CORE
Metadata, citation and similar papers at core.ac.uk
Provided by Elsevier - Publisher Connector

ELSEVIER
Biochimica et Biophysica Acta 1468 (2000) 381–395
BIOCHIMICA ET BIOPHYSICA ACTA
BBA
www.elsevier.com/locate/bba

The interaction between lipid derivatives of colchicine and tubulin: Consequences of the interaction of the alkaloid with lipid membranes

Stéphane Mons [a], Françoise Veretout [b], Marie-France Carlier [b], Inge Erk [c], Jean Lepault [c], Eric Trudel [d], Christian Salesse [d], Pierre Ducray [a], Charles Mioskowski [a], Luc Lebeau [a,*]

[a] *Laboratoire de Synthèse Bioorganique associé au CNRS, Université Louis Pasteur de Strasbourg, 67401 Illkirch, France*
[b] *Laboratoire d'Enzymologie et Biochimie Structurales du CNRS, 91198 Gif-sur-Yvette, France*
[c] *Centre de Génétique Moléculaire du CNRS, 91198 Gif-sur-Yvette, France*
[d] *Centre de Recherche en Photobiophysique de l'Université du Québec à Trois-Rivières, Trois-Rivières, Que., Canada G9A 5H7*

Received 10 April 2000; received in revised form 20 June 2000; accepted 6 July 2000

Abstract

Colchicine is a potent antimitotic poison which is well known to prevent microtubule assembly by binding tubulin very tightly. Colchicine also possesses anti-inflammatory properties which are not well understood yet. Here we show that colchicine tightly interacts with lipid layers. The physical and biological properties of three different lipid derivatives of colchicine are investigated parallel to those of membrane lipids in the presence of colchicine. Upon insertion in the fatty alkyl chains, colchicine rigidifies the lipid monolayers in a fluid phase and fluidifies rigid monolayers. Similarly X-ray diffraction data show that lecithin–water phases are destabilized by colchicine. In addition, an unexpectedly drastic enhancement of the photoisomerization rate of colchicine into lumicolchicine in the lipid environment is observed and further supports insertion of the alkaloid in membranes. Finally the interaction of colchicine with lipids makes the drug inaccessible to tubulin. The possible in vivo significance of these results is discussed. © 2000 Elsevier Science B.V. All rights reserved.

Keywords: Colchicine derivative; Photoisomerization; Lipid membrane; Fluorescence; Tubulin immobilization; Electron microscopy

1. Introduction

Colchicine, a heterocyclic alkaloid isolated from *Colchicum automnale* [1], is a well known antimitotic poison (Fig. 1) and is also commonly used for its anti-inflammatory properties. Its antitumor activity derives from its tubulin binding activity [2,3]. Colchicine binds specifically to tubulin αβ heterodimers with a high affinity [4,5]. Extensive studies on the kinetics of tubulin–colchicine binding have indicated that the binding reaction occurs as a two step process, the initial fast and reversible bimolecular binding reaction being followed by a slow monomolecular reaction [6–10]. The resulting 1/1 tight complex dissociates very slowly [11–13]. The binding of colchicine to tubulin induces a GTPase activity at the E-

Abbreviations: MES, 2-(*N*-morpholino)ethanesulfonic acid; PIPES, 1,4-piperazinediethanesulfonic acid; EGTA, ethylene glycol-bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; DMPC, dimyristoyl-phosphatidylcholine; DOPC, dioleoyl-phosphatidylcholine; eggPE, egg phosphatidylethanolamine; DPPE, dipalmitoyl-phosphatidylethanolamine; GTP, guanosine triphosphate; a.u, arbitrary units

* Corresponding author. Fax: +33-388-67-88-91; E-mail: lebeau@aspirine.u-strasbg.fr

0005-2736/00/$ – see front matter © 2000 Elsevier Science B.V. All rights reserved.
PII: S0005-2736(00)00279-0

site (β tubulin) [14]. Colchicine binding is linked to a conformational change of tubulin dimers which inhibits their assembly into microtubules both in vivo and in vitro [7,14–23]. However, the tubulin–colchicine complex binds actively to microtubule ends in an end poisoning fashion and can be incorporated, to a low extend, into microtubules [24–26], pending an appreciable loss in stability. In addition, the tubulin–colchicine complex can self-assemble into non microtubular polymers with thermodynamic characteristics similar to those of microtubules [20,22]. The atomic structure of the αβ tubulin dimer has been solved by electron crystallography of Zn-induced tubulin sheets [27]. An atomic model of the microtubule has recently been proposed [28,29]. The colchicine binding site on the β subunit, in close proximity of the nucleotide E-site, accounts for the biochemical properties of tubulin–colchicine. Although colchicine is a water soluble compound and shows a high affinity and selectivity for tubulin, difficulties have been met in its clinical use, suggesting that other unknown targets might interfere with tubulin binding.

In the course of our work on the two-dimensional crystallization of the tubulin–colchicine complex on lipid layers at the air–water interface, we examined the possibility to immobilize the protein at monolayers spread from colchicine-anchored lipids. We faced different problems that led us to investigate into details the colchicine–lipid and tubulin–colchicine interactions. A set of water soluble and lipid derivatives of colchicine has been prepared and herein we present a series of protein binding experiments, spectroscopic measurements, X-ray diffraction and transmission electron microscopy data that provide strong evidence for the tight interaction of colchicine with lipid boundaries. The results are discussed in view of the technical problems linked to the understanding of the in vivo effects of colchicine.

2. Materials and methods

2.1. Chemicals

Compounds **1–10** (Fig. 1) were synthesized as described elsewhere [30,31].

Firstly, four colchicinoids (compounds **1–4**), differing in their hydrophobicity, were prepared in order

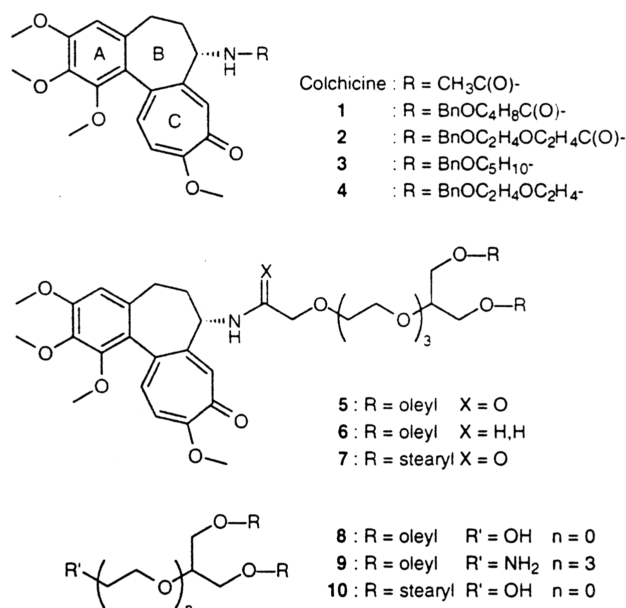

Fig. 1. Chemical structures of colchicine and colchicine derivatives analyzed in the present work. Four water soluble derivatives of colchicine have been studied (**1–4**). The initial *N*-acetyl group on the B-ring in colchicine is replaced with either a *N*-acyl linker (compounds **1** and **2**) or an *N*-alkyl linker (compounds **3** and **4**). Linkers are terminated with a benzyl group so as to sterically mimic the bottom part of the further lipids (see **5–7**). Introduction of an additional ether function in the linker chain (compounds **2** and **4**) aimed to decrease the hydrophobicity in the close neighborhood of the modified alkaloid. Three different lipid derivatives of colchicine (**5–7**) were used to investigate the possible insertion of colchicine into lipid aggregates (monolayers or vesicles) and the interaction between tubulin and colchicine in a hydrophobic environment. Some of them were used as mixtures with diluting lipids **8–10**.

to check whether structural modifications at the B-ring have crucial influence on the complexation of the alkaloid by tubulin. The original acetyl group on the B-ring of colchicine has been replaced by either a *N*-acyl linker (compounds **1** and **2**) or a *N*-alkyl linker (compounds **3** and **4**). To decrease the hydrophobicity of the close neighborhood of the modified alkaloid, a hydrophilic ether function has been introduced in the linker chain (compounds **2** and **4**). The hydrophilicity of the compounds increases from **1** to **4**, and compound **1** will be referred to as the more hydrophobic colchicinoid while **4** will be referred to as the more hydrophilic one. In all cases, the linker is terminated with a benzyl group in order to sterically mimic the later attached lipid. Many properties of these compounds are similar.

