Introduction

The resistance of cancer cells to antimicrotubule agents is a serious clinical problem in the successful treatment of cancer. This resistance is considered to be a multifactorial phenomenon involving several mechanisms such as overexpression of the P-glycoprotein (P-gp) drug efflux pump (1), structural alterations to tubulin (2–4), altered expression of microRNAs (6), and impaired apoptotic pathways (7). Alterations in the tubulin/microtubule system, specifically in the β-tubulin subunits, are prominent mechanisms for resistance of cancer cells to antimitotic agents (2–4).

In humans, β-tubulin exists as at least 7 distinct isotypes, with the most significant differences occurring at the carboxyl terminus region (8). These 7 isotypes have been characterized for their tissue-specific expressions. For example, βI (class I) and βIVb (class IVb) are constitutively expressed in all tissues; βII (class II), βIII (class III), and βIVa (class IVa) are expressed mainly in brain tissue; βV is expressed constitutively, but at low levels in all tissues; and βVI (class VI) is restricted to hematopoietic tissues (8–10). A strong correlation between overexpression of β-tubulin isotypes and drug resistance to tubulin-targeting compounds has been reported in cultured cancer cell lines and in the clinic (2, 3), although the clinical significance of β-tubulin mutations is still uncertain (11, 12). Among β-tubulin isotypes, high abundance of βII and βIII isotypes has been highlighted as a resistance mechanism of cancer cells to various tubulin-binding and DNA-damaging agents (2, 3). To date, however, there are only a limited number of studies that have directly investigated the functional significance of these isotypes in antimitotic drug resistance. Some of these studies used cell lines that were selected with taxoid or vinca site drugs or that naturally overexpressed βII- or βIII-tubulin. Hence, the significance of these isotypes in cancer cell resistance to other classes of microtubule-active agents remains largely unknown. Understanding the role of βII- and βIII-tubulin in the resistance to a different class of tubulin-binding agents would help to further clarify the complex mechanisms of action of the β-tubulin isotypes in...
contributing to survival of cancer cells in the presence of diverse chemotherapeutic agents.

Peloruside A (PLA) and laulimalide (Fig. 1) are 2 marine organism–derived natural products that have shown promising anticancer activity in a panel of different mammalian cancer cell lines (13–15). The compounds bind to a similar or overlapping non-taxoid site on β-tubulin (16–22) and enhance tubulin polymerization. This action inhibits microtubule dynamics and blocks cell-cycle progression at G2-M phase, promoting cell death (14, 15). Studies in cells have shown that PLA and laulimalide retain their cytotoxicity in paclitaxel (PTX)- and epothilone-resistant cancer cell lines that have mutations in the taxoid site of β-tubulin (16, 17). PLA and laulimalide are also poor substrates for the P-gp drug efflux pump, overexpression of which significantly reduces the inhibitory activity of PTX and vinblastine (Fig. 1) on cancer cell growth (16, 17).

We recently showed that selection of a human ovarian carcinoma cell line, 1A9-L4 (L4), in the presence of high concentrations of laulimalide led to multiple β-tubulin alterations that included a βI-tubulin structural mutation R306H/C, in addition to increased abundance of βII- and βIII-tubulin isotypes (22). This cell line was highly resistant to laulimalide and PLA but not to other tubulin-targeting drugs that bind to the taxoid, vinca, or colchicine sites on β-tubulin (22). The mutated βI-tubulin residue R306 had earlier been modeled by computer docking and shown to be an essential site for PLA and laulimalide binding to β-tubulin (19–21), suggesting that this βI-tubulin mutation was likely to be one of the key resistance mechanisms of L4 cells to the 2 compounds. The importance of the βII- and βIII-tubulin overexpression in the resistant cells, however, has not been directly determined. Therefore, the aim of this study was to examine the role of βII- and βIII-tubulin overexpression in the resistance phenotype of L4 cells by investigating the effect of short interfering RNA (siRNA) knockdown of these 2 isotypes on the sensitivity of L4 cells to the compounds.

