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Taxanes with high potency inducing tubulin assembly overcome tumoural cell resistances.

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Summary

We have found that four taxanes with chemical modifications at positions C10 and C13 were active against all types of taxane resistant cell lines, resistant by p-Gp overexpression, by mutations in the β -tubulin binding site or by overexpression of the highly dynamic β III-tubulin isotype.

We have characterized the interaction of taxanes with high activity on chemotherapy resistant tumoural cells with microtubules, and also studied their cellular effects. The biochemical property enhanced in comparison with other taxanes is their potency at inducing tubulin assembly, despite the fact that their interactions with the microtubule binding sites (pore and luminal) are similar as studied by NMR and SAXS. A differential interaction with the S7-S9 loop (M-loop) is responsible for their enhanced assembly induction properties. The chemical changes in the structure also induce changes in the thermodynamic properties of the interaction, indicating a higher hydrophilicity and also explaining their properties on p-Gp and β III overexpressing cells and on mutant cells.

The effect of the compounds on the microtubular network is different from those observed with the classical (docetaxel and paclitaxel) taxanes, inducing different bundling in cells with microtubules being very short, indicating a very fast nucleation effect and reflecting their high assembly induction power.

Introduction

One of the main problems in the chemotherapeutic treatment is the development of resistance to the compounds used. Although many tumours show a favourable response in the early stages of treatment, after exposure to the chemotherapeutic agents, the tumour often develops resistance to the treatment. Antitumour agents targeting tubulin have a great efficacy in the treatment of solid (paclitaxel, docetaxel, ixabepilone) or blood (vinblastine, eribulin) cancers,¹⁻³ however, their highly systemic toxicity compromises the treatments based on these agents. Nevertheless, these compounds have also an advantage over the kinase inhibitors.⁴⁻⁵ Since they block tubulin which is involved in several essential cell functions and is a major constitutive protein, there is no possible alternative pathway to substitute the functions of tubulin (the main reason for the development of resistance against kinase inhibitors⁶⁻⁷). Thus, resistance through alternative pathways is not possible in the case of these compounds.

Resistance to antitumoral agents targeting tubulin is possible through three different mechanisms:⁸ (a) overexpression of membrane pumps, (b) mutations in the ligand binding site and (c) overexpression of tubulin isotypes less sensitive to the drug employed.

One of the membrane pumps usually involved in resistance to taxanes is p-Glycoprotein (p-Gp), a protein involved in detoxification mechanism. It is assumed that its major role is to restrict the entrance of toxics through the gastrointestinal track or to prevent their access from blood stream to fetus and sensitive organs such as brain and testis.⁹ In cells overexpressing p-Gp, two opposite mechanisms control the intercellular concentration of drug, i.e., p-Gp decreases the internal concentration of a drug to reduce its effective cytotoxicity, but on the other hand, the target protein binds to a drug with high affinity to accumulate the drug inside the cell. The effectiveness of these resistance

mechanisms depends on the relative affinity of a drug to p-Gp and the target. An increased affinity to the target or a decreased affinity to p-Gp will overcome p-Gp-mediated resistance.¹⁰

The effect of variations in the affinity of taxanes on the resistance level of p-Gp overexpressing cells have been previously studied in our group and reported.¹⁰⁻¹¹ These studies have shown that very high affinity taxanes (chitax-40) are able to overcome p-Gp-mediated resistance. The same effect has been observed for other compounds which bind covalently to tubulin.¹²⁻¹⁴

Second-generation taxanes with modifications at C2, C3'N and C10 positions have also been shown to be extremely effective against cells resistant to taxanes by p-Gp overexpression.¹⁵⁻¹⁷ Although there were previous studies on the relation of changes in the taxane structure with their biological activities,¹⁸⁻¹⁹ measurements of the microtubule-taxane binding affinities have been performed only recently, which allows a rigorous examination of the relationship between binding affinity and cytotoxicity in drug-sensitive cells and drug-resistant cells overexpressing p-Gp.^{10,20-21} The study of the relationship between binding affinity and cytotoxicity (Figure 5) by Matesanz et al. in 2008¹⁰ indicates that there are factors that modulate the cytotoxicity in cells although both variables are related, i.e., there are compounds showing cytotoxicities on sensitive cells higher than expected from their binding affinity (paclitaxel, docetaxel, chitax-18), while some compounds exhibit higher cytotoxicity on resistant cells than those predicted from their binding affinity (chitax-19, chitax-21, chitax-35).

The second way that tumour cells resists paclitaxel and its derivatives is by changing their tubulin. Although it is difficult for human cells to mutate in response to a chemical agent, it is possible for them to use the genetic diversity of the tubulin isotypes to reduce its sensitivity to certain compounds. Most vertebrate isotypes fall into six

categories, i.e., β I, β II, β III, β IV, β V and β VI,²² with most of the differences in the clusters 124-129 and 237-240, as well as the C-terminal sequence. Although all 6 isotypes are able to carry out two of the three canonical functions of microtubules (forming the mitotic spindle and the interphase network), the strong conservation of the tubulin isotypes implies that they should have specific individual functions. From these isotypes, β III is the one mostly involved in resistance to chemotherapy. β III differs from the other main tubulin isotypes, β I, β II, and β IV, with the changes S126C and C241S, and in the presence of a lysine and a phosphorylated serine at the C-terminal.²³

β II, β III, β IV isotypes have been isolated from bovine brain²⁴ and it was found that the properties of the $\alpha\beta$ III dimer differs significantly from the other two in the binding of several microtubule modulating agents.²⁵ It was also found that the $\alpha\beta$ III microtubules are less prone to inhibition of their dynamic behavior by docetaxel.²⁶ Cells overexpressing β III tubulin are resistant to paclitaxel,²⁷ and it is known that high levels of β III tubulin are found in docetaxel-resistant tumors. Thus, overexpression of β III tubulin is an important prognosticator for the development of drug resistance.²⁸⁻³¹

There are different hypotheses to explain the molecular basis of paclitaxel-resistance due to overexpression of β III tubulin. Two plausible explanations are: (a) $\alpha\beta$ III dimers are the ones that are most dynamic,²⁶ so that the cell compensates for the decrease in dynamics due to microtubule-stabilising agents by increasing the amount of these dimers, and (b) there are small but critical differences in the pore type I site (the initial external site for the binding of microtubule-stabilising agents on their way to the luminal site) between β I and β III tubulins.^{12,32} Some difference in the pore type site I should result in a lower binding affinity to the initial pore site and then much lower internalization rate towards the inner site³³, which may cause weakening of microtubule stabilisation.

