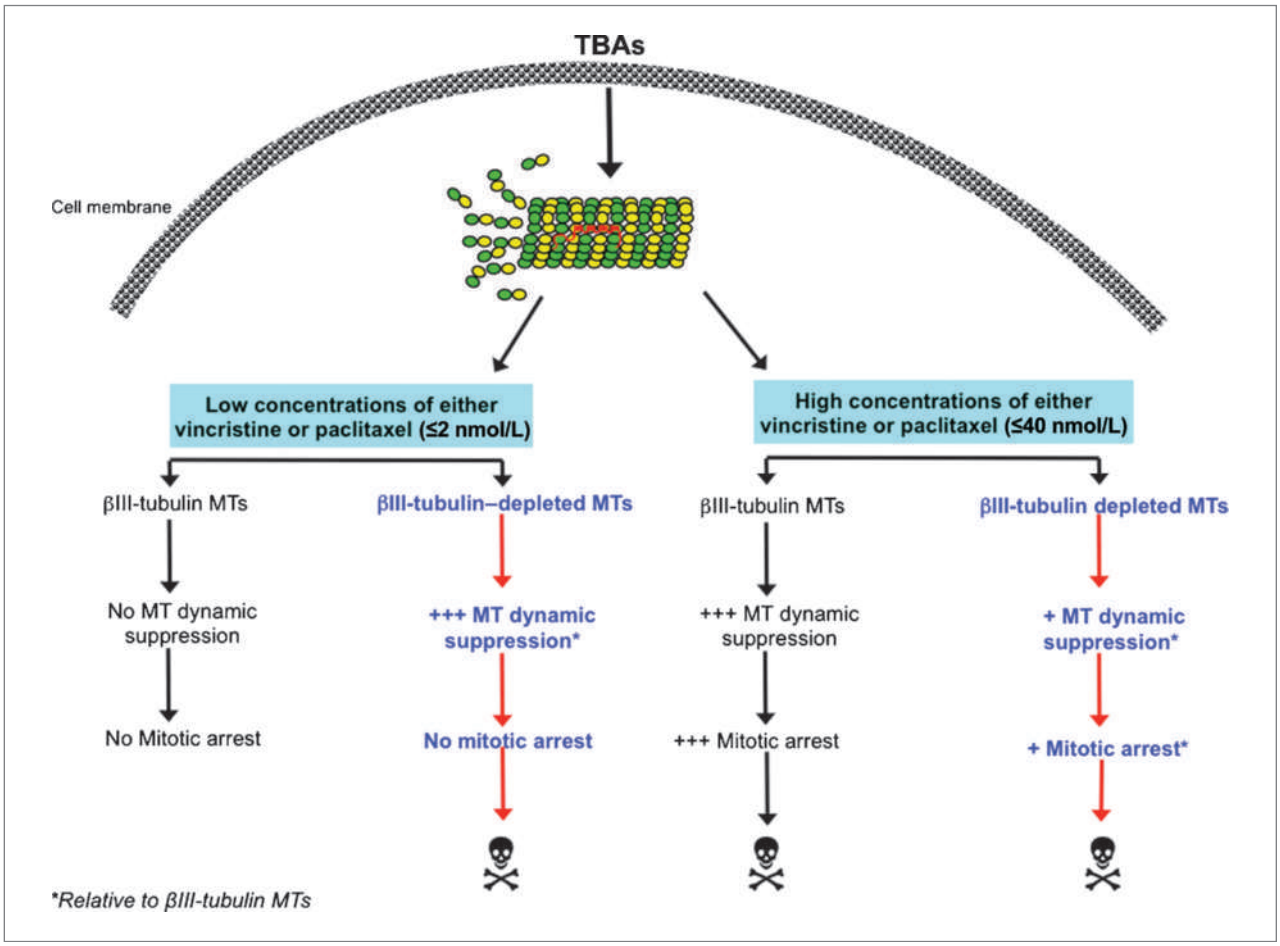

Figure 4. Proposed model of effects of βIII-tubulin on microtubule dynamics, drug-induced mitotic arrest, and cell death. At low concentrations of vincristine and paclitaxel (≤2 nmol/L, IC_{50} from clonogenic assay), βIII-tubulin depleted microtubules suppressed microtubule dynamics and induced apoptosis. In contrast, at concentrations ≤40 nmol/L of vincristine and paclitaxel, βIII-tubulin–depleted microtubules suppressed microtubule dynamics significantly less than controls and underwent mitosis-independent cell death. Scale for relative degree of change: +, small; ++, medium; +++, large. MT.

