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Differential binding to the α/β-tubulin dimer of vinorelbine and vinflunine revealed by nuclear magnetic resonance analyses

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Abstract

The binding of two antitumour alkaloids, vinorelbine and vinflunine, to the α/β-tubulin dimer has been investigated at equilibrium by nuclear magnetic resonance (NMR) spectroscopy. Tubulin stability and assembly induced by these drugs has been checked under NMR experimental conditions, and tubulin spirals were found in majority. Then, using increasing ligand concentrations, the alkaloids were titrated against tubulin. A non-specific binding of both compounds to tubulin ($K_d > 10^{-5}$ M) was characterised by broad NMR ligand signal at 4 and 30°. The tubulin dimer exhibited also 2.7 (σ: 0.3) and 2.6 (σ: 0.6) binding sites with a $K_d < 10^{-5}$ M for vinorelbine at 4 and 30°, respectively. In contrast, if the tubulin dimer exhibited 2.7 (σ: 0.2) binding sites for vinflunine at 4°, these sites were not detected at 30°. This NMR study revealed for the first time the presence of specific binding sites and a clear differential affinity of vinorelbine and vinflunine to the tubulin dimer at physiological temperatures which could possibly account for their differential cytotoxicity.
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Keywords: Vinorelbine; Vinflunine; NMR; Equilibrium binding; Tubulin; TRNOE

1. Introduction

The *Vinca* alkaloids vinblastine and vincristine are present in the leaves of *Catharanthus roseus*. These substances and some of their derivatives like vindesine and vinorelbine are used in cancer therapy against leukemia and several solid tumours [1]. Vinflunine was obtained from vinorelbine by fluorination [2]. Both compounds (Fig. 1) show antitumour properties, but while vinflunine exhibited a relatively low *in vitro* cytotoxic potency against various tumour cells compared to the other *Vinca* alkaloids [3], it has superior *in vivo* activity against a series of murine and human tumour experimental models [4]. The cytotoxicity of the various *Vinca* alkaloids correlates with their action on the interphase and mitotic microtubule cytoskeleton [5]. Minor modifications of the dynamics of the mitotic spindle affect the arrangement of chromosome bivalents and prevent the formation of a normal chromosomal plate. Lagging bivalents located outside the chromosomal plate maintain the activation of the mitotic checkpoint and prevent cell progression in post-metaphase stages. Occurrence of a defective mitotic checkpoint in some cancer cells could account, at least partially, for the antitumour activity of these mitotic poisons [6].

In agreement with the action of the antitumour *Vinca* alkaloids on the microtubule cytoskeleton these compounds prevent purified tubulin assembly in microtubules. In all cases, this *in vitro* inhibitory effect occurred in the μM range [3]. The binding of vinblastine and vincristine to tubulin has been documented, although the exact number of binding sites on the tubulin molecule is not yet very clear because of the limitations of standard methods for affinity determination in the submicromolar range [7]. Due to these limitations, it has not been possible to demonstrate

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Abbreviations: PIPES, piperazine-*N*,*N*′-bis(2-ethanesulfonic acid); EGTA, ethylene glycol-bis(2-aminoethylether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; GTP, guanosine 5′-triphosphate; GDP, guanosine 5′-diphosphate; FID, free induction decay; DW, dwell time; TRNOE, transferred nuclear overhauser effect.

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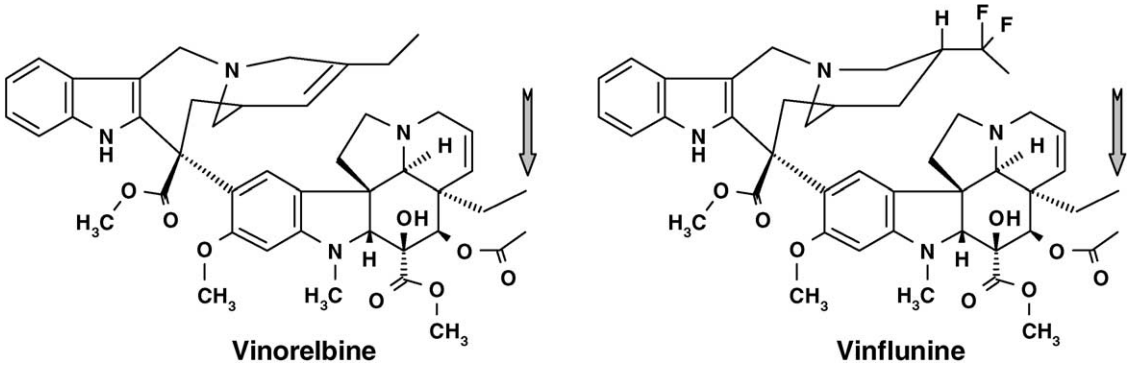

