Molecular mobility of nitroxyl-labelled taxol during tubulin assembly

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Abstract Taxol[1] is an important natural anticancer agent that binds to β-tubulin and suppresses microtubule depolymerization. We have used electron paramagnetic resonance (EPR) spectroscopy to analyze the molecular motion of three novel nitroxyl free radical taxol analogues. Taxol was chemically modified at C2 or C7 carbon of the taxane ring with the TEMPO free radical to yield two spin-labelled taxols and concurrently at C2′ and 3′N of the side chain to yield a spin-labelled taxol biradical. Nitroxyl moieties attached to the taxane ring are significantly restricted in their molecular motion during microtubule assembly, and they show no molecular restriction upon binding to tubulin. We conclude that taxol binds to tubulin in a way such that the taxane ring is not constrained by the dimer structure. However, the taxane ring is strongly immobilized after polymerization of tubulin, i.e. it is incorporated into the structure of microtubule. In contrast, the nitroxy moieties of the taxol biradical show strong immobilization upon attachment to tubulin. The nitroxyl energy exchange is restricted prior to the assembly of microtubules, and no differences associated with the process of polymerization were detected. The taxol side chain resides in a region that is not significantly constrained during polymerization.

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Key words: Taxol; Tubulin; Electron paramagnetic resonance

1. Introduction

Taxol is a diterpenoid that can be obtained from bark, leaves and stems of yew trees and has been isolated from cell cultures of *Taxus* species [1]; it is now used as an antimitotic drug [2] approved for treatment of metastatic breast cancer [3] and ovarian cancer [4]. Taxol binds to cellular microtubules [5–8] and stabilizes microtubule assembly. It interacts predominantly with the β-tubulin subunit of the α,β-tubulin heterodimer [9]. The photoaffinity method was used to identify 31 amino acids near the N-terminus as a taxol binding site [10]. More detailed study localized a peptide containing amino acid residues 217–231 of β-tubulin as a major photolabelled domain [11]. The structure of tubulin depicted recently from electron crystallography of zinc-induced two-dimensional crystals of tubulin verified the location of the taxol binding site [12]. One taxol binding site is identified for each heterodimer, in agreement with the known stoichiometry of taxol and tubulin [13]. The location of bound taxol in proximity to interprotofilament contacts is known, as well as its effect on stabilization of microtubules; however, the fundamental details governing taxol-microtubule stabilization are not known [14], and a molecular level description of the tubulin-taxol interaction remains to be fully developed.

We have synthesized nitroxyl-labelled taxol analogues and used these to obtain, for the first time, information about the orientation of taxol at its binding site and its rotational mobility during microtubule assembly using electron paramagnetic resonance spectroscopy (EPR).

2. Experimental results and discussion

The nitroxyl-taxol analogue, 7-(4-carboxy-2,2,6,6-tetramethyl-1-piperidinyloxy) taxol referred subsequently to as SP-C7 has the nitroxyl paramagnetic centre covalently attached to C7 carbon of the taxane ring. The ability of SP-C7 to initiate tubulin polymerization, at 310°K in the presence of GTP, as taxol does [15], was confirmed by turbidity measurements (data not shown). After polymerization, the EPR spectra of SP-C7 (Fig. 1A) is modified in comparison to the EPR spectra of the same radical in buffer recorded at the same temperature (Fig. 1B). The polymerization of tubulin into microtubules, initiated by increased temperatures 310–313°K drastically altered the EPR spectra. The low magnetic field resonance line broadened and split; a new maxima appeared at lower and higher magnetic fields; the distance between outer extrema $2T_{II}$ increased from 33.78 ± 0.25 G to $2T_{II} = 62.96 \pm 0.46$ G. The immobilized component of SP-C7 spectrum at 310°K corresponds to the spin-labelled molecule strongly restricted in its molecular motion. In comparison, the EPR spectra of SP-C7 in buffer, recorded at the same temperature shows neither broadening nor splitting of the resonance lines, and corresponds to a fast tumbling nitroxyl radical with a rotational correlation time of $\tau_c = 1 \times 10^{-10}$ s. The data show a strong interaction between SP-C7-taxol and tubulin; otherwise, SP-C7 would show an EPR spectrum typical of its presence in an aqueous environment as shown in Fig. 1B. Secondly, the nitroxyl radical attached to C7 carbon of the taxol molecule is notably affected by the increased temperature, that is known to induce tubulin polymerization. The modifications in the EPR spectra, however, are characteristic of an immobilization of the nitroxyl moiety, i.e. the assembly of tubulin heterodimers into filaments imposes an immense restriction on the molecular rotation of the nitroxyl group of SP-C7 attached to carbon-7. Data suggest spin-labelled taxol, SP-C7 is interacting with microtubules and is immobilized in its molecular motion.

