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Porphyrins affect the self-assembly of tubulin in solution

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ABSTRACT

Self-assembly of tubulin heterodimers in solution has been studied in the past to predict the effects that ligands and/or conformational changes have on the formation of tubulin filaments. Self-assembly of tubulin in solution has produced formations similar to cellular microtubules (MTs). The present study reports on the effects that two porphyrins (protoporphyrin IX, PPIX and tetrakis(4-sulfonatophenyl)porphyrin, TPPS) produce on the self-assembly of tubulin α,β-heterodimers in buffer solution. The study shows that, when incubated simultaneously with MT-stabilizing ligands (i.e., paclitaxel and guanosine triphosphate, GTP), porphyrins do not affect the ability of tubulin to form MT. However, if paclitaxel and GTP are added after tubulin has been allowed to self-assemble in the presence of either porphyrin, the ability to form MT-like structures is reduced or suppressed. We suggest that this effect is due to the formation of porphyrin-mediated aggregates that cannot be broken or elongated by the addition of GTP or paclitaxel.

1. Introduction

Microtubules (MTs) are elongated anisotropic assemblies of tubulin heterodimers (mostly α and β monomers [1]). MTs are essential components of all eukaryotic cells and vital factors in cell functions such as intracellular signaling, cellular morphology and mitosis [2–5]. *In vivo*, the assembly of tubulin into MTs is regulated by the GTPase activity of tubulin [2,6–12] and is assisted by other factors such as the binding of microtubule-associated proteins (MAP) [13]. Because of the key role played by MTs in so many cell functions, they have become a prime target for biomedical research [10,14–17] especially in regard to their interaction with drugs [17–21] as well as neurotoxic agents [22]. Among the ligands that were shown to affect MT formation were porphyrins [23–25]. Some studies suggest that porphyrins co-localize with MTs, *in vivo*, stabilize them, and trigger apoptosis [5,26]. Other studies indicated a destabilization of microtubules *in vitro* which was linked to the neurotoxicity of some porphyrins [25,27]. The interaction of porphyrins with tubulin has been investigated because of its relevance for the biomedical applications of these tetrapyrroles, both for the potential use of tubulin as a target in porphyrin-mediated phototherapy but also for some of the side effects of administering the photoactive drug. Most of these studies [5,25–27] probed porphyrin–tubulin interactions with phenomenological approaches through cellular effects. However, the studies neglected the molecular details and effects of the binding. These details are important to understand whether porphyrins can be used to target proteins with a mechanism similar to chromophore-assisted laser inactivation (CALI) in living cells. In a previous study we showed that two porphyrins with different chemical and physical properties bind α,β tubulin dimers [28] at separate sites. In the current study we have investigated whether their binding induces or modifies the self-assembly of MT in solution with or without the assistance of MT-inducing ligands such as paclitaxel and GTP. Such study has a two-fold rationale. On the one hand it investigates whether the binding of porphyrins promotes or affects the ability of tubulin to assemble into MT; on the other hand it also probes whether docking competition exists with established ligands, such as GTP or paclitaxel. The latter aspect would provide more direct information regarding the location of the porphyrin docking site on the protein, since the locations of the docking sites for GTP and paclitaxel are well established. Like most globular proteins of similar size, tubulin offers many possible binding sites for a variety of ligands [29], and for this reason can be very vulnerable to ligand-induced inhibition of structure and function. The ligand binding properties of monomeric and dimeric tubulin as well as of MTs can lead to disastrous consequences for the cells [30], however it can also be exploited to attack abnormal cells.

The effect of ligands on the formation of MT is a particularly accessible information since MT-like filaments and protofilaments can be reconstituted *in vitro* under proper conditions of buffer and temperature [17,30,31]. This property enables one to investigate how ligands may affect the formation of MTs [15,18,32,33].

We studied the self-assembly rate of tubulin using the turbidity assay and we assessed the final size and shape of the aggregates using electron microscopy.

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2. Materials and methods

2.1. Chemicals

Tetrakis(4-sulfonatophenyl)porphyrin (TPPS) (Frontier Scientific, UT), protoporphyrin IX (PPIX) sodium salts (Sigma-Aldrich, St Louis, MO) and microtubule competent, >99% pure, bovine α,β-tubulin (TL238, Cytoskeleton, Denver, CO) were used without further purification. Paclitaxel (LC Laboratories, Woburn, MA), and guanosine triphosphate (GTP) (Sigma-Aldrich, St Louis, MO) were used as received. Spectroscopic grade DMSO was also purchased from Sigma-Aldrich and used without further purification.

2.2. Buffers

Modified PIPES buffer at pH 6.9 was prepared using deionized water with 80 mM PIPES, 4 mM $MgCl_2$, 5% glycerol and 0.5 mM EGTA.

2.3. Methods

All ligands used in the study, except paclitaxel, were prepared as 1 mM stock solutions in the modified PIPES buffer. Their concentration was established from the optical density of the solution using the following molar extinction coefficients [28,34]: $\varepsilon^{PPIX}_{405nm} = 2.42\times10^5\,M^{-1}cm^{-1}$, $\varepsilon^{TPPS}_{413nm} = 5.10\times10^5\,M^{-1}cm^{-1}$, and $\varepsilon^{GTP}_{254nm} = 1.37\times10^4\,M^{-1}cm^{-1}$. The concentration of tubulin was also determined from the optical density in solution using [35] $\varepsilon^{Tubulin}_{280nm} = 1.15\times10^5\,M^{-1}cm^{-1}$. Because of its insolubility in aqueous solutions, paclitaxel was weighed and dissolved in DMSO to produce a stock solution of 1 mM. The stock was then diluted into 0.5 ml of the tubulin solution to yield 10 μM concentration of the ligand. This dilution ensured that the final concentration of DMSO in PIPES buffer was kept <2% v/v, in order to avoid DMSO-induced polymerization of tubulin.

2.4. Incubation

The tubulin solutions were kept at 5 °C during addition and incubation of the ligands. The use of cold solutions allows the binding to occur without the simultaneous formation of MTs or other filaments [36,37]. The final tubulin:ligand molar ratio in was 1:1 or 1:20 depending on the experiments. The turbidity assay was carried out differently in 1:1 and 1:20 solutions (see below), whereas the optical density of the solutions was not crucial in electron microscopy experiments (STEM). The 1:20 solutions were used because this molar ratio is the one suggested by the manufacturer for reconstituting microtubules using GTP. Incubation of the ligands and tubulin proceeded for 30 min in the dark.