Fig. 1. Structure of vinorelbine and vinflunine. Arrows indicate the methyl groups whose resonances have been monitored during titration.

directly the binding of vinflunine to tubulin [3,8]. In order to further characterise the binding of these two compounds with the tubulin molecule, we explored interactions at equilibrium in the mM–μM K_d range using nuclear magnetic resonance (NMR). The molecular weight of the tubulin heterodimer does not allow a direct study of the tubulin-bound conformation of *Vinca* alkaloids. However, it is possible to determine the number of binding sites by titration of the tubulin heterodimer with increasing amounts of ligand.

2. Materials and methods

2.1. Purification of tubulin

Microtubule proteins were prepared from sheep brain by two cycles of assembly and disassembly, then pure tubulin, obtained by cation exchange chromatography (SP Pharmacia) [9], was stored in liquid nitrogen. Tubulin purity was checked by SDS–PAGE. Protein concentrations were determined using Bradford assay with bovine serum albumin as standard and from UV absorption at 280 nm using an extinction coefficient of 1.2 L/g cm [8]; both assays gave the same concentration within experimental errors. For calculation of molar concentrations a molecular weight of 97,700 was used, assuming an average of five glutamates and six glycines as post-translational modifications [10].

2.2. Microtubule assembly or disassembly

In vitro microtubule assembly or disassembly was followed turbidimetrically by the variations in absorbance at 400 nm using a spectrophotometer (DU-64, Beckman) equipped with thermostatically-controlled cells [11]. Turbidity experiments were conducted with 7 mg/mL purified tubulin in assembly buffer containing 100 mM piperazine-*N*,*N*′-bis(2-ethanesulfonic acid) (PIPES), pH 6.9, 1 mM $MgCl_2$, 1 mM ethylene glycol-bis(2-aminoethylether)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA) and 1 mM guanosine 5′-triphosphate (GTP) was added at the beginning of the measurement.

2.3. Differential ultracentrifugation

Utracentrifugation was performed on TL100 (Beckman). Tubulin (80 μM) was incubated, at 4 and 30°, with *Vinca* alkaloids (molar ratio of 1:15) for 1 and 15 hr. Then, tubulin paracrystals and tubulin spirals induced by *Vinca* alkaloids were sedimented in a TL100 Beckman for 5 min at 18,000 rpm (13,300 *g*) and for 30 min at 65,000 rpm (178,000 *g*), respectively [12] and the amount of sedimented proteins were determined in the pellets.

2.4. NMR experiments

All measurements were performed on a Bruker DMX-500 spectrometer, operating at the proton resonance frequency of 500.13 MHz. The length of a 90° pulse was ca. 9 μs. ^{1}H-1D spectra were recorded on 8K points with 512 scans per free induction decay (FID), with the relaxation delay set to 3 s. For temperature-dependent experiments (Fig. 4E), a 30 min equilibration period was used before each acquisition. The spectral bandwidth covered 10 ppm (DW = 100 μs) and was centred on the H_2O signal at 4.75 ppm. During the relaxation delay the solvent signal was continuously pre-saturated at low power (16 mW). To minimise baseline distortion, the initial acquisition delay DE was set to DW/2 (50 μs). Data were processed on an SGI Indy workstation equipped with the XWINNMR program. The FIDs were apodised with a 90° shifted sine-bell-squared function and zero-filled to 32K points before the Fourier transformation. Peak integrals were obtained with the Felix module of MSI NMRchitect Software (MSI, 1993). An initial phase correction and a fifth order polynomial baseline correction were applied to entire spectra before integration. A second baseline correction was applied when needed to symmetrise and flatten the reference baseline in the vicinity of the integrated peak. A well resolved peak, characterised by a high signal-to-noise ratio (the CH_3 group at 0.46 ppm for vinorelbine and at 0.73 ppm for vinflunine), was integrated by fitting to a Gaussian curve. Attempts to fit to a Lorentzian curve, characteristic for liquids, always failed, whereas the Gaussian minimisation, specific for solids and anisotropic liquids, gave correct