Finally, it has been observed that mutations in β -tubulin rapidly appear at the paclitaxel or laulimalide binding sites³⁴⁻³⁶ in cell cultures exposed to microtubule-stabilizing agents, which suggests that a mutational mechanism of resistance is possible. However, despite early reports on the relevance of tubulin mutations in patients with non-small cell lung carcinoma,³⁷ sequencing of tumors does not show any evidence that β -tubulin mutations could play a major role in the resistance of tumors to taxanes.³⁸⁻⁴⁴ Nevertheless, a few mutations^{41, 45-46} have been found to result in effective resistance of the mutated cells to paclitaxel.⁴⁷ This finding indicates that these mutations can play an effective role in some specific cases and thus drugs that are effective against tumors mutated in the β -tubulin gene could be of clinical interest.

In this paper we investigate the reasons for the effectiveness of a series of modified taxanes previously reported to be highly effective on p-Gp-overexpressing cells^{15,16,17} by performing complete characterizations of their cellular effects, as well as their interaction with microtubules. These characterizations include the assessment of the NMR, thermodynamic and biochemical parameters of the microtubule assembly, and the analysis of the structural effects of these taxanes on microtubules using small angle X-ray scattering.

Materials and Methods

Ligands and chemicals

Chitax-4 and chitax-40 were synthesized as previously reported.¹⁰ SB-T-1102, SB-T-1214, SB-T-12854 and SB-T-121303 were synthesized using the methods previously reported.¹⁵⁻¹⁷ Flutax-2 was synthesized as previously described.⁴⁸ Epothilone B was a kind gift from Prof. Karl-Heinz Altmann (ETH, Zürich). The rest of the chemicals employed were commercially available with analytical grade or better.

Human cell methods.

Cytotoxicity evaluation was performed with A2780, A2780AD, (overexpressing p-Gp)⁴⁹, PTX10, PTX22, A8 (A2780-derived)⁵⁰ human ovarian carcinoma cell lines, HeLa and HeLa β III transfected cells⁵¹ with the MTT assay modified as previously described.¹¹ Indirect immunofluorescence was performed in A549 cells⁵² that had been cultured overnight in 12 mm round coverslips and incubated further for 24 h in the absence (drug vehicle: DMSO) or in the presence of different ligand concentrations. Attached cells were permeabilized with Triton X100 and fixed with 3.7% formaldehyde. Microtubules were specifically stained with DM1A α -tubulin monoclonal antibodies and DNA with Hoechst 33342 as previously described.⁵³ The preparations were examined using a Zeiss axioplan epifluorescence microscope and the images were recorded in a Hamamatsu 4742-95 cooled CCD camera. Cell cycle analysis was performed as previously described.⁵⁴

Tubulin preparation and tubulin assembly induction measurements.

Calf brain tubulin was purified as described in the literature.⁵⁵ Glutaraldehyde-stabilized microtubules with active paclitaxel binding sites were prepared as described.³² Critical tubulin concentrations of tubulin in PEDTA4-GTP (10 mM sodium phosphate, 1 mM EDTA, 4 mM MgCl₂, 1 mM GTP, pH 6.7) in the presence of ligands were measured as previously described.⁵⁴ The polymers formed were adsorbed onto formvar/carbon coated copper grids, stained with 1% uranyl acetate, and observed with a JEOL 1230 electron microscope operated at 100 kV and equipped with a Gatan Bioscan 702 CCD camera.

Binding of taxanes to unassembled tubulin and microtubules.

The binding constants of SB-T-1102, SB-T-1214 and SB-T-12854 were measured as previously described.⁵⁶ To measure the binding affinity of SB-T-121303, 1 mL samples of 1 μ M glutaraldehyde-stabilized MTs, 1.1 μ M Epothilone B and 1.1 μ M of SB-T-121303 in GAB (glycerol assembly buffer; 3.4 M glycerol, 10 mM sodium phosphate, 6 mM MgCl_2 , and 1 mM EGTA pH 6.5) with 0.1 mM GTP were incubated for 30 min at the desired temperature in polycarbonate centrifuge tubes (Beckman Coulter, Inc., Fullerton, CA). The samples were then centrifuged at 90,000 g for 20 min at the desired temperature in a TLA-100.2 rotor employing a Beckman Optima TLX ultracentrifuge.

The supernatants were collected by pipetting, and the pellets were resuspended in 10 mM phosphate buffer (pH 7.0). One micromolar paclitaxel was added as the internal standard. Both the pellets and the supernatants were extracted three times with an excess volume of dichloromethane, dried in vacuo, and dissolved in 30 μ L of methanol. The samples were analyzed by HPLC. Binding constants for compounds reversibly displacing flutax-2 or epothilone B were calculated using Equigra v5⁵⁷. The thermodynamic parameters (apparent ΔG_0 , ΔH_0 , and ΔS_0) were calculated as previously described.⁵⁴

The binding of the compounds to unassembled tubulin was measured by ultracentrifugation. Samples (200 μ L) containing 50 μ M tubulin and 50 μ M compound in 10 mM Sodium Phosphate and 0.1 mM GTP at pH 7.0 were incubated for 20 min at 25°C, centrifuged at 100,000 rpm for 2 h at 25 °C in a TLA100 rotor, and the upper half and lower half of the solution as well as pellet were separated. Then, 25 μ L of each sample was taken, diluted to 1:20 with 10 mM sodium phosphate 1% SDS at pH 7.0, and the tubulin concentration in each part of the tube was measured by absorbance at

275 nm.⁵⁸ Paclitaxel (5 μ M, internal standard) was added to the rest of the samples and the samples were extracted three times with 1 volume of dichloromethane, dried, redissolved in 30 μ L methanol/water, and analyzed with an Agilent 1100 series HPLC, employing a ZORBAX Eclipse XDB-C18 column and acetonitrile/water gradient from 50 to 80% at 1 mL/min in 30 min.

Preparation of NMR samples.

SB-T-12854 (300 μ M) was added to a 20 μ M solution of tubulin in 10 mM KPi with 0.1 mM GMPCPP and 6 mM MgCl_2 at pH 6.7 (effective measurement of a pH meter in the D_2O buffer) prepared as previously described.⁵⁹

NMR Experiments

NMR spectra were recorded at 310 K (polymeric tubulin samples) in D_2O on a Bruker AVANCE 500 MHz spectrometer as previously described.⁵⁹ STD and TR-NOE experiments were performed also as previously described⁵⁹.

Conformational search and docking of ligands.

The paclitaxel derivative structures were built from the paclitaxel template based on electron crystallography and NMR TR-NOESY experiments.⁶⁰ The internal binding site structure was taken from 1JFF,⁶¹ while the external binding site used the one previously reported by us.⁶⁰ The β III binding site was constructed by homology modeling from 1JFF. SB-T-1102, SB-T-1214, SB-T-12854, SB-T-121303 and paclitaxel were docked in both the internal and external sites of microtubules as previously described.⁶⁰ Structures located in the internal site were minimized using Macromodel 9.6, with several steps of Polak-Ribière conjugate gradient (PRCG) until

the energy gradient became lower than $0.005 \text{ kJ } \text{\AA}^{-1} \text{ mol}$. The poses in the external binding site of microtubules were refined by using molecular dynamics (MD) simulations performed with AMBER 11 (500ps equilibration time and 1ns acquisition time). For each compound, 100 structures were saved along the last nanosecond of the MD trajectory.

