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Molecular Recognition of Peloruside A by Microtubules. The C24 Primary Alcohol is Essential for Biological Activity

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Peloruside is a microtubule-stabilizing agent that targets the same site as laulimalide. It binds to microtubules with a 1:1 stoichiometry and with a binding affinity in the low-μM range; thereby reducing the number of microtubular protofilaments in the same way as paclitaxel. Although the binding affinity of the compound is comparable to that of the low-affinity stabilizing agent sarcodictyin, peloruside is more active in inducing microtubule assembly and is more cytotoxic to tumor cells; this suggests that the peloruside site is a more effective site for stabilizing microtubules. Acetylation of the C24 hydroxyl group results in inactive compounds. According to molecular modeling, this substitution at the C24 hydroxyl group presumably disrupts the interaction of the side chain with Arg320 in the putative binding site on α-tubulin. The binding epitope of peloruside on microtubules has been studied by using NMR spectroscopic techniques, and is compatible with the same binding site.

Introduction

Peloruside A (**1**), a polyketide anticancer compound isolated from the New Zealand marine sponge *Mycale hentscheli*,[1] is one of several known microtubule-targeting agents.[2] The compound acts by binding to and stabilizing the polymerized form of tubulin, a protein that has several important cellular functions, including separation of sister chromatids during cellular division; this makes it an attractive target for cancer therapy.[3,4]

Microtubules are formed from α- and β-tubulin, which associate as dimers and organize themselves into filaments that comprise the tubulin macrostructure. The microtubule is dynamic and is constantly lengthening and shortening in order to fulfill its cellular functions. Microtubules are an important target in cancer therapy, and currently there are several drugs that perturb its dynamic properties. Taxol® (paclitaxel, **2**) is an important antimitotic agent currently used for the treatment of metastatic breast and ovarian cancer, as well as Kaposi's sarcoma, and non-small cell lung cancer.[5] A close analogue of paclitaxel is Taxotere® (docetaxel, **3**), which is used clinically for the treatment of lung and metastatic breast cancer.[6] The taxane drugs act by binding to the β-tubulin subunit of microtubules and thus stabilize the polymer.[7–9] Two examples of microtubule-destabilizing agents, which exert their effects by promoting the depolymerization of tubulin, are vinblastine (**4**) and vinorelbine (**5**).[10] These drugs are primarily used against non-small cell lung cancer.

Despite the success of these drugs, toxicity and development of resistance (in particular to the taxanes) threatens their effective clinical use. Over the past few years, a structurally diverse set of natural products from a variety of sources have been discovered that share the ability of the taxanes to stabilize the polymeric microtubules. These include, but are not limited to, discodermolide (**6**),[11] dictyostatin (**7**),[12] peloruside (**1**), laulimalide (**8**),[13–15] the sarcodictyins (**9**),[16,17] and the epothilones (**10**).[18] This latter class of agents, isolated from the myxobacterium *Sorangium cellulosum*, have been intensively researched[19] leading to Ixempra® entering the market for the treatment of advanced breast cancer.[20]

A major threat to the effective targeting of the microtubule as a mechanism for chemotherapy is the development of resistance by the neoplastic tissue. Although many tumors initial-

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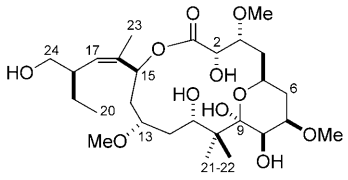
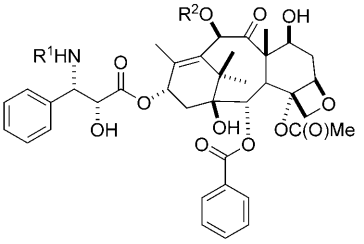
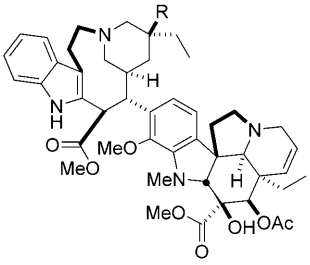


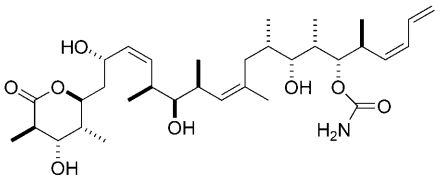
1: peloruside A



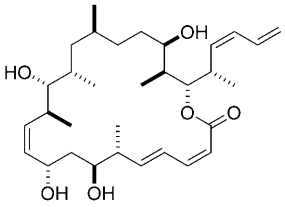
2: paclitaxel, R^1 = PhCO, R^2 = Ac
3: docetaxel, R^1 = *t*-BuOCO, R^2 = H



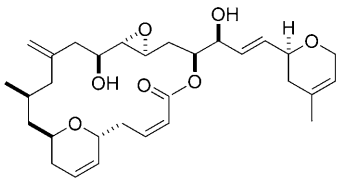
4: vinblastine, R = OH
5: vinorelbine, R = H



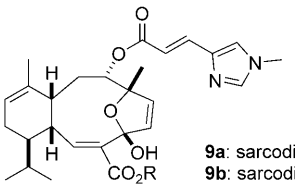
6: discodermolide



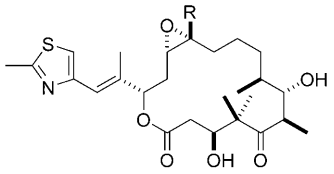
7: dictyostatin



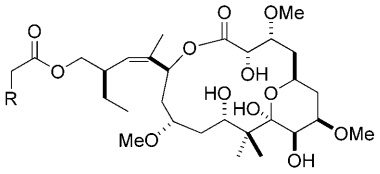
8: laulimalide



9a: sarcodictyin A, R = Me
9b: sarcodictyin B, R = Et



10a: epothilone A, R=H
10b: epothilone B, R=Me



11: 24-*O*-(chloro)acetyl peloruside, R= Cl
12: 24-*O*-acetyl peloruside, R = H

ly respond favorably to treatment, effectiveness is limited by two main causes. Firstly, overexpression of the MDR-1 gene, which encodes for the drug efflux pump P-glycoprotein (P-gp), is often apparent in resistant tissues. P-gp, a member of a group of transmembrane proteins of the ATP binding cassette (ABC) family, has a broad substrate specificity and is responsible for the efflux of a wide range of drugs including anticancer agents, HIV protease inhibitors, and peptides.[21] It has been shown that the extent of drug resistance in human tumors correlates well with P-gp expression.[22] The net result of this hyperexpression is the reduction of the intracellular drug concentration. Although cells overexpressing P-gp remain sensitive to the taxanes, they require much higher concentrations of either paclitaxel or docetaxel to achieve the same therapeutic result.[23] As a consequence of this, normal, non-cancerous cells are put at adverse risk because they no longer can be differentially spared due to their lower division rate; this leads to serious side effects.