results in agreement with the observed line widths, reaching 30 Hz (Fig. 3B). Identical results were obtained using other ligand resonances, or the average over several resonances. A control experiment of non-specific binding was performed using bovine serum albumin (Sigma).

3. Results

The validity of the observations obtained from the NMR approach relies upon the stability of the compounds used. NMR experiments require both high protein concentrations (5–10 mg/mL) and acquisition times of several hours. The 97 kDa α/β-tubulin heterodimer has a limited stability when stored in solution [13]. The loss of its capacity to assemble into microtubules and to hydrolyse GTP into guanosine 5′-diphosphate (GDP) are the first signs of denaturation. Under the experimental conditions used, the capacity of tubulin to assemble into microtubules remained unchanged over a period of 15 hr at 0°, as judged both by the kinetics and the plateau of microtubule assembly (Fig. 2). Although tubulin was still capable of assembly after an incubation of 24 hr at 0°, the kinetics were slow and exhibited a weak amplitude implying that some of the tubulin molecules were no longer in their native state. The capacity of tubulin to assemble microtubules after different incubation times at 30° required first the disassembly of the microtubules formed at 30° by a 30 min incubation at 0° and then the usual assay of assembly at 37°. The tubulin preparation exhibited clear signs of denaturation after 6 hr. Hence all NMR experiments were performed over less than 6 hr at 30° and less than 15 hr at 0°.

Although μM concentrations of *Vinca* alkaloids prevent the assembly of tubulin heterodimers into microtubules at 30–37°, higher concentrations of alkaloids induce the formation of spiraled tubulin assemblies, which can further aggregate to form paracrystals [12]. Since NMR experiments are performed in the presence of *Vinca* alkaloids in the 0.1–3 mM range, we characterised whether we were dealing with these tubulin assemblies. After 1 hr incubation in the presence of vinorelbine and vinflunine, less than 3% of the tubulin heterodimers were present in paracrystalline structures, while at least 78% were assembled as spirals at both 4 and 30°.

The linewidths of NMR signals depend strongly on the molecular mobility of vinorelbine and vinflunine (Fig. 3A). Free molecules (L_f) are highly mobile and their signals are easily observed in high-resolution NMR spectra. But when these molecules bind strongly to tubulin spirals (L_{sb}), their mobility becomes identical to the low mobility of the tubulin assemblies and the signals of the bound ligand become too broad to be observed. Hence strongly-bound ligands do not contribute to the observable signals. In NMR experiments, these binding sites correspond to