Therefore, for the sake of clarity, we will refer to these molecules as colchicinoids, unless differences have to be described.

Secondly, colchicinoids were chemically bound to different lipid structures. That was required for further two-dimensional crystallization trials with the microtubule component. In order to provide the colchicinoid with sufficient accessibility, three oxyethylene units were inserted as a linker between the alkaloid and the lipid moieties (compounds **5–7**). The aliphatic chains of the lipids are either unsaturated (compounds **5** and **6**) or saturated (compound **7**). Due to the intrinsic mechanical properties of the compounds resulting from the structure of the chains, these lipids will be referred to as the fluid neutral (**5**), fluid-charged (**6**), and rigid neutral (**7**) colchicinoid-anchored lipids. MES was purchased from Calbiochem; PIPES and EGTA were from Sigma; colchicine was from Prolabo; DMPC, DOPC, eggPE-fluorescein and DPPE-rhodamine B were from Avanti Polar Lipids.

2.2. Tubulin purification

Tubulin was purified from pig brain through three cycles of assembly–disassembly according to Shelanski [32] followed by phosphocellulose chromatography [33] in 25 mM MES pH 6.8, containing 0.125 mM $MgCl_2$ and 0.25 mM EGTA. Pure tubulin was concentrated by ultrafiltration (Amicon) and stored at −80°C in 50 mM MES pH 6.8, 3.4 M glycerol, 0.5 mM $MgCl_2$, 1 mM EGTA, and 0.1 mM GTP (buffer A).

2.3. Binding of colchicine and colchicinoids to tubulin

Binding of colchicine and colchicinoids to tubulin was monitored by the associated increase of the colchicine fluorescence [7,13]. Tubulin was equilibrated in 0.1 mM PIPES pH 6.8, 1 mM $MgCl_2$, 1 mM EGTA, 0.1 mM GTP (buffer B) by Sephadex G-25 chromatography (PD-10, Pharmacia). Binding assays were then typically performed at 37°C with a 3–5 μM colchicine concentration. The time course of fluorescence change was monitored with a Spex Fluorolog 2, equipped with a shutter that was closed between measurements in order to prevent bleaching of the sample.

2.4. Polymerization of tubulin–colchicine and tubulin–colchicinoid complexes

The kinetics of assembly of tubulin–alkaloid complexes was monitored turbidimetrically as previously described [34]. Formation of the tubulin–colchicine (or tubulin–colchicinoid) complex was carried out by incubating tubulin (62 μM) with 250 μM colchicine (or colchicinoid) in buffer A at 37°C for 30 min, followed by removal of the free ligand by activated charcoal (3 mg/ml) and rapid centrifugation in an Eppendorf microcentrifuge. The tubulin concentration was determined by UV absorption as previously described [35]. The concentration of bound ligand was determined spectrophotometrically using an extinction coefficient of 16 600 M^{-1} cm^{-1} at 350 nm. One molecule of colchicine was found to be bound per tubulin dimer.

Polymerization of tubulin–colchicine and tubulin–colchicinoid complexes was monitored at 37°C in buffer A supplemented with 1 mM GTP and 10 mM $MgCl_2$. The critical concentration for assembly was determined by measuring the concentration of unpolymerized tubulin present in the supernatant of samples centrifuged at $400\,000 \times g$, at 37°C for 5 min at the end of the assembly process.

2.5. GTP hydrolysis measurements

The rate of GTP hydrolysis by tubulin–colchicinoid complexes was assayed as previously described [20] in buffer A containing 0.625 mM γ-^{32}P-labeled GTP at 37°C. The concentration of the tubulin–colchicine and tubulin–colchicinoid complexes was 15 μM.

2.6. Colchicine and colchicinoids photoisomerization experiments

The method previously described by Roigt and Leblanc was used [36]. The isomerization reaction was monitored by measuring the optical density at 350 nm, corresponding to the absorption of the tropone moiety which is absent in lumicolchicines. The residual absorption coefficient of lumicolchicines at this wavelength has been determined to be 1650 cm^{-1} M^{-1}, i.e. 1/10 that of colchicine [37]. Colchicine samples were prepared either in water, methylene chlo-

ride or hexane/ethanol (9/1; v/v) at a concentration of 51 μM. Colchicinoid-anchored lipid **5** was solubilized in hexane/ethanol (9/1; v/v), at the same concentration. Spectra were recorded on a Hewlett Packard 8452A diode array spectrophotometer using 1 cm quartz cuvettes. Photoisomerization experiments were realized with samples in the same quartz cuvettes, using a bench UV-lamp (Bioblock Scientific, 365 nm, 6 W).

2.7. Film balance experiments

Surface pressure isotherms of the colchicinoid-anchored lipids were measured using a home-made Langmuir trough made of aluminum covered with Teflon (76.8 × 253.4 mm). Surface pressure was measured with a Wilhelmy plate coupled to a linear transducer as previously described by Albrecht [38]. Monolayers were spread from 0.5 mM lipid solutions in hexane/ethanol (9/1; v/v). Volumes ranging from 100 to 300 μM were delivered at several locations across the water surface with a Hamilton microsyringe. At least 15 min were allowed for the spreading solvent to evaporate before monolayer compression. The subphase consisted of purified water (pH 5.35, Millipore filtration system, Barnstead, NANO pure 11) with a resistivity of 18.2 10^6 Ωcm. Surface pressure versus molecular area curves were recorded at 21.0 ± 0.2°C with a compression speed of 2–5 $Å^2$ per lipid molecule per minute. All measurements were carried out in triplicate. Specific areas A_s, lift-off areas A_{lo}, and collapse pressures π_c were determined as previously described [39].

2.8. Fluorescence microscopy of the monolayers

Fluorescence microscopy experiments were performed on a dedicated film balance of local design equipped with a Nikon epifluorescence microscope. This equipment is very similar to that used by Meller [40], and has been thoroughly described elsewhere [41]. Colchicinoid-anchored lipid samples containing either eggPE-fluorescein or DPPE-rhodamine B (1 mol%) were spread on pure water (pH 5.35, 21°C). After spreading, the lipid layers were in the gas-analog state (2–5 nm^2/molecule) and were observed with a SIT camera attached to the microscope. The images were recorded on a videotape and printed by a Sony video printer.

2.9. Tubulin binding to colchicinoid-anchored lipid layers

All the experiments were carried out under red light to avoid photodegradation of the alkaloid bound to the lipids. Typically, buffer B (13 μl) was placed in a small Teflon well (4 mm diameter, 1 mm deep) and a colchicinoid-anchored lipid (**5**–**7**, 1 μl, 0.5 mg/ml in hexane/ethanol 9/1; v/v) was spread at the surface of the aqueous solution. The spreading solvent was allowed to evaporate for 15 min before tubulin (4 μl, 4 mg/ml in buffer B) was injected beneath the lipid layer. The whole device was sealed to prevent water evaporation and stored in the dark. The protein was allowed to incubate with the lipid film over periods ranging from a few minutes to several hours at 4, 20, or 37°C. The lipid layer and the bound protein were picked up onto a carbon-coated electron microscopy grid, negatively stained with uranyl acetate, and examined on a CM12 (Philips) electron microscope. Micrographs were taken at a magnification of 35 000 using 80 kV electrons.

2.10. Colchicinoids fluorescence measurements

Samples of colchicine and colchicinoids **2** and **4** were prepared in water. Vesicles were prepared by mixing compound **5** or **6** (10 nmol) with the fluid neutral lipid **8**, and compound **7** (10 nmol) was mixed with the rigid neutral lipid **10**, in a 4/1 molar ratio in chloroform. Solvent was then removed in vacuo and pure water was added (2.0 ml). Samples containing **5** and **6** were sonicated in the dark with a sonication probe for 45 min at room temperature whereas sonication of **7** was performed at 70°C. Unless otherwise stated, all samples were prepared to have a final colchicine or colchicinoid concentration of 5 μM. Fluorescence measurements were performed in 1 cm quartz cuvettes, with a Jobin Yvon JY3D spectrofluorimeter.