2.5. Turbidity assay (light scattering)

Polymerization/aggregation of tubulin was recorded using the turbidity assay [38,39] which measures the apparent increase in optical density (O.D.), due to increased scattering, at a wavelength (e.g., 340 nm) where the protein and the porphyrins do not absorb. Scattering increases with the mass of the aggregates in solution [40]. The concentration of tubulin for these experiments (~10 μM) has been shown to be sufficient for most tubulin polymerization studies [31]. After incubation with a single ligand, or a combination of ligands (see below), 0.5 ml of the samples was transferred into a quartz cell placed inside the temperature-controlled holder of the spectrophotometer, pre-set at 37 °C. The O.D. of the sample at 340 nm was recorded vs. time for 60 min. Readings were taken every 30 s with a 1 s integration time. In samples containing 200 μM of PPIX or TPPS, the scattering of the solution was probed at 600 nm. We carried out control experiments on the absorption spectra of the two porphyrins bound to tubulin at various temperatures and determined that in the 580–610 nm region the contribution of porphyrin absorption was minimized (<0.01 in optical density) even upon binding to the protein. The time profile of the scattering signal (i.e., the kinetics of polymerization) was analyzed using the nucleation and elongation model described in detail by Hall [31]. According to this model the scattering time profile can be described by:

$$\ln\left(1-\frac{T(t)}{T_{\max}}\right) = -kt \tag{1}$$

where $T(t)$ is the scattering at time t, $T_{\max}$ is the maximum scattering and k is the apparent rate of polymerization. The effect of porphyrin on filament formation was also probed by addition of MT-inducing ligands (GTP, paclitaxel) after the porphyrin/tubulin complexes were allowed to assemble for 60 min.

2.6. Electron microscopy

At the end of the polymerization process approximately 20 μl of the solution is deposited onto formvar-coated copper grids while gently drying with filter paper placed at the edge of the grid. The deposition process is carried out in approximately 20 s and the remaining solution is allowed to air dry for 3 min. The grid is then immediately mounted on the microscope holder and images are recorded immediately. The dimensions of the aggregates that are formed were analyzed using Image J (free downloadable software of the National Institutes of Health).

2.7. Fluorescence measurements

The binding isotherms that describe the docking of PPIX and TPPS to tubulin were obtained from the porphyrin fluorescence spectra [28] in solutions obtained by adding aliquots of a 10 μM tubulin stock to a porphyrin stock solution whose optical density at the maximum of the Soret band was 0.1 (approximately 5 μM PPIX and 0.8 μM TPPS). After each addition of the tubulin stock the fluorescence of the porphyrin is recorded in the 580–750 nm spectral range ($\lambda_{ex}=405$ for PPIX, $\lambda_{ex}=413$ for TPPS). The effects of GTP and paclitaxel were investigated by addition of tubulin that had been previously incubated

Table 1
Polymerization rate, k ($\times10^5\,s^{-1}$) for the formation of tubulin filaments/aggregates.

		+ PPIX		+ TPPS	
		Simultaneous[b]	Preceding[c]	Simultaneous[b]	Preceding[c]
Tubulin[a] alone	2.1 ± 0.1	4.2 ± 0.4 (10 μM) 29 ± 3 (200 μM)		84 ± 11 (10 μM) 52 ± 4 (200 μM)	
Tubulin[a] + paclitaxel (10 μM)	343 ± 111	273 ± 21	148 ± 8	350 ± 71	4.1 ± 0.8
Tubulin[a] + GTP (10 μM)	17 ± 2			13 ± 5	
Tubulin[a] + GTP (200 μM)	377 ± 85	44 ± 15		18 ± 5	23 ± 7

[a] Tubulin concentration was always 10 μM.
[b] Porphyrins and paclitaxel (or GTP) incubated simultaneously with tubulin.
[c] Porphyrins incubated and allowed to self-assemble at 37 °C for 1 h before addition of paclitaxel (or GTP).

(following the procedure described above) with GTP or paclitaxel. The binding parameters of PPIX and TPPS in the presence and absence of GTP and paclitaxel were obtained with methods described previously [28] using the equation:

$$\frac{[Porphyrin]_b}{[Tubulin]} = \frac{\frac{nK_b}{\Phi_b}[Porphyrin]}{1 + K_b[Porphyrin]} \quad (2)$$

where $[Porphyrin]_b$ and $[Porphyrin]$ are the bound and total concentration of porphyrin in solution as determined with fluorescence spectroscopy, $[Tubulin]$ is the total concentration of tubulin, K_b is the binding constant whereas n and ϕ_b are the number of bound porphyrin molecules and the fluorescence quantum yield of the bound porphyrin respectively. The fraction of bound porphyrin was obtained using the Gaussian fitting method explained previously [28]. To prevent artifacts due to the formation of filaments during the fluorescence experiments, these were carried out at 20 °C where we had previously verified that filament formation is negligible.

2.8. Instrumentation

Absorption spectra and turbidity measurements were recorded with a dual beam Evolution 300 spectrophotometer (Thermo Fisher Scientific, Waltham, MA). Spectra were recorded with 1.5 nm resolution and 240 nm/min speed. Porphyrin spectra were recorded between 250 and 700 nm while tubulin absorption spectra were recorded between 220 and 350 nm. Appropriate baseline and reference cells were used for each scan.

Fluorescence spectra were recorded using an Aminco Bowman-2 (AB2) double monochromator fluorimeter (Thermo Fisher Scientific, Waltham, MA). Temperature in the cell was controlled with a home-made circulating water system, and the temperature of the solution was probed before and after the experiments with a thermocouple. Spectra were recorded at 1 nm/s with a 4 nm bandwidth.

Electron microscopy was performed on a S5500 cold field emission scanning electron microscope (Hitachi, Pleasanton, CA). Images were recorded at 1 KV.