The second mechanism by which tumors become resistant to taxane chemotherapy is by the overexpression of other tubulin isotypes with lower sensitivity to the taxanes. In humans, there are six β-tubulin isotypes and of these class III β-tubulin (Hβ4 gene product) is the least sensitive to paclitaxel.[24] In normal human and murine cells, class I β-tubulin is the major isotype and accounts for approximately 70% of the total β-tubulin in all tissues,[25,26] In contrast, class III β-tubulin is not usually expressed in normal cells but has been found to be upregulated in taxane-resistant tumor cells.[27] Of the different classes of tubulin isotypes, microtubules composed of class III β-tubulin are the most dynamically unstable[28] and therefore it is unsurprising that this composition is the least sensitive to the stabilizing taxanes.[29]

Peloruside and laulimalide are microtubule-stabilizing agents that are synergistic with paclitaxel and some of its biomimetics (for example, discodermolide), both in isolated tubulin[30] and in cells.[31] They bind to an as yet undefined common, or overlapping site distinct from the paclitaxel site in the tubulin dimer.[32,33] Both compounds are poor substrates for P-gp and are therefore appropriate lead compounds for the design of ligands targeting microtubules as part of a combination therapy with the taxanes, as well as for the treatment of multidrug-resistant tumors.[32,33] Moreover, although the actual binding site of peloruside and laulimalide is unknown, it is understood that these compounds do not interact with the type I pore site (the taxoid site on β-tubulin)[34] and therefore any changes to the microtubule here (for example, in αβIII microtubules) will not adversely influence their activity. The synergy they display with paclitaxel and its biomimetics highlights these compounds as promising leads for drug development, warranting further investigation into structure–activity relationship (SAR) and mechanistic studies.

Whereas some information is available on the SAR of laulimalide,[35,36] little is known about peloruside, except that reductive opening of the six-membered pyranose ring leads to a tenfold decrease in cytotoxicity.[2] Any further information regarding which functional groups and stereochemical features are essential for peloruside's biological activity would therefore be important. Moreover, in order to determine the binding properties of peloruside, derivatives bearing fluorescent, reactive, or radioactive groups would be of great value. With this in mind, we pursued the preparation of 24-*O*-(chloro)acetyl peloruside (**11**). From a chemical perspective, the primary alcohol at position C24 is the simplest to target and therefore the intuitive starting point for our investigations.

A common approach for obtaining a ligand–protein conjugate is through the incorporation of an electrophilic site (for example, as included in structure **11**) on the ligand. This can

then react with a nucleophilic residue in the protein, for example the thiol of a cysteine.[37] After cross-linking is achieved, digestion and mass spectrometry experiments are used to determine which segment of the protein the ligand binds. Coupled with detailed information already known about the protein structure, a preliminary active site model can be constructed. Information on this binding site is of critical importance for the design of more efficacious peloruside-based drug candidates and is the key motivation behind this work.

Results

Ligand binding to microtubules

The stoichiometry of peloruside binding to cross-linked and native microtubules was studied by using centrifugation techniques. Peloruside was determined to bind to cross-linked stabilized microtubules (Figure 1A) with a stoichiometry of 0.95 ± 0.06 molecules of peloruside per paclitaxel binding site. This indicated that the peloruside binding site is preserved in cross-linked stabilized microtubules and that peloruside has a 1:1 relationship with the tubulin dimer, as is the case for microtubule-stabilizing agents that target the taxoid site. Under conditions that normally prevent native tubulin assembly into microtubules, peloruside was able to promote microtubule assembly (1.04 ± 0.08 molecules of peloruside bound per tubulin dimer, Figure 1B).

Analogue binding to cross-linked microtubules was also studied by centrifugation techniques. Whereas peloruside was determined to bind to cross-linked microtubules with μM affinity (Figure 1C and Table 1), neither 24-*O*-(chloro)acetyl peloruside nor 24-*O*-acetyl peloruside (**12**) were found bound to microtubules when incubated with 50 μM binding sites. No appreciable decrease in the supernatant concentrations of the ligands was observed, thus indicating no covalent binding of 24-*O*-(chloro)acetyl and 24-*O*-acetyl peloruside (data not shown).

Table 1. Thermodynamic parameters of peloruside binding to microtubules. Binding affinity.

Affinity	20 °C	25 °C	30 °C	35 °C	40 °C
$\times 10^6\ M^{-1}$	3.5 ± 1.2	3.2 ± 0.8	2.4 ± 1.1	2.6 ± 0.5	2.1 ± 0.3

$\Delta H = -19 \pm 4\ kJ\,mol^{-1}$. $\Delta S = 61 \pm 14\ J\,mol^{-1}\,K^{-1}$.

Microtubule assembly induction and modulation

Strong microtubule-stabilizing agents are able to promote microtubule assembly under conditions that are normally hostile to tubulin assembly.[38] Peloruside was able to induce microtubule assembly with a critical concentration[39] of 3.1 μM tubulin in 10 mM sodium phosphate, 1 mM EDTA, 1 mM GTP, 4 mM $MgCl_2$, pH 6.7 (PEDTA4), indicating an assembly-induction power greater than those of paclitaxel and docetaxel under the same conditions, despite peloruside's lower affinity (critical concentration of paclitaxel = 5.4 μM, critical concentration of docetaxel = 1.5 μM, K_a paclitaxel 35 °C = $1.4 \times 10^7\ M^{-1}$ K_a docetaxel 35 °C = $3.9 \times 10^7\ M^{-1}$[40]). As expected, both esterified peloruside derivatives **11** and **12** were unable to induce microtubule assembly under these conditions.