2.11. X-Ray diffraction experiments

Samples were analyzed with a Guinier camera

mounted on a sealed X-ray generator operated at 850 W. Shortly, the X-ray beam was focused with a quartz monochromator. The specimens were loaded in a cell equipped with mica windows. The temperature of the cell was monitored by a circulating water bath in the range −10–60°C (±1°C). The X-ray patterns were recorded on photographic films.

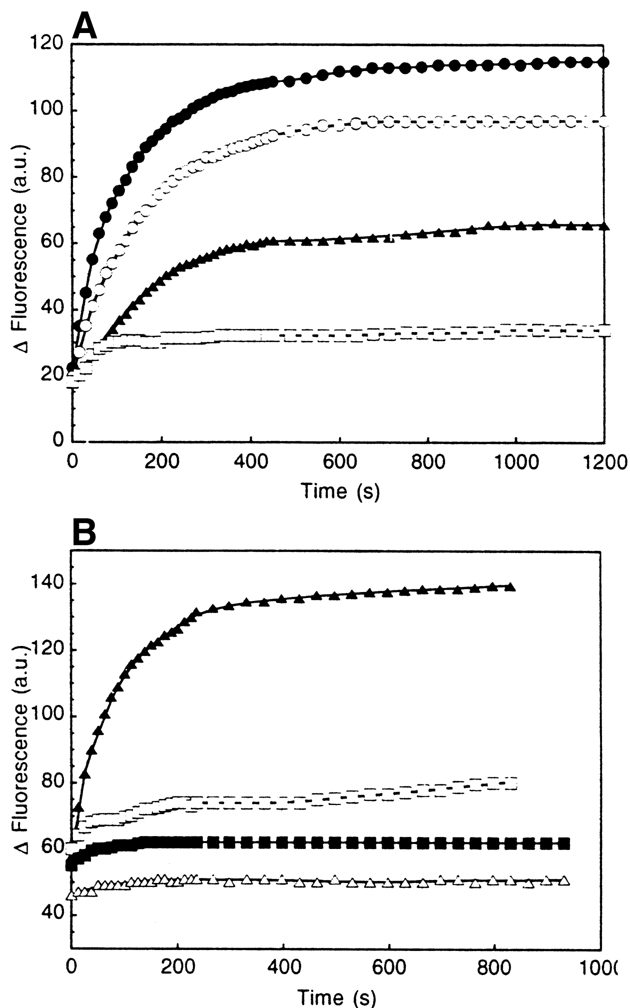

Fig. 2. Binding of colchicine derivatives to tubulin in competition with colchicine. (A) Fluorescence change for the formation of the tubulin–colchicine complexes (▲) and the tubulin–colchicinoid complexes (tubulin-**1**: ●; tubulin-**2**: ○; tubulin-**3** or tubulin-**4**: □, superposable curves). The solid line is a theoretical exponential fit of the data. Concentration of colchicine and analogues was 50 μM and of tubulin 3.3 μM. The experiments were carried out in buffer B at 37°C. Excitation wavelength was 370 nm; emission wavelength was 460 nm. (B) Inhibition of colchicine binding to tubulin by colchicinoids **3** and **4**. Colchicine (100 μM) and the competitor (50 μM; compound **3**: □, compound **4**: ■, podophyllotoxin: △) were added to the tubulin solution (3 μM), then mixed to start the reaction. Buffer conditions are the same as in (A). The ▲ data set corresponds to the fluorescence recorded in the absence of competitor.

3. Results

3.1. Binding of colchicinoids to tubulin

The binding of colchicinoids **1**–**4** to pure tubulin was monitored by fluorescence spectroscopy. Compounds **1** and **2** possess a *N*-acyl linker on the alkaloid B-ring; they exhibit a characteristic fluorescence increase upon binding to tubulin (Fig. 2A) [11,19,42]. Because colchicinoids **3** and **4** have a *N*-alkyl group on the B-ring, they exhibit a very small fluorescence change upon addition of tubulin. This data are in agreement with the previously reported correlation between the fluorescence change and the presence or not of a *N*-acyl substituent on the B-ring of colchicine [11]. However as podophyllotoxin, a well known competitor of colchicine for the binding to tubulin, colchicinoids **3** and **4** prevent the binding of colchicine to tubulin (Fig. 2B). We conclude that compounds **3** and **4** bind to tubulin at the colchicine site.

The rate constants for colchicine and colchicinoid binding to tubulin were derived from the concentration dependence of the pseudo first order rate constant of the fluorescence increase. Very similar results were obtained for colchicine and colchicinoids **1** and **2**. Since the fluorescence enhancement observed with colchicinoids **3** and **4** is very low, the kinetic parameters could not be determined accurately for these compounds and the corresponding experiments were not carried out.

3.2. Assembly properties of tubulin–colchicinoid complexes

The tubulin–colchicinoid complexes were found capable of spontaneous assembly at 37°C in buffer A containing 10 mM $MgCl_2$ and 1 mM GTP, as described for tubulin–colchicine complexes [20,43]. Fig. 3 shows typical turbidimetric recordings of the assembly processes. The self-assembly process of tubulin–colchicinoid complexes exhibits a shorter initial lag time in the turbidity increase than the tubu-

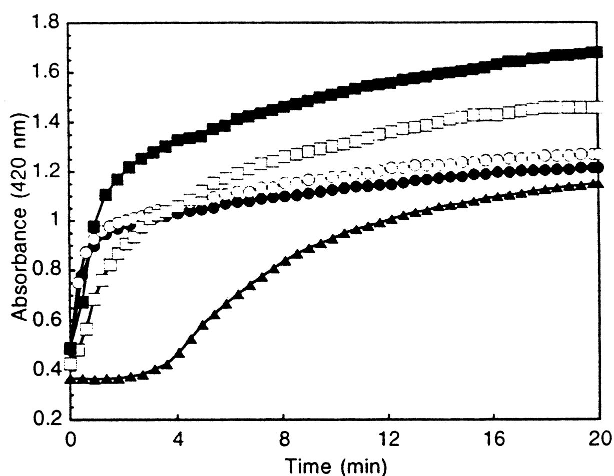

Fig. 3. Polymerization of the tubulin–colchicine and tubulin–colchicinoid complexes. Polymerization process was monitored turbidimetrically at 420 nm under the following conditions: buffer A containing 10 mM $MgCl_2$, 37°C, 15.0 mM tubulin–colchicine (▲), 14.3 μM tubulin-**1** (●), 13.9 μM tubulin-**2** (○), 14.0 μM tubulin-**3** (□), and 16.0 μM tubulin-**4** (■).

lin–colchicine complexes, indicating that the free energy of nucleation is lower. In all cases, the assembly process is biphasic in a large range of tubulin–colchicinoid concentrations. The extent of turbidity change per mol of assembled tubulin is larger for tubulin–colchicinoid than for tubulin–colchicine complexes. In agreement with the kinetic data, the critical concentrations for assembly of tubulin–colchicinoid complexes is lower than for tubulin (Fig. 4). Values of 12 μM for tubulin–colchicine, 2.0 μM for tubulin-**1**, 2.2 μM for tubulin-**2**, and 3.8 μM for both tubulin-**3** and tubulin-**4** were obtained. These data suggest that the more or less hydrophobic arm attached to the colchicine core facilitates the interactions between tubulin–colchicinoid complexes which lead to self-assembly.

