Peloruside A Does Not Bind to the Taxoid Site on β-Tubulin and Retains Its Activity in Multidrug-Resistant Cell Lines

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Abstract

Peloruside A (peloruside), a microtubule-stabilizing agent from a marine sponge, is less susceptible than paclitaxel to multidrug resistance arising from overexpression of the P-glycoprotein efflux pump and is not affected by mutations that affect the taxoid binding site of β-tubulin. In vitro studies with purified tubulin indicate that peloruside directly induces tubulin polymerization in the absence of microtubule-associated proteins. Competition for binding between peloruside, paclitaxel, and laulimalide revealed that peloruside binds to a different site on tubulin to paclitaxel. Moreover, laulimalide was able to displace peloruside, indicating that peloruside and laulimalide may compete for the same or overlapping binding sites. It was concluded that peloruside and laulimalide have binding properties that are distinct from other microtubule-stabilizing compounds currently under investigation.

Introduction

Peloruside A (peloruside), a potent cytotoxin isolated from a New Zealand marine sponge (1, 2), is available both synthetically (3) and from the aquacultured sponge (unpublished observations). It is active at nanomolar concentrations and has been shown to induce apoptosis in cultured cells (2). A total synthesis of peloruside has recently been reported (3). Studies in our laboratory have shown that peloruside works in a manner similar to paclitaxel by stabilizing the polymerized form of microtubules (4). Microtubule-stabilizing compounds can be classified into three groups: (a) the terpenoids, including paclitaxel, taxotere, and eleutherobin/sarcodictyin; (b) the macrolides, including peloruside, epothilone, and laulimalide; and (c) the polyhydroxylated alkalatetraene lactones, including discodermolide. Paclitaxel and taxotere are currently used for the treatment of solid tumors of the breast, ovary, and lung (5). Because of its lipophilic nature, paclitaxel has two major disadvantages as an anticancer drug. First, it must be delivered in a vehicle, Cremophor, that causes major undesirable side effects (6). Second, it is effectively pumped out of cells that overexpress the P-glycoprotein (P-gp) efflux system (7). Activation of the P-gp efflux pump (MDR-1) in tumor cells leads to the development of multidrug resistance (MDR; Ref. 7). In addition to overexpression of P-gp, some cells become resistant to paclitaxel as a result of M40 human β-tubulin gene mutations that affect the taxoid binding site (8–11). It is therefore important to identify drugs that have similar or improved pharmacological properties to paclitaxel that are also effective in paclitaxel-resistant cancer cells. Most of the microtubule-stabilizing agents have been examined for their susceptibility to MDR, and three look particularly promising because of their lack of interaction with the P-gp pump: epothilone (9–12); discodermolide (13); and laulimalide (11, 14). Recently, it was shown that laulimalide is not only more toxic than paclitaxel in cells with a MDR phenotype, but it also binds to a different site on tubulin to paclitaxel, epothilone, and discodermolide (11). Because laulimalide does not compete with paclitaxel for binding, it was also found to be effective in paclitaxel-resistant cells that have β-tubulin gene mutations that modify the taxoid binding site (11), thus providing additional evidence that the laulimalide binding site is unique to that of paclitaxel. Interestingly, epothilone and discodermolide, both of which compete with paclitaxel for binding to β-tubulin, have different sensitivities to particular mutations of the β-tubulin gene (9). The taxoid binding site has been modeled in detail, but nothing is known about the newly described second site to which laulimalide binds (11). The aim of the present study was to investigate the cytotoxicity of peloruside in paclitaxel and epothilone B-resistant cell lines that either overexpress the P-gp efflux pump or have mutations in the β-tubulin gene. A second aim was to demonstrate that peloruside polymerizes microtubules by binding directly to tubulin rather than binding indirectly through other proteins such as microtubule-associated proteins. A third aim was to use competitive binding experiments to determine whether peloruside binds to either the paclitaxel or laulimalide binding sites or to a unique site of its own.