Next, it was examined whether the modified compounds were at least weak microtubule-stabilizing agents able to enhance microtubule assembly under conditions in which tubulin assembles by itself (namely, 3.4 M glycerol, 1 mM EGTA, 10 mM sodium phosphate, 6 mM $MgCl_2$, 1 mM GTP at pH 6.5 (GAB)). Under these conditions, tubulin was able to assemble with a critical concentration of 3.3 ± 0.3 μM. Paclitaxel, docetaxel, and peloruside were found to strongly stabilize microtubules, decreasing the critical concentration to 0.38 ± 0.06, 0.26 ± 0.05, and 0.24 ± 0.09 μM, respectively, indicating that in these conditions peloruside is at least as strong a microtubule-stabilizing agent as the taxanes. Additional-

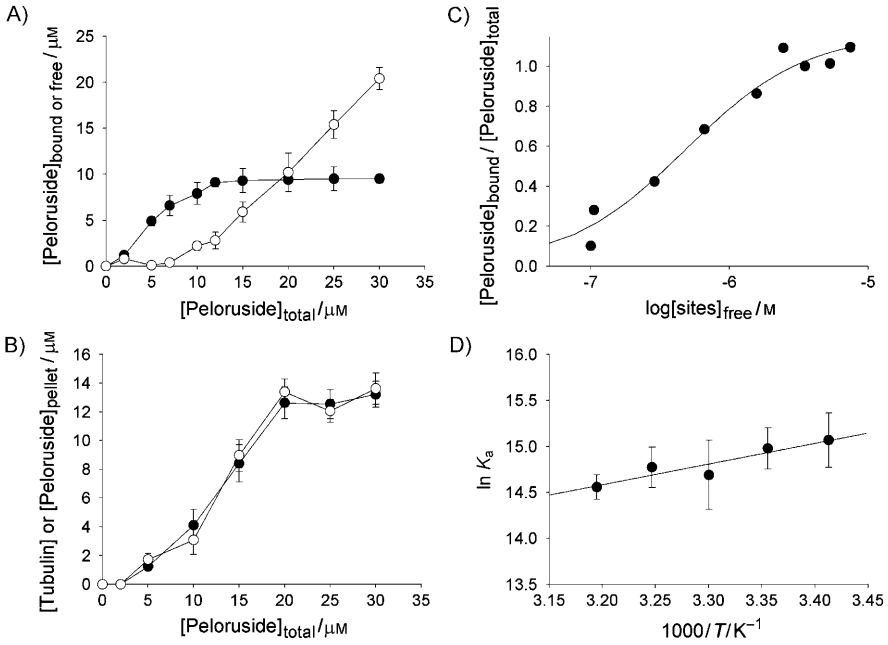


Figure 1. Biochemical studies of the interactions of peloruside with microtubules. A) Co-sedimentation of peloruside with paclitaxel sites in cross-linked stabilized microtubules (10 μM); ●: pelleted peloruside, ○: peloruside in supernatant. B) Stoichiometry of peloruside-induced tubulin assembly; ●: tubulin concentration, ○: peloruside bound to the microtubules. C) Titration curve of peloruside (0.5 μM) with stabilized microtubules at 25 °C. D) van't Hoff plot of the binding of peloruside to microtubules.

ly, the esterified peloruside derivatives **11** and **12** did not significantly promote further microtubule stabilization (critical concentration for both = 3.3 ± 0.3 μM), thus indicating that they do not exhibit microtubule-stabilizing activity.

Cellular activity of the compounds

The IC_{50} of the derivatives **11** and **12** compared with peloruside were determined in A2780 and A2780AD ovarian carcinoma cells. Peloruside was found to be cytotoxic in these cell lines as previously described.[33] Surprisingly, 24-*O*-(chloro)acetyl peloruside was found to be almost as active as peloruside, whereas 24-*O*-acetyl peloruside was tenfold less active than the former (Table 2). Treatment of A549 lung carcinoma cells for 24 h with either 24-*O*-(chloro)acetyl peloruside (1 μM), or 24-*O*-acetyl peloruside (10 μM) compared to peloruside (1 μM), gave rise in all cases to characteristic cytoplasmic microtubule bundles as well as aberrant mitosis (multiple asters) and micronucleated interphasic cells (Figure 2B –D). This indicates that despite their lack of in vitro binding, the esterified compounds are microtubule-stabilizing agents. When A549 cells were incubated for 20 h in the presence of serial concentrations of these drugs, maximal cell accumulation (more than 90%) was observed in the G2/M phase of the cell cycle with 20 nM paclitaxel (not shown), 80 nM peloruside, 160 nM 24-*O*-(chloro)acetyl peloruside, and 1.6 μM 24-*O*-acetyl peloruside (Figure 2F–H). Because these biological results were inconsistent with those obtained in vitro with the peloruside derivatives, we sought to determine the reasons for it.

Table 2. Cytotoxicity of 24-*O*-(chloro)acetyl and 24-*O*-acetyl peloruside on the growth of two human ovarian carcinomas.[a]

Drug	A2780 [nM][b]	A2780AD [nM]	*R/S*[c]
paclitaxel	1.6 ± 0.72	1100 ± 50	687.5
peloruside	19.2 ± 0.69	880 ± 100	45.8
24-*O*-(chloro)acetyl peloruside	26.6 ± 1.8	2950 ± 450	110.9
24-*O*-acetyl peloruside	256.6 ± 25	3100 ± 70	12.1

[a] IC_{50} of the ligands determined in ovarian carcinomas A2780 and P-glycoprotein-overexpressing A2780AD. [b] IC_{50} values [nM] are the mean ± standard error of three independent assays. [c] The relative resistance of the A2780AD cell line obtained by dividing the IC_{50} of the resistant cell line by that of the parental A2780 cell line.