Materials and Methods

Cell culture

Human 1A9 ovarian carcinoma cells, derived from the A2780 cell line, were obtained from NIH. The laulimalide/
PLA-resistant cell line, L4, was a gift from Dr. Paraskevi Giannakakou of Weill Medical College of Cornell University, NY. Neither cell line was directly authenticated in our laboratory, but both L4 and L4 cells retained their epithelial phenotype throughout the study. The derivation of the L4 cell line was as previously described (22). Cells were cultured in RPMI-1640 medium (Invitrogen) supplemented with 10% fetal calf serum (Invitrogen), 0.25 units/mL insulin (Sigma Chemical Co.), 100 units/mL penicillin, and 100 units/mL streptomycin (Invitrogen). The cells were maintained in a humidified incubator in a 5% CO2 in air atmosphere at 37°C.

siRNA transfection

siRNAs designed to target βII- or βIII-tubulin were purchased from Dharmaco (ON-Target plus SMART pool reagent; Supplementary Table S1). An siRNA negative control (MISSION; Sigma) that has no specificity to any human genes was used as the negative transfection control throughout the experiments. L4 cells were transfected with the siRNAs using Lipofectamine 2000 reagent (Invitrogen) following the manufacturer’s instructions. Briefly, the cells were seeded in 35-mm dishes and allowed to attach for 24 hours, then transfected with βII or βIII siRNA for 48 hours. The medium in the dishes was replaced after 48 hours with complete cell culture medium to remove any free siRNAs. The cells were then processed for mRNA and protein quantification. To select the optimal siRNA concentration that gave high target knockdown, but caused minimal toxicity to the cells, each siRNA was titrated for its knockdown effect by transfecting L4 cells with 2-fold serial dilutions of siRNA. For simultaneous silencing of both βII- and βIII-tubulin expression, multiplexes of siRNAs were transfected in the same way as singleplex transfection.

Drugs

PLA and laulimalide were isolated from the marine sponges Mycale hentschelii (New Zealand) and Cacospongia mycofijiensis (Tonga), respectively, and stored as a 1 mmol/L stock solution in absolute ethanol at –80°C. PTX and vinblastine were purchased from Sigma Chemical Company. Cisplatin was purchased from EBewe Pharma, and ixabepilone was purchased from Bristol-Myers Squibb (Fig. 1).

Quantitative real-time PCR

The effects of βII- and βIII-tubulin siRNAs on the mRNA expression of the isotypes were examined by quantitative real-time PCR (qRT-PCR) after 48 hours of siRNA transfection using methodology and specific primers as described previously (22).

Western blot

Total cellular proteins were extracted at 72 hours or 144 hours posttransfection, and Western blotting was done as previously described (22). Mouse monoclonal βIII-tubulin (1:1,000, MMS-422P, clone-7B9; Covance), βIII-tubulin (3:1,000, T8660, clone-SDL.3D10; Sigma), β-tubulin (3:1,000, T4026, clone-TUB.21; Sigma) and β-actin (1:3,000, A2228, clone-AC74; Sigma) primary antibodies and a Cy5-conjugated anti-mouse secondary antibody (1:2,500, PA45010; GE Healthcare) were used to detect the protein bands using a Fujifilm FLA-5100 imaging system (Fuji Photo Film Co. Ltd). The densities of the protein bands were quantified with ImageJ software (NIH) and normalized to the β-actin band density.

Cytotoxicity assay

After 48 hours siRNA transfection, the cells were harvested and transferred to a 96-well plate (5,000 cells per well) and cultured for 24 hours. The cells were then treated with microtubule-stabilizing and microtubule-destabilizing agents for 72 hours, and cell proliferation was assessed by colorimetric reduction of the dye MTT by viable cells. The IC50 of the drugs was determined from a concentration–response curve using Sigma Plot software version 8 (Systat Software Inc.).

Intracellular tubulin polymerization assay

After 72 hours siRNA transfection, cells were treated with various concentrations of PLA or laulimalide for 16 hours, and the polymerized and nonpolymerized tubulins were extracted as described elsewhere (23). The relative intracellular levels of soluble (nonpolymerized) and pelletted (polymerized) tubulin fractions were assessed by immunoblotting against α-tubulin. A rabbit polyclonal primary antibody to α-tubulin (1:1,000, ab18251; Abcam) in conjunction with a Cy5-conjugated goat antirabbit secondary antibody (1:2,500, PA45011V; GE Healthcare) was used to detect the protein bands.