Materials and Methods

Materials. Peloruside (548.3 Da; protonated form, Fig. 1) was isolated from the marine sponge, Mycale hentscheli, collected in Pelorus Sound off the northern coast of the South Island, New Zealand (1). Peloruside was stored at −20°C as a 1 mm solution in absolute ethanol or as a 10 mm solution in DMSO. Paclitaxel and epothilone B were purchased from Sigma Chemical Co. (St. Louis, MO). Laulimalide was kindly provided by Dr. Arun K. Ghosh (University of Illinois, Chicago, IL). Docetaxel was kindly provided by Rhône Poulenc Rorer (Aventis, Schiltigheim, France). Flutax-2 (7-O-[N-(2,7-difluoro-4’-fluoresceincarbonyl)-1-allyl]paclitaxel) was provided by Dr. Francisco Amat-Gerri, dissolved in DMSO, and its concentration measured spectrophotometrically \( \epsilon_{eox} = 49100 \text{ cm}^{-1} \text{M}^{-1} \) in 50 mm sodium phosphate, 0.5% SDS (pH 7.0).

The parental (AUXB1) and MDR-overexpressing (CHRS54) Chinese hamster ovary (CHO) cell lines (15) were a gift from Dr. Michael Berridge (Malaghan Institute for Medical Research, Wellington, New Zealand), and the parental (A2780) and P-gp-overexpressing (A2780AD) human ovarian carcinoma cell lines (13) were a gift from Dr. Steven Williams (Fox Chase Cancer Center, Philadelphia, PA). The parental (1A9) and β-tubulin mutant (PTX10, 5063

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PTX22, A8, and B10) human ovarian carcinoma cell lines (8) were a gift from Dr. Paraskevi Giannakakou (Emory University, Atlanta, GA).

Cell Culture and Cell Proliferation Assay. CHO and human ovarian carcinoma cell lines were cultured at 37°C in 5% CO2 in air atmosphere using standard techniques. The CHO auxotroph cells were cultured in α-MEM medium supplemented with 10% FCS, 10 μM hypoxanthine, 10 μM glycine, 10 μM thymidine, 100 μM adenine, 100 units/ml penicillin, and 100 μg/ml streptomycin. The ovarian carcinoma cells were cultured in RPMI-1640 medium supplemented with 10% FCS, 0.25 units/ml insulin, 100 units/ml penicillin, and 100 μg/ml streptomycin. A cell proliferation assay was used as previously described (2), involving the reduction of the dye 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyterazolium bromide (MTT) by viable cells.

Ligand-Induced Microtubule Assembly. Samples of purified GTP-bound tubulin, free from microtubule-associated proteins, were prepared in the appropriate buffer as described previously (16). Before microtubule assembly, the ligand was added at 0–2°C, and the critical concentration (Cr) of ligand-induced tubulin assembly was measured in 10 mM sodium phosphate, 1 mM EDTA, 4 mM MgCl2, and 1 mM GTP (pH 7.0) buffer. Control experiments showed that tubulin at concentrations up to 200 μM did not assemble in the absence of exogenous ligands (16). Increasing concentrations of GTP-tubulin up to 60 μM were incubated for 45 min at 37°C in the presence of the ligand at a concentration 10% in excess over tubulin (molar ratio of 1:1). The polymerized tubulin was centrifuged at 90,000 × g for 10 min in a TLA 100 rotor (Beckman Instruments, Fullerton, CA) pre-equilibrated at 37°C. The supernatants were collected by pipette, and the pellets were resuspended in 10 mM sodium phosphate, 1% SDS (pH 7.0) buffer. Tubulin concentrations in the supernatants and pellets were measured fluorometrically, using tubulin as a standard. The Cr of tubulin was calculated from a graph of tubulin concentration in the supernatants and pellets versus total tubulin (Fig. 2; Refs. 17, 18).

Flutax-2 Displacement Test. The binding of ligands to the paclitaxel binding site of microtubules was tested by competition with the fluorescent taxoid probe, Flutax-2, using cross-linked microtubules (19, 20). The displacement isotherms of the ligands were measured several times in different plates by the anisotropy of the fluorescence, employing a fluorescence polarization microplate reader at 37°C, as described previously (11, 21, 22). The binding constant of the reference ligand Flutax-2 has been determined in earlier studies to be 2.2 × 107 M−1 (19, 22). Increasing competitor concentrations up to 100 μM were set up against 50 nM Flutax-2 and 50 nM cross-linked microtubules in buffer consisting of 10 mM sodium phosphate, 3.4 M glycerol, 1 mM EGTA, 6 mM MgCl2, and 0.1 mM GTP (pH 6.5; GAB-0.1 mM GTP buffer).