The expected STD effects of SB-T-12854 bound to microtubules were calculated using the CORCEMA-ST program.⁶² For these full relaxation matrix calculations, the overall rotational motion correlation time τ_c for the free state was always set to 0.75 ns (values between 0.25 and 1 ns were tested), since NOESY cross peaks for the free molecule were basically zero at room temperature and 500 MHz. The τ_c value for the bound state was set to 60 ns for non-polymerized α/β -heterodimers (τ_c estimated with HYDROPRO⁶³) and 100 ns for microtubules (optimized in the CORCEMA-ST calculation). An order parameter, $S^2 = 0.85$, was employed to account for the fast rotation of the methyl groups, as implemented in CORCEMA-ST.

In order to fit the experimental STD effects and TR-NOE intensities, off-rate constants between $5\text{-}100,000 \text{ s}^{-1}$ were tested. Optimal agreement was achieved using $k_{\text{off}} = 100 \text{ s}^{-1}$. Conformational search calculations were performed using the MacroModel/Batchmin⁶⁴ package (version 9.6) and the OPLS2005 all-atom force field as previously described.⁵⁹

Docking of the ligand was performed using the AutoDock 4.0 program,⁶⁵ employing the additional CORCEMA-ST⁶⁶-based scoring function as previously illustrated.⁵⁹ The predicted STD values of each structure were obtained with CORCEMA-ST and the structure of lowest energy value of these 100 structures was considered for comparison against the experimental data.

X-ray scattering measurements.

Tubulin was equilibrated in 10 mM sodium phosphate buffer with 1 mM EDTA and 0.1 mM GTP at pH 7.0 through a Sephadex G-25 medium column (25x9 mm), and the protein was centrifuged for 20 min at 90,000xg in a TLA120.2 rotor in a Optima TLX centrifuge (Beckman) to remove aggregates. The tubulin concentration was then determined spectrophotometrically as previously described.⁵⁸ MgCl₂ (7 mM) and up to 1 mM GTP were added to the sample (final pH 6.7) and the desired ligand or DMSO (vehicle) in a 10% stoichiometric excess over the protein concentration was added. The samples were incubated for 20 min at 37 °C and kept at 25 °C before recording the scattering patterns.

SAXS data collection was performed on a Bruker NANOSTAR system coupled to an X ray tube ($\lambda = 1.54\text{\AA}$), wherein the camera was set to cover the scattering vector range, defined as reciprocal Bragg spacing i.e. $s = 2 \sin \theta / \lambda$, from 0.013 to 0.22 nm⁻¹. Calibration of the scattering vector was obtained by reference to the orders of diffraction of silver behenate.⁶⁷ The temperature of the samples was set to 25 °C and the X-Ray scattering profile was recorded for 2 h in 30-min frames with a Bruker Hi-Star multiwire area detector. Data analysis was performed using the Bruker software provided or the FIT2D software package provided by ESRF (<http://www.esrf.eu/computing/scientific/FIT2D/>). Raw data were normalized for the incoming intensity and detector response before averaging and subtracting the buffer scattering pattern. Time frames in which the data significantly differed from the original pattern due to radiation damage were removed before averaging.

The low angle X-ray scattering pattern of microtubules in solution can be described as the Fourier transform of a hollow cylinder. To a first approximation, the

intensity is given by the zero'th order Bessel function $|J_0(qR)|^2$, q being the scattering

angle and R , the cylinder radius.⁶⁸ The position of the first scattering maximum is therefore a sensitive measure for the radius of the microtubule, via relation $J_{01} = (1.22/2R)$, since these are not distorted in a solution scattering pattern due to overlap between the higher order diffraction maxima of the helical lattice⁶⁹⁻⁷⁰

The differences in diameter between microtubules assembled in the presence of different taxanes can be interpreted, to a good approximation, as changes in the average number of protofilaments making up the cylinder wall.

RESULTS

Cell biology

In order to have a rigorous comparison between the cytotoxicity of the compounds and those previously determined,¹⁰ the compounds were tested for their cytotoxicity under the same conditions against the same cell lines (Table I). The compounds showed a very good activity on A2780AD cells (resistant to paclitaxel by the MDR mechanism), hence confirming all previous results.¹⁵⁻¹⁷

Then, we wanted to know if the compounds were also effective in cells resistant to paclitaxel by other mechanisms (mutations in the β -tubulin binding site and overexpression of β III-tubulin isotype). The compounds showed a very good activity in cells overexpressing β III-tubulin isotype (Table II). While β III-tubulin transfected HeLa cells have a resistance index of 11 against paclitaxel, the resistance index is decreased to 2.5 for SB-T-1102 and to 5.3 for SB-T-1214. Moreover the modified cells are neither resistant to SB-T-12854 (R/S 1.4) nor to SB-T-121303 (R/S 0.8). A similar result was obtained for the toxicity of the compounds in cells mutated in the paclitaxel binding site. PTX10 cells and PTX22 cells are A2780 cells resistant to paclitaxel through mutations in the paclitaxel binding site F272V and A366T, while A8 are resistant to

epothilone through another mutation T276I (involved in the interaction with the thiazole side chain of epothilone⁷¹) but not to paclitaxel.⁵⁰ While PTX10 and PTX22 are resistant to paclitaxel with resistance indexes of 20.8 and 8.4, respectively, they are sensitive to all compounds assayed (Table III).

The results are fully compatible with those previously published for p-Gp-overexpressing cells and indicate that these taxanes are able to overcome resistance by all known mechanisms. It is worth mentioning that the compounds do not have a large increase in cytotoxicity as compared to paclitaxel (in fact they are less cytotoxic than paclitaxel in the A2780 and Hela non-resistant cells). This is compatible with previously reported results,¹⁰ which indicate that a minimal amount of ligand molecules should be bound to the cellular microtubules to produce the cytotoxic effect. Thus, the drug concentration should be over a certain minimum which is the minimal IC₅₀ that can be measured with the method employed. As shown in Figure 5A of Matesanz et al.,¹⁰ the maximum cytotoxicity in A2780 cells possible for taxanes under the condition of the experiment is in the order of nM.

We also studied the effects of the compounds in the cell cytoskeleton and in the cell cycle (Figure 2) to compare them with paclitaxel and docetaxel. The immunofluorescence experiments indicate a differential effect of the SB-T taxanes, chitax-4 and chitax-40. Although all compounds arrest the cell cycle at G2/M (Figure 2, lower panel), the morphology of the changes induced in the cytoskeleton is different from those induced by paclitaxel. (*Note:* SB compounds, chitax-4 and chitax-40 cause the cell cycle arrest at similar concentrations for A2780 and A2780AD cells, which confirms the IC₅₀ results.) While paclitaxel (Figures 2C, 2E and 2G) induces the typical MSA bundles of microtubules which accumulate close to the membrane and the

nucleus, the SB-T compounds, chitax-4 and chitax-40 induce micronucleated cells with much shorter microtubule bundles distributed all over the cytoplasm.