3. Results

3.1. Polymerization of tubulin

The polymerization rate studied with light scattering reveals the effects of PPIX and TPPS on the ability of paclitaxel and GTP to induce the formation of tubulin filaments. Tubulin alone at 10 μM in the modified PIPES buffer has a negligible rate of filament formation ($<3\times10^{-5}s^{-1}$) (Table 1). Equally negligible is the one in the presence of PPIX at 10 μM (Fig. 1A and Table 1). Conversely, PPIX at 200 μM induces a faster rate (Table 1). The optical observation of the polymerization reveals that, after an initial increase, the apparent optical density plateaus quickly, indicating the formation of small aggregates that do not grow further (Fig. 2). A similar result is observed upon addition of TPPS at both 10 μM and 200 μM. With TPPS, however, a larger rate of initial polymerization was recorded (Fig. 2 and Table 1). The addition of GTP at 10 μM produces a slight increase

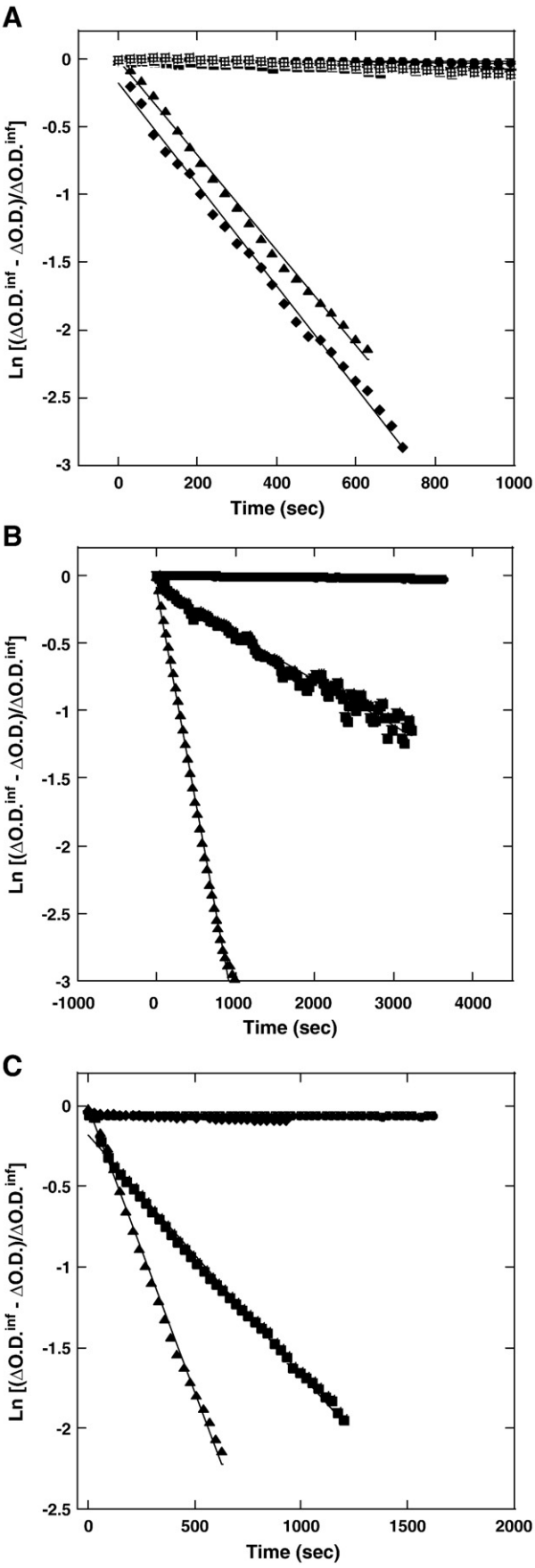

Fig. 1. The assembly kinetics of tubulin (10 μM) in modified PIPES buffer at pH 6.9 and 37 °C as represented according to Eq. (1). (A) Tubulin alone (●), tubulin incubated with GTP (10 μM) (▲), tubulin incubated with GTP (200 μM) (♦), tubulin incubated with paclitaxel (10 μM) (■), tubulin incubated with PPIX (10 μM) (▼), and tubulin incubated with TPPS (10 μM) (). (B) Tubulin incubated simultaneously with PPIX (10 μM) and GTP (10 μM) (●), tubulin incubated simultaneously with PPIX (200 μM) and GTP (200 μM) (■), tubulin incubated simultaneously with PPIX (200 μM) and paclitaxel (10 μM) (▲). (C) Tubulin incubated simultaneously with TPPS (200 μM) and GTP (200 μM) (●), tubulin incubated and assembled with PPIX (200 μM) before addition of GTP (200 μM) (♦), tubulin incubated simultaneously with TPPS (200 μM) and paclitaxel (10 μM) (▲), and tubulin incubated and assembled with PPIX (200 μM) before addition of paclitaxel (10 μM) (■).

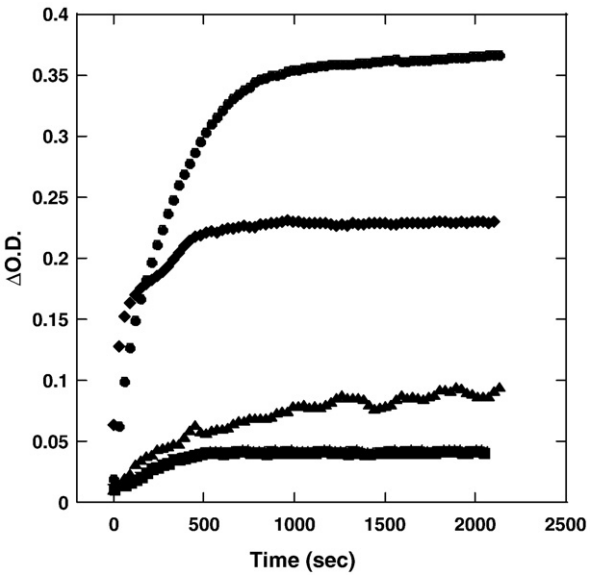

Fig. 2. The assembly kinetics of tubulin (10 μM) in modified PIPES buffer at pH 6.9 and 37 °C directly recorded from the turbidity assay represented as changes in the optical density of the samples at 340 nm. Turbidity assay of tubulin incubated with paclitaxel (10 μM) (●), tubulin incubated with TPPS (200 μM) (▲), tubulin incubated with GTP (200 μM) (◆), and tubulin incubated with PPIX (200 μM) (■). The curves for tubulin/PPIX and tubulin/TPPS samples are multiplied 5×.

in the polymerization rate (Table 1), however, when GTP is added at 200 μM the rate of formation increases to $>250\times10^{-5}s^{-1}$ (Fig. 1A and Table 1) and the optical density measurements reaches a much larger plateau typical of elongated filaments [17,31] (Fig. 2). The presence of paclitaxel (10 μM) produces a very large rate ($>320\times10^{-5}s^{-1}$ (Fig. 1A and Table 1) and a clear elongation of the filaments (Fig. 2). What is the effect of the porphyrins on the formation of GTP- and paclitaxel-induced tubulin filaments? The simultaneous incubation of tubulin with either porphyrin or paclitaxel does not substantially affect the rate at which the latter ligands form tubulin filaments (Table 1); conversely the presence of PPIX and TPPS reduces substantially the rate of the GTP-induced MT (Table 1).