A decrease of the sample temperature back to 283°K, after a 30 min incubation at 313°K, did not restore the expected initial EPR spectrum (Fig. 1C). $2T_{II}$ at this temperature was determined to be 66.84 ± 0.68 G, close to the rigit-limit value. This indicates that SP-C7 stabilizes microtubule proteins against depolymerization by cold treatment, as does taxol.

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[1]'Taxol' is a registered trademark of Bristol-Myers Squibb, that offers 'paclitaxel' as a substitute name.

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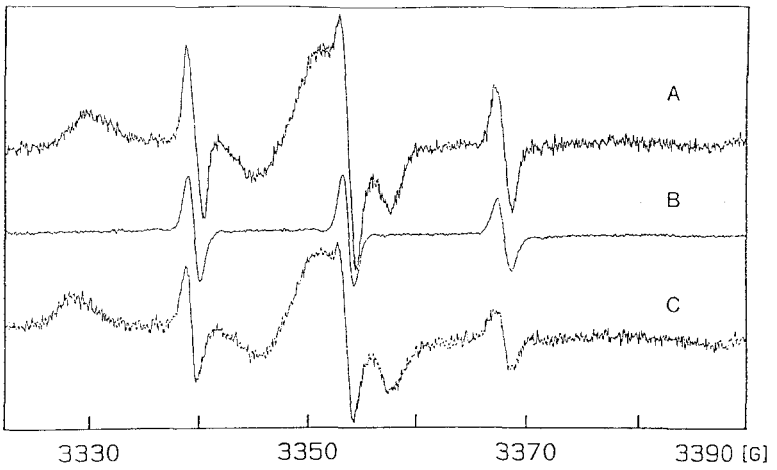

Fig. 1. ESR spectra of 40 μM SP-C7 (taxol modified at carbon-7 of the taxane ring with 2,2,6,6-tetramethyl-1-piperidinyl-*N*-oxyl) in a reaction mixture of 2.5 mg/ml (25 μM) tubulin, 0.1 M MES, pH 6.9, 250 μM GTP, 4% (v/v) DMSO recorded after incubation for 15 min at 310°K. The concentration of tubulin was calculated according to [17], and controlled by optical absorption [15]. Tubulin assembly was followed by ESR, and by turbidity measurements at 350 nm using a Hewlett Packard 8452A Diode Array Spectrophotometer. EPR spectra were recorded on a Bruker ECS-106 EPR spectrometer equipped with a model B-VT2000 temperature controller. Spectral parameters are: microwave frequency 9.4 GHz, microwave power 12 mW, magnetic field 3360 G, modulation frequency 50 KHz, modulation amplitude 0.5 G. B: EPR spectra of SP-C7 at 310°K in a reaction mixture without tubulin. C: Sample A recorded after incubation at 283°K for 30 min.

Once polymerization has been initiated it could not be stopped or reversed by cold treatment.