Effects on tubulin assembly.

To seek the reasons for the high activity of the compounds in cells resistant to classical taxanes, we studied the biochemistry of their interactions with their target tubulin. It has been shown that SB-T-1102, SB-T-1214, SB-T-121303 and SB-T-12854 are extremely effective in increasing the rate of microtubule assembly. In the presence of these taxoids the assembly rate (even in the absence of GTP in the media) is dramatically greater than that in the presence of paclitaxel or GTP.¹⁶⁻¹⁷ Also, the assembly proceeds without an appreciable induction period and is complete within 2 min. Since the assembly induction in this work¹⁶⁻¹⁷ was monitored by turbidity and this technique was affected by the morphology of the polymers, we wanted to know if the effect of these compounds on the microtubule assembly was limited to an increase in the rate of the assembly or if it also increased the mass of polymer formed. Thus, we tested the critical concentrations of tubulin required for assembly induction in the presence of the compounds using centrifugation and quantification of the polymer formed. All ligands induced tubulin assembly with much higher potency than paclitaxel and docetaxel, indicating that not only the rate of assembly was faster but also more polymer was formed (Table IV).

Binding constant and thermodynamic parameters of the binding of the compounds to microtubules and dimeric tubulin.

In order to correlate the observed cytotoxic effect of the compounds with their affinity constant to microtubules, the binding constants of SB-T-1102, SB-T-1214 and

SB-T-12854 at different temperatures were measured using the flutax-2 displacement method^{54,56} (Table V). Since the binding constant of SB-T-121303 exceeded the sensitivity of the flutax-2 displacement test,⁵⁷ its affinity constant was obtained using the epoB displacement method¹⁰ (Table V).

Using the binding affinity data, the thermodynamic parameters of the interaction, i.e., free energy of the binding (ΔG) and the enthalpy (ΔH) and entropy (ΔS) contributions to ΔG (Table VI), could be calculated.

The effect of single substituent changes on the binding affinity of the compounds could easily be quantified. The change at C13, which eliminated the aryl group at position C3' and introduced an isobutyl moiety resulted in no change in the binding affinity, i.e., SB-T1102 ($2.4 \pm 0.3 \times 10^7 \text{ M}^{-1}$) was found to have nearly the same binding constant at 35°C as that of paclitaxel ($1.43 \pm 0.17 \times 10^7 \text{ M}^{-1}$) and docetaxel ($3.93 \pm 0.27 \times 10^7 \text{ M}^{-1}$) ($\Delta\Delta G \text{ SB-T1102:paclitaxel} = -1.4 \text{ kJ mol}^{-1}$; $\Delta\Delta G \text{ SB-T1102:docetaxel} = +1.3 \text{ kJ mol}^{-1}$). SB-T-1102 behaves thermodynamically in a manner similar to the other reversibly binding taxanes, wherein its binding affinity decreases with increase in temperature. However, the decrease in its binding affinity with increase in temperature is much less marked than other taxanes. The result indicates that its binding is much less exothermic with a large decrease in the enthalpy of binding ($\Delta H_{\text{SB-T1102}} = -8 \pm 3 \text{ kJ mol}^{-1}$ vs. $-42.1 \pm 0.3 \text{ kJ mol}^{-1}$ for paclitaxel and $-44.8 \pm 0.2 \text{ kJ mol}^{-1}$ for docetaxel), but this decrease in the enthalpy of binding is compensated by a substantial increase in the entropy of binding ($\Delta S_{\text{SB-T1102}} = -115 \pm 11 \text{ kJ mol}^{-1} \text{ K}^{-1}$ vs. $-29 \pm 13 \text{ kJ mol}^{-1} \text{ K}^{-1}$ for paclitaxel and $-26 \pm 8 \text{ kJ mol}^{-1} \text{ K}^{-1}$ for docetaxel), which suggests significant differences in the binding mechanism.

Since SB-T-1214 contains two structural differences as compared to SB-T1102 and docetaxel, it is not possible to directly compare the effect of the introduction of the

double bond in the 2-methylpropenyl moiety at C3' on the binding affinity with that of an isobutyl or phenyl moiety. However, the effect is positive on the binding affinity, i.e., SB-T-1214 ($8\pm3\times10^7\text{ M}^{-1}$) has twice the binding affinity of docetaxel, three times that of SB-T-1102 and five times that of paclitaxel at 35 °C ($\Delta\Delta G_{\text{SB-T-1214:docetaxel}} = -1.8\text{ kJ mol}^{-1}$; $\Delta\Delta G_{\text{SB-T-1214:SB-T-1102}} = -3.1\text{ kJ mol}^{-1}$). Given the contribution of the substituents in position C10 (lower than 2 kJ mol^{-1}),¹⁰ it is not possible to distinguish which one of the modifications, i.e., the 2-methylpropenyl moiety at C13 or the cyclopropanecarbonyl moiety at C10, is responsible for the increase in the binding affinity. The comparison of SB-T-1102 and SB-T-1214 with compounds with single point modifications,¹⁵ indicates that, for both of them, the cytotoxicity decreases when the double bond is reduced and when the acetyl group is replaced by a cyclopropanecarbonyl group, which suggests that the favorable contribution to the binding arises from the 2-methylpropenyl group. Interestingly, the binding profile of SB-T-1214 is substantially different as compared with SB-T1102. The binding process becomes more endothermic similar to classical taxanes ($\Delta H_{\text{SB-T1214}} = -32\pm2\text{ kJ mol}^{-1}$ vs. $-8\pm3\text{ kJ mol}^{-1}$ for SB-T1102; $-42.1\pm0.3\text{ kJ mol}^{-1}$ for paclitaxel; $-44.8\pm0.2\text{ kJ mol}^{-1}$ for docetaxel), while keeping a strong entropy contribution ($\Delta S_{\text{SB-T1214}} = 47\pm6$ vs. $115\pm11\text{ kJ mol}^{-1}\text{ K}^{-1}$ for SB-T1102; $-29\pm13\text{ kJ mol}^{-1}\text{ K}^{-1}$ for paclitaxel; $-26\pm8\text{ kJ mol}^{-1}\text{ K}^{-1}$ for docetaxel).

The further introduction of fluorines or a double bond does not significantly modify the binding parameters of taxanes. Compound SB-T-12854¹⁷ binds to microtubules in a manner very similar to SB-T-1214 ($\Delta\Delta G_{\text{SB-T-12854:SB-T-1214}} = -0.5\text{ kJ mol}^{-1}$, $\Delta H_{\text{SB-T12854}} -28\pm3\text{ kJ mol}^{-1}$, $\Delta S_{\text{SB-T12854}} 64\pm10\text{ kJ mol}^{-1}\text{ K}^{-1}$), indicating a weak influence of the fluorine atoms on the binding.