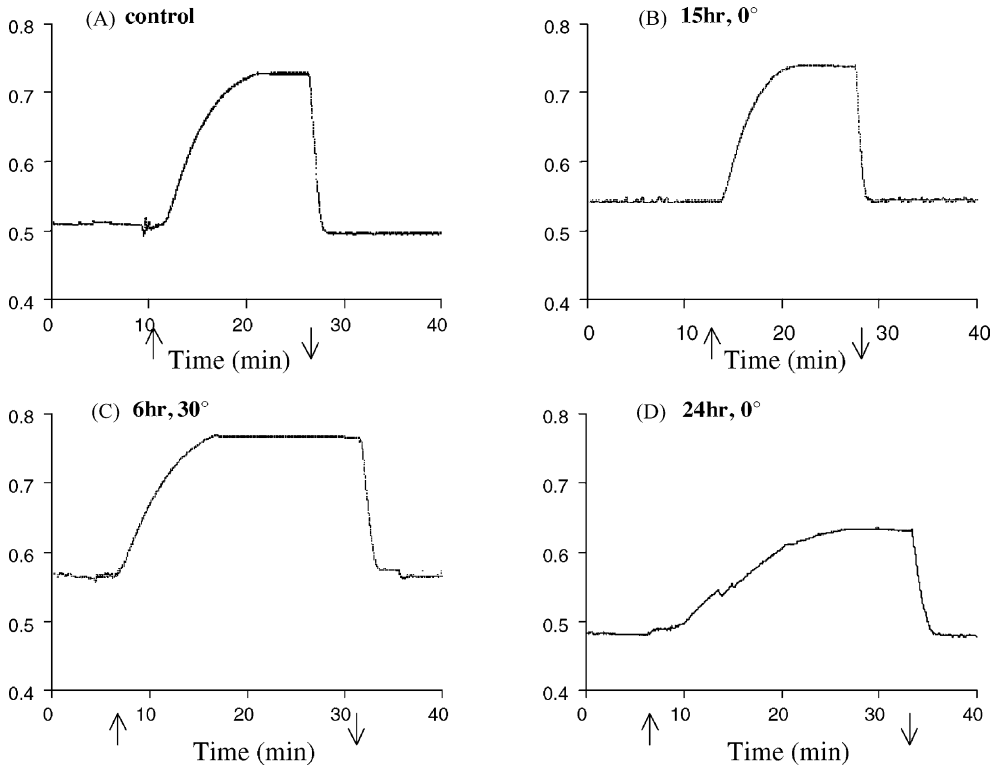

Fig. 2. Stability of tubulin preparation at 0 and 30°. Variation of the absorbance at 400 nm of the tubulin preparation (7 mg/mL) was measured according to temperature change (0°↓ and 37°↑). Tubulin was pre-incubated during 15 hr (B) or 24 hr (D) at 0° or for 6 hr (C) at 30° before the turbidimetry assays were performed. The microtubule formation kinetics is identical to control in condition B and C, whereas a reduction in amplitude and an increase in time constant is observed in condition D, indicative of a reduction in tubulin activity.