To assess whether the 24-*O*-(chloro)acetyl peloruside derivative was the species responsible for the observed activity, we incubated 1 μM of the compound either with cells or with only culture medium for 24 h. The medium of each well was harvested, cells were recovered with PBS–EDTA, pelleted after washing with PBS, and lysed. Compounds were extracted from the cells and analyzed by MS-HPLC. The analysis (Figure 3) revealed that 24-*O*-(chloro)acetyl peloruside was hydrolyzed into the active compound peloruside after a 24 h incubation period, either in the presence or in the absence of cells. The higher cellular activity observed for 24-*O*-(chloro)acetyl peloruside compared to 24-*O*-acetyl peloruside is presumably due to the greater susceptibility of the chloroacetoxy group to hydrolysis owing to its increased electrophilicity. This kind of hydrolysis was also observed previously in the case of 2-acetyl flutax.[41]

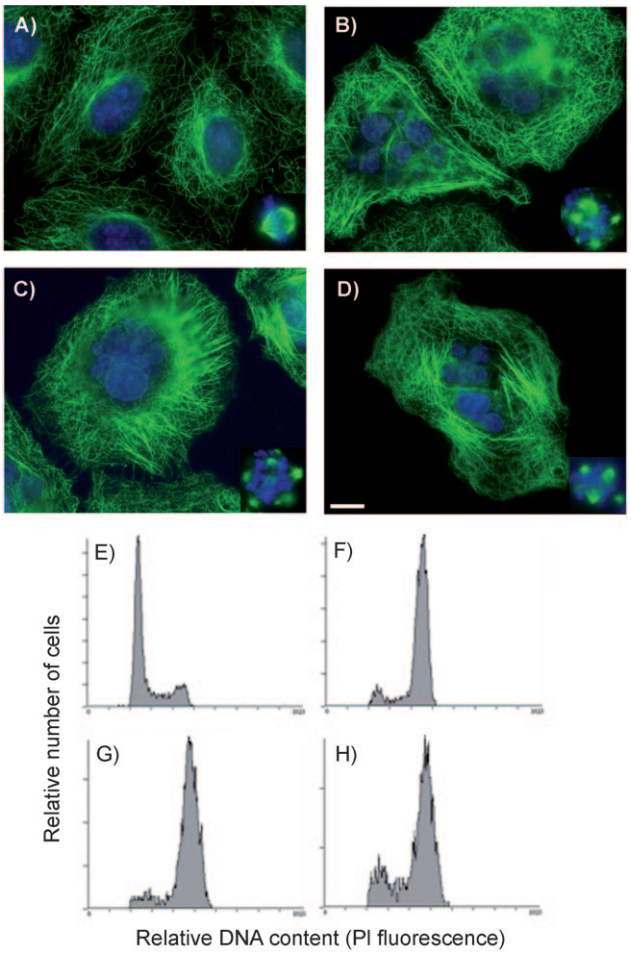


Figure 2. Cellular effects of 24-*O*-(chloro)acetyl and 24-*O*-acetyl peloruside on the microtubule network, nucleus morphology and cell cycle of A549 carcinoma cells. A549 cells were incubated for 24 h with DMSO (A), 1 μM peloruside (B), 1 μM 24-*O*-(chloro)acetyl peloruside (C) and 10 μM 24-*O*-acetyl peloruside (D). Microtubules were immunostained with α-tubulin monoclonal antibodies and DNA was stained with Hoechst 33342. Insets are mitotic spindles from the same preparation. The scale bar represents 10 μm. All panels have the same magnification. Effect of DMSO (E), 80 nM peloruside (F), 160 nM 24-*O*-(chloro)acetyl peloruside (G) and 1.6 μM 24-*O*-acetyl peloruside (H) on the cell cycle of A549 cells.

Molecular modeling

The different behavior of peloruside and its derivatives in their interaction with tubulin was explored by docking these ligands into the structure of the proposed peloruside binding site on the α-subunit of tubulin, under the B9-B10 loop.[42,43] All three compounds were predicted to predominantly bind the B9-B10 site in a conformation similar to the one previously reported (Figure 4A).[43] In the resulting complexes, the C24-hydroxyl of peloruside forms a hydrogen bond with Arg320 (Figure 4B) which, as expected, is lost in the case of the esterified com-

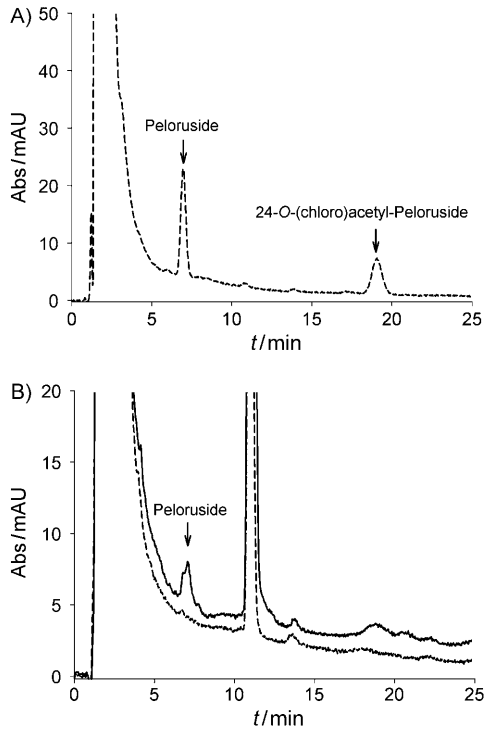


Figure 3. Hydrolysis of 24-*O*-(chloro)acetyl peloruside in the cell culture medium. A) HPLC analysis of a standard solution containing peloruside (50 μM) and 24-*O*-(chloro)acetyl peloruside (50 μM). B) HPLC analysis of the cell medium containing 24-*O*-(chloro)acetyl peloruside (1 μM, solid line) or DMSO (vehicle, dashed line) after 24 h of incubation.

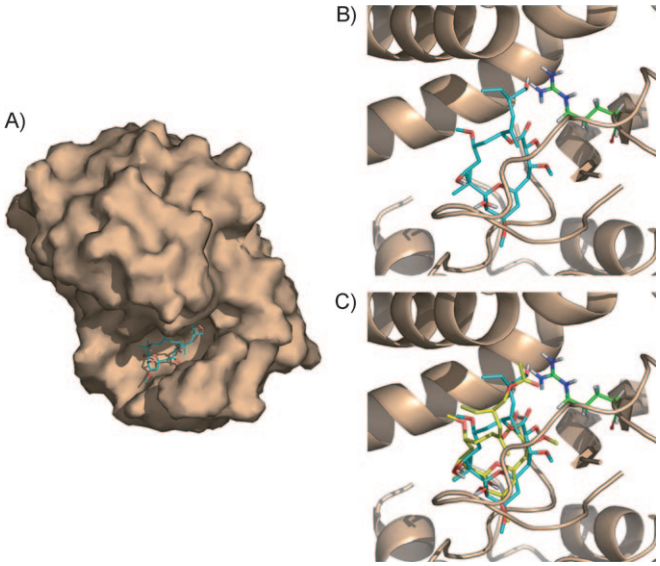


Figure 4. Docking of the compounds into the putative peloruside binding site on α-tubulin. A) Overall view of peloruside bound at the α-subunit cleft. B) Detail of the peloruside–tubulin interaction. C) Effect of the acetylation of the C24-OH on the peloruside interaction with the site blue-red (peloruside), yellow-red (24-*O*-acetyl peloruside).