Significant effects can be observed if GTP or paclitaxel is added after tubulin is allowed to assemble in the presence of the porphyrins. Even at 200 μM, GTP is unable to induce polymerization of tubulin ($k<25\times10^{-5}s^{-1}$) after this has been allowed to assemble in the presence of PPIX and TPPS (Table 1 and Fig. 1B and C). Similarly, the assembly induced by either porphyrin reduces the ability of paclitaxel (10 μM) to stabilize the formation of MT. The optical density data however show its residual ability to form and elongate some filaments.

Addition of paclitaxel or GTP to tubulin that was allowed to assemble without previous incubation with porphyrins, produced the same polymerization rate as that produced by samples incubated with either ligands. Control experiments revealed that (i) DMSO by itself at the same concentration resulting from the addition of paclitaxel does not prompt formation of MT, and (ii) addition of 10 μM paclitaxel or 200 μM GTP to solutions of tubulin left at 37 °C for 1 h (to reproduce the condition of the experiments where GTP (200 μM) and paclitaxel (10 μM) are added at the end of porphyrin-induced polymerization) still yielded polymerization rates (300 ± 80 for paclitaxel; 275 ± 52 for GTP) comparable to those of Table 1 where the two ligands were incubated at 4 °C. This ensures that changes in the polymerization rates are due to porphyrin-induced effects and not to decay of tubulin.

3.2. Electron microscopy

Scanning electron microscopy (SEM) confirms the results obtained with optical measurements. When tubulin alone is allowed to assemble

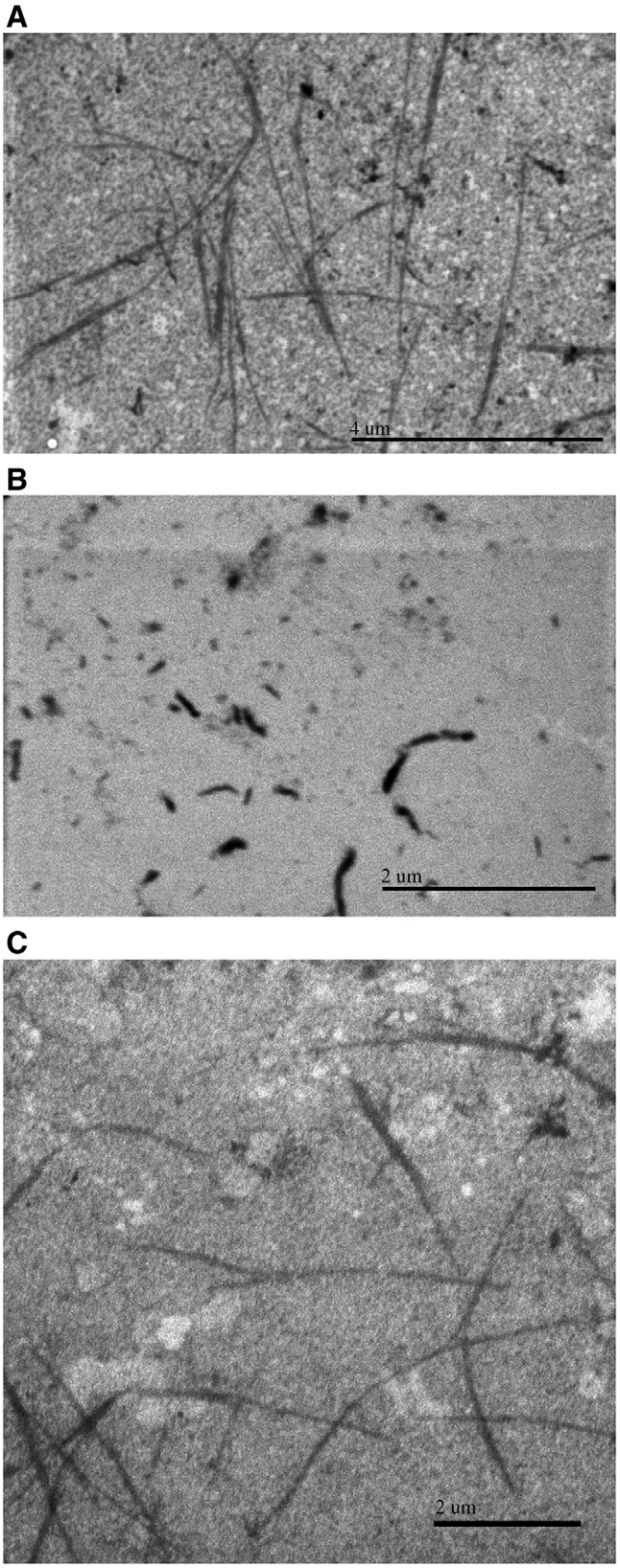

Fig. 3. (A) Paclitaxel-induced filaments (bar = 4 μM), (B) GTP-induced filaments (bar = 2 μM), (C) residual paclitaxel-induced filaments in tubulin self-assembled with TPPS (bar = 4 μM).