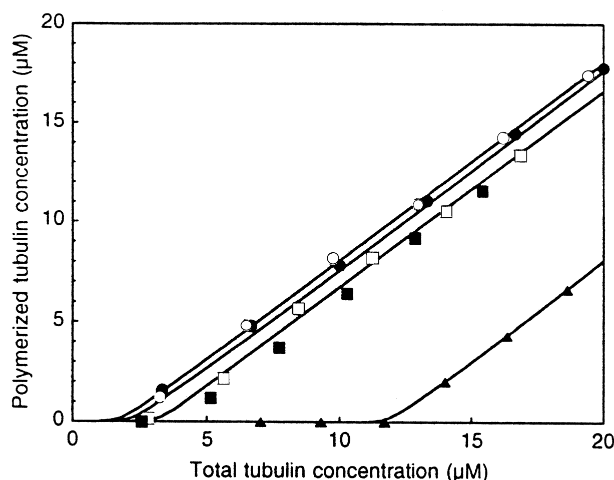

Fig. 4. Critical concentration for self-assembly of tubulin–colchicine and tubulin–colchicinoid complexes. Tubulin–colchicine (▲) and tubulin–colchicinoid complexes (symbols ●, ○, □, and ■ refer to **1**–**4** respectively) were polymerized at different concentrations, and sedimented at the end of the polymerization process. The amount of polymerized material in each sample was determined as described in Section 2.

3.3. GTPase activity of tubulin–colchicinoid complexes

Binding of colchicine to tubulin provides the protein with a spontaneous slow GTPase activity [14,18], which appears uncoupled from the polymerization process. Data, displayed in Table 1, show that tubulin–colchicinoid complexes hydrolyze GTP at a twofold faster rate than the tubulin–colchicine complex. The higher catalytic efficiency of tubulin–colchicinoid compared to tubulin–colchicine may be attributed to the greater ability of these complexes to undergo self-association and is possibly related to the overall hydrophobicity of the ligand neighborhood. The conclusion of these experiments is that derivatization of colchicine with an acyl or an alkyl chain does not impair its binding to tubulin and does not cause gross changes in the biochemical properties of the tubulin–colchicine complex.

From these three sets of experiments, it can be unambiguously concluded that the derivatized alkaloid fully retains its biological activity towards tubulin. Preliminary binding experiments with the micro-

Table 1
GTPase activity of the tubulin–colchicinoid complexes

Complex	k (min^{-1})
Tubulin	0.012 ± 0.002
Tubulin–colchicine	0.107 ± 0.015
Tubulin-**1**	0.203 ± 0.011
Tubulin-**2**	0.209 ± 0.006
Tubulin-**3**	0.163 ± 0.005
Tubulin-**4**	0.163 ± 0.005

The kinetic parameters of GTP hydrolysis by the tubulin–colchicine and tubulin–colchicinoids complexes were determined at 37°C in 25 mM MES pH 6.8 (see Section 2).

tubule component and lipid layers spread from compound **7** however indicated that the protein did not interact with the lipid film (data not shown). At first sight that result is not consistent with those described just above. Two possible explanations were proposed, the first one involving a complete photodegradation of the alkaloid moiety, and the second one putting forward the hypothesis of some particular lipid–alkaloid interaction. Each of these two points has been carefully examined.

3.4. *Photoisomerization of colchicine and lipid-anchored colchicine*

Photodegradation of colchicine in solution has been thoroughly investigated since it was discovered in 1865 [44]. The degradation products have been identified as colchicine isomers and were named α-, β- and γ-lumicolchicines [37,45,46]. Unlike colchicine, the photoisomerization products do not bind tubulin [47–52]. Early studies on the absorption and photochemistry of colchicine solutions have shown that the rate of the photoisomerization process depends on the value of the dielectric constant of the solvent [36,53]. Indeed, although the alkaloid is fairly stable in water when irradiated, it decomposes slowly in alcohols and much faster in haloalkanes. This observation prompted us to investigate the photoisomerization rate of colchicine when linked to a lipid matrix. We found that isomerization of colchicine in hexane/ethanol (9/1; v/v) proceeds sevenfold faster than in water (Fig. 5). When colchicine is anchored to a lipid, degradation is drastically enhanced and the fluid neutral colchicinoid-anchored lipid (**5**) photoisomerizes fivefold faster than colchicine in hexane/ethanol, i.e. 35 fold faster than colchicine in water. These observations indicate that colchicine behaves differently in pure water and in presence of lipids and thus suggest that the alkaloid interacts with the aliphatic chains of the lipids. Consequently, all further experiments involving colchicine and lipids were realized under red light. A new series of binding experiments with tubulin and colchicinoid lipid monolayers under non-isomerizing conditions however failed and suggested that the alkaloid might be deeply buried in between the aliphatic chains of the lipid used. That point has been addressed through lipid monolayer experiments, fluorescence measurements, and X-ray diffraction studies.

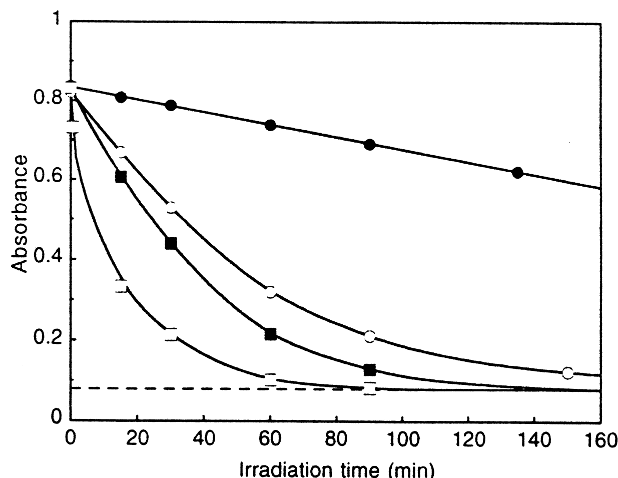

Fig. 5. Photoisomerization of colchicine and lipid-anchored colchicine in different solvents. Samples were irradiated at 365 nm and optical density was measured at λ_{max}: colchicine in water (51.07 μM, $\lambda_{max} = 353$ nm, ●), methylene chloride (51.07 μM, $\lambda_{max} = 345$ nm, ○), hexane/ethanol (9/1) (51.07 μM, $\lambda_{max} = 351$ nm, ■), and **5** in hexane/ethanol (9/1) (50.77 μM, $\lambda_{max} = 346$ nm, □). The horizontal line corresponds to the theoretical residual absorption after complete photoisomerization.

3.5. *Monolayer behavior of colchicinoid-anchored lipids*

The accessibility of the colchicine moiety to tubulin in colchicinoid-anchored lipid layers might vary depending on the conformation of the lipid aliphatic chains. To tackle that problem, we performed film balance experiments.

The surface pressure versus area isotherms of fluid and rigid colchicinoid-anchored lipids **5**–**7** are shown in Fig. 6. The isotherms of the precursor lipid matrices **8** (fluid) and **10** (rigid) are displayed for reference. As expected, the values for the lift-off area A_{lo} are much higher for the colchicinoid-anchored lipids (**5**–**7**) than for the corresponding non-derivatized lipids (**8** and **10**). This clearly indicates that when present, the colchicine moiety inserts between the lipids fatty chains. With fluid lipids (**5** and **6**), the alkaloid remains partly buried in the chains even at high surface pressure and, when the monolayers enter the collapse region, the areas per molecule are still much higher than that recorded with lipid **8** (Table 2). In addition, the A_s value for the fluid-

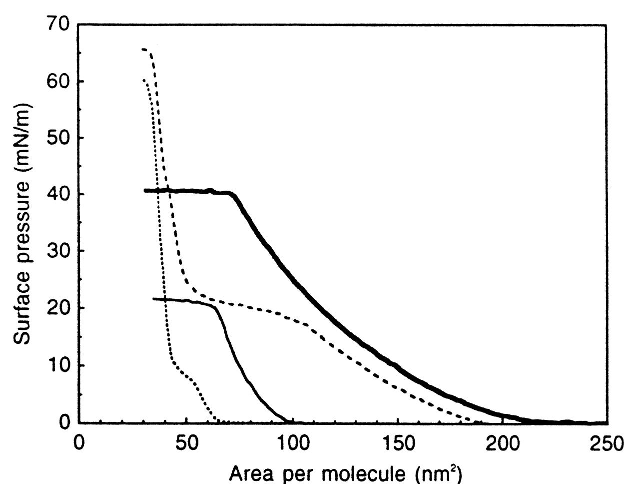

Fig. 6. Monolayer behavior of the different lipid compounds. Pressure versus area curves were obtained with compounds **5** (–), **7** (- - -), **8** (–) and **10** (.....) spread at the air–water interface at 21°C. Compound **6** exhibits a pressure versus area curve that superimposes on that of **5** except that collapse occurs at 42 mN/m (see Table 2).