Mass Spectrometry Detection of Ligand Binding to Microtubules. Samples containing ligand and either stabilized cross-linked microtubules or native ones were incubated for 45 min at 37°C in Beckman polycarbonate centrifuge tubes in GAB-0.1 mM GTP buffer. The samples were centrifuged at 90,000 × g for 10 min at 37°C. The supernatants were collected by pipette and the pellets resuspended in 10 mM phosphate (pH 7.0) buffer. Both the pellets and supernatants were extracted three times with an excess volume of dichloromethane, dried in vacuum, and dissolved in 30 μl of a methanol/water (v/v:70:30) mixture. Mass spectra were generated by laser desorption/ionization experiments performed in a BIFLEX III time-of-flight instrument (Bruker-Franzen Analytik, Bremen, Germany) operated in the positive mode. Samples were analyzed in the reflection mode, with typically 100 laser shots summed into a single mass spectrum. External calibration was performed using the monoisotopic peaks of angiotensin (m/z 1046.5) and the matrix α-cyano-4-hydroxycinnamic acid (m/z 379) recorded in a single spectrum. Aqueous 2,3-dihydrobenzoic acid (10 mg/ml) was used as the sample matrix. Equal volumes (0.5 μl) of the sample solution and the matrix were spotted on the target grid and air-dried. Peaks corresponding to laulimalide+Na+ (m/z: 537.8), laulimalide+K+ (m/z: 554.9), paclitaxel+K+ (m/z: 876.8), and peloruside+Na+ (m/z: 571.3) were observed in the mass spectra.

Electron Microscopy. Aliquots of ligand-induced microtubule solutions were adsorbed onto Formvar/carbon-coated 300 mesh copper grids, stained with 2% uranyl acetate, and observed with a Jeol 1230 transmission electron microscope (JEOL, Tokyo, Japan).

**Results**

**Peloruside Cytotoxicity in MDR Cell Lines.** Peloruside IC50 values in parental and MDR cell lines are presented in Table 1. In CHO cells, the ratios of activities of peloruside and epothilone B in CHP55 cells relative to AuxB1 cells were not significantly different from each other; however, when paclitaxel was tested, the CHP55 MDR cells were found to be 312-fold more resistant than the parental cell line (P < 0.015; Kruskal-Wallis nonparametric test). The peloruside and epothilone B resistance ratios of 26 and 15, respectively, indicate that both compounds are transported to some extent by the P-gp efflux pump. Sensitivity to verapamil, an inhibitor of the P-gp pump, confirmed that most of the resistance in the MDR cell line was due to the action of the efflux pump, particularly with paclitaxel, which showed the greatest resistance ratio of the three (P < 0.01; Kruskal-Wallis nonparametric test) but that some P-gp-related resistance or a direct toxic effect of verapamil unrelated to P-gp was also present in the parental cell line (P < 0.05; Kruskal-Wallis nonparametric test). Similar results were obtained for the human ovarian cancer cell line A2780 and its MDR derivative, A2780/Ad. Again, the MDR cells were somewhat more resistant to peloruside than to epothilone B (Table 1). As with the CHO cells, however, there was a much larger difference between paclitaxel and either peloruside or epothilone B (P < 0.001; Kruskal-Wallis nonparametric test). The verapamil results in the human ovarian cell line suggest that, unlike the situation with AUXB1 CHO cells, there is little P-gp activity in the parental cell line, but, as with the CHP55 cells, there is a large verapamil-induced decrease in IC50 in the MDR cell line (P < 0.05; Kruskal-Wallis nonparametric test). Interestingly, we found that, although peloruside at the highest concentrations killed all of the A2780/Ad cells in the cultures with and without verapamil, neither
pelotaxil nor epothilone B were able to completely kill the cells, even at concentrations in the micromolar range. The greatest inhibition of cell proliferation, measured by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide dye reduction, in A2780AD cells compared with untreated controls was found to be only 82% (68% for epothilone B in cells treated with verapamil (n = 5; 8 preparations, duplicate wells at each concentration/96-well plate). The effect of verapamil (10 μM), an inhibitor of the P-gp efflux pump, was tested concurrently in paired, duplicate wells for the MDR cell lines, and the IC50 ratio of [drug/drug plus verapamil] is given in parentheses below the drug IC50 values. The tubulin mutant cell lines were tested in two sets of five experiments (set one = 1A9; PTX10, A8; set two = 1A9, PTX22, B10).