In summary, at the IC_{50} of both drugs that inhibit the proliferation of βIII-tubulin knockdown cells but not control siRNA cells (i.e., 2 nmol/L vincristine and 1.6 nmol/L paclitaxel), knockdown of βIII-tubulin increased apoptosis and increased the TBA-induced suppression of microtubule dynamics. Paradoxically, with increasing drug concentrations (≥40 nmol/L vincristine and ≥6 nmol/L paclitaxel), βIII-tubulin knockdown cells reduced the ability of either drug to suppress microtubule dynamicity compared with control siRNA cells. This is reflected in a reduction in the extent of mitotic block induced by either vincristine or paclitaxel in the βIII-tubulin knockdown cells. Despite the decrease in mitotic arrest, there was an increase in apoptosis in the βIII-tubulin knockdown cells.

Discussion

Microtubule dynamics are tightly regulated because microtubule behavior is critical to cell survival. Suppression of microtubule dynamics leading to cell cycle arrest and consequently inhibition of cell proliferation or apoptosis is thought to be the most potent mechanism of action of TBAs (14). Our finding that siRNA-mediated knockdown of βIII-tubulin in NSCLC cells does not significantly affect the intrinsic dynamics of microtubules is of interest, as based on the cell-free microtubule assembly studies using purified βIII-tubulin (5, 6), we had anticipated an effect on microtubule dynamics. Of interest is a study by Kamath et al. (9) who found that overexpression of βIII-tubulin in Chinese hamster ovary cells failed to alter the intrinsic properties on the microtubule dynamics. The differences in the effect of βIII-tubulin on microtubule dynamics between the *in vitro* studies using microtubules assembled from purified tubulin and *in vivo* cell studies may be due to additional cellular regulatory factors that are present in cells but are absent from *in vitro* systems.

The suppressive effects of vincristine on microtubule dynamics in cells have not been reported previously.

Published OnlineFirst May 4, 2010; DOI: 10.1158/1535-7163.MCT-09-0679

Importantly, we have shown that vincristine and paclitaxel strongly suppress microtubule growth and shortening in βIII-tubulin knockdown H460 cells during interphase at concentrations that inhibit their proliferation (2 and 1.6 nmol/L for vincristine and paclitaxel, respectively). In contrast, none of these parameters were detectably affected at these concentrations in the control cells with unaltered βIII-tubulin expression. Further, both vincristine and paclitaxel significantly reduced the overall dynamicity of βIII-tubulin depleted microtubules by 31.2% and 20.9% respectively, compared with only ≤6.5% in the control siRNA cells. In addition, cell death was significantly increased in the βIII-tubulin knockdown cells compared with the control cells. These results highlight the fact that βIII-tubulin knockdown hypersensitizes NSCLC cells to TBA-induced cell death through the suppression of microtubule dynamics at lower drug concentrations. Paradoxically, as the concentrations of vincristine and paclitaxel were increased to concentrations that arrested mitotic progress in the control siRNA cells, suppression of microtubule dynamicity was approximately 2- to 3-fold weaker in the βIII-tubulin knockdown cells than in control cells. Despite the fact that vincristine and paclitaxel have diverse structures and bind to distinct sites on tubulin/microtubules (2), their interactions with different β-tubulin isotypes are poorly understood. Our results highlight that βIII-tubulin may mediate sensitivity to both *Vinca* alkaloids and taxanes through a similar mechanism of action, suggesting a potential common pathway mediated by this isotype on microtubules and the action of TBAs (Fig. 4).