Finally, the highest affinity compound, SB-T-121303,¹⁶ was studied. This compound closely resembles chitax-40 with a high activity with MDR cells¹⁰. This taxane has a methoxy group at the *meta* position of the C-2 benzoate instead of an azido group in chitax-40 and 2-methylpropenyl in the C13 side chain instead of a phenyl group in chitax-40. Concomitantly its thermodynamic profile of binding to tubulin is nearly identical to that of chitax-40, i.e., $\Delta H_{\text{SB-T121303}} = -33 \pm 2 \text{ kJ mol}^{-1}$ vs. $-26 \pm 24 \text{ kJ mol}^{-1}$ for chitax-40; $\Delta S_{\text{SB-T121303}} = 77 \pm 7$ vs. $99 \pm 80 \text{ kJ mol}^{-1} \text{ K}^{-1}$ for chitax-40).

It is difficult to know the exact contribution of the structural changes on the binding affinity because there are at least two differences between the compounds and any other taxanes analyzed, but if we consider the differences in binding energy to the paclitaxel site, the effect of the modifications at C10 appears to be negligible.¹⁰ The observed effects of structural changes on binding affinity can be quantified as follows: (i) 3'-Aryl $\rightarrow$ 3'-isobutyl = $+1.3 \text{ kJ mol}^{-1}$; (ii) introduction of the double bond is slightly favorable in terms of binding based on 3'-Aryl $\rightarrow$ 3'-(2-methylpropenyl) = -1.8 kJ mol^{-1} (docetaxel $\rightarrow$ SB-T-1214); (iii) further introduction of a $-\text{OCH}_3$ group at the *meta* position of the C2 benzoyl group results in a large increase of the free energy of binding (nearly -10 kJ mol^{-1}), which arises from an entropic contribution similar to that described previously¹⁰ for the introduction of a methoxy or azide substituent at this position; (iv) introduction of fluorines does not have any appreciable effect on binding affinity, i.e., 3'-(2-methylpropenyl) $\rightarrow$ 3'-(2-difluorovinyl) = -0.5 kJ mol^{-1} (SB-T-1214 $\rightarrow$ SB-T-12854).

In order to examine if the fast nucleation effect may come from higher affinity of the compounds to unassembled tubulin, the binding of chitax-4, chitax-40, SB-T-1102, SB-T-1214 and SB-T-12854 to dimeric tubulin was studied. Under the conditions of the study (50 μM tubulin and 50 μM compound), we were unable to detect

accumulation of taxanes in the lower part of the centrifuge tube, which indicates that their binding constants to dimeric tubulin are lower than 10^3 M^{-1} , as in the case of classical taxanes.⁷²

NMR studies.

The bound conformation of the highly active C3'-substituted family of taxanes was studied, employing SB-T-12854. STD-NMR experiments are widely employed to detect ligand binding to protein receptors and to characterize the ligand binding epitope, while TR-NOESY experiments are used to assess the conformational features of the compound bound to microtubules (it is worth mentioning that these techniques require particularly fast kinetic conditions of the interaction. Thus, very slow dissociating ligands cannot be studied with these techniques).⁷³⁻⁷⁴ Both the TR-NOESY spectra of the compound bound to microtubules which would provide the bioactive conformation (Figure 3A) and the STD spectra which would provide the binding epitope (Figure 3B) were obtained, although the signals obtained were of low intensity, as expected from a compound with a high affinity to the target and thus with slow dissociation kinetics.

The TR-NOESY data showed clearly cross peaks between the benzoyl protons at *ortho* position with respect to the ester group and the *tert*-butyl protons of the side chain, indicating that the bound conformation is essentially identical to that previously determined for docetaxel¹⁰ and thus this conformation could be used to build the structures of the taxanes examined.

NMR-guided modeling of the bioactive conformations.

The STD and TR-NOESY data were then employed to derive a detailed 3D model for the tubulin-bound conformations of the ligands. Although the intensities of

the cross peaks were weak, the STD information obtained allowed us to model SB-T-12854 into the pore binding site as previously described.⁵⁹ NRMSD of the STD fitting was 14.4%, which is excellent and indicates that the compound fits in the pore binding site in the same way as docetaxel does (Figure 4A).

Considering that the conformation and fitting in the pore binding site are identical to those described for docetaxel, it is reasonable to hypothesize that the interaction of the compounds with tubulin may be essentially identical to that previously described.⁵⁹ The taxanes in this study were thus docked into the binding site and the interaction was optimized (Figures 4B-E) in order to seek the reasons for their increased assembly induction power, which was the main cellular and biochemical difference observed. Recent crystallographic data⁷¹ indicate that microtubule-stabilizing agents of the paclitaxel site induce microtubule assembly using an allosteric mechanism. They bind to the paclitaxel binding site, inducing a strong stabilization of the S7-H9 loop (M-loop). This loop is involved in contacts with the H2-S3 and the S2-S2' loops of the adjacent subunit, and this stabilization results in stronger lateral contacts between adjacent subunits, leading to microtubule stabilization.

From the comparison of potency of the compounds in assembly induction, the compound with an acetyl group at C10 (paclitaxel) exhibits the weakest assembly induction power (higher Cr) of 4.2 μM . SB-T1102, the compound with the acetyl group at this C10 position, also shows the worst Cr (1.6 μM) in the series of taxanes examined in this study. The compound with fluorines, SB-T-12854, exhibits the strongest assembly induction power with Cr 0.3 μM .

Effect on microtubule structure.

In order to examine the effect of the compounds on microtubule structure, the

diameter of the ligand-induced microtubules was measured using SAXS.^{60, 69-70} The size of all induced microtubules (Figure 5) were between those of paclitaxel and docetaxel, with an average number of protofilaments ranging between 13.2 (SB-T-1214) and 13.9 (SB-T-12854) (Table VII).

In a previous paper⁶⁰ we reported that the diameter of taxane-induced microtubules was dominated by the substituents at C7 and C10. We have observed that bulky groups at C7 increase the number of protofilaments of microtubules, while bulky groups at C10 decrease the number of protofilaments. However, modifications at C13 have little or no effect on microtubule structure, while the introduction of an azide group at the *meta* position of the phenyl ring at C2 slightly increases the microtubule diameter.

All the compounds examined have small substituents at C10 and subsequently induce microtubules with smaller diameter than that of docetaxel, which does not have substituents at C7 and C10. Also, as expected from the introduction of the methoxy group at C2, SB-T-121303 induced the microtubules with the largest diameter observed. However, none of the taxanes studied induced a major modification in the structure of the microtubules, which indicates that the effect on the structure is not the cause of the higher activity observed. Nevertheless, the fact that the changes in the structures reflect the same pattern as those previously reported¹⁰ reinforces the hypothesis that the interaction of the compounds with the binding site is essentially the same as that of other taxanes.