### Pezoluside Cytotoxicity in β-Tubulin Mutant Cell Lines

The effects of mutant β-tubulins on IC50 values for pezoluside, epothilone B, and paclitaxel were tested (Table 1). The paclitaxel-resistant cell lines PTX10, PTX22, and A8 showed high resistance ratios (133-, 18-, and 9-fold, respectively) for paclitaxel compared with the parental cell line 1A9 (P < 0.01; Kruskal-Wallis nonparametric test). Although these resistance ratios are less than those seen with cells that overexpress P-gp (312- and 1417-fold; Table 1), the values are similar to those measured by others in these cell lines (8, 9, 11, 13). As reported previously by Pryor et al. (11) and as seen in our study, A8 and B10 cells are more resistant to epothilone B than PTX10 and PTX22 cells. Neither A8 nor B10 cells showed any resistance to peloruside. The resistance to epothilone B in A8 cells was similar to the resistance to paclitaxel (9-fold); thus, the amino acid substitution in these cells (βT274I; Ref. 9) presumably affects binding of both epothilone B and paclitaxel but not peloruside binding (P < 0.01; Kruskal-Wallis nonparametric test). The amino acid changes in PTX10 cells (βP270N) and PTX22 cells (βA364T; Ref. 8), however, have only small effects on either peloruside or epothilone B cytotoxicity but cause major increases in resistance to paclitaxel (Table 1).

From these results, it can be seen that, similar to the situation already reported for laulimalide by Pryor et al. (11), neither PTX10, PTX22, nor A8 cells display major resistances to peloruside (resistance ratios of 3.5, 0.8, and 1.2, respectively) compared with their large resistance ratios to paclitaxel, although these three cell lines have β-tubulin mutations that affect the taxoid binding site. The epothilone-resistant cell line B10 (11-fold resistance to epothilone B) with an amino acid change of βR282Q showed low resistance to paclitaxel (4-fold) and no resistance to peloruside (P < 0.005; Kruskal-Wallis nonparametric test).

### Ligand-Induced Microtubule Assembly

The biochemical interactions between peloruside and microtubules were investigated. The structure of peloruside is given in Fig. 1. We first checked whether peloruside was able to induce tubulin polymerization in PDETA4-1 mM GTP buffer. In this buffer, tubulin is unable to self-assemble in the absence of a microtubule stabilizing agent (Cr > 200 μM; Ref. 16). Peloruside induced microtubule assembly at a Cr of 12.0 ± 1.3 μM (Fig. 2), indicating that it is a weaker microtubule stabilizing agent than paclitaxel (Cr = 4.2 ± 0.2 μM) and taxotere (Cr = 1.5 ± 0.1 μM). The polymerized microtubules showed typical morphology on transmission electron microscopy (Fig. 2, inset). The lateral projections of negatively stained microtubules assembled in the presence of peloruside are characteristic of 12 protofilament microtubules, similar to those induced by paclitaxel (23).

### Flutax-2 Displacement Test

Because peloruside was able to induce tubulin assembly in a paclitaxel-like manner, we next checked whether peloruside was able to bind to the paclitaxel site, using a Flutax-2 displacement test (Fig. 3; Refs. 11, 20–22). Concentrations up to a 2000-fold excess of peloruside over Flutax-2 (100 μM against 50 nm) were used for this test. Peloruside behaved in the same way as laulimalide; thus, neither ligand displaces Flutax-2 from its binding site.