pounds. However, not only is this single hydrogen bond lost, but the steric effect of the acetyl and chloroacetyl appears to distort the resulting complex in a way that the remaining interactions of peloruside are weakened or lost altogether (Figure 4C). In addition, docking simulations were also performed for the alternative putative peloruside binding site on the β-subunit of tubulin, near residues 292–340.[44] This time the docking simulations predicted that the acetoxy and chloroacetoxy derivatives of peloruside should retain their activity because the three compounds showed similar binding modes with the two structures of tubulin evaluated (Figure 5).

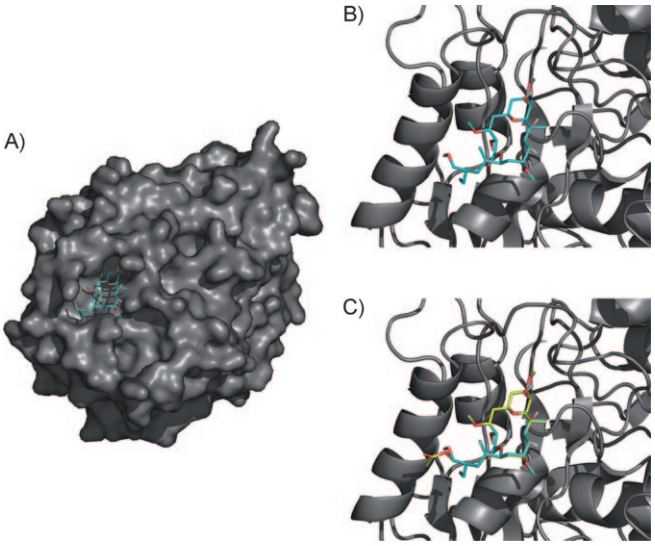


Figure 5. Docking of the compounds into the putative peloruside binding site on β-tubulin. A) Overall view of peloruside bound at the β-subunit cleft. B) Detail of the peloruside–tubulin interaction. C) Effect of the acetylation of the C24-OH on the peloruside interaction with the site blue-red (peloruside), yellow-red (24-*O*-acetyl peloruside).

Effect of peloruside on microtubule structure

In order to gain insight into the mode of action of peloruside in microtubules, the small-angle X-ray scattering profile of peloruside-induced microtubules was compared with those of microtubules assembled in the absence of drugs and with those induced by docetaxel and paclitaxel.[45,46] Solutions of glycerol, paclitaxel, docetaxel, and peloruside-induced microtubules were found to give characteristic differences in their X-ray scattering profiles. These consist of displacements of the maxima of the J_0 Bessel function (corresponding to the low-resolution Fourier transform of the excess electron density of the microtubule hollow cylinder), which indicates perturbation in the diameter of the polymers and therefore reflect changes in the number of protofilaments that compose the microtubules[47] (Table 3).

As previously described by Andreu et al.,[45] binding of paclitaxel to microtubules results in an average decrease of 0.9 in the number of protofilaments that comprise the microtubules (from 13.6 to 12.7), whereas docetaxel leaves the microtubule structure unchanged.[46] Peloruside produces a more significant effect in altering the microtubule structure than paclitaxel, reducing the average number of protofilaments by 1.2 (from 13.6 to 12.4).

Table 3. Characteristics of the X-ray scattering profiles of microtubules of different compositions. Deviations in the measurements of the positions of the J_0 maxima are typically 0.001 nm^{-1}.

Ligand	J_{01} [nm^{-1}]	J_{02} [nm^{-1}]	Mean radius [nm]	Protofilament number
none	0.049	0.091	12.53	13.6
paclitaxel	0.052	0.099	11.51	12.7
docetaxel	0.049	0.090	12.45	13.7
peloruside	0.054	0.101	11.04	12.4

Binding epitope of peloruside to microtubules

In order to gain insight into the interaction mode of peloruside with microtubules, we studied the binding of the compound to microtubules by using saturation-transfer difference (STD) analysis (Figure 6). STD-NMR spectroscopic experiments detect magnetization transferred from the protein to a bound ligand. Only bound ligands show STD effects and the effect is dependent on the distance between the protons, thus STD-NMR spectroscopy is a useful tool to detect the regions of the ligand that interact with the protein.

STD from microtubules to peloruside have been previously observed[43] (Figure S5 in the Supporting Information), although the data obtained were of low quality and precluded the determination of the binding epitope. Here, high-quality spectra were obtained and allowed us to identify two regions of the molecule that appear to be part of the tubulin-binding epitope and key protons (those with higher saturation), which are expected to be involved in direct interaction with the binding site. These protons are H8, H17, OMe-3, OMe-13, Me-20 and Me-22 (inset Figure 6), and include regions of the side chain and the methyl groups incorporated on the macrocyclic portion of the molecule.