Immunocytochemistry

After 48 hours of siRNA transfection, cells were harvested and plated onto glass coverslips in a 35-mm dish, and the cells were allowed to attach for another 24 hours. The cells were then treated with various concentrations of PLA for 16 hours and fixed with ice-cold methanol-acetone (1:1) for 5 minutes. Nonspecific antibody binding was blocked with 5% bovine serum albumin in 0.25% Triton X-100 in PBS. The cells were then incubated at room temperature for 1 hour with rabbit polyclonal primary antibody to α-tubulin (1:1,000, ab18251; Abcam) and an Alexa Fluor 488–conjugated antirabbit secondary antibody (1:1,000, A11012; Invitrogen) for 1 hour in the dark. After washing 3 times with Triton-PBS, the cells were incubated with Alexa Fluor 594–conjugated antirabbit secondary antibody (1:1,000, A11012; Invitrogen) for 1 hour in the dark. After washing 3 more times, the cells were costained with mouse monoclonal primary antibodies to either βII-tubulin (1:1,000, MMS-422P, clone-7B9; Covance) or βIII-tubulin (1:1,000, T8660, clone-SDL.3D10; Sigma) and a rabbit polyclonal primary antibody to β-actin (1:1,000, A2228; Sigma). After washing with PBS3 times, the coverslips were mounted onto glass slides in Prolong Gold Antifade with 4′,6-diamidino-2-phenylindole (DAPI) to stain the nuclei (Invitrogen). Fluorescent staining was examined with an Olympus Fluoview...
FV1000 confocal laser scanning microscope (inverted model IX81) using a 60 x or 100 x oil-immersion objective with the following settings: filter, Dichroime; wavelength range, DAPI (425–465 nm), Alexa Fluor 488 (485–545 nm), Alexa Fluor 594 (575–620 nm); imaging mode, sequential.

Flow cytometry
L4 cells were seeded at a density of 5 x 10⁴ cells per well in a 24-well plate and transfected with different siRNAs. Seventy-two hours after transfection, the cells were treated with various concentrations of PLA and laulimalide for 16 hours, harvested, and stained with propidium iodide to analyze their cell-cycle distribution by flow cytometry, as previously described (22).

Results
Isotype-specific siRNAs silence βII- and βIII-tubulin mRNA and protein expression in L4 cells
To determine the role of βII- and βIII-tubulin isotypes in the chemoresistance to PLA and laulimalide, isotype-specific siRNAs were used to silence the expression of βII- and/or βIII-tubulin in L4 cells. The isotype mRNA expression levels in L4 cells have been reported previously to be 55% of total β-tubulin for βII isotype and 19% for βIII isotype, compared with 7% each for both βII and βIII in parental 1A9 cells (22). The siRNA knockdown was optimized by transfecting the cells with 2-fold serial dilutions of siRNA. A final concentration of 25 nmol/L, which gave the highest βII- and βIII-tubulin mRNA and protein knockdown and showed the least adverse effect on cell proliferation, was selected for further experiments (Supplementary Fig. S1). Equivalent concentrations of a nonsilencing siRNA control (negative control) or Lipofectamine-only control (mock control) were also paired with each transfection experiment. The cells were transfected with either singleplex or multiplexes in a 1:1 (25:25 nmol/L) ratio of βII- and βIII-tubulin siRNAs. With singleplex transfection, βII- and βIII-tubulin mRNA levels were decreased by 80% compared with the negative control- or the mock-transfected L4 cells (Fig. 2A). The knockdown of mRNA was correlated with a decrease in the protein levels of the isotypes (Fig. 2B and C), and the knockdown effect lasted at least 6 days (Supplementary Fig. S1). With multiplex (βII+βIII) transfection, the mRNA and protein expression levels of βII- and βIII-tubulin were decreased by approximately 60% each (Fig. 2). This lower silencing efficiency with multiplex transfection was possibly caused by some loss in function of the siRNAs in the multiplex mixture compared with when they are used separately. Although singleplex siRNAs are highly functional, having a high affinity for the RNA-induced silencing complex (RISC), in a multiplex experiment, the siRNAs may compete with each other for loading on the RISC. The mRNA expression and protein abundance of other β-tubulin isotypes were not affected by the βII- and βIII-tubulin siRNA transfections. Total β-tubulin abundance decreased significantly after knockdown, and this was correlated with the decreased expression of βII- and βIII-tubulin (Fig. 2). The decrease in total β-tubulin was not expected because of the tendency for tubulin levels to autoregulate in cells (24); however, tubulin autoregulation may have reduced the magnitude of the decrease in total tubulin in the siRNA-treated cells. Silencing βII and βIII isotype expression in the parental 1A9 cells was not carried out because these tubulin isotypes are not expressed in significant amounts in the cells (22).