In evaluating nuclear morphology, we noted that mitotic cells were rarely observed in TBA-treated βIII-tubulin knockdown cells compared with similarly treated control siRNA cells with unaltered βIII-tubulin expression. Despite the reduced TBA-induced mitotic arrest in βIII-tubulin knockdown cells, there was a significant increase in the number of cells showing chromosome condensation and fragmentation (morphologic features characteristic of apoptosis) following exposure to vincristine or paclitaxel. One limitation of this study is that one cell line was evaluated; however, this finding is consistent with previous data showing increased growth inhibition in two independent paclitaxel-treated βIII-tubulin NSCLC knockdown cell lines and increased apoptosis using Annexin V staining (10). Little is known about how mitotic arrest links to apoptosis; however, mitotic arrest may not be an absolute prerequisite for growth inhibition and cell death, and TBAs may induce apoptotic cell death independent of cell cycle arrest (15, 16). Alternatively, the time dependence for induction of cell death and/or mitotic slippage may differ after the knockdown of βIII-tubulin. It seems that βIII-tubulin knockdown may alter the potential to bypass or to slip through the mitotic arrest checkpoint and to induce apoptosis as a final consequence. Apoptosis may be a consequence of conflicting cell survival signals leading to failure to transverse the cell cycle ("better dead than wrong" principle). Hence, our results suggest that βIII-tubulin–mediated inhibition of cellular proliferation correlates with the suppression of microtubule dynamics and increased cell death. As mentioned previously, TBAs induce the suppression of microtubule dynamics leading to cell cycle arrest and apoptosis. Importantly, we found that the knockdown of βIII-tubulin leads to TBA-induced apoptosis independent of suppression of microtubule dynamics and mitotic arrest at concentrations that arrested mitosis in the control siRNA–treated cells. This finding implies that βIII-tubulin has a function or activity that sensitizes cells to apoptosis that may be independent of its function in microtubule biology, an important possibility that is currently under investigation.

Microtubule dynamics are known to change dramatically between interphase and mitosis, with mitotic cells exhibiting approximately 20 to 100 times more dynamic microtubules than interphase cells. Because microtubule dynamics were measured in interphase cells in this study, the question arises about whether these measurements accurately reflect the effects of βIII-tubulin knockdown in combination with drug treatment on microtubules in the mitotic spindle. Although this question has not been addressed, multiple studies have previously shown that, in cancer cells, TBAs including paclitaxel, vinblastine, and epothilone B all suppress interphase microtubule dynamics at concentrations that coincide with the concentrations that induced mitotic block (11, 17, 18). Further, studies on centromere dynamics in human osteosarcoma cells after TBA treatment also revealed that mitotic block induced by TBAs is a result of kinetic suppression of centromere dynamics (19, 20). Therefore, TBAs seem to suppress microtubule dynamics relatively similarly or proportionally between interphase and mitotic cells.

In summary, the mechanism of action of βIII-tubulin seems to be more complex than previously envisioned. At low concentrations of TBAs, suppression of microtubule dynamics and increased cell death play a major role in their ability to inhibit cancer cell proliferation in βIII-tubulin knockdown cells. However, βIII-tubulin increases apoptosis induction independent of effects on microtubule dynamics and mitotic arrest seems to play a more prominent role at higher concentrations of two distinct classes of TBAs. Collectively, this study supports a broader role for βIII-tubulin as a cellular survival factor, with a yet unknown mechanism of action that requires further investigation.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

We thank Dr. Eddy Pasquier for the helpful suggestions and critical reading of this manuscript and Tanya Dwarte for technical support.

Published OnlineFirst May 4, 2010; DOI: 10.1158/1535-7163.MCT-09-0679

Grant Support

Children's Cancer Institute Australia for Medical Research, which is affiliated with the University of New South Wales and Sydney Children's Hospital, and by grants from the National Health and Medical Research Council (M. Kavallaris), Cancer Council New South Wales (M. Kavallaris), and NIH CA 57291 (M.A. Jordan). P.P. Gan was supported by an Endeavour International Postgraduate Research Scholarship and International cancer technology transfer fellowships generously offered by the International Union Against Cancer. M. Kavallaris is supported by a National Health and Medical Research Council Senior Research Fellowship.

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Received 07/24/2009; revised 03/01/2010; accepted 03/14/2010; published OnlineFirst 05/04/2010.

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Published OnlineFirst May 4, 2010; DOI: 10.1158/1535-7163.MCT-09-0679

Molecular Cancer Therapeutics

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Mol Cancer Ther 2010;9:1339-1348. Published OnlineFirst May 4, 2010.