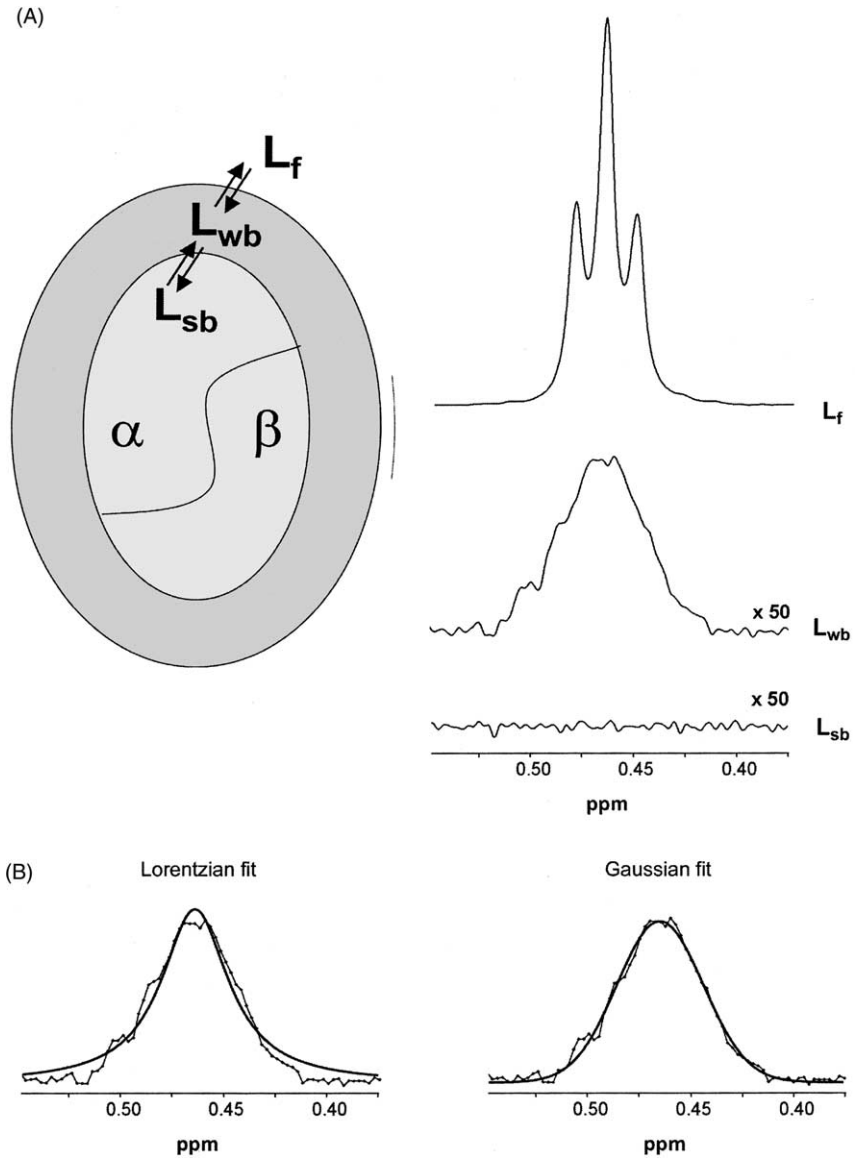

Fig. 3. Ligand–protein interaction as seen by NMR. (A) Free ligand in solution is highly mobile and gives high-resolution NMR spectra (L_f). Formation of a protein–ligand complex due to specific binding implies a drastic reduction in the ligand's mobility, resulting in the loss of its signal (L_{sb}). In the intermediate case a shell of weakly-bound ligand molecules encapsulates a tubulin dimer. Partial immobilisation of ligand molecules results in large NMR linewidths (L_{wb}). The CH_3 signal at 0.46 ppm (see Fig. 1) has been recorded for vinorelbine at 4° for the following ligand/protein molar ratios: 1.5 (L_{sb}), 15 (L_{wb}) and 40 (L_f). (B) Integration of peaks involved fitting to Lorentzian and Gaussian curves. The latter gave more satisfactory results in terms of the fitting quality. The correlation coefficients (r^2) and normalised sums of squared deviations (SSD) between the experimental and calculated profiles for the examples in the figure are: $r^2 = 0.96$, SRSD = 4.2 for the Lorentzian fit and $r^2 = 0.99$, SSD = 1.0 for the Gaussian fit.

$k_{off} < 100$ Hz and $K_d < 10^{-5}$ M (assuming a diffusion limited rate $k_{on} = 10^7$ s^{-1}). Low affinity binding (L_{wb}), characterised by a fast exchange rate between the bound and free forms is detected on the free ligand spectrum by a line-broadening proportional to the bound fraction. It is thus possible to calculate the number of strongly-bound ligand molecules by determining the amount of *Vinca* alkaloid which is no longer detectable by NMR. The dependence of the NMR signal integral on the ratio of the alkaloid and the tubulin heterodimer concentrations allows a determination of the number of ligand molecules strongly interacting with the protein. Schematically, a reference spectrum was obtained with pure tubulin (9 mg/mL, i.e. ~82 μM), and then vinorelbine (or vinflunine) was added sequentially in order to obtain the spectra corresponding to various alkaloid/tubulin ratio. The alkaloid methyl resonance at 0.46 ppm was used as a signal of ligand concentration quantification.

In the range of concentrations used in this study (l/p ratio ranging from 1 to 30) the ligand signals exhibited by vinorelbine or vinflunine were not identical to that obtained with free alkaloid. Their linewidths were broader (~30 Hz) than when these alkaloids were free in solution (~2 Hz). A fast exchange between bound and free alkaloid could account for this effect. In this case the chemical shift of the integrated peak as well as its width should depend on