βII- and βIII-tubulin silencing sensitizes L4 cells to PLA and laulimalide
The effect of knockdown of βII- and βIII-tubulin on the resistance phenotype of L4 cells was examined by comparing the IC₅₀ values determined from the MTT assay. The IC₅₀ values of the cells transfected with βII-, βIII-, or βII+βIII-siRNAs were significantly less than those of their negative control siRNA–transfected cell IC₅₀ values (Fig. 3). The mock control-transfected cells, as expected, were not significantly different from the negative control-transfected cells for any of the drugs. For PLA, the fold increase in sensitivity in βII, βIII, and βII+βIII siRNA-transfected cells was 1.18, 1.65, and 1.88, respectively (Fig. 3). Similar results were obtained for laulimalide, with βII, βIII, and βII+βIII siRNA-transfected L4 cells showing 1.19, 1.37, and 1.44-fold increases in sensitivity, respectively (Fig. 3, Supplementary Table S2). These results indicated that siRNA-mediated silencing of βII- and βIII-tubulin expression partially restored the sensitivity of L4 cells to PLA and laulimalide. Full sensitivity similar to the parental 1A9 cells would require approximately an 18-fold change for PLA and a 25-fold change for laulimalide. Thus, although sensitivity was partially restored to the 2 compounds, the siRNA-transfected L4 cells were still highly resistant to the drugs. In contrast, the bioactivities of PTX, vinblastine, ixabepilone (an epothilone B analog), and cisplatin were not affected at all by knockdown of the β-tubulin isotypes in L4 cells (Fig. 3). Epothilone B is another microtubule-stabilizing agent that binds to the taxoid site on β-tubulin. In a previous study, we showed that the IC₅₀ for growth inhibition of L4 cells by epothilone B was the same as for the parental 1A9 cells (22).

Silencing βII- and βIII-tubulin expression enhances intracellular PLA- and laulimalide-induced tubulin polymerization
To determine whether βII- and βIII-tubulin silencing affects tubulin polymerization in situ, relative levels of soluble (S) and polymerized (P) tubulin after PLA or laulimalide treatment were evaluated using a cell-based in situ tubulin polymerization assay. Knockdown of βII- and βIII-tubulin resulted in a significant increase in drug-induced microtubule assembly compared with the negative control siRNA-treated L4 cells (Fig. 4). Treatment with 100 nmol/L PLA in βII-, βIII-, and βII+βIII-tubulin–silenced L4 cells induced 19%, 35%, and 46% polymerization, respectively (Fig. 4A). In
contrast, PLA at 100 nmol/L failed to induce comparable levels of polymerized tubulin in negative control siRNA-treated cells (only 1% polymerized tubulin; Fig. 4A). Similar results were obtained for laulimalide. In negative control siRNA–transfected cells, 100 nmol/L laulimalide induced only 2% polymerized tubulin; whereas, in βII-, βIII-, and βII+βIII-tubulin knockdowns, laulimalide at 100 nmol/L induced 24%, 39%, and 48% polymerized tubulin, respectively (Fig. 4B). Thus, there was a strong correlation with the cytotoxicity results (Fig. 3) in which the βII, βIII, and βII+βIII knockdowns increased the sensitivity of the cells to PLA and laulimalide, compared with the negative control siRNA–transfected L4 cells.