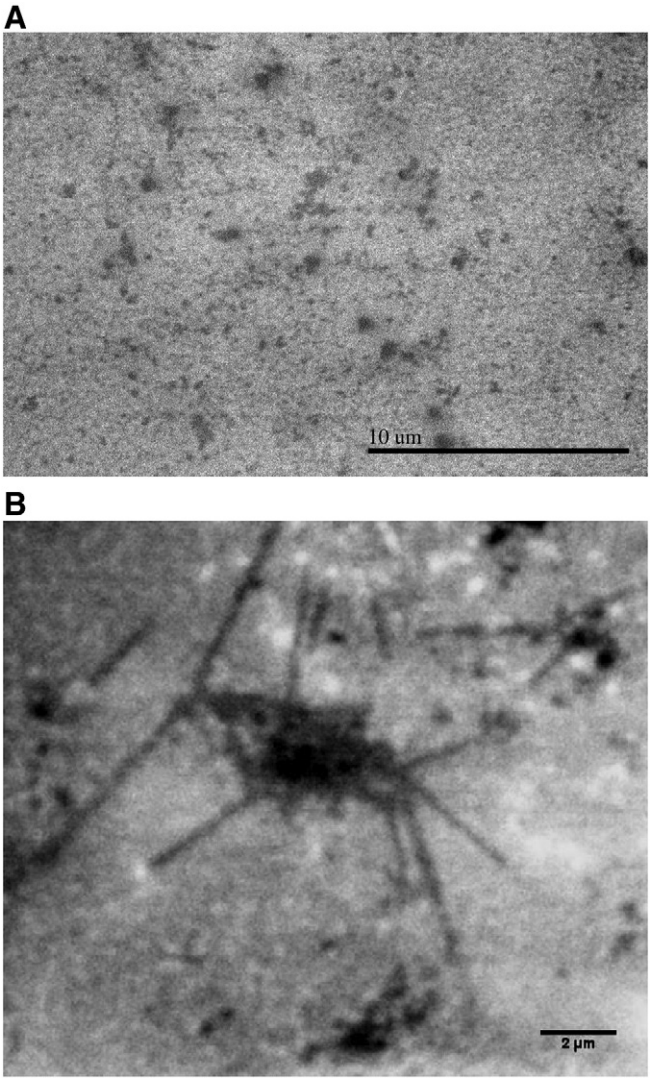

Fig. 4. (A) Lack of filaments in formation upon addition of GTP after assembly of tubulin with TPPS (bar = 10 µm), (B) residual filaments induced by paclitaxel added at the end of PPIX/tubulin assembly (bar = 2 µm).

at 37 °C for up to 2 h, there was no detectable formation of aggregates. The same result was observed after incubation with TPPS and PPIX, at 10 µM and 200 µM, as well as GTP at 10 µM. Incubation with 10 µM paclitaxel does instead produce filaments that are 2–4 µM in length and ~25 nm in width (Fig. 3A). Polymerization after incubation with 200 µM GTP produced filaments of shorter length (<1 µM) (Fig. 3B). When paclitaxel is incubated simultaneously with TPPS or PPIX it retains the ability to prompt the formation of MT whose length and width is similar to the one in the absence of the porphyrins (Supplemental Information) even though with PPIX fewer filaments were detected. When paclitaxel is added at the end of the TPPS-induced aggregation of tubulin it shows residual ability to promote the formation of filaments (Fig. 3C) even though the density of the filaments detected is lower. This is consistent with the turbidity data (Table 1) where there was a decrease in the rate of filament formation and a decrease in the final scattering intensity. This indicates that MTs still form to the same final size but fewer of them form under these conditions. However, if tubulin is allowed to incubate

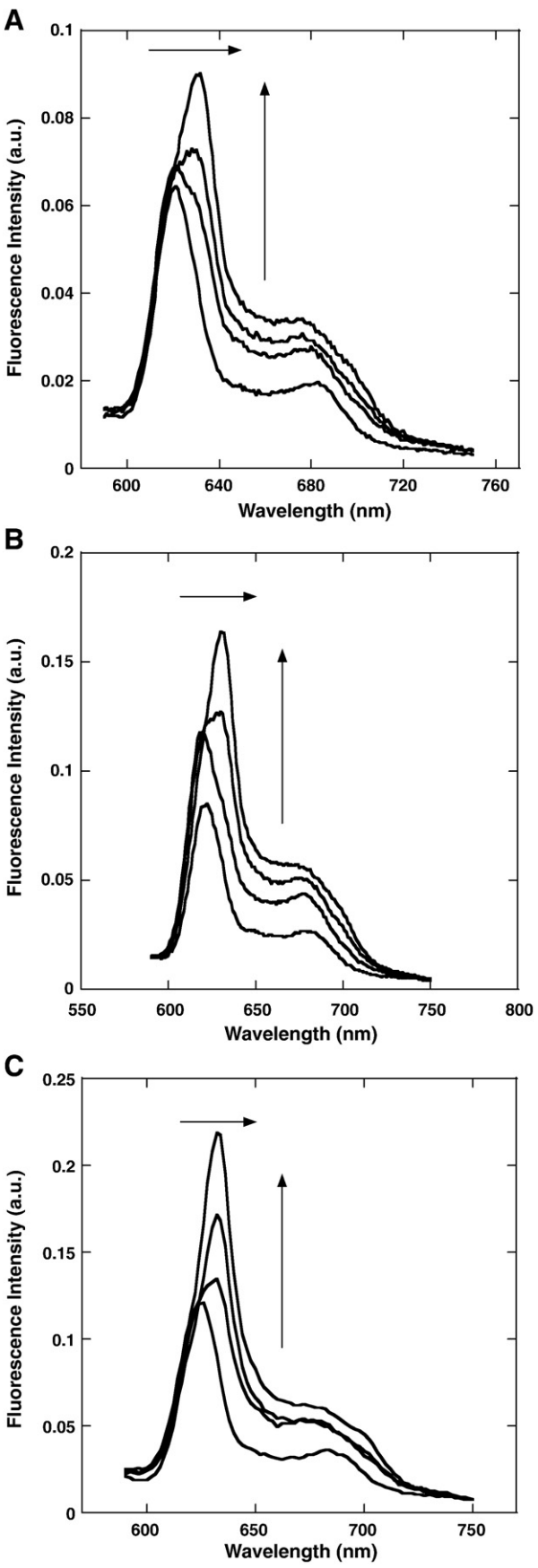

Fig. 5. (A) Fluorescence spectra of PPIX as a function of tubulin concentration. (B) Fluorescence spectra of PPIX as a function of the concentration of tubulin/GTP complexes. (C) Fluorescence spectra of PPIX as a function of the concentration of tubulin/paclitaxel complexes. $\lambda_{ex} = 405$ nm. The arrows indicate the behavior of the spectra as tubulin concentration is increased from 0 to 3.6 µM.

with TPPS alone for a longer period of time (90 min), the paclitaxel-induced MTs, formed after a 1 h assembly of the tubulin/TPPS complex, are much shorter (<1 μM) and more sparse (Supplemental Information). When GTP is added after assembly of TPPS/tubulin aggregates the formation of MT appears to be suppressed (Fig. 4A).