In the temperature region 273–283°K, before tubulin polymerization, the EPR spectrum of SP-C7 in reation mixture with tubulin has characteristics close to those of the EPR spectrum of SP-C7 dissolved in buffer and recorded at the same temperature. The protein is in a dimeric state [16], and as shown by Sengupta et al. [17], the taxol binding affinity is lower than that when tubulin is in the polymeric state. However, the presence of tubulin in a reaction medium in a concentration higher than the binding stoichiometry should insure that at least part of the labelled taxol is associated with the protein. The comparison of EPR spectra shows that if the attachment of SP-C7 to tubulin occurs it does not alter EPR spectra parameters significantly, i.e. the protein does not impose any additional restriction on the mobility of the nitroxyl moiety attached at position C7 prior to the assembly of microtubules.

The assembly of tubulin molecules into microtubules brings a tubulin/SP-C7 complex in close approximity to a β-tubulin subunit from a neighbouring protofilament, and as shown from our experiments, with a consequent steric crowding in the immediate vicinity of the nitroxyl group. This results in an EPR spectrum with broad lines that are observed neither at low temperatures, nor from the control experiments (SP-C7 in reaction mixture without tubulin, and SP-C7 in reaction mixture without GTP) at any temperature. The hindrance is not due to the binding of SP-C7 to tubulin, because it does not occur in the absence of tubulin polymerization. Most probably, the restricted mobility of the nitroxyl moiety is due to the increased lateral interactions of adjacent protofilaments [12,18]. Furthermore, the restriction of the nitroxyl molecular

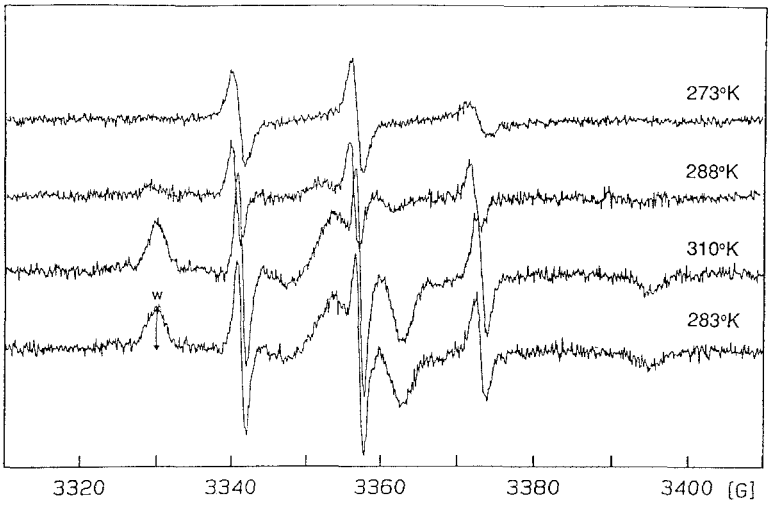

Fig. 2. ESR spectra of 40 μM SP-C2 (taxol modified at carbon-2 of the taxane ring with 2-debenzoyl-2-(4-carboxyl,2,6,6-tetramethyl-1-piperinyloxy/taxol) in a reaction mixture as in Fig. 1. The sample was sealed under N_2 and EPR spectra were recorded at the temperatures indicated. EPR instrument parameters were the same as in Fig. 1.