### Binding of Peloruside to Microtubules

Binding of peloruside to microtubules was checked by mass spectrometry. Samples containing ligand and cross-linked stabilized microtubules were incubated for 45 min at 37°C in GAB-0.1 mM GTP buffer. After centrifugation, the pellets and supernatants were extracted with dichloromethane, dried, and dissolved in methanol/water. A peak corresponding to peloruside-sodium was observed in the extracted pellets (Fig. 4A), confirming that peloruside is able to bind to stabilized microtubules. As a control of the method, the binding of laulimalide to cross-linked microtubules was also confirmed (Fig. 4B). Control experiments were performed with non-cross-linked microtubules, and peloruside was again able to bind to the polymerized tubulin (data not presented).

Because peloruside does not bind to the paclitaxel site on the microtubule, we tested whether laulimalide was able to displace peloruside from its binding site. This would provide information on whether peloruside binds to the same (or an overlapping) site as paclitaxel.
lauimalide. For this test, samples were prepared with laulimalide added at 10-fold excess to both the peloruside and stabilized microtubule concentrations. No peloruside was seen in the pellet, only in the supernatant (Fig. 4C), indicating that laulimalide competes with peloruside for binding. As a control, samples of stabilized microtubules with a similar excess of paclitaxel over peloruside were prepared. Both peloruside and paclitaxel were found in the pellet (Fig. 4D), confirming that paclitaxel does not compete for the same site as peloruside.

Discussion

Peloruside Cytotoxicity in MDR Cell Lines. The IC_{50} results in cell lines that overexpress the P-gp efflux pump indicate that peloruside, as with epothilone B, discodermolide, and laulimalide, is not as good a substrate for the P-gp pump as paclitaxel. Thus, peloruside remains cytotoxic at low concentrations in MDR-overexpressing cells that are resistant to paclitaxel. This property is important in the development of peloruside as an anticancer drug because tumor cells that become resistant to paclitaxel over prolonged treatment periods would be sensitive to peloruside, thus reestablishing drug-induced tumor regression that is lost when cells take on the MDR phenotype. Peloruside appeared to be somewhat less effective than epothilone B, however, in both MDR cell lines, particularly the A2780AD human ovarian cancer cells, although in another study, an epothilone B resistance ratio of 15 was reported for A2780AD cells (11). This value is considerably higher than the 2-fold increased resistance that we found for epothilone B in these cells but is similar to the value of 21 that we measured for peloruside. The actual IC_{50} values for epothilone B and paclitaxel were similar in both cell lines (11). When compared with laulimalide, which is reported to have an increased resistance of 8-fold in this same cell line (11), peloruside is a slightly better substrate for the P-gp pump in both CH34C5 cells (26-fold) and A2780AD cells (21-fold). In a different study, Mooberry et al. (14) tested laulimalide in the human ovarian carcinoma cell line SK-VLB and found a MDR resistance of 105-fold. Interestingly, isolauimalide, the degradation product of laulimalide, is only cytotoxic at micromolar concentrations and is not affected by an increased level of P-gp expression, suggesting that the epoxide moiety of laulimalide is required for its interaction with the MDR efflux pump (14). A potential advantage of peloruside over laulimalide as a drug for additional anticancer development is the fact that peloruside is more stable than laulimalide, which may convert to its less potent isolauimalide form (14). This has implications in terms of the pharmacokinetic effects that control the duration of action of laulimalide in vivo.

Fig. 3. Inability of peloruside or laulimalide to displace Flutax-2 from its binding site. Inhibition of the binding of Flutax-2 (50 nM) to paclitaxel stabilized binding sites by paclitaxel (up to 5 μM; ○), docetaxel (up to 5 μM; □) but neither by laulimalide (up to 25 μM; ▼) nor peloruside (up to 100 μM; ●). Data are presented as the mean ± SE (n = 4 experiments).