Discussion

We have studied the differences in the cellular effects and the interaction with microtubules for a series of taxanes highly active in MDR cells, with the intention to

unveil the characteristics that result in the ability to overcome all known mechanisms employed by tumour cells to resist taxanes (p-Glycoprotein-mediated multidrug resistance, mutations in the taxane binding pocket at β -tubulin, and β III tubulin isotype overexpression).

Celular and biochemical differential effects of the SB-T compounds. Structural basis for the higher assembly induction power.

Three biochemical and biophysical parameters for the interaction of the compounds with microtubules, i.e., (a) their binding affinity for the paclitaxel site, (b) their potency for inducing tubulin assembly under conditions in which tubulin is not able to assemble by itself, and c) their effect on the structure of the microtubules, have been characterized with the intention to find out which one of these parameters explains the activity of the compounds in resistant cell lines.

The main difference in the cellular effects is the morphological changes induced in the cytoskeleton which are different from those induced by paclitaxel. While paclitaxel (Figures 2C, 2E and 2G) induces the typical MSA bundles of microtubules which accumulate close to the membrane and the nucleus, the compounds studied induce much shorter microtubule bundles distributed all over the cytoplasm. These differences in morphology suggest a much faster and intense assembly induction by the taxanes examined.

Concerning the biochemical parameters of the taxane-site interaction, the two main significant differences observed are the higher assembly induction power as compared with classical and other high affinity taxanes (as expected from the cellular results), and the higher entropic contribution to the free energy of binding.

From the comparison of the entropy changes of taxane-binding previously obtained,¹⁰ it has been found that the entropy change in the binding process is determined by two parameters, i.e., the hydrophobicity of the ligand and the molecular volume which determines the entropy of the bound ligand. When the volume of a certain side chain is decreased and the functional group incorporated has more sp^3 carbons, the conformational freedom of the side chain increases which leads to the increase in the entropy of the bound species. This is the case for all the SB-T compounds examined, in which the phenyl moiety at the C13 side chain has been replaced with an aliphatic side chain, which justifies the higher entropic contribution to the free energy of binding.

The comparison of the binding affinities of the taxanes assayed with the observed assembly induction power (Cr) shows that all the SB-T compounds assayed induce tubulin assembly much more effectively than paclitaxel and docetaxel as well as the high-affinity taxanes, chitax-4 and chitax-40. Since the main biochemical effect observed is a large increase in assembly induction power, we examined the NMR-directed models to seek the structural reason for the high assembly induction power.

Recent structural data have shown that the main structural effect of taxane-binding site ligands (zampanolide and epothilone)⁷¹ is the strong structural stabilization of the S7-H9 loop (M-loop). This loop is disordered in the absence of ligand and becomes structured in the presence of ligands. The models indicate that the conformation and binding mode of the high potency taxanes remain unchanged (Figure 4 A-F), and thus only minor changes in the H-bonding and conformation in the binding pocket would occur. The main difference between the structure of the binding site in the zampanolide- or epothilone-bound tubulin⁷¹ and the model of paclitaxel-bound tubulin in the luminal site (Figure 4b) is the formation of an H-bond between the carboxyl

group at C10 and R284. In the crystal structure determined by Prota et al.⁷¹ (4I4T), this residue is involved in interactions with the H9 loop, stabilizing the structure of the M-loop. Although there is not yet any crystallographic structure of tubulin with a taxane molecule in the binding site, it is logical to assume that the main structural effect of the taxanes would be the stabilization of the M-loop and that the differences in assembly induction power should be related to differential influences on the M-loop.

The main chemical differences between paclitaxel and the ligands studied are related to the C13 side chain and the C10 moiety. From the ligands studied, SB-T-1102 is the one with the lowest affinity (similar to paclitaxel) and also with the lowest assembly induction power (although still significantly higher than that of paclitaxel). In this case, the structural differences with paclitaxel are in the C13 side chain which has aliphatic groups instead of aryl moieties, while the group at C10 is an acetyl group that is same as that in paclitaxel. The main visible difference in the binding site is the slight rotation of the ligand which disrupts the H-bond with R284, inducing a different conformation to the M-loop. The same effect is observed in the more powerful ligands, SB-T-1214 (Figure 4C) and SB-T-12854 (Figure 4D), in which a bulkier substituent at C10 points toward the luminal space of the microtubule. The last ligand, SB-T-121303 (Figure 4F), has the highest binding affinity, but is not the one with the highest assembly induction power. In this case, the binding pose is dominated by the interaction of the methoxy group at the *meta* position of the benzoyl group at C2 with H229, as in the case for chitax-40,⁶⁰ wherein the interaction with R284 is also formed, but the M-loop has the same conformation as that in the presence of paclitaxel.

From the data available, we propose that the enhancement of the tubulin assembly power is caused by (i) the different interactions of the compounds with the M-loop, resulting in higher potencies in the absence of the interaction with R284, and (ii)

by the higher binding affinity to the site (in the case of SB-T-121303), as observed in the cases for other MSAs (Figure 4).⁵⁴ The compounds that do not interact with R284 (SB-T-1102, SB-T-1214 and SB-T-12854) have much higher assembly induction potency than the classical taxanes, as well as high-affinity taxanes (chitax-4 and chitax-40), although these compounds have affinities to the binding site similar to that of paclitaxel. SB-T-121303, with 50 times higher binding affinity by interacting with R284, is significantly less potent in inducing microtubule assembly than SB-T-12854.

Rationalization of the effect of the compounds on resistant cells.

How could these changes observed be related to their effect on resistant cells? From the view point of the p-Gp-mediated multidrug resistance, which is not tubulin mediated, the effect can only be ascribed to either an increase in affinity to tubulin (high affinity compounds or covalent binders to tubulin are able to overcome p-Gp-mediated MDR in tumoural cells^{10, 12, 75}) or to a decrease in affinity to the membrane efflux pump. The former cannot be the case, because the effects of SB-T-1102, SB-T-1214 and SB-T-12854 have similar or only slightly higher binding affinities than paclitaxel and docetaxel, except for the case of SB-T-121303, which has a very high affinity to tubulin due to the *meta*-methoxy group in the C2 benzoyl moiety.

Lower R/S ratios have been previously observed for other medium-affinity ($K_a = 10^7 \text{ M}^{-1}$) taxanes halogenated in the C2 benzoyl ring (chitax-30, 34 and 35: $K_a = 1.76 \times 10^7 \text{ M}^{-1}$, $1.20 \times 10^7 \text{ M}^{-1}$ and $0.88 \times 10^7 \text{ M}^{-1}$, respectively), and these compounds also have favorable entropic contributions to the free energy of binding, as observed in the cases of SB-T-1102, SB-T-1214, SB-T-12854 and SB-T-121303. Considering the structural changes in the substituents at positions C10 and C13, it is logical to propose that they should decrease the hydrophobicity of the compounds (as the introduction of

halogen atoms should do), this decrease in hydrophobicity may turn them into weaker substrates for p-Glycoprotein, which results in the same effect as that by the increase in the binding affinity for tubulin¹⁰ (see Supplemental Information). In this case, the binding affinity to tubulin is not modified because the lower entropic cost required to transfer a less hydrophobic ligand out of the water compensates the higher enthalpy cost for placing it into the hydrophobic binding pocket without affecting binding affinity to tubulin.