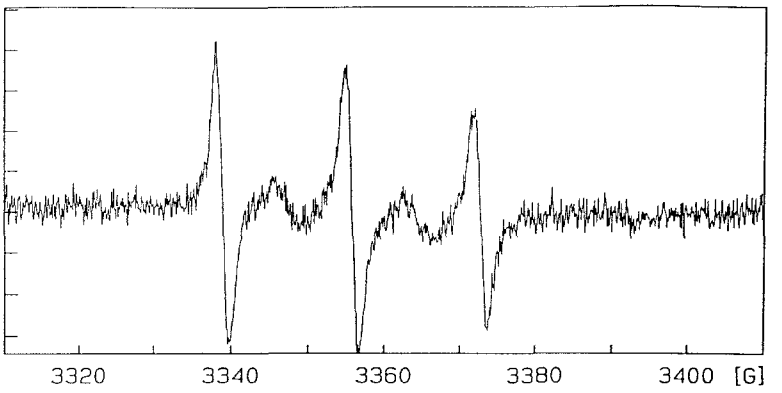

Fig. 3. EPR spectra of the taxol biradical SP-2′3′ (4-carboxyl,2,6,6-tetramethyl-1-piperinyloxy/taxol): 0.25 mg/ml in 0.1 M MES, and 0.5 mM $MgCl_2$ recorded at 310°K.

freedom upon tubulin polymerization shows that this moiety is located in the microtubule structure, and not exposed in the liquid phase.

The nitroxyl-taxol analogue, 2-debenzoyl-2-(4-carboxy-2,2,6,6-tetramethyl-1-piperidinyloxy) taxol, referred to as SP-C2 bears the nitroxyl moiety at position C2 of the taxane ring. The spin probe rotational correlation time, $\tau_c = 0.8 \times 10^{-10}$ s at room temperature in buffer is typical of a freely tumbling nitroxyl moiety. The EPR spectra of SP-C2 in the range 273–283°K in the reaction medium with and without tubulin are comparable. SP-C2 and tubulin concentrations in the reaction mixture suggest a possibility of spin probe protein interactions. The similarity of the ESR spectra shows that if the association of SP-C2 with tubulin occurs it does not induce any additional restriction on the molecular motion of the nitroxyl moiety. However, at 310°K, the temperature which induces microtubule assembly, the EPR spectrum is modified. The low magnetic field resonance line is split and the hyperfine splitting constant $2T_{II}$ increases from 33.35 ± 0.37 G to 65.56 ± 0.99 G (Fig. 2). The perturbation of the EPR spectrum continued at 313°K. In buffer the increase in temperature increases the molecular mobility of SP-C2. In contrast, in the presence of tubulin in the reaction mixture, with the increase of temperature the resonance lines of SP-C2 have broadened, their amplitude have decreased, and the outer resonance lines have split, i.e. the molecular mobility of the nitroxyl moiety decreases. These data show that the taxane ring (at carbon-2) is interacting with the microtubules, and that a restriction is imposed on the mobility of SP-C2 simultaneously with the assembly of tubulin into protofilaments.

A nitroxyl-taxol analogue, 2,3′*N*-di-(4-carboxy-2,2,6,6,-tetramethyl-1-piperidinyloxy) taxol referred to as SP-2′3′ is a biradical. Its EPR spectrum has three or five resonance lines depending on spin-spin energy exchange. According to Parmon and Zhidomirov [19] the shape of EPR spectra of dynamic biradicals depends on the rate and the strength of the intramolecular exchange interaction. The additional resonance lines were recorded from SP-2′3′ in buffer (Fig. 3), and their intensity increases with increased temperature, indicative of a fast energy exchange rate between the radicals. The EPR spectrum of SP-2′3′ in a reaction medium with tubulin shows a strong immobilization after tubulin polymerization (Fig. 4). The spectrum has three resonance lines, not five, and shows a nitroxyl radical restricted in its molecular motion. The EPR spectra modifications of SP-2′3′ indicate a change in the molecular configuration. Indeed, the exchange integral is sensitive to the separation between the orbitals containing the unpaired electrons and will vary with the alteration of the molecular configuration. The outer lines splitting demonstrates the immobilization of the nitroxyl moieties. The additional resonance lines are not recorded, and this reports on the increased distance between the two nitroxyl moieties, and suppressed energy exchange (Fig. 3). Two radicals interacting with tubulin are immobilised and separated from each other. Therefore, the interaction and energy exchange is restricted. It is possible that one of the nitroxyl moieties, buried within tubulin creates