the ligand concentration. Since this effect was not observed (data not shown), it is likely that there was no free alkaloid at low concentrations and that the recorded signals originated from a layer of non-specifically bound alkaloid. This effect could account for the increased linewidth and the modified lineshape which is Gaussian rather than Lorentzian. The variation in the integral of the peak at 0.46 ppm (methyl resonance indicated by an arrow in Fig. 1) as a result of the titration was determined at 4 and 30° for vinorelbine (Fig. 4A). In both cases the different titration points defined two almost identical straight lines which exhibited a similar slope and which extrapolated to a ratio corresponding to 2.7 (σ: 0.34) and 2.6 (σ: 0.62) bound vinorelbine molecules per tubulin heterodimer, respectively. Fig. 4C and D visualise the spreading of the stoichiometric ratio on the curves themselves by plotting two limiting straight lines at 95% confidence limit ($\sim 2\sigma$). Hence the number of bound vinorelbine molecules appeared to be independent of the temperature of incubation. To obtain an independent verification of the significance of these results, we performed a titration of bovine serum albumin, a protein that has no particular binding site

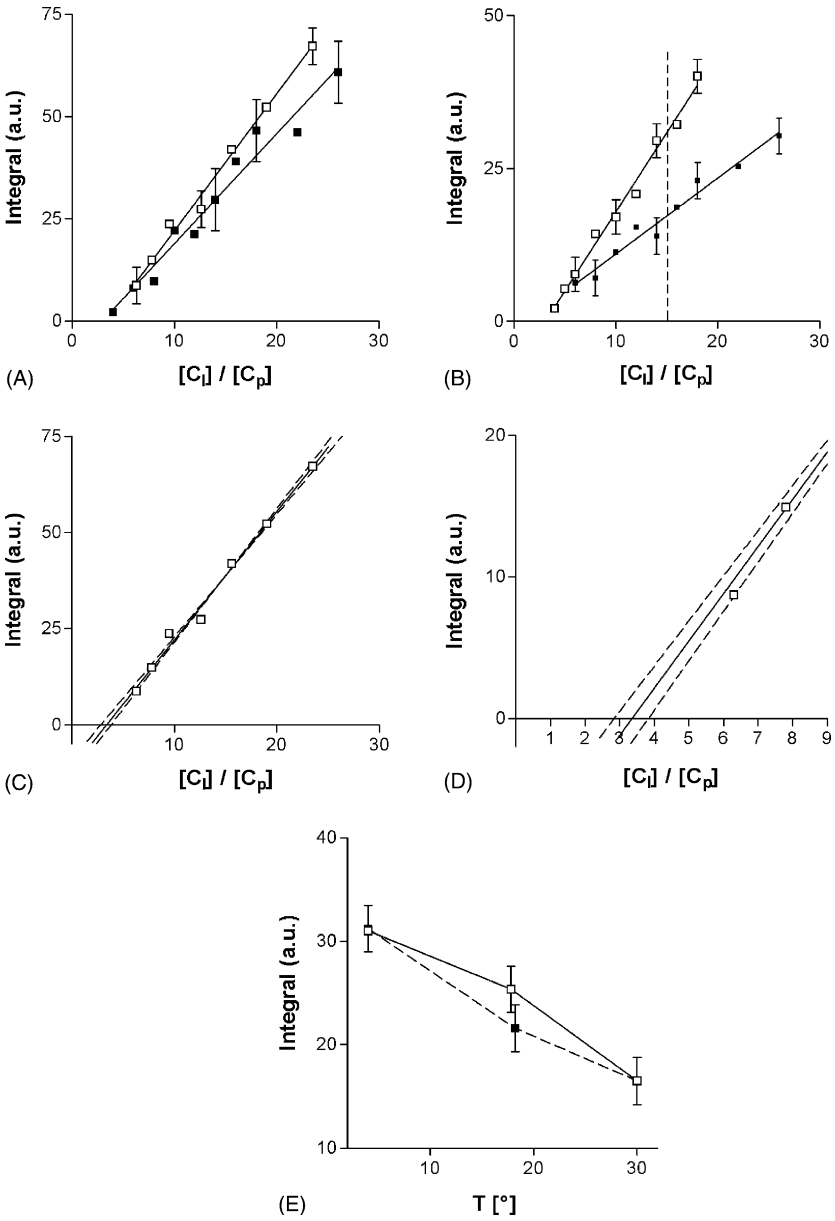

Fig. 4. NMR titration experiments: amplitude of NMR signals as a function of the ligand/protein concentration ratio. The signal integral as a function of ligand to protein ratio can be fitted by a straight line whose intercept at zero integral provides the number of strongly-bound ligands. The integrated peaks appeared at 0.46 ppm (vinorelbine) and at 0.73 ppm (vinflunine), and were plotted for a temperature of 4° (open squares) at 30° (solid squares). For clarity, error bars are placed on selected points only. (A) Vinorelbine at 4 and 30°. (B) Vinflunine at 4 and 30°. (C) Uncertainty in determination of the *X*-axis intercept is illustrated using the data for vinorelbine at 4°. The two straight lines shown represent limiting values of slopes and intercepts, drawn at 95% confidence limit ($\sim 2\sigma$). (D) Zoom of C at the region of the *X*-axis intercept. (E) Vinflunine at the C_l/C_p ratio of 15 (vertical dashed line in part B): starting from 4°, the temperature was increased up to 30° (solid line), then decreased back to 4° (dashed line).

for *Vinca* alkaloids, by vinorelbine. Here again a straight line was obtained when plotting the integral vs. C_l/C_p (data not shown). The origin intercept in this case was 0.4 (σ: 0.31) at 4° and 0.1 (σ: 0.66) at 30° in agreement with the absence of specific binding in this case.

The number of vinflunine molecules bound to tubulin was determined by the same procedure at 4 and 30° (methyl resonance at 0.73 ppm indicated by an arrow in Fig. 1). At both temperatures the different titration points defined two straight lines (Fig. 4B), but in contrast to the observations made with vinorelbine, they extrapolated to different values: at 4° an intercept of 2.7 (σ: 0.24) vinflunine bound per tubulin heterodimer was found, a value which was similar to the value obtained for vinorelbine, while multiple experiments performed at 30° led to values varying from −0.9 to 1.0 molecule of vinflunine per tubulin heterodimer. The average of all the experiments performed at 30° suggested the absence of strongly-bound vinflunine since the straight line extrapolated to 0.0 (σ: 0.82). This observation was further assessed by recording a series of spectra of the same sample of tubulin corresponding to a ratio of vinflunine to tubulin of 15 according to the following schema: 4, 18, 30, 18, and 4°. Due to the limited stability of the tubulin heterodimer at this temperature (6 hr maximum), only five temperatures could be tested in this experiment. The result, shown in Fig. 4E, confirmed that the number of molecules of vinflunine bound to tubulin varied according to the temperature and indicated that the variations of binding were fully reversible.

4. Discussion

Determination of the number of *Vinca* alkaloids bound to the tubulin heterodimers has been previously determined by the association of a radioactive-derivative using gel filtration or ultracentrifugation methods. Although efficient for high affinity interactions, they have led to controversial numbers of *Vinca* alkaloid binding sites vinblastine [7,14,16] and have been ineffective for other *Vinca* alkaloids such as vinorelbine and vinflunine most likely due to K_d values higher than the μM range [3]. In contrast, NMR has proven to be a useful tool for the analysis of protein structure, dynamics and protein–ligand interactions in the mM to μM range. In the field of tubulin it has allowed to reveal internal dynamics of microtubule proteins [17], to determine the solution structure of fragment peptides [18], and to characterise complexes with a variety of ligands including microtubule associated peptides [19], GTP and ATP [20], colchicine [21], paclitaxel [22]. It usually requires high drug and high tubulin concentrations, apparently higher than average physiological and pharmacological concentrations. But it is necessary to underline that intracellular concentrations of *Vinca* alkaloids are at least two orders of magnitude higher than the extracellular concentrations [23]. Moreover, the concentrations encountered locally in subcellular compartments are unknown and in most cell lines the extracellular concentrations leading to cytotoxicity (IC_{50}) of vinorelbine and vinflunine are higher than the concentrations of vinblastine and vincristine, reaching a difference of one order of magnitude in the case of vinflunine [24].

One particularly interesting NMR technique is transferred NOE (TRNOE), which potentially allows the determination of the protein-bound conformation of a weakly-bound ligand ($k_{off} > 100\ s^{-1}$) [25]. Transferred NOEs have been detected previously between vinblastine and tubulin [26] and we have confirmed this observation (data not shown). However, our data show that the tubulin–vinorelbine/vinflunine complexes present several slowly exchanging states which do not give rise to TRNOEs, and numerous, non-saturable rapidly exchanging states, which are responsible for the observed TRNOEs, but which, contrary to previous expectations, cannot be used safely for any structure determination and are probably biologically irrelevant.

The NMR titrations have been performed over a large range of ligand/protein concentration ratio so that a statistical analysis of the data could be performed, thus giving a realistic assessment of the accuracy on the number of binding sites. This was classically expressed in terms of σ values, intervals of 2σ corresponding to a confidence interval of 95%. NMR determinations performed at 4° suggest that the number of vinorelbine and vinflunine binding sites on the tubulin heterodimer are both around 2.7 ± 0.6 in agreement with the similarities of their chemical structure. These values are consistent with the presence of three vinorelbine and vinflunine binding sites per tubulin heterodimer in the tubulin spirals at 4°. The presence of one high affinity binding site for vinorelbine was expected from the classical determinations performed with vinblastine and vinorelbine [7,14–16], but the presence of other sites is somewhat unexpected from the values reported for other *Vinca* alkaloids [7]. Concerning vinflunine, specific binding of [^{3}H]-vinflunine to tubulin was undetectable by the standard centrifugal gel filtration method [3]. These discrepancies arise from the difficulty to observe low affinity sites by previous methods, although technical problems such as the exact concentration of tubulin and the limited stability of tubulin could possibly be a source of error. Alternatively, several authors have emphasised that they could also result from a strong modulation by the state of tubulin self-association [27] and medium conditions such as the presence of magnesium ions [14]. The NMR determinations do not imply the presence of three *equivalent* sites with the same K_d, these data suggest simply the presence of three binding sites with a K_d inferior to 10^{-5} M. One site for vinblastine was proposed to be located on residues 175–213 of β-tubulin [16]. The other ones could be on one or both monomers which share only 41% amino acid sequence identity, or even at the interface between monomers or else at the

interface between two dimers, since under the experimental conditions used (high concentrations of *Vinca* alkaloids: 0.1–3 mM), we are not dealing with free tubulin heterodimers but mostly with tubulin spirals.

When the same determinations were performed with vinorelbine at 30°, a value of 2.6 ± 1.2 was obtained. This value was consistent with the value obtained at 4°, although it is less precise. In contrast, when it was performed with vinflunine at 30°, the data (0.0 ± 1.6) suggests the absence of (or possibly the presence of at most one) vinflunine binding site. This number is significantly different from the values calculated at 4° for vinflunine and from the values obtained at 4 and 30° for vinorelbine. The experimental data do not allow to distinguish vinorelbine and vinflunine by their number of binding sites at 4°, but these two *Vinca* alkaloids differ significantly by their number of binding sites at 30°. This unexpected observation was further confirmed in submitting the tubulin spirals formed at 4° to an increase of temperature to 18 and 30°, and then by decreasing the temperature to 18 and 4° (Fig. 4E). This experiment confirmed that the number of vinflunine bound to tubulin spirals decreased upon increasing the temperature. The effect was shown to be fully reversible so that it is not due to tubulin denaturation.

Since we demonstrated the presence of non-saturable weak binding sites for vinorelbine and vinflunine ($K_d > 10^{-4}$ M deduced from the linewidth analysis, see Section 3) it is likely that the apparent disappearance of the vinflunine binding sites at 30° results from an increase of their K_d by one or two orders of magnitude. Such an effect of temperature is not surprising in the case of the tubulin heterodimers which, in standard conditions, do not assemble at 0° and need higher temperatures to polymerise. The difference of affinity of the tubulin binding sites for vinorelbine and vinflunine at 30°, which was shown clearly for the first time, may account for the need of higher concentrations of vinflunine than vinorelbine in order to obtain the same cytotoxicity on cultured cells [3,24], without implying a different mechanism of action between these two substances in agreement with *in vivo* and *in vitro* observations.

Acknowledgments

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