Knockdown of βII- and βIII-tubulin affects the abundance of PLA-induced microtubule aberrations

A reduced ability of PLA and laulimalide to induce microtubule aberrations such as microtubule bundles and multiple asters has been shown in L4 cells (22). To determine whether the increased expression of βII- and βIII-tubulin was partially responsible for this altered drug–tubulin interaction, PLA-induced microtubule bundle formation was examined in siRNA-treated L4 cells using
immunocytochemistry and confocal microscopy. There was no difference in the microtubule morphology between the drug-untreated βII-, βIII-, and βII+III-tubulin knockdowns and the negative control siRNA–transfected cells (Fig. 5). However, in agreement with the tubulin polymerization results, 100 nmol/L PLA significantly increased the formation of microtubule bundles in βII (31.8 ± 0.5%), βIII (47.8 ± 2.6%), and βII+III (57.6 ± 2.0%) silenced L4 cells compared with the negative control siRNA–treated cells (19.5 ± 0.7%; Fig. 5, Supplementary Table S3). The negative control siRNA-treated cells needed at least 500 nmol/L PLA to obtain an equivalent proportion of microtubule bundles to that seen in the knockdown cells.

Inhibition of βII- and βIII-tubulin expression promotes PLA- and laulimalide-induced G2-M block

To assess whether silencing of βII- and βIII-tubulin influences antimitic activity of PLA and laulimalide in L4 cells, cell-cycle analysis was done. Tubulin isotype siRNA transfection itself had no effect on the percentage of cells in G2-M in the absence of drug treatment (23%–25%; Fig. 6). There were also no differences in cell-cycle effects of the drugs between negative control siRNA–transfected and siRNA-untransfected, Lipofectamine-treated L4 cells (data not presented). However, lower concentrations of PLA and laulimalide were needed to induce G2-M block in the isotype-specific siRNA–transfected cells compared with negative control siRNA–treated cells. Cells transfected with βII-, βIII-, or βII+III-tubulin siRNAs arrested in their G2-M phase at 300 nmol/L PLA or 80 nmol/L laulimalide; whereas the negative control siRNA–transfected cells showed no significant G2-M block at these concentrations (Fig. 6). There was no significant G2-M arrest at the lower concentrations of PLA and laulimalide tested. The concentrations of drugs used in Fig. 6 (300 nmol/L PLA and 80 nmol/L laulimalide) were lower than the threshold concentrations needed in previous studies on L4 cells (500 nmol/L PLA and 200 nmol/L laulimalide; ref. 22). In βII+III-tubulin–silenced cells, a greater G2-M block was seen (58% with PLA and 200 nmol/L laulimalide) compared with βII-tubulin–silenced cells (52% with PLA and 51% with laulimalide) and βII-tubulin–silenced cells (44% with PLA and 43% with laulimalide; Fig. 6). These differences were consistent with the effects of silencing seen on cell growth and aberrant microtubule morphology, indicating again that the increased abundance of βII- and βIII-tubulin alters cell-cycle responses to PLA and laulimalide, and the
effects of knockdown of the 2 isotypes are somewhat additive.

Discussion

Resistance phenotype

βII- and βIII-tubulin knockdown partially sensitized resistant L4 cells to both PLA and laulimalide. Knockdown of βII-tubulin had less of an effect than that of βIII-tubulin, even though it was previously shown that expression of βII isotype in L4 cells was enhanced more than βIII isotype (7.4- vs. 5.6-fold; ref. 22). Simultaneous knockdown of both isotypes increased the sensitivity more than knockdown of either isotype on its own. Although the changes in sensitivity were small compared with the total resistance of the L4 cell line, they