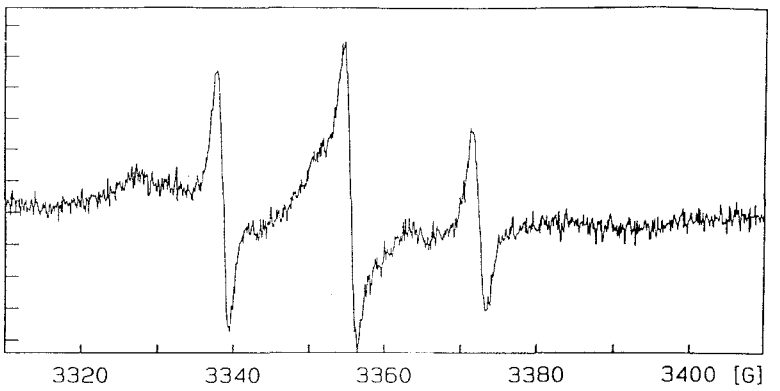

Fig. 4. ESR spectrum of 8.4 μM SP-2′3′ and 25 μM unlabelled taxol in 0.1 M MES, pH 6.9, 2.6 mg/ml tubulin and 250 μM GTP, 4% v/v DMSO after incubation for 40 min at 310°K.

very broad, undetectable resonance lines due to its severe immobilization. The second nitroxyl radical, restricted in its motion yields an immobilized spectrum shown in Fig. 4. This spectrum is not significantly different from the EPR spectrum recorded prior to microtubule assembly, i.e. the assembly of tubulin into microtubules imposed no additional hindrance to an already immobilized side chain of SP-2′3′. This observation may be interpreted to suggest that the side chain of SP-2′3′ lies within a region of β-tubulin that is not constrained during microtubule assembly.

3. Conclusion

Spin-labelled taxol analogues act in a fashion similar to that of taxol, since tubulin polymerization is not impeded by the presence of the spin probes, and it proceeds under identical conditions as those used for taxol-induced polymerization [16]. Our data show that nitroxyl-taxol analogues SP-C7 and SP-C2, when bound to tubulin, show no molecular restriction of the nitroxyl moieties. The immobilizations occur simultaneously with the polymerization of tubulin. The experiments have been performed with 40 μM of spin-labelled taxol analogues and 25 μM of tubulin, which could be considered below the suprastoiometric concentration; therefore, it is correct to assume that taxol completely suppressed the dynamics of microtubules with high polymeric mass [20]. The binding site is located near inter-protofilament contacts of β-tubulin [18]. Our data suggest that an adjacent protofilament, most likely, restricts the rotational mobility of SP-C7 and SP-C2 attached to its β-tubulin binding site. Absence of any modification before polymerization shows that the taxane ring is not embedded within the tubulin structure. During temperature-induced polymerization, the EPR spectra of SP-C7 and SP-C2 display strong rotational restrictions, which suggests that the taxane ring is engulfed within the microtubule. The two immobilized spectra of SP-C7 and SP-C2 (Fig. 1CFig. 2), differ and this difference can be assigned to the differences in the position of the nitroxyl groups as well as to the difference in molecular dimensions of the spin-labelled molecules. Indeed, in SP-C7, the C7 carbon had its hydroxyl moiety replaced with a much larger group.

The side chain of SP-2′3′-taxol assumes its binding site on tubulin, and subsequently imposes strong immobilization on both nitroxyl radicals and suppressing the energy exchange between them prior to polymerization. The assembly of protofilaments, however, impose no further restrictions to the mobility of the nitroxyl moieties. We conclude that the side chain of SP-2′3′-taxol is located in a region that is not additionally constrained during polymerization of tubulin. Although many questions remain unanswered, this study provides the most detailed picture to date of the conformation and the orientation of spin-labelled taxol analogues bound to tubulin. The side chain spin-labelled taxol analogue is immobilized prior to polymerization of the tubulin dimers, and the C2 and C7 analogues become constrained upon microtubule assembly.

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