For β III-overexpressing cells, the effect has to be related to the binding affinity to the protein. The β III-overexpressing cells have been shown to be resistant to paclitaxel²⁷ and high levels of β III tubulin are found in docetaxel-resistant tumors.²⁸⁻³¹ However, there are no differences in the paclitaxel-binding site between β I (the major isotype) and β III, the only changes observed there are S227A and C241S close to the binding site. These changes should result in a global change in the binding site, i.e., slightly displacing the global volume of the binding site towards the east (Figure 6). This change is due to the loss of the H-bonds formed between S277 and S280, as well as Q281 and a small opening of the loop directly in contact with the α -subunit due to the C241S mutation. This increase in space affects the position C13 in particular with the phenyl ring being displaced 2.5 Å towards the east inside a larger binding pocket (Figure 6). This change should increase the desolvation energy for classical taxanes, decreasing their affinity. The effect can be compensated with an increase in affinity, since from the cytotoxicity data, high affinity compounds are able to overcome the resistance due to β III-overexpression (chitax-4 and chitax-40). However the SB-T compounds studied are more effective in these cells than the chitax compounds with much higher affinity. The reason for this should be the larger entropic freedom of the side chain, which increases the entropy of the bound state and compensates the increase

in desolvation energy. In fact, the compound with the highest conformational freedom in the C13 side chain, SB-T-1102, is more effective in β III-overexpressing cells than SB-T-1214 with lower conformational freedom.

The SB-T compounds are also effective in PTX10 and PTX22 cells which contain mutations in the paclitaxel binding site, β F272V and β A366T. The effectiveness of compounds in the cells with mutation F272V can also be ascribed to their smaller and more flexible C13 side chain. F272 is directly involved in the interaction with the C13 side chain (Figure 7). Thus, when it is changed to a smaller residue, the volume of the binding pocket (Figure 7 grey surfaces) increases by nearly 40 Å³, which leads to the increase in the desolvation energy of the binding. The smaller and flexible groups of the SB-T compounds at C13 compensate this effect by the entropy gain in the bound-state and thus it can overcome the resistance.

Finally, the molecular modeling also indicates that the resistance of cells containing the A366T mutation is due to their indirect effect on the M-loop. The mutation results in two new interactions in the binding site, i.e., T366 with β -M295 and β -F319, as well as a steric repulsion with A273. M295 is placed in the α -helix (H9), while A273 is in the β -sheet (S7) adjacent to the M-loop. Both new interactions should affect M-loop structure, decreasing the effect of paclitaxel on tubulin. Thus, we propose that the efficacy of the SB-T compounds in PTX-22 cells should be ascribed to their differential interaction with the M-loop, as well as their higher assembly induction power.

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Table I.-Cytotoxicity^a of the compounds employed in the study on the growth of nonresistant and resistant by p-Gp overexpression cells.

Compuestos	A2780 (nM)	A2780AD (nM)	R/S	MCF7 (nM)	MCF7-R (nM)	R/S
Paclitaxel	0.4±0.1	1244±164	3110	1.7 ^c	299 ^c	176 ^c
Docetaxel	0.6±0.2 ^b	290±16 ^b	483 ^b	1.0 ^c	235 ^c	235 ^c
Chitax-4	2.7±0.6 ^b	14.0±3.8 ^b	5.2 ^b	ND	ND	ND
Chitax-40	7.0±1.0 ^b	9.1±0.45 ^b	1.3 ^b	1.9±0.3 ^d	153±11.6 ^d	81 ^d
SB-T-1102	3.7±1	29.1±2.6	7.9	0.35 ^e	2.8 ^e	8 ^e
SB-T-1214	5.2±1.2	10.9±1.8	2.1	0.20 ^e	2.1 ^e	10.5 ^e
SB-T-12854	3.5±0.6	15.1±3	4.3	0.13 ^e	4.3 ^e	33 ^e
SB-T-121303	3.4±0.6	10.5±1.2	3	0.36 ^f	0.33 ^f	0.91 ^f

^aIC₅₀ values determined for this work (nM) are the mean ± SEM of three independent assays.

^bData from¹⁰

^cData from¹⁵

^dData from⁷⁶

^eData from¹⁷

^fData from¹⁶

Table II. Cytotoxicity of different SB-T taxanes on the growth of human cervical carcinoma cells HeLa and β III transfected cell line (wild-type β III) cells^a.

Compound	HeLa (nM)	HeLa transfected (nM)	R/S ^b
Paclitaxel	0.7±0.2	7.7±0.9	11
Chitax-4	3.2±1.9	10.25 ± 1.9	3.2
Chitax-40	9.2±0.3	16 ± 2	1.7
SB-T-1102	4.4±1.0	11.2±2.6	2.5
SB-T-1214	2.1±0.5	11.2±2.2	5.3
SB-T-12854	2.5±0.6	3.5±0.9	1.4
SB-T-121303	5.7±1.6	4.8±0.9	0.8

^aIC₅₀ values (nM) are the mean ± SEM of three independent assays.

^bThe relative resistance values were calculated dividing the IC₅₀ of the β III transfected cell line (wild-type β III) by the IC₅₀ of the parental HeLa cell line.

Table III. Cytotoxicity of SB-T taxanes on the growth of ovarian carcinoma cell lines sensitive (1A9) and resistant to paclitaxel (Ptx10 and Ptx22) and to epothilone B (A8) by tubulin mutations^a.

Compound	1A9	PTX10	R/S ^b	PTX22	R/S	A8	R/S
Taxol	3.9±1.3	81.4±5.4	20.8	32.8±1.9	8.4	4.2±0.7	1.1
Chitax-4	2.3±0.6	11.6±3.7	5	1.9±0.4	0.83	2±0.04	0.9
Chitax-40	3.1±0.7	2.4±0.5	0.4	12±1.41	1.9	3.8±0.6	1.2
SB-T-1102	2.4±1.4	1.75±0.9	0.7	4.95±0.35	2	11.5±1	4.7
SB-T-1214	5±1.7	10.8±1.4	2.2	7.9±2.3	1.6	12.2±0.5	2.4
SB-T-12854	3.2±0.4	5.25±0.05	1.6	7	2.2	4±0.8	1.2
SB-T-121303	3.3±1.15	2.9±0.76	0.9	4.9±0.8	1.5	4±0.2	1.2

^aIC₅₀ values of the ligands were determined in parental ovarian carcinoma cells 1A9 and the tubulin mutant cell lines ^bIC₅₀ values (nM) are the mean ± SEM of three independent assays.