Fig. 4. Displacement of peloruside from its binding site by laulimalide. Mass spectra of extracted microtubule pellets and supernatants. A, peloruside (5 μM) and stabilized microtubules (5 μM). B, laulimalide (5 μM) and stabilized microtubules (5 μM). C, peloruside (5 μM), laulimalide (50 μM), and stabilized microtubules (5 μM; the supernatant spectrum in C is scaled down by a factor of 2 for presentation purposes). D, peloruside (5 μM), paclitaxel (Taxol; 50 μM), and stabilized microtubules (5 μM; the pellet spectrum for paclitaxel in D is scaled up by a factor of 2 for presentation purposes). Lauimalide + Na^+ (m/z) = 537.8; laulimalide + K^+ (m/z) = 554.9; peloruside + Na^+ (m/z) = 571.3; paclitaxel + K^+ (m/z) = 876.8.
Peloruside Cytotoxicity in β-Tubulin Mutant Cell Lines. Tumor cells can become resistant to paclitaxel as a result of mutations in the β-tubulin gene that alter the taxoid binding site. In this regard, peloruside and laulimalide have an advantage over other microtubule-stabilizing agents such as paclitaxel, epothilone, or discodermolide because neither peloruside nor laulimalide binds to the taxoid site. Pryor et al. (11) confirmed that ovarian cancer cells with β-tubulin mutations affecting the toxicity of paclitaxel (PTX10, PTX22, and A8) and epothilone B (A8 and B10; Refs. 8, 9) were not resistant to laulimalide. The PTX10 and PTX22 cell lines were derived from the I.A9 parental cell line with mutations at amino acid positions β270V and β364T, respectively (8). The A8 and B10 cell lines, also derived from I.A9, have mutations at amino acid positions β274T and β282Q, respectively (9). We found similar results with peloruside to those reported for laulimalide (11). None of the paclitaxel or epothilone-resistant cell lines expressing single amino acid mutations in the β-tubulin gene were resistant to peloruside. In PTX10 and PTX22 cells, resistance to epothilone B, as with peloruside and laulimalide, is not greatly increased relative to the parental cell line, presumably because the specific amino acid substitutions are not directly involved in epothilone B binding to the paclitaxel site. Amino acid substitutions at β1–31, β271–233, β270, β274, β282, and β364 have all been identified as important areas for paclitaxel binding and cluster close to the known binding site (9). Amino acid β270 (PTX10 cells), however, affects the resistance of the cells to paclitaxel but not to epothilone B, whereas amino acid β282 (B10 cells) affects the resistance to epothilone B but not paclitaxel. These results suggest that although paclitaxel and epothilone B compete for the same site on β-tubulin, their specific amino acid interactions differ. In other cell lines, mutations at amino acid positions β292 (A549.epoB40 cells) and β422 (HeLa.EpoB1.8 cells) reduce both epothilone B and paclitaxel cytotoxicities but have little effect on discodermolide (24). Discodermolide is also insensitive to mutations at amino acids β270 (PTX10 cells) and β364 (PTX22 cells; Ref. 13). Thus, discodermolide is unaffected by a number of β-tubulin mutations that increase resistance of cells to paclitaxel and/or epothilone, despite the fact that discodermolide competes with paclitaxel for binding to microtubules (11, 13). The results of the present study with peloruside highlight the uniqueness of peloruside to paclitaxel and epothilone B. Pryor et al. (11) found a similar pattern of resistances with laulimalide as we found with peloruside, indicating that both peloruside and laulimalide cytotoxicities are largely unaffected by the specific β-tubulin mutations in PTX10, PTX22, A8, or B10 cells. Thus, the studies with laulimalide (11) and our own results with peloruside provide independent support for the conclusion that peloruside and laulimalide do not exert their cytotoxic effects by binding to the taxoid site on β-tubulin.

The Peloruside Binding Site. The peloruside binding results in the present study indicate that peloruside does not bind to the paclitaxel site and suggest that peloruside and laulimalide bind to the same or overlapping sites, supporting the notion that the peloruside/laulimalide site for microtubule stabilization is a functional site that can bind different compounds. Both peloruside and laulimalide cytotoxicities are largely unaffected by the specific β-tubulin mutations in PTX10, PTX22, A8, or B10 cells. Thus, the studies with laulimalide (11) and our own results with peloruside provide independent support for the conclusion that peloruside and laulimalide do not exert their cytotoxic effects by binding to the taxoid site on β-tubulin. Future efforts will be directed toward understanding the biochemical and structural features of the peloruside binding site. This will help define the characteristics of this new class of tubulin-stabilizing agents and promote the search for new compounds that bind to this site and show potential anticaner activity.

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