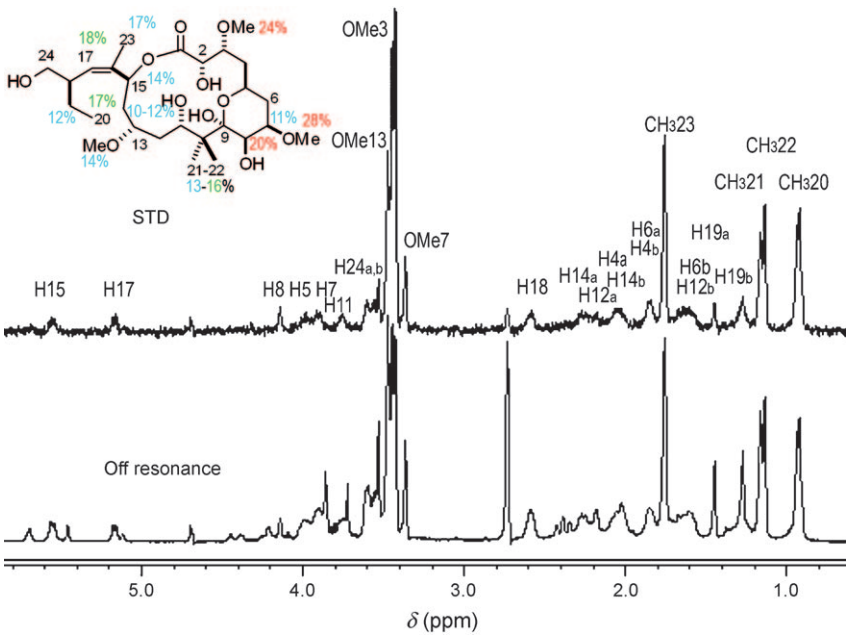


Figure 6. Saturation transfer difference spectrum of peloruside bound to microtubules. Inset: structure of peloruside indicating the percentage of saturation of the protons within the molecule, figures in red and green indicate those protons with high saturation.

Discussion

The peloruside binding site in assembled microtubules is increasingly becoming an important focus in the field of anticancer drug research. Although peloruside targets tubulin and produces the same microtubule-stabilizing effect as paclitaxel, its binding site is different.[32,33] Little is known about the actual thermodynamic properties of the interaction or the structure–activity relationship between peloruside and tubulin. Some molecular modeling studies have suggested the α-tubulin subunit as the molecular target for peloruside,[42,43] whereas others suggest the β-tubulin subunit.[44,48]

Peloruside binds to microtubules and induces assembly in a similar way to paclitaxel

The results of this study firmly establish that peloruside binds to microtubules in the same way as microtubule-stabilizing agents that interact with the paclitaxel site. Mild fixation of microtubules preserves both the paclitaxel and the peloruside site enabling stabilized microtubules to be used to study ligand binding at the peloruside site.[49] A 1:1 binding stoichiometry of preformed assembled microtubules and peloruside has been determined.

The stoichiometry and the link between peloruside binding and assembly have been confirmed in native microtubules (Figure 1B), under conditions at which tubulin itself does not assemble (that is, in the absence of glycerol). No ligand-free tubulin was pelleted, and no more than one peloruside molecule was bound per assembled tubulin molecule, which is similar to the paclitaxel-induced assembly.[38]

From a thermodynamic perspective, peloruside binds microtubules with low affinity (K_a 35 °C 2.6×10^6 M^{-1}), significantly weaker than most of the paclitaxel binding site ligands, and is comparable to sarcodictyin A **9a** (K_a 35 °C 1.8×10^6 M^{-1}), which is a known low-affinity binder.[50] The thermodynamic parameters of peloruside binding are moderately favorable in the enthalpic ($\Delta H=-19\pm4$ kJ mol^{-1}) and entropic ($\Delta S=61\pm14$ J mol^{-1} K^{-1}) terms (Figure 1D) of the free energy of binding, and are similar to those determined for sarcodictyin A (**9a**; $\Delta H=-24\pm3$ kJ mol^{-1} and $\Delta S=43\pm19$ J mol^{-1} K^{-1}). In spite of this, peloruside is much better at in-

ducing assembly of GTP-tubulin (in 10 mM sodium phosphate, 1 mM EDTA, 4 mM $MgCl_2$, 1 mM GTP pH 6.7 buffer) with a critical concentration of 3.1 μM than sarcodictyin, which is unable to induce tubulin assembly under these buffer conditions.[50] Peloruside is thus comparable to strong assembly inducers like paclitaxel and docetaxel, but with a binding affinity of up to tenfold weaker. Additionally, the cellular effects of peloruside (1 μM) are similar to those of the high-affinity paclitaxel-site microtubule-stabilizing agents. Bundles of microtubules are observed as well as micronuclei, multiple asters and cell accumulation in G2/M phase of the cell cycle. In comparison, sarcodictyin (10 μM) induced fewer bundles made up of long microtubules, multipolar asters, as well as multinucleated cells.[50] Low-affinity peloruside-site ligands are determined to be better at promoting microtubule assembly than those with comparable affinity for the paclitaxel site, as in the case of sarcodictyin.

Location of the peloruside binding site in microtubules

Two different binding-site locations on microtubules have previously been proposed for peloruside based either on the docking of the bioactive peloruside conformation[43] or by data-directed modeling (mass shift perturbation studies).[44] The former suggests the binding site is located on the α-subunit of tubulin, under the B9–B10 loop, in a location equivalent to the luminal site of paclitaxel in the β-subunit.[9] The latter methodology suggests the binding site is located on the β-subunit close to the type II pore site. Both possible models are discussed in light of the experimental results presented in this work.

The SAR results indicate that the primary alcohol at position C24 is essential for tubulin binding because both the chloroacetoxy (**11**) and acetoxy (**12**) derivatives were unable to bind to microtubules or induce microtubule assembly in vitro. Although they were cytotoxic to tumor cells, this activity arises from hydrolysis of the ester bond in the cell culture media, a phenomenon previously observed in the case for 2-acetyl flutax.[41]

The effect of the acetylation of the primary alcohol at position C24 has been explored by using molecular modeling. On the basis of previous docking studies, which indicated that peloruside-site ligands bind to the α-tubulin subunit,[42,43] the introduction of the acetoxy or chloroacetoxy groups at position C24 appears to severely perturb binding. This would not be the case for the alternative putative β-tubulin site.[44]

The small-angle X-ray scattering experiments indicate that peloruside binding narrows the angle between microtubule protofilaments more strongly than paclitaxel. Peloruside binding reduces the average number of protofilaments by 1.2, thus indicating a similar structural influence. Microtubules are hollow cylinders composed of equally spaced protofilaments, with an average number of 13 protofilaments. However, this is a relatively flexible property of tubulin assembly. Transitions in the number of protofilaments have been observed within individual in vitro assembled microtubules[51] and preassembled microtubules can rapidly change their number of protofilaments in response to ligand binding.[52] Changes in the protofilament number necessarily implies that the angle of interaction between the protofilaments is altered, reducing the number of protofilaments if the angle is narrower and increasing the number of protofilaments if the angle is wider. Although, in principle, the reduction observed indicates a similar microtubule stabilization mode as paclitaxel, this appears to be inconsistent with the mechanism of stabilization mode proposed by Huzil et al.[44] for peloruside. The peloruside mode of action they suggest involves the interdimer interface and includes contributions from the α/β-tubulin intradimer interface and protofilament contacts. However, given that the proposed β-tubulin binding site is proximal to the type II pore site and the interprotofilament space, peloruside binding to this site (proposed by Huzil et al.) appears to be consistent with the measured reduction in protofilament number.