Figure 4. PLA- and laulimalide-induced intracellular tubulin polymerization in siRNA-transfected L4 cells. L4 cells were transfected with the negative control or βII, βIII, and βII + βIII siRNAs for 72 hours, then treated with PLA or laulimalide for 16 hours, and the drug-induced tubulin polymerization was determined by an intracellular tubulin polymerization assay. Immunoblots of PLA (n = 3 independent experiments; A) and laulimalide (n = 2 independent experiments; B) are shown. The percent soluble tubulin (S) and polymerized tubulin (P) are presented below each protein band. LAU, laulimalide.
were highly significant. We showed previously that PLA and laulimalide have a reduced ability to induce cellular tubulin polymerization, microtubule bundling, and G2-M block in L4 cells compared with the parental 1A9 cells (22). Given the βII-tubulin mutation R306H/C in L4 cells (22) and its location in the proposed binding site for PLA and laulimalide (19–21), it is likely that most of the resistance of the cell line to these drugs is a result of this structural mutation; however, this still needs to be directly tested. The sensitivity of the cell lines was tested using a cell proliferation assay that monitors growth and death of cells by changes in cell metabolism. Use of a clonogenic assay instead might have enhanced the sensitivity of the assay to the silencing effects of the siRNA, although given that the siRNA knockdown persisted for up to 6 days (Supplementary Fig. S1), the MTT assay used was well within the window of the silencing effect.

**βIII-Tubulin role in resistance**

βIII-Tubulin overexpression has been correlated with resistance to microtubule-stabilizing and microtubule-destabilizing drugs (2). βIII-Tubulin seems to have a role in microtubule dynamics and in opposing the ability of tubulin-binding agents to suppress spindle dynamics. For example, Panda and colleagues (25) showed that microtubules assembled from purified αβIII isotype were considerably more dynamic than microtubules made from the αβII or αβIV isotypes or from unfractionated tubulin that consisted of a mixture of α- and β-tubulin isotypes. Consistent with this, Derry and colleagues (26) showed that microtubules composed of purified αβIII-tubulin were 7.4-fold less sensitive to the effects of PTX than microtubules assembled from unfractionated tubulin. In another study, overexpression of βIII-tubulin in transfected Chinese hamster ovary (CHO) cells decreased microtubule assembly and conferred resistance to PTX (27). Removal of βIII-tubulin from the tubulin pool led to more rapid PTX-induced tubulin assembly (28). In a PTX-resistant lung cancer cell line, A549-T24, that is 17-fold resistant to PTX and has a 4-fold increase in βIII-tubulin expression compared with the parental A549 cells, siRNA-mediated inhibition of βIII expression caused a 39% increase in sensitivity to PTX (29). A more recent study by Gan and colleagues (30) showed that βIII-tubulin

![Figure 5. PLA-induced microtubule bundling in siRNA-transfected L4 cells. Seventy-two hours after siRNA transfection, L4 cells were treated for 16 hours with PLA and costained with antibodies against βII- or βIII-tubulin and α-tubulin. Nuclei were stained with DAPI. For better visualization of microtubule bundles, only α-tubulin staining is shown in the image. White arrows point to microtubule bundles. The full-color fluorescence images of the βII-, βIII-tubulin, and nuclear staining of the cells are presented in Supplementary Fig. S2.](image-url)
knockdown in non-small cell lung carcinoma cells enhanced the suppression of microtubule dynamics at low concentrations of PTX or vincristine. These results support the earlier studies (26–28) showing that overexpression of βIII-tubulin reduces the ability of PTX to inhibit microtubule dynamic instability. High levels of expression of βIII-tubulin have been correlated with resistance to docetaxel in breast (31) and prostate (32) cancer cells as well.

βIII-Tubulin is a multifunctional protein that, when suppressed, increases the in vitro and in vivo sensitivity of cells to tubulin-binding and DNA-damaging agents, such as cisplatin, through enhanced apoptosis and decreased tumorigenesis (33, 34). The involvement of βIII-tubulin in mediating the sensitivity to DNA-damaging agents (33) suggests that βIII isotype overexpression, in addition to its destabilizing activity, might have a role as a cellular survival factor against chemotherapy. This is supported by the studies of Raspaglio and colleagues (35) who showed that cellular stresses, such as hypoxia, can induce the expression of βIII-tubulin. Cicchillitti and colleagues (36) also showed that βIII-tubulin overexpression was associated with adaptation to oxidative stress and glucose deprivation. The mechanism by which βIII-tubulin might alter these cell stress pathways, however, is not known. More recently, De Donato and colleagues (37) showed that βIII-tubulin can act as a cytoskeletal gateway for prosurvival signals.