Table 2
Surface behavior of the colchicinoid-anchored lipids

Compound	A_{lo} (nm^2)	A_s (nm^2)	π_c (mN/m)
5	2.30	0.73	41
6	2.05	0.70	42
7	1.90	0.33	66
8	1.05	0.67	22
9	1.55	0.60	38
10	0.70	0.33	58

Monolayer parameters of compounds **5**–**10** at the air–water interface were determined at 21°C. Compressions were carried out as described in Section 2.

charged lipid (**6**) is found smaller than that for the neutral one (**5**). This is due to the protonation of the amine group in the colchicinoid moiety that makes it more hydrophilic. Consequently, the alkaloid goes further into the aqueous phase. In the case of the rigid colchicinoid-anchored lipid (**7**), the main-phase transition plateau [54] appears around 20 mN/m (to be compared to 9 mN/m for compound **10**) due to some disorder induced by the alkaloid insertion in the monolayer. Once the lipid enters the crystalline phase, the alkaloid moiety is progressively expelled towards the aqueous phase and the monolayer collapses with the same A_s value as that measured for the non-derivatized saturated lipid **10**, consistent with a total exclusion of the colchicinoid from the aliphatic chains region.

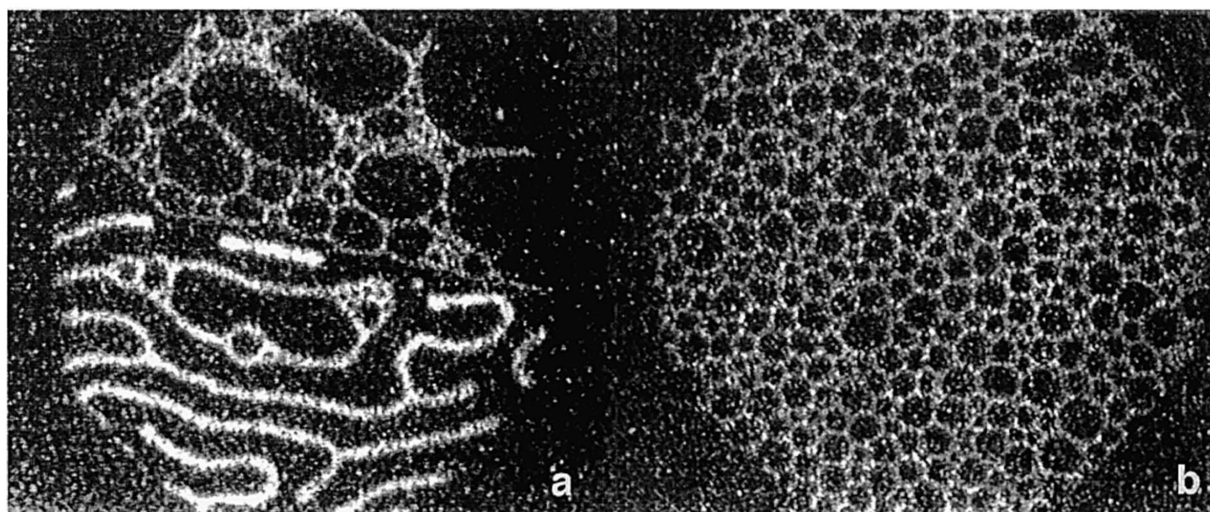

Fig. 7. Fluorescence of a steady-state monolayer of colchicinoid-anchored lipids. The micrographs correspond to the fluid neutral colchicinoid-anchored lipid (**5**) incorporating eggPE-fluorescein (1 mol%) spread at the air–water interface at 550 Å^2/molecule. The bright regions indicate the liquid expanded phase, while the dark regions correspond to the gas phase, as provided by the identification in the literature [58,59]. The thermodynamically unstable filamentous bright regions that form initially (a, 2 min) disappear over an abnormally long period of time (b, 12 h). In the same experimental conditions, the fluid lipid **8** reaches the equilibrium state as shown in b within 5–10 min (data not shown).

Accordingly, fluorescence microscopy experiments show that the fluid colchicinoid-anchored lipid monolayers (from **5** and **6**) are abnormally rigid and cohesive. Firstly, vigorous streaming normally observed in fluid monolayers at the air–water interface is drastically reduced with these lipids, and then sharp images are obtained even with extended exposure times. Secondly, whereas a classical fluid monolayer reaches the equilibrium within a few minutes following complete evaporation of the spreading solvent [54–57], compounds **5** and **6** spread into steady-state monolayers over several hours (Fig. 7 [58,59]). We propose that the insertion of colchicine in the fatty alkyl chains accounts for such a behavior. The alkaloid inhibits some of the chain molecular motion, hence the monolayer rigidification. This interpretation is consistent with another observation, when colchicine is introduced into the aqueous phase beneath a preformed lipid monolayer, the surface pressure varies slowly with time. While a fluid monolayer evolves toward a more condensed state (Fig. 8a), a rigid one gives rise to a surface pressure increase (Fig. 8b). As can be seen in Fig. 6, the expelling of colchicine from a saturated film only occurs at high surface pressure (above 40–45 mN/m, see the isotherm obtained for compound **7**). Thus at 18 mN/m the surface pressure is not high enough to efficiently prevent the insertion of the alkaloid in the monolayer, even though the later is in the crystalline phase. Due to the compact organization of the film, the alkaloid insertion then provokes an increase in the surface tension. This surprising dual effect of colchicine on lipid layers depending on whether they are fluid or rigid is indeed similar to that of cholesterol, a widespread compound of cell membranes [60–62].

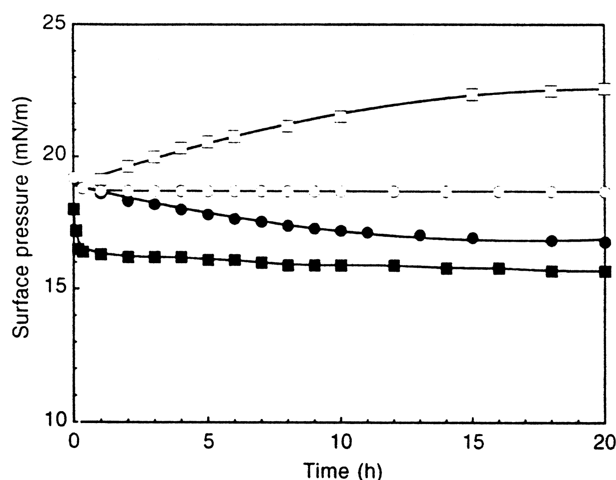

Fig. 8. Time-dependent insertion of colchicine into lipid monolayers. Compound **8** (○) was spread at the air–water interface and compressed to 19 ± 0.5 mN/m. the resulting fluid monolayer appeared quite stable after a short period of time (20 min). When 0.5 mM colchicine from a 0.5 M stock solution is injected beneath the monolayer (●), the surface pressure slowly decreases over an extended period of time. During the first minutes, the surface pressure variations are governed by the rate of mixing of the alkaloid solution to the bulk water in the trough, and revealed poorly reproducible (data not shown). The same experiment was realized with compound **10** (■), compressed to 18 ± 0.5 mN/m. The crystalline monolayer obtained is not stable as evidenced by the initially fast then slower decrease of the surface pressure. When colchicine is injected beneath the rigid monolayer (□), the interaction of the alkaloid with the lipid film provokes an increase of the surface pressure.

3.6. Fluorescence measurements on vesicles of colchicinoid-anchored lipids

Fluorescence of colchicine is extremely low at room temperature [63]. In polar solvents (dielectric constant $\varepsilon > 15$) fluorescence arises from the immobilization of the alkaloid due to solvent viscosity or steric hindrance [64]. In apolar solvents, on the other hand, fluorescence is highly sensitive to the dielectric constant and increases more than threefold when ε decreases from 15 to 7.5 [64]. Hence, we used the fluorescence of colchicine to probe the insertion of the colchicine moiety in the aliphatic chains when colchicinoid-anchored lipids are displayed at an interface. A high sensitivity CCD camera (100 times as sensitive as the spectrofluorimeter) failed to detect any significant fluorescence signal in monolayers of the compounds at the air–water interface, even with integration times extending over 10 min. To increase the signal over noise ratio, experiments were performed on lipid vesicles.