The addition of paclitaxel at the end of the self-assembly of PPX/tubulin complexes shows the residual formation of sparse filaments (Fig. 4B), whereas the addition of GTP does not prompt any residual polymerization (Supplemental Information).

3.3. Fluorescence spectroscopy

In agreement with previous results [28], fluorescence spectra show that PPIX and TPPS bind tubulin in the absence of other ligands. Porphyrins appear to dock GTP/tubulin and paclitaxel/tubulin non-aggregated complexes, as shown by the red-shift and the increase in intensity of porphyrin emission which are the characteristic fluorescence "signatures" of porphyrin binding [41] (Fig. 5 and Supplemental Information). The analysis of the binding isotherms (Supplemental Information) revealed that the presence of GTP and paclitaxel reduced the affinity of PPIX and TPPS for the protein (Table 2).

Conversely, our results also show that the quenching constant, retrieved from Stern–Volmer plots (Fig. 6), for the addition of GTP or paclitaxel to tubulin/PPIX and tubulin/TPPS complexes remained virtually unchanged (Table 3).

An overall remark has to be devoted to the fact that the binding constants obtained with this study are smaller than the ones we reported earlier [28]. At the moment we do not have an explanation for the discrepancy.

Unfortunately fluorescence cannot be used as a quantitative tool to detect the binding of the ligands to filaments/aggregates since their formation would interfere with the emission measurements.

4. Discussion

On the basis of the data presented above it is evident that both PPIX and TPPS have an effect on the self-assembly of tubulin in solution. In the absence of porphyrins, paclitaxel rapidly produces MT (Fig. 3) whose structure is in agreement with the one reported by many other groups [30,32,42]. The rates and structures observed for GTP-induced assembly in the absence of porphyrins (Fig. 3) are also in agreement with previous reports [12,37].

Simultaneous incubation with porphyrins and paclitaxel produces a decrease in the polymerization rate (Table 1) and shows that filaments, although still present, are reduced in number (Fig. 3). The effect is similar upon simultaneous incubation with GTP.

When tubulin and porphyrins are allowed not only to incubate but to remain at 37 °C for 1 h before the addition of paclitaxel or GTP, the results are more dramatic. First of all the polymerization assay shows that both porphyrins prompt the formation of tubulin aggregates (with slower kinetics than GTP or paclitaxel (Table 1)), and that these aggregates do not elongate but remain much smaller than the ones prompted by GTP or paclitaxel (the value of O.D. at plateaus is about 10-fold smaller). The small size of these aggregates is confirmed by the fact that they could not be detected by SEM.

Subsequent addition of GTP or paclitaxel shows two separate effects. Paclitaxel can still prompt the formation of filaments at a rate which is at least one order of magnitude smaller than it is with tubulin alone. The formation of GTP-induced filaments, instead, is suppressed.

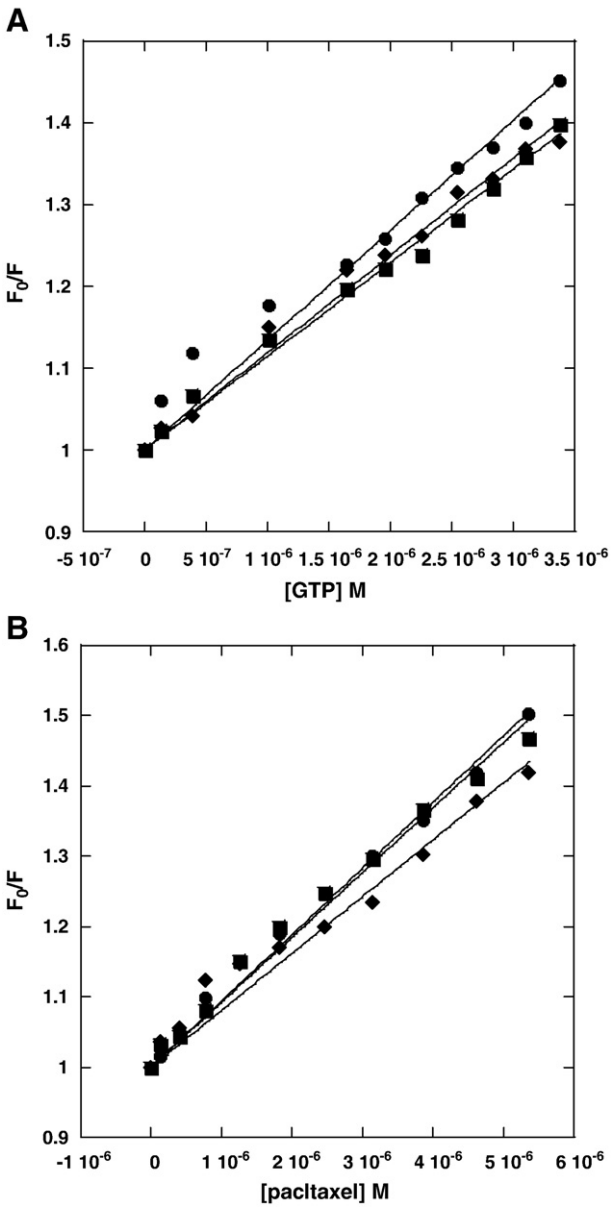

Fig. 6. Stern–Volmer plots of the quenching of tubulin fluorescence by GTP and paclitaxel. (A) Quenching produced by GTP on tubulin (■), tubulin/TPPS complexes (●) and tubulin/PPIX complexes (♦). (B) Quenching produced by paclitaxel on tubulin (■), tubulin/TPPS complexes (●) and tubulin/PPIX complexes (♦).

Thus, the formation of porphyrin-induced aggregates prevents paclitaxel and GTP from forming filaments. Paclitaxel is such a strong MT stabilizer, that residual filaments are formed (and detected) with the tubulin that is not aggregated (probably because it is not bound to PPIX or TPPS).

TPPS-induced aggregation of globular proteins is known and due to the partial neutralization of surface charges in the protein [43]. PPIX, however, is not known to prompt aggregation of proteins. The fluorescence data provide some additional clues. Although a direct interaction with the aggregates and filaments could not be detected with this

Table 2
Binding constant, K_b (M^{-1}), obtained from the binding isotherm (Eq. (2)).