In this study, we showed a clear decrease in the resistant phenotype after siRNA-mediated silencing of βIII isotype, yet the silencing of βIII had no effect on the normal sensitivity to PTX, vinblastine, ixabepilone, or cisplatin. Possible reasons for not finding resistance of L4 cells to PTX, vinblastine, and cisplatin in our study are discussed below.

Figure 6. G2-M block induced by PLA and laulimalide in siRNA-transfected L4 cells. [βII, βIII, and βII+βIII siRNA-transfected cells were treated with PLA or laulimalide for 16 hours, and the DNA content in each phase of the cell cycle was analyzed using flow cytometry. Representative histograms are shown in A. The percentage of cells in each phase of the cell cycle is presented at the top of each histogram. A summary of the percentage of cells in G2-M phase following treatment with PLA and laulimalide is given in B and C. Results are based on 4 independent experiments, bars = SEM; *P < 0.01 for the βII+βIII siRNA knockdowns for both PLA and laulimalide. Single knockdowns of βII and βIII were not significant; Kruskal–Wallis test compared negative control to target siRNA knockdown. Using a Dunn multiple comparison test, the only significant differences were for the double knockdowns for PLA at 300 nmol/L and laulimalide at 80 nmol/L. *P < 0.01. LAU, laulimalide.]
βII-tubulin role in resistance

The mechanisms by which βII-tubulin overexpression confers resistance on cells are poorly understood. Purified vertebrate βII-tubulin has different assembly and drug-binding properties to a mixture of β-tubulin isotypes (38). Using monoclonal antibodies specific for βII, Banerjee and colleagues (39) prepared isotypically pure αβII tubulin dimers from bovine brain and examined their assembly properties in the presence of microtubule-associated proteins (MAP) and PTX. They found that, in the presence of MAPs, the αβII dimers assembled into microtubules considerably faster than unfractonated tubulin dimers. Derry and colleagues (26) measured the effects of PTX on the dynamics of microtubules composed of purified αβII-tubulin isotypes and showed that microtubules composed of purified αβII were 1.6-fold less sensitive to the effects of PTX than microtubules assembled from unfractonated tubulin. Consistent with this, several other studies in cells have reported high levels of βII-tubulin isotype in PTX-resistant ovarian (40), murine J774.2 (41), and DTX-resistant breast cancer cell lines (42). Whereas Gan and Kavallaris (43) showed that siRNA-mediated knockdown of βII-tubulin hypersensitized the lung cancer cell lines NCI-H460 and Clau-6 to vinca alkaloids, no change in the sensitivity to PTX was seen in these cell lines following knockdown of the βII-tubulin isotype.

In this study, we show that knockdown of βII-tubulin decreases the resistance of L4 cells to PLA and laulimalide by about 15%, and this effect is correlated with decreases the resistance of L4 cells to PLA and laulimalide with tubulin. It would be interesting to sequence the βII-tubulin gene in L4 cells to see whether it has a mutation confers 10- to 15-fold resistance to PLA in 1A9 ovarian carcinoma cells. Importantly, the human βII isotype also has a serine residue at position 296. Thus, in the case of PLA and laulimalide, the overexpression of βII-tubulin in L4 cells to see whether it has a role in reducing the suppressive effect of PTX on microtubule dynamics used in vitro purified tubulin (26).