The fluorescence intensities measured for colchicine and colchicinoids **2** and **4** in aqueous solutions were found similar, indicating that the structural modifications of the alkaloid do not drastically affect its fluorescence properties (Table 3). The measurements realized on vesicles of the colchicinoid-anchored lipids **5**, **6** and **7** clearly indicate that immobilization of the colchicine moiety at the lipid interface led to a fluorescence enhancement. More-

Table 3
Fluorescence of colchicine derivatives

Sample	Concentration (μM)[a]	λ_{max} (nm)	Intensity (a.u.)[b]
Colchicine	5.01	421	1.00
Colchicine	10.02	421	0.62
Colchicine	105.80	411	0.05
2	5.03	412	0.92
4	5.30	411	0.95
5[c]	5.08	412	4.63
6[c]	5.01	400	1.29
7[d]	4.98	417	2.20

The insertion of colchicine in between the lipid fatty chains is acknowledged by an increase in fluorescence intensity.
[a]Concentration of colchicine or colchicine derivative.
[b]Fluorescence intensities have been normalized (to 1 for colchicine) and are corrected for the discrepancies in concentrations.
[c]The sample contains 20% of compound **8**.
[d]The sample contains 20% of compound **10**.

over, that fluorescence enhancement is likely to be partly reduced by a fluorescence quenching phenomenon [65,66]. As a matter of fact, the local surface concentration of the colchicine moiety on the vesicles is much higher than the 5 μM considered for the bulk sample. It is not possible however to make correlations with the results of measurements realized on colchicine samples at higher concentration (10 and 105 μM) and leading to a complete fluorescence quenching.[1] Assuming that the colchicine moieties are similarly distributed in the samples (i.e. the local concentrations of the alkaloid are the same), the differences observed in fluorescence intensity can be attributed either to partial immobilization of the alkaloid structure, or to a difference in the dielectric properties of the colchicine neighborhood, or to both of the effects. In all cases, the results are fully consistent with those of the monolayer study. Indeed, insertion of the colchicine moiety between the aliphatic chains of the lipids both restricts its rotational relaxation and decreases the local value of the dielectric constant [67–69]. More precisely, the deeper the position of colchicine in the membrane, the greater its structural constraint and the lower the value of ε, leading to the higher fluorescence intensity on the whole. Accordingly the fluid neutral lipid sample (**5**) is by far the most fluorescent (Table 3). On the other hand, the fluorescence intensity measured for the fluid charged lipid sample (**6**) was expected to be intermediate between those for the fluid neutral and the rigid neutral lipid samples (**7**). That is not the case and can be interpreted in terms of difference in the local dielectric constants. In the experimental conditions, compound **6** is protonated at the colchicine moiety which thereby is better solvated by water molecules than in compound **7**. The resulting higher local ε value thus causes a decrease in the fluorescence intensity of the compound.

[1] A rough estimate of local alkaloid concentration at the surface of liposomes would give 1–2 M.

3.7. X-ray diffraction study of lecithin-aqueous phases in the presence of colchicine

To further investigate the interaction between colchicine and lipids, X-ray diffraction experiments were realized using dimyristoyl lecithin (DMPC), the major constituent of biological membranes. Aqueous lecithin samples exhibit characteristic phases which can be easily analyzed by X-ray diffraction studies. The structural polymorphism of lecithins mainly depends on the nature of the aliphatic chains (length, saturation), the concentration of the lipid and the temperature. To assess the effects of colchicine on the phase diagram of lecithins, we studied DMPC samples containing 25% water. Such DMPC samples exhibit three different conformations in the temperature range 0–30°C (Lα, Pβ′ and Lβ′) which have characteristic X-ray diffraction patterns [70]. The transition temperatures are equal to 15 and 24°C for Lβ′-Pβ′ and Pβ′-Lα, respectively. A DMPC sample containing an equimolar amount of colchicine displays a similar phase diagram (Fig. 9). The X-ray diffraction patterns of DMPC–colchicine mixtures show the characteristic reflections of lamellar phases plus additional ones. These additional reflections are present in the X-ray diffraction pattern of a concentrated aqueous colchicine solution, revealing the presence of colchicine aggregates (Fig. 9d). However, the two phase transition temperatures are significantly lowered. While the second lipid phase transition temperature (Pβ′-Lα) is slightly shifted by colchicine (−2°C), the first transition temperature (Lβ′-Pβ′) is lowered by more than 10°C. Similar results have been obtained for lipid–colchine aqueous

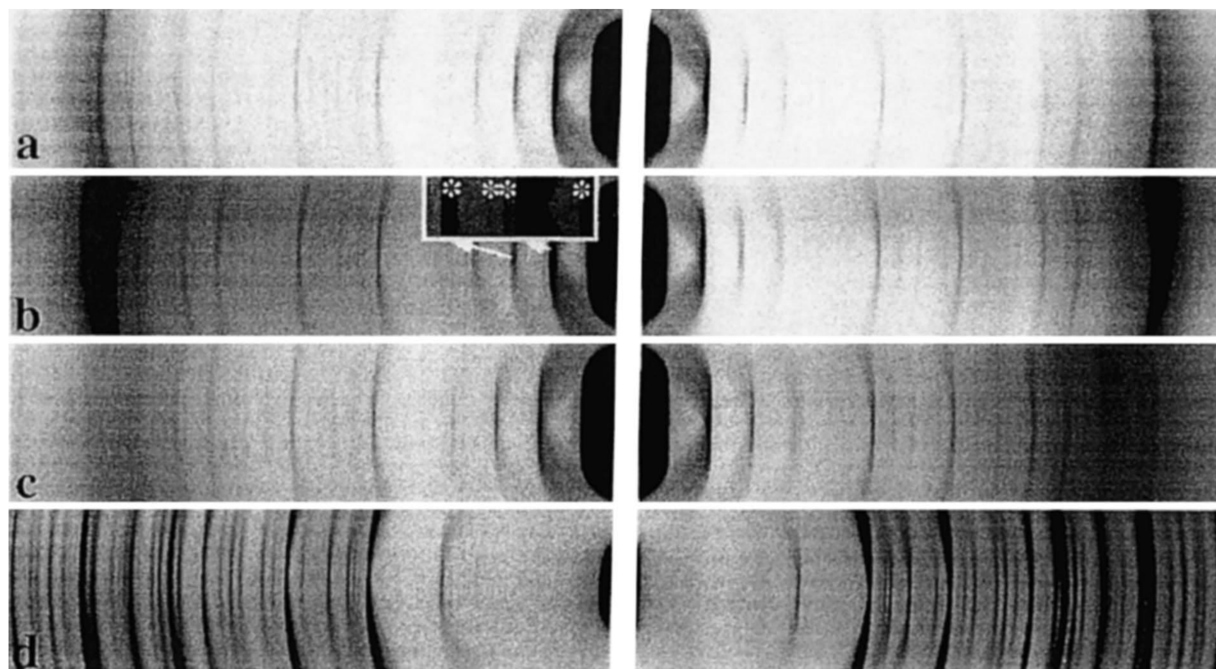

Fig. 9. X-Ray diffraction patterns of DMPC–colchicine aqueous phases. Equimolecular amounts of colchicine were added to DMPC samples containing 25% water. After stirring, the samples were let to equilibrate for at least 24 h at 60°C. The X-ray diffraction patterns of the DMPC–colchicine samples show the characteristic reflections of lamellar phases [70], plus additional ones (a: 30°C; b: 5°C; c: −5°C). These last reflections are present in the X-ray diffraction pattern of a colchicine sample lacking DMPC (d: 20°C). This indicates that colchicine microcrystalline aggregates are present in the lipid phases. This is further supported by some effect on the lamellar phase transition. Depending upon the temperature, the lipid lamellar phases have a Lβ′ (a), Pβ′ (b), or Lα (c) conformation. The low angle pattern is enlarged and inserted in b, showing the characteristic reflections of the Pβ′ phase (*, [70]). The temperatures of the lipid phase transitions are lowered by the presence of colchicine. While the first transition (Lβ′-Pβ′) is lowered by more than 10°, the second (Lβ′-Lα) is lowered by a few degrees.