^bThe relative resistance of the cell line was obtained by dividing the IC₅₀ of the resistant cell line by that of the parental 1A9 cell line.

Table IV- Free concentration of tubulin required for microtubule assembly in the presence and absence of the drugs (Critical concentration)

	Critical concentration (μM) of tubulin in PEDTA4
DMSO (vehicle)	> 200
Paclitaxel	4.2 ± 0.2^a
Docetaxel	2.4 ± 0.3^c
Chitax-4	2.9 ± 0.4^c
Chitax-40	3.6 ± 1.1^c
SB-T-1102	1.6 ± 0.5
SB-T-1214	0.9 ± 0.2
SB-T-12854	0.3 ± 0.1
SB-T-121303	0.6 ± 0.1

^aData from ⁵⁶

^bData from ⁵⁴

^cData from ¹⁰

Table V: Equilibrium constants of binding of compounds to the paclitaxel site in microtubules ($\times 10^7 \text{ M}^{-1}$)

Compound	26°C	27°C	30°C	32°C	35°C	37°C	40°C	42°C
Paclitaxel ^a	2.64±0.17	2.19±0.05	1.83±0.09	1.81±0.21	1.43±0.17	1.07±0.11	0.96±0.14	0.94±0.23
Docetaxel ^a	6.95±0.42	6.57±0.52	5.42±0.42	4.89±0.38	3.93±0.27	3.09±0.22	2.89±0.17	2.38±0.11
Chitax-4	124±39 ^b	ND	85±11	ND	87±19 ^b	ND	52±27 ^b	ND
Chitax-40 ^b	612±228 ^b	ND	748±152	ND	628±15 ^b	ND	355±56 ^b	ND
SB-T-1102	3.0±0.4	2.8±0.5	2.7±0.4	2.7±0.4	2.4±0.3	2.3±0.5	2.7± 0.5	2.5±0.4
SB-T-1214	12±2	11±1	10±3	9±3	8±2	7±1	7±2	6±1
SB-T-12854	15±3	14±5	13±4	13±3	10±3	10±1	10±2	8±2
SB-T-121303	731±82	ND	545±70	ND	478±47	ND	318±83	ND

^aData from ⁵⁶

^bData from ¹⁰

Table VI: Thermodynamic parameters of binding of taxanes to its site.

Compound	ΔG 35°C (kJ/mol)	ΔH (kJ/mol)	ΔS (kJ/mol K ⁻¹)
Paclitaxel ^a	-42.1±0.3	-51±4	-29±13
Docetaxel ^a	-44.8±0.2	-53±2	-26±8
Chitax-4 ^b	-52.7±0.5	-46±13	19±44
Chitax-40 ^b	-57.7±0.1	-26±24	99±80
SB-T-1102	-43.5±0.3	-8±3	115±11
SB-T-1214	-46.6±0.6	-32±2	47±6
SB-T-12854	-47.1±0.7	-28±3	64±10
SB-T-121303	-57.0±0.2	-31±2	87±7

^aData from⁵⁶^bData from¹⁰

Table VII. Structural data of the ligand induced microtubules.

Compound	J01 (nm ⁻¹)	Mean helical radius (nm)	Average pf number
Paclitaxel ^a	0.0518±0.0004	11.8±0.1	13.0±0.1
Docetaxel ^a	0.0480±0.0005	12.7±0.2	14.0±0.2
Chitax-4 ^a	0.0443±0.0008	13.8±0.3	15.2±0.3
Chitax-40 ^a	0.0521±0.0005	11.7±0.2	12.9±0.2
SB-T-1102	0.0497±0.0005	12.3±0.2	13.6±0.2
SB-T-1214	0.0507±0.0004	12.0±0.1	13.2±0.1
SB-T-12854	0.0493±0.0007	12.4±0.3	13.7±0.3
SB-T-121303	0.0486±0.0003	12.6±0.2	13.9±0.2

Figure Legends

Figure 1. Chemical structures of the compounds employed in this study.

Figure 2. Effect of the SB-T taxanes on the cytoplasmic MTs and DNA morphology of A549 cells. Cells were incubated with either drug vehicle (DMSO) or serial concentrations of the different drugs. A549 cells were incubated for 24 h with either DMSO (A,B); 50 nM (C,D), 100 nM (E,F) or 500 nM (G,H) of paclitaxel; 60 nM (I,J) or 120 nM (K,L) of SB-T-1102; 60 nM (M,N) or 120 nM (O,P) of SB-T-1214; 60 nM (Q,R) or 120 nM (S,T) of SB-T-12854; 60 nM (U,V) or 120 nM (W,X) of SB-T-121303. Microtubules are stained with α -tubulin antibodies (A, C, E, G, I, K, M, O, Q, S, U, W), whereas DNA (B, D, F, H, J, L, N, P, R, T, V, X) was stained with Hoechst 33342. The scale bar represents 10 μ m.

Lower panel: Effect of the SB-T taxanes on the cell cycle of A2780, A2780AD and A549 cells. Cells were incubated for 20 h with either drug vehicle (DMSO) (control cells) or with serial concentrations of each drug. The concentrations shown are those at which the maximal accumulation of cells in the G2/M phase was observed. PI denotes propidium iodide.

Figure 3. (A) TR-NOESY spectrum of SB-T-12854 in the presence of microtubules. (B) STD spectrum of SB-T-12854 in the presence of microtubules.

Figure 4. (A) Comparison between experimental and calculated STD data (CORCEMA-ST) of SB-T-12854 bound to microtubules in the pore site: Experimental STD (solid line and circles); calculated STD (dashed line and squares). Solutions found

for the docking of the luminal site bound form of paclitaxel (B); SB-T-1102 (C); SB-T-1214 (D); SB-T-12854 (E); SB-T-121303 (F).

Figure 5. SAXS profiles of the ligand-induced microtubules. Paclitaxel (black line), docetaxel (red line), SB-T-1102 (green line), SB-T-1214 (blue line), SB-T-12854 (brown line), SB-T-121303 (pink line).

Figure 6. Model of paclitaxel bound to the binding site in β I (light blue) or β III (brown) tubulin. The colored surfaces represent the calculated volume of the binding site.

Figure 7. Effect of the F272V mutation and the modifications in the C13 side chain of the SB-T taxanes on the occupied volume in the paclitaxel binding site. (Sticks) β 272 residue; (grey) surface paclitaxel binding site volume, (blue surface) taxane-occupied volume. (A, B) Paclitaxel; (C,D) SB-T-1214; (E-F) SB-T-12854; (G-H) SB-T-121303.

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Paclitaxel

Docetaxel

Chitax-4

Chitax-40

SB-T1102

SB-T-1214

SB-T-12854

SB-T-121303

Figure 1

Figure 2

Figure 3

Figure 4

Figure 5

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