Lack of resistance of L4 cells to PTX, vinblastine, and cisplatin

An important finding in this study was that, despite overexpression of βI and βIII tubulin, L4 cells retained a normal sensitivity to PTX, vinblastine, and cisplatin. Although the studies described above link overexpression of these isotypes to resistance to these drugs (2, 3), some other studies have found results similar to ours. In a phase III clinical trial with docetaxel and doxorubicin on patients with locally advanced or metastatic breast cancer, tumors with higher levels of βIII-tubulin had an increased probability of showing a response to docetaxel (44). High abundance of βIII-tubulin has also contributed to an increased sensitivity to epothilone B in human ovarian cancer cells (45). In an in vivo study, Nicolletti and colleagues (46) reported no correlation between isotype expression and PTX sensitivity. In another study using CHO cells, overexpression of βII-tubulin had no effect on the sensitivity of the cells to PTX (47). Thus, overexpression of βII- or βIII-tubulin does not always confer cancer cell resistance to PTX or vinblastine. Although, in vitro systems have shown a microtubule-destabilizing property of βIII-tubulin, its involvement in the resistance to vinca site drugs and DNA-damaging agents suggests that these drugs can also implement different cellular survival pathways against specific chemotherapies. This might in part explain the fact that in L4 cells, βII- or βIII-tubulin induced a resistance mechanism that specifically affect PLA and laulimalide, but not PTX, vinblastine, or cisplatin. Recently, Wilmes and colleagues (48) showed in the human HL-60 promyelocytic leukemia cell line that there were differences in the proteomic effects of PLA and PTX, particularly with regard to apoptotic proteins. This suggests that PLA and PTX may activate different apoptotic pathways that are differentially affected by βII and βIII expression levels.

Another possible explanation for the different effects on PLA/laulimalide and PTX/vinblastine/cisplatin is that tumor cells may acquire resistance to tubulin-binding agents by overexpressing specific isotypes to which the drugs have a reduced binding affinity. Using digital signal processing, Chen and colleagues (49) modeled the binding affinities of PLA, PTX, and vinblastine to β, βI, and βII-tubulin isotypes: βI, βII, and βIV. The authors predicted that the order of binding affinity of PTX and vinblastine was βI>βIV>βIII and βIV>βI>βIII, respectively. They predicted that PLA would have a reduced affinity for βI- and βIII-tubulin isotypes, compared with that for the βIV-tubulin isotype. This altered drug-binding affinity is further supported by a more recent study by Begaye and colleagues (50) who showed that an A296S mutation confers 10- to 15-fold resistance to PLA in 1A9 ovarian carcinoma cells. Importantly, the human βII iso- type also has a serine residue at position 296. Thus, in the case of PLA and laulimalide, the overexpression of βI isotypes with lesser affinities for the 2 drugs could limit the binding of the drugs and reduce their potency in cells overexpressing these isotypes. For βII-tubulin, it seems that its function in other cell lines that mediates changes in sensitivity to PTX, vinblastine, and cisplatin (2) is not active in the L4 cells. Either the βII-tubulin mutation in L4 cells is inhibiting or compensating for this function of the βIII isotype, or βIII-tubulin itself is altered or mutated in L4 cells such that it no longer affects sensitivity to PTX, vinblastine, and cisplatin but reduces the interaction of PLA and laulimalide with tubulin. It would be interesting to sequence the βIII-tubulin in L4 cells to see whether it has comutated with βII-tubulin.

Conclusions

This study clearly showed that knockdown of βII- and βIII-tubulin increased the sensitivity of resistant L4 cells to PLA and laulimalide, and that this increased sensitivity was associated with an increase in drug-tubulin...
interactions in the cells, as evidenced by intracellular tubulin polymerization, formation of microtubule bundles, and G2-M block. The exact mechanism by which βII- and βIII-tubulin affect L4 cell resistance is, however, not known. The lack of resistance of L4 cells to PTX, vinblastine, and cisplatin suggests that the effect of these isotypes on resistance is specific for PLA and laulimalide compared with other microtubule-targeting and DNA-damaging agents. This difference might be related to the distinct PLA- and laulimalide-binding site on β-tubulin and differences in the mechanisms of polymerization or killing by these drugs.

Disclosure of Potential Conflicts of Interest

P.T. Northcote and J.H. Miller have a patent on Peloruside A.

References


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βII-Tubulin and βIII-Tubulin Mediate Sensitivity to Peloruside A and Laulimalide, but not Paclitaxel or Vinblastine, in Human Ovarian Carcinoma Cells

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