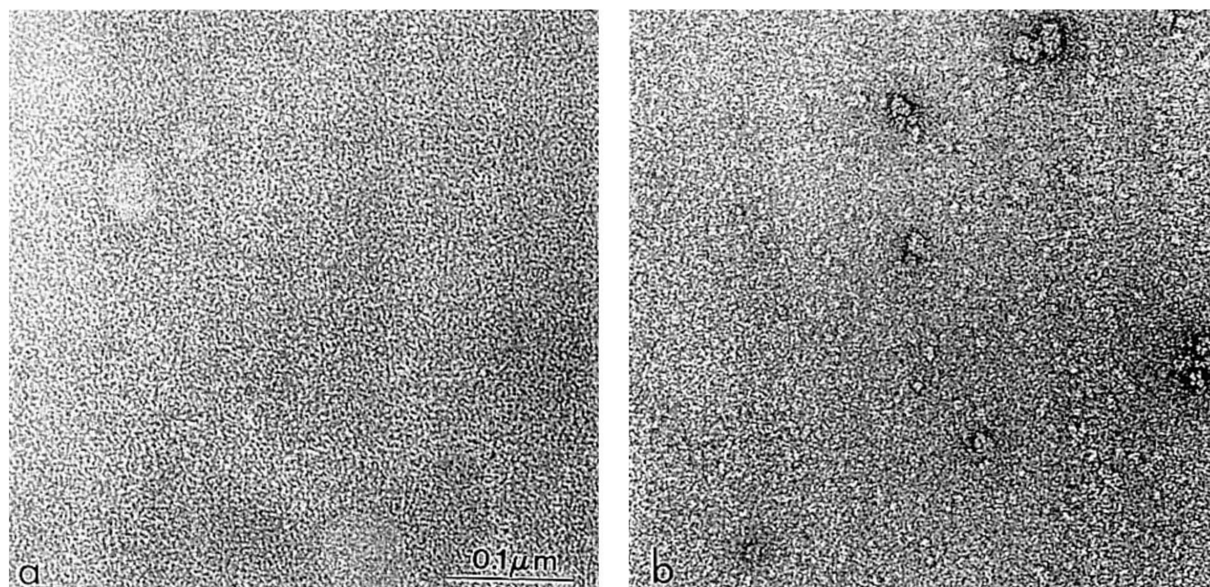

Fig. 10. Micrographs of colchicinoid-anchored lipid monolayers. Samples were negatively stained with a 2% uranyl acetate solution. Prior to incubation with tubulin, the lipid film spread from **7** appears very smooth and homogeneous (a). When the lipid film is deposited at the surface of a tubulin solution, the protein adsorbs scarcely at the interface (b). This adsorption is not reduced when the film is incubated with the tubulin–colchicine complex which reveals a purely non-specific electrostatic interaction.

mixtures with a different lipid/colchicine molar ratio (5 and 10, data not shown). This result indicates that colchicine interacts with the DMPC molecules and affects the stability of the lecithin-aqueous phases whatever the organization of the lipid chains may be (Lα, Pβ′ or Lβ′).

3.8. *Interaction between tubulin and colchicinoid-anchored lipids*

No binding of tubulin could be detected to any one of the colchicine-anchored lipids spread at the air–water interface, even with extended incubation times, at high temperature, and under red light (Fig. 10). Compound **7** spreading into a rigid monolayer and thus mechanically expelling the alkaloid toward the aqueous phase gave the same results as **5** and **6** and could not fix the protein. Occasionally isolated tubulin molecules could be seen on the surface of the monolayer. However, the binding was most likely aspecific since it could not be inhibited when colchicine was added to the protein prior to incubation with the lipid films. These last pieces of data demonstrate that when it interacts with lipids, colchicine is not accessible to tubulin.

4. Discussion

This work has been focused on the interaction of colchicine with lipid boundaries. All the results convey the view that colchicine interacts tightly with a hydrophobic neighborhood. This conclusion is at variance with the one derived from previous ESR experiments [71]. The colchicine–membrane interaction results in significant alterations of the properties of both the alkaloid and the lipid membrane. Such alterations are likely to affect biological activities of colchicine that are independent of its interaction with tubulin and its effect on microtubule dynamics [72–81].

The general behavior of the alkaloid in a lipid environment is consistent with its high octanol/water partition coefficient (12.6) [82]. The direct interaction of colchicine with the cell membrane has been a subject of controversy several years ago [83–87] but has not been investigated into details until very recently [88]. The results presented here establish that the alkaloid interacts with lipid layers, whether they are in the fluid or in the crystalline phase. The association of colchicine with lipids in a membrane provokes a modification of the mechanical properties of the membrane. This can be paralleled with the action of local anesthetics on membrane fluidity and by the way some studies have reported physiological effects of colchicine similar to those elicited by anesthetics [89–97].

Certainly one of the most important results in this work is that colchicine interacting with the lipid membrane may be unavailable for its cytosolic receptor tubulin. This is clearly demonstrated by the absence of protein binding to the films observed in the electron microscopy experiments. In the case of the crystalline lipid film (spread from compound **7**) though the alkaloid is fully displayed in the aqueous media it does not allow tubulin binding. This rather unexpected result could be explained considering the alkaloid lying flat on the lipid film what might impede its recognition by the protein. Another plausible explanation would reside in the poorly documented property of the alkaloid to form dimers or higher order aggregates in solution [98,99]. Thus it has been established that in a 15 mM colchicine aqueous solution, 48% of the alkaloid is involved in the formation of dimers, larger aggregates predominate at higher concentration. The thermodynamic parameters of the self-association process are not in agreement with the classical picture of hydrophobic interactions and rather correlate with stacking interactions as calculated for a number of dyes and bases. Considering the specific area measured for compound **7** when spread into a monolayer, we can estimate the local concentration of the alkaloid at the interface to be around 8.7 M what might definitely prevent the existence of any monomer available for protein complexation.[2]

Our results suggest that when colchicine is used in vivo, it should primarily widely distribute in the membranes of cells so most of the alkaloid molecules are not able to directly interact with tubulin. In a second step of the drug delivery process, the alkaloid is slowly released into cytosol being mostly irrever-

[2] Volume per alkaloid molecule: $V = 0.33^{3/2} = 0.19$ nm^3. Local alkaloid concentration: $c = 1/(0.19\ 10^{-24} \times 6.02\ 10^{23}) = 8.7$ M.

sibly captured by tubulin. Though the concentration investigated herein is several orders of magnitude higher than the serum concentration obtained when a recommended total therapeutic dose of 2 mg of colchicine is administered to a 70 kg human, it is likely that our observations reflect part of the development of the alkaloid in the cell. Thus there should be some clinical implications as colchicine is highly toxic and the therapeutic margin very narrow (7 mg colchicine has proved fatal [100]). The picture is in agreement with the scarce pharmacokinetic information on the drug actually available [74,101] and with the high concentrations excreted in bile [102]. May be the way colchicine is used in clinical environment at present should be basically reconsidered. As an example, liposomal formulations of colchicine have been described very recently [103]. However the true location of the drug was not investigated. The authors worked on the assumption that the alkaloid is encapsulated inside the liposomes (e.g. located in the liposome internal aqueous volume) whereas it is probably mostly incorporated in the liposomes bilayer. No pharmacokinetic data are provided and clinical efficacy is not claimed what seems to be another argument matching our results.

Even if colchicine widely distributes in membranes, its soluble part, as low as it can be, may interact with tubulin. As that interaction is very poorly reversible it can be supposed that at steady-state in cell a significant amount of the alkaloid is bound to the protein and exerts antimitotic activity. Additional work is required to determine quantitatively how much colchicine is in the cytosol within a cell and how much is membrane-associated. In the end that provides a picture in which cell membranes serve as a depot for colchicine, regulating in a passive manner the intracellular alkaloid concentration. Their physical properties are therefore altered and the consequences are still to be understood.

Colchicine is a very old drug that comes from immemorial empirical traditions. As early as in the Greek mythology, the poisoner Medee used *Colchicum* extracts to achieve some dark plans and along history, colchicine has been used in turn as poison and remedy. After many centuries however, much is still to be learned about the drug and the ways it interacts with biological systems.