Finally, the peloruside-binding epitope determined by NMR spectroscopic experiments indicates that protons H8, H17, OMe-3, OMe-13, Me-20, and Me-22 should be strongly involved in the interaction with microtubules. When the saturation values of the protons measured are compared with the degree of exposure in both binding models (Table 4), binding to the putative α-subunit site seems to be favored because all the protons with high or medium saturation values are pointing inside the proposed binding site or are in close contact with the protein. This is not the case for the putative binding site on the β-subunit tubulin.

Table 4. Saturation transfer difference of the peloruside protons compared with the predicted binding epitopes of both proposed binding sites.

Proton number	Saturation [%]	α-Subunit site[42,43]	β-Subunit site[44]
H7	11	inside	inside
H8	20	inside	exposed
H14	10–12	inside	exposed
H15	14	exposed	exposed
H17	18	strong	exposed
H19a	12	strong	exposed
Me-20	17	strong	exposed
Me-21	13	inside	exposed
Me-22	16	inside	exposed
Me-23	17	strong	inside
OMe-3	24	inside	strong
OMe-7	28	inside	exposed
OMe-13	14	inside	exposed

Inside = protons into the binding site, exposed = protons exposed to the solvent, strong = protons in close contact with the protein.

From the experimental data (SAR, similar influence of the compounds on the microtubule structure to paclitaxel and binding epitope), the proposed binding site on the α-subunit of tubulin seems to be favored. However, the experimental evidence previously provided by mass shift perturbation studies lends support to the β-tubulin site (close to the type II pore site, which would more easily influence microtubule diameter).[44] Furthermore, the α-tubulin binding site (within the 373–383 region) is also protected from deuterium exchange after peloruside binding, as previously demonstrated by Huzil

et al.[44] In light of this, we speculate that peloruside binding involves a two-step binding mechanism equivalent to that proposed for the taxanes.[34,49] This mechanism would involve an initial binding event, which could be on the pore β-tubulin site, with subsequent transportation of the ligand to the internal α-tubulin site.

Conclusions

Peloruside is a novel microtubule-stabilizing agent that targets a new binding site on the microtubule, different from that of the clinically used drugs paclitaxel and docetaxel. Peloruside induces microtubule assembly with the same biochemical assembly-binding-linked mechanism as paclitaxel, by binding to preformed microtubules with a 1:1 stoichiometry relative to tubulin dimers, and with an affinity of the order of μM. Although the binding affinity of the compound and the apparent biochemical mechanisms are comparable to that of the low-affinity microtubule-stabilizing agents, peloruside is both more active in inducing microtubule assembly and cytotoxic to tumor cells. This suggests that the peloruside site might be a more effective site for stabilizing microtubules. In investigations towards the preparation of reactive peloruside molecular probes, two C24 esterified derivatives were synthesized in order to assess this position as a useful handle for elaboration. Unlike the parent compound, the derivatives are unable to bind to microtubules, although they are active in cells because they are hydrolyzed back to peloruside in the cell media and recover their biological activity. The C24 hydroxyl group was determined to be essential for peloruside's biological function and precludes this position for the introduction of fluorescent or cross-linking labels. Molecular modeling of the interaction of peloruside with its putative binding site on α-tubulin indicates that substitution at the C24 hydroxyl group disrupts the interaction of the side chain with Arg320, thus destabilizing the side-chain binding. This SAR data, the effect of the compounds on the microtubule structure and the binding epitope determined by using NMR spectroscopic techniques are more compatible with the putative α-tubulin subunit binding site,[42,43] than the alternative β-tubulin binding site.[44,48] Further investigations are underway to uncover the important molecular recognition features of the peloruside–microtubule interactions.

Experimental Section

Proteins and ligands: Purified calf brain tubulin and chemicals were used as previously described.[7] Taxotere (docetaxel) was kindly provided by Rhône Poulenc Rorer, Aventis (Schiltigheim, France). The 24-*O*-(chloro)acetyl peloruside derivative was prepared by treating natural peloruside with chloroacetic anhydride and pyridine (47% yield). Excessive cooling (−100 °C) was required to ensure selective substitution at the primary position. Similarly, reaction with acetyl chloride and pyridine at −100 °C produced the 24-*O*-acetyl derivative (62% yield). Both synthetic products were purified by HPLC (see the Supporting Information for a full description of the synthetic procedures). Extensive NMR spectroscopic studies confirmed derivatization at the desired position and showed that the rest of the molecule remained intact and unaffected. All the compounds were diluted in 99.8% [D_6]DMSO (Merck) to a final concentration of 20 mM and stored at −20 °C.

Ligand binding to microtubules: Peloruside, 24-*O*-(chloro)acetyl peloruside or 24-*O*-acetyl peloruside (0.5 μM) were incubated in 3.4 M glycerol, 1 mM EGTA, 0.1 mM GTP, 6 mM $MgCl_2$ at pH 6.5 with increasing concentrations (up to 10 μM) of cross-linked stabilized microtubules prepared as described previously.[53] Samples were incubated for 30 min in a water bath at 25 °C. Microtubules were pelleted by centrifugation (50 000 rpm for 20 min in a TLA120.2 rotor in an Optima TLX centrifuge) and the supernatant was carefully collected and the pellet was resuspended in sodium phosphate 10 mM pH 7 buffer (1 mL). Docetaxel (10 μM) was added to the samples as an internal standard. The ligands were extracted from the samples with 3×1 volume of CH_2Cl_2. The CH_2Cl_2 extract was dried, and the sample was resuspended in 75% MeOH/H_2O (40 μL). Peloruside and analogue contents were analyzed in an Eclipse XDB C-18 column on an Agilent 1100 HPLC by using a two-step isocratic system, 10 min at 60% MeOH/H_2O followed by another 10 min at 75% MeOH/H_2O, running at a flow rate of 1 mL min^{-1}.