	Tubulin alone	Tubulin/GTP	Tubulin/paclitaxel
PPIX	3.7×10^5	1.9×10^5	1.4×10^5
TPPS	1.8×10^6	6.8×10^5	6.9×10^5

Table 3
Quenching constant, K_S (M^{-1}), of GTP and paclitaxel for tubulin.

Quencher	Tubulin alone	Tubulin + PPIX	Tubulin + TPPS
GTP	$1.63\ (\pm0.28)\times10^5$	$1.59\ (\pm0.27)\times10^5$	$2.04\ (\pm0.35)\times10^5$
Paclitaxel	$0.93\ (\pm0.16)\times10^5$	$1.35\ (\pm0.28)\times10^5$	$1.03\ (\pm0.18)\times10^5$

method, the emission data of Fig. 6 (and Supplemental Information) reveal that there is no direct competition between porphyrins and GTP or paclitaxel for the binding to tubulin, although the change in affinity produced by tubulin/GTP and tubulin/paclitaxel complexes, may indicate that these ligands force the porphyrins to bind to a secondary site. Thus, the inability of GTP and paclitaxel to promote filament formation in porphyrin-aggregated tubulin is not due to a competition for the same binding sites. It is more likely that the aggregates cannot be separated and re-assembled into filaments by GTP and paclitaxel probably because of the strong interactions and geometrical constraints produced by the porphyrin-induced tubulin aggregation.

In conclusion, we have reported an effect that had not been observed so far: two porphyrins with very different physico-chemical properties may be able to aggregate tubulin to a level that prevents formation of MTs by either paclitaxel or GTP. Further investigations are ongoing to understand the structural details of this mechanism. Our findings may have a potential repercussion for the biomedical applications of porphyrins as well as for their neurotoxicity. We showed that MT could be a target of properly designed porphyrins during PDT. Neither PPIX nor TPPS would be indicated to target MT *in vivo*, but their effects on MT formation show that porphyrins formulated to dissolve in the cytoplasm could affect MT thus providing a possible target for photodynamic therapy. On the other hand our results also indicate that porphyrins could provide neurotoxicity by disrupting MT formation in neurons.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bpc.2009.09.006.