Acknowledgements

This work was supported by CNRS (PCV) and ARC.

References

[1] F. Chemnitius, J. Prakt. Chem. 118 (1928) 29–32.
[2] R.L. Margolis, L. Wilson, Nature 293 (1981) 705–711.
[3] P. Dustin, Microtubules, Springer-Verlag, New York, 1984.
[4] L. Wilson, S. Meza, J. Cell. Biol. 58 (1973) 709–719.
[5] M.L. Shelanski, E.W. Taylor, J. Cell. Biol. 38 (1968) 304–315.
[6] A. Lambeir, Y. Engelborghs, Eur. J. Biochem. 109 (1980) 619–624.
[7] J.M. Andreu, S.N. Timasheff, Biochemistry 21 (1982) 6465–6476.
[8] J.M. Andreu, S.N. Timasheff, Biochemistry 21 (1982) 534–543.
[9] F.J. Diaz, J.M. Andreu, J. Biol. Chem. 266 (1991) 2890–2896.
[10] C. Dumortier, Q.Y. Yan, S. Bane, Y. Engelborghs, Biochem. J. 327 (1997) 685–688.
[11] S. Hastie, Biochemistry 28 (1989) 7753–7760.
[12] A. Lambeir, Y. Engelborghs, J. Biol. Chem. 256 (1981) 3279–3282.
[13] D.L. Garland, Biochemistry 17 (1978) 4266–4271.
[14] T. David-Pfeuty, C. Simon, D. Pantaloni, J. Biol. Chem. 254 (1979) 11696–11702.
[15] E. Taylor, J. Cell. Biol. 25 (1965) 145–149.
[16] J.B. Olmsted, G.G. Borisy, Biochemistry 12 (1973) 4282–4286.
[17] D.L. Garland, Biochemistry 17 (1978) 4266–4272.
[18] J.M. Andreu, S.N. Timasheff, Arch. Biochem. Biophys. 211 (1981) 151–157.
[19] H.W.I. Detrich, R.C.J. Williams, L. Wilson, Biochemistry 21 (1982) 2392–2400.
[20] D. Saltarelli, D. Pantaloni, Biochemistry 21 (1982) 2996–3006.
[21] J.M. Andreu, S.N. Timasheff, Proc. Natl. Acad. Sci. USA 79 (1982) 6753–6756.
[22] J.M. Andreu, T. Wagenknecht, S. Timasheff, Biochemistry 22 (1983) 1556–1566.
[23] R. Audenaert, L. Heremans, K. Heremans, Y. Engelborghs, Biochim. Biophys. Acta 996 (1989) 110–115.
[24] H. Sternlicht, I. Ringle, J. Biol. Chem. 254 (1979) 10540–10550.
[25] R.L. Margolis, L. Wilson, Proc. Natl. Acad. Sci. USA 74 (1977) 3466–3470.
[26] R.L. Margolis, C.T. Rauch, L. Wilson, Biochemistry 19 (1980) 5550–5557.
[27] E. Nogales, S.G. Wolf, K.H. Downing, Nature 391 (1998) 199–203.

[28] K.H. Downing, E. Nogales, Eur. Biophys. J. 27 (1998) 431–436.
[29] K.H. Downing, E. Nogales, Curr. Opin. Cell Biol. 10 (1998) 16–22.
[30] L. Lebeau, P. Ducray, C. Mioskowski, Synth. Commun. 27 (1997) 293–296.
[31] P. Ducray, L. Lebeau, C. Mioskowski, Helv. Chim. Acta 79 (1996) 2346–2352.
[32] M.L. Shelanski, F. Gaskin, C.R. Cantor, Proc. Natl. Acad. Sci. USA 70 (1973) 765–768.
[33] M.D. Weingarten, A.H. Lockwood, S.Y. Hwo, M.W. Kirshner, Proc. Natl. Acad. Sci. USA 72 (1975) 1858–1862.
[34] F. Gaskin, C.R. Cantor, M.L. Shelanski, J. Mol. Biol. 119 (1974) 737–758.
[35] H.W. Detrich, R.C. Williams, Biochemistry 17 (1978) 3900–3907.
[36] H. Roigt, R.M. Leblanc, Can. J. Chem. 51 (1973) 2821–2827.
[37] R. Grewe, W. Wulf, Chem. Ber. 84 (1951) 621–625.
[38] O. Albrecht, Thin Solid Films 99 (1983) 227–232.
[39] L. Lebeau, C. Mioskowski, P. Oudet, Biochim. Biophys. Acta 939 (1988) 417–429.
[40] P. Meller, Rev. Sci. Instrum. 59 (1988) 2225–2231.
[41] K.M. Maloney, M. Grandbois, D.W. Grainger, C. Salesse, K.A. Lewis, M.F. Roberts, Biochim. Biophys. Acta 1235 (1995) 395–405.
[42] J.M. Andreu, S.N. Timasheff, Biochemistry 21 (1982) 6465–6476.
[43] D. Saltarelli, D. Pantaloni, Biochemistry 22 (1983) 4606–4614.
[44] M. Hübler, Arch. Pharm. 171 (1865) 205–209.
[45] E.J. Forbes, J. Chem. Soc. (1955) 3864–3870.
[46] F. Santavy, Coll. Czech. Chem. Commun. 16 (1951) 665–675.
[47] J.C. Paulson, W.O. McClure, J. Cell. Biol. 67 (1975) 461–467.
[48] P. Banks, R. Till, J. Physiol. Lond. 252 (1975) 283–294.
[49] C. Patzelt, A. Singh, Y.L. Marchand, L. Orci, B. Jeanrenaud, J. Cell. Biol. 66 (1975) 609–620.
[50] T.A. Pfeffer, C.F. Asnes, L. Wilson, J. Cell. Biol. 69 (1976) 599–607.
[51] S. Gordon, Z. Werb, Proc. Natl. Acad. Sci. USA 73 (1976) 872–876.
[52] P. Cappuccinelli, B.D. Hames, Biochem. J. 169 (1978) 499–504.
[53] H. Roigt, R.M. Leblanc, Can. J. Chem. 50 (1972) 1959–1961.
[54] M. Lösche, E. Sackmann, H. Möhwald, Ber. Bunsenges. Phys. Chem. 87 (1983) 848–852.
[55] D.Y. Kwok, D. Vollhardt, R. Miller, D. Li, A.W. Neumann, Colloid Surf. A 88 (1994) 51–58.
[56] F. Caruso, F. Grieser, A. Murphy, P. Thistlethwaite, R. Urquhart, M. Almgren, E. Wistus, J. Am. Chem. Soc. 113 (1991) 4838–4843.
[57] R. Peters, K. Beck, Proc. Natl. Acad. Sci. USA 80 (1983) 7183–7187.
[58] H. Möhwald, Thin Solid Films 159 (1988) 1–9.
[59] H. Lösche, H. Möhwald, Colloid Surf. 10 (1984) 217–224.
[60] D. Chapman, S.A. Penkett, Nature 211 (1966) 1304–1305.
[61] D. Chapman, in: Biological Membranes, vol. II, Academic Press, New York, 1973.
[62] R.M. Williams, D. Chapman, in: R.T. Holman (Ed.), Progress in the Chemistry of Fats and Other Lipids, Pergamon Press, New York, 1970.
[63] T. Arai, T. Okuyama, Seikagaku 45 (1973) 19–29.
[64] B. Bhattacharyya, J. Wolff, J. Biol. Chem. 259 (1984) 11836–11843.
[65] R. Croteau, R.M. Leblanc, J. Lumin. 15 (1977) 353–356.
[66] R. Croteau, R.M. Leblanc, Photochem. Photobiol. 28 (1978) 33–38.
[67] H. Brockman, Chem. Phys. Lipids 73 (1994) 57–79.
[68] L.A. Bagatolli, G.G. Montich, M. Ravera, J.D. Perez, G.D. Fidelio, Chem. Phys. Lipids 78 (1995) 193–202.
[69] E. Von Kitzing, D.M. Soumpasis, Biophys. J. 71 (1996) 795–810.
[70