The stoichiometry of peloruside binding to crosslinked microtubules was measured by incubating paclitaxel binding sites (10 μM) in stabilized microtubules with increasing amounts of peloruside (up to 25 μM) in glycerol (3.4 M), sodium phosphate (10 mM), EGTA (1 mM), GTP (0.1 mM), and $MgCl_2$ (6 mM) for 30 min at pH 6.5 and 25 °C. Microtubules and bound ligand were separated from unbound ligand by centrifugation at 50 000 rpm by employing a TLA100 rotor in an Optima TLX ultracentrifuge. The supernatant was carefully collected, and the pellet was resuspended in 10 mM sodium phosphate pH 7.0 buffer. Docetaxel (10 μM) was added to the samples as an internal standard. The ligands were extracted from the samples with 3 volumes of CH_2Cl_2. The CH_2Cl_2 extract was dried, and the sample was resuspended in 75% MeOH/H_2O (40 μL). Peloruside contents were analyzed on a Eclipse XDB C18 column in an Agilent 1100 HPLC by using a two-step isocratic system, 10 min at 60% MeOH/H_2O followed by another 10 min at 75% MeOH/H_2O, running at a flow rate of 1 mL min^{-1}. The tubulin concentration of the supernatants and pellets were measured by using the Bradford method.[54] Peloruside was quantified by comparison of the integrated areas of the HPLC peak with that of the weighted standards.

Additionally, tubulin (20 μM) in PEDTA6 was incubated for 30 min at 37 °C with increasing amounts (up to 30 μM) of peloruside. The microtubules that formed were sedimented as described above, and the tubulin and ligand concentrations in the pellet and supernatants were quantified as described above.

The binding affinity of peloruside to microtubules was determined by incubating peloruside (0.5 μM) with concentrations up to 8 μM of cross-linked stabilized microtubules at different temperatures. Microtubules and bound ligand were separated from unbound ligand and analyzed as described above.

Tubulin assembly induction: Tubulin (20 μM) equilibrated in PEDTA4 was incubated at 37 °C for 30 min with increasing concentrations of peloruside, 24-*O*-(chloro)acetyl peloruside or 24-*O*-acetyl peloruside. The samples were centrifuged at 50 000 rpm for 20 min employing a TLA100 rotor in an Optima TLX ultracentrifuge. The supernatant was carefully collected and the pellets resuspended in cold 10 mM pH 7.0 phosphate buffer. The tubulin concentration of the supernatants and pellets were measured by using the Bradford method.[54]

Alternatively, tubulin (15 and 25 μM) was equilibrated in GAB and were incubated with 20 or 30 μM concentrations of the ligands, respectively. The samples were centrifuged at 50000 rpm for 20 min employing a TLA100 rotor in an Optima TLX ultracentrifuge. The supernatant was carefully collected and diluted 1:5 in 1% SDS 10 mM pH 7.0 sodium phosphate buffer, the pellets resuspended in 1% SDS 10 mM pH 7.0 sodium phosphate buffer and diluted 1:5 in the same buffer. Tubulin concentration of the supernatants and pellets were measured fluorometrically ($\lambda_{exc}=280$ nm and $\lambda_{exc}=323$ nm) employing spectrophotometrically measured tubulin samples as a standard.

Cell biology: Human ovarian carcinomas A2780 and A2780AD (MDR overexpressing P-glycoprotein) and A549 non-small-cell lung carcinoma were cultured as previously described.[34]

Indirect immunofluorescence and cell cycle analysis was performed as described previously.[50] Cytotoxicity assays were performed with the MTT assay, which was modified as described previously.[55]

Hydrolysis of the modified compounds in the cell culture media was analyzed by incubating 1 mL of cell culture media containing DMSO and peloruside (1 μM) or 24-*O*-(chloro)acetyl peloruside (1 μM) for 24 h in the presence or absence of 150000 A549 cells. Plates were centrifuged, the supernatants were harvested and the adherent cells were removed by treatment with PBS–EDTA. After washing with PBS, the cell pellets were then lysated with 70% MeOH (Merck analytical grade). The supernatants and pellets were frozen at −80 °C and lyophilized. The lyophilized samples were extracted with abs MeOH (1 mL), and the extracts were dried. The residues were resuspended in 70% MeOH (100 μL) and analyzed by HPLC by using an Agilent 1100 series instrument employing a Supercosil, LC18 DB, 250×4.6 mm, 5 mm bead diameter column developed with 55% MeOH/H_2O at a flow rate of 1 mL min^{-1}, by following the absorbance at $\lambda=200$ nm. The identity of the peaks was confirmed by mass spectrometry by using a Thermo instrument Finnigan LXQ coupled to a Surveyor chromatograph and employing Supercosil, LC18 DB, 250×4.6 mm, 5 mm bead diameter column developed as described above.

X-ray scattering by microtubule solutions: Measurements were made at station 2.1 of the Daresbury Laboratory Synchrotron Radiation Source, UK. Instruments employed, data acquisition and processing, and interpretation of the microtubule X-ray scattering, have been described previously.[52]

NMR spectroscopy experiments: NMR spectra were recorded at 310 K in D_2O on a Bruker AVANCE 500 MHz spectrometer. STD were performed as described previously,[56] by using a 30:1 ligand/receptor molar ratio with 0.5, 1, and 2 s saturation time (concatenation of 50 ms Gaussian pulses).

Molecular modeling: The complexes of peloruside (**1**) and its esterified derivatives **11** and **12** with tubulin were evaluated with AutoDock 3.05.[57] The solution conformation of peloruside was chosen as the starting structure for the three compounds studied, which were relaxed with MacroModel 8.5 (AMBER*/H_2O/GBSA)[58] before docking over the molecular-dynamics-generated structure proposed for the binding site of peloruside (based on the 1JFF[59] structure deposited in the PDB). The paclitaxel site was also included in the searched region. To evaluate the complexes at the putative β-subunit peloruside proposed binding site,[44] a search box of 27 Å was centered over residues 292–340 (72×72×72 points, grid spacing of 0.375 Å) of the tubulin structures complexed with epothilone and paclitaxel (1TVK[60] and 1JFF entries of the PDB respectively). Previously validated parameters for these protein and similar ligands were used in our simulations:[42] Lamarckian genetic algorithm as search algorithm, 100 individuals, 10000 generations, 10^{10} energy evaluations, 100 LGA runs and a grid spacing of 0.75 Å.

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