References

[1] S.K. Dutcher, The tubulin fraternity: alpha to eta, Curr. Opin. Cell Biol. 13 (2001).
[2] K.L. Richards, K.R. Anders, E. Nogales, K. Schwartz, K.H. Downing, D. Botstein, Structure–function relationships in yeast tubulins, Mol. Biol. Cell 11 (2000) 1887–1903.
[3] K.H. Downing, E. Nogales, Tubulin structure: insights into microtubules properties and functions, Curr. Op. Struct. Biol. 8 (1998) 785–791.
[4] G.L. Gabor Miklos, M.T. Yamamoto, R.G. Burns, R. Maleszka, An essential cell division gene of *Drosophila*, absent from *Saccharomyces*, encodes an unusual protein with tubulin-like and myosin-like peptide motifs, Proc. Natl. Acad. Sci. U. S. A. 94 (1997) 5189–5194.
[5] C. Berg, J. Moan, Lysosomes and microtubules as targets for photochemotherapy of cancer, Photochem. Photobiol. 65 (1997) 403–409.
[6] L.A. Amos, J. Lowe, How Taxol stabilises microtubule structure, Chem. Biol. 6 (1999) R65–R69.
[7] J. Avila, Microtubule dynamics, FASEB J. 4 (1990) 3284–3290.
[8] J. Lowe, H. Li, K.H. Downing, E. Nogales, Refined structure of αβ-tubulin at 3.5 A resolution, J. Mol. Biol. 313 (2001) 1045–1057.
[9] R. Bai, X.F. Pei, O. Boye, Z. Getahun, S. Grover, J. Bekisz, N.Y. Nguyen, A. Brossi, E. Hamel, Identification of cysteine 354 of β-tubulin as part of the binding site for the A ring of colchicine, J. Biol. Chem. 271 (1996) 12639–12645.
[10] L. He, G.A. Orr, S.B. Horwitz, Novel molecules that interact with microtubules and have functional activity similar to Taxol, Drug Discov. Today 6 (2001) 1153–1164.
[11] L.M. Rice, E.A. Montabana, D.A. Agard, The lattice as allosteric effector: structural studies of alphabeta- and gamma-tubulin clarify the role of GTP in microtubule assembly. Proc. Natl. Acad. Sci. 105 (2008) 5378–5383.
[12] R.D. Vale, C.M. Coppin, F. Malik, F.J. Kull, R.A. Milligan, Tubulin GTP hydrolysis influences the structure, mechanical properties, and kinesin-driven transport of microtubules, J. Biol. Chem. 269 (1994) 23769–23775.
[13] K.J. Rosenberg, J.L. Ross, H.E. Feinstein, S.C. Feinstein, J. Israelachvili, Complementary dimerization of microtubule-associated tau protein: implications for microtubule bundling and tau-mediated pathogenesis, Proc. Natl. Acad. Sci. 105 (2008) 7445–7550.
[14] Y.L. Chen, S.Z. Lin, J.Y. Chang, Y.L. Cheng, N.M. Tsai, S.P. Chen, W.L. Chang, H.J. Harn, In vitro and in vivo studies of a novel potential anticancer agent of isochaihulactone on human lung cancer A549 cells, Biochem. Pharmacol. 72 (2006) 308–319.
[15] C. Rappl, P. Barbier, V. Bourgarel-Rey, C. Gregoire, R. Gilli, M. Carre, S. Combes, J.P. Finet, V. Peyrot, Interaction of 4-arylcoumarin analogues of combretastatins with microtubule network of HBL100 cells and binding to tubulin, Biochemistry 45 (2006) 9210–9218.
[16] P. Giannakakou, R. Gussio, E. Nogales, K.H. Downing, D. Zaharevitz, B. Bollbuck, G. Poy, D. Sackett, K.C. Nicolaou, T. Fojo, A common pharmacophore for epothilone and taxanes: molecular basis for drug resistance conferred by tubulin mutations in human cancer cells, Proc. Natl. Acad. Sci. 97 (2000) 2904–2909.
[17] R. Geney, L. Sun, P. Pera, R.J. Bernacki, S. Xia, S.B. Horwitz, C.L. Simmerling, I. Ojima, Use of the tubulin bound paclitaxel conformation for structure-based rational drug design, Chem. Biol. 12 (2005) 339–348.
[18] M. Banerjee, A. Poddar, G. Mitra, A. Surolia, T. Owa, B. Bhattacharyya, Sulfonamide drugs binding to the colchicine site of tubulin: thermodymanic analysis of the drug–tubulin interactions by isothermal titration calorimetry, J. Med. Chem. 48 (2005) 547–555.
[19] S.K. Chatterjee, J. Laffray, P. Patel, R. Ravindra, Y. Qin, M.E. Kuehne, S.L. Bane, Interaction of tubulin with a new fluorescent analogue of vinblastine, Biochemistry 41 (2002) 14010–14018.
[20] S. Gupta, M. Banerjee, A. Poddar, A. Banerjee, G. Basu, D. Roy, B. Bhattacharyya, Biphasic kinetics of the colchicine–tubulin interaction: role of amino acids surrounding the A ring of bound colchicine molecule, Biochemistry 44 (2005) 10181–10188.
[21] R.F. Luduena, M.C. Roach, V. Prasad, M. Banerjee, Y. Koiso, Y. Li, S. Iwasaki, Interaction of ustiloxin A with bovine brain tubulin, Biochem. Pharmacol. 47 (1994) 1593–1599.
[22] P.W. Baas, H.C. Joshi, Gamma–tubulin distribution in the neuron: implications for the origins of neuritic microtubules, J. Cell Biol. 119 (1992) 171–178.
[23] K. Berg, The unpolymerized form of tubulin is the target for microtubule inhibition by photoactivated tetra(4-sulfonatophenyl)porphine, Biochim. Biophys. Acta 1135 (1992) 147–153.
[24] K. Berg, H.B. Steen, J.W. Winkelman, J. Moan, Synergistic effects of photoactivated tetra (4-sulfonatophenyl)porphine and nocodazole on microtubule assembly, accumulation of cells in mitosis and cell survival, J. Photochem. Photobiol. B 13 (1992) 59–70.
[25] K. Boekelheide, J. Eveleth, A.H. Tatum, J.W. Winkelman, Microtubule assembly inhibition by porphyrins and related compounds, Photochem. Photobiol. 46 (1987) 657–661.
[26] K. Berg, J. Moan, Mitotic inhibition by phenylporphines and tetrasulfonated aluminium phtalocyanine in combination with light, Photochem. Photobiol. 56 (1992) 333–339.
[27] J.W. Winkelman, G.H. Collins, Neurotoxicity of tetraphenylporphinesulfonate $TPPS_4$ and its relation to photodynamic therapy, Photochem. Photobiol. 46 (1987) 801–807.
[28] F. Tian, E.M. Johnson, M. Zamarripa, S. Sansone, L. Brancaleon, Binding of porphyrins to tubulin heterodimers, Biomacromol. 8 (2007) 3767–3778.
[29] Y. Han, H. Malak, A.G. Chaudhary, M.D. Chordia, D.G.I. Kingston, S. Bane, Distances between the paclitaxel, colchicine and exchangable GTP binding sites, Biochemistry 37 (1998) 6636–6644.
[30] C. Madiraju, M.C. Edler, E. Hamel, B.S. Raccor, R. Balachandran, G. Zhu, K.A. Giuliano, A. Vogt, Y. Shin, J.H. Fournier, Y. Fukui, A.M. Bruckner, D.P. Curran, B.W. Day, Tubulin assembly, taxoid site binding, and cellular effects of the microtubule-stabilizing agent dictyostatin. Biochemistry 44 (2005) 15053–15063.
[31] D. Hall, The effects of Tubulin denaturation on the characterization of its polymerization behavior, Biophys. Chem, 104 (2003), 655–682.
[32] R.M. Buey, I. Barasoain, E. Jackson, A. Meyer, P. Giannakakou, I. Paterson, S. Mooberry, J.M. Andreu, J.F. Diaz, Microtubule interactions with chemically diverse stabilizing agents: thermodynamics of binding to the paclitaxel site predicts cytotoxicity, Chem. Biol. 12 (2005) 1269–1279.
[33] J. Zhou, P. Giannakakou, Targeting microtubules for cancer chemotherapy, Curr. Med. Chem. Anticancer Agents 5 (2005) 65–71.
[34] M. Beltramini, P.A. Firey, F. Ricchelli, M.A.J. Rodgers, G. Jori, Steady-state and time resolved spectroscopic studies in the hematoporphyrin–lipoprotein complex, Biochemistry 26 (1987) 6852–6858.
[35] C.M. Waterman-Storer, E.D. Salmon, How microtubules get fluorescent speckles, Biophys. J. 75 (1998) 2059–2069.
[36] S. Sharma, T. Ganesh, D.G. Kingston, S. Bane, Promotion of tubulin assembly by poorly soluble taxol analogs, Anal. Biochem. 360 (2007) 56–62.
[37] B. Vulevic, J.J. Correia, Thermodynamic and structural analysis of microtubule assembly: the role of GTP hydrolysis, Biophys. J. 72 (1997) 1357–1375.
[38] D. Hall, A.P. Minton, Turbidity as a probe of tubulin polymerization kinetics: a theoretical and experimental re-examination, Anal. Biochem. 345 (2005) 198–213.
[39] P. Horowitz, V. Prasad, R.F. Luduena, Bis(1, 8-anilinonaphtalenesulfonate). A novel and potent inhibitor of microtubule assembly, J. Biol. Chem. 259 (1984) 14647–14650.
[40] F. Gaskin, C.R. Cantor, M.L. Shelanski, Turbidimetric studies of the in vitro assembly and disassembly of porcine neurotubules, J. Mol. Biol. 89 (1974) 737–755.
[41] L. Brancaleon, H. Moseley, Effects of photoproduct on the binding properties of protoporphyrin IX, Biophys. Chem. 96 (2002) 77–87.
[42] I. Arnal, R.H. Wade, How does taxol stabilize microtubules? Curr. Biol. 5 (1995) 900–908.
[43] I.E. Borissevitch, T.T. Tominaga, H. Imasato, M. Tabak, Fluorescence and optical absorption study of interaction of two water soluble porphyrins with bovine serum albumin. The role of albumin and porphyrin aggregation, J. Luminesc. 69 (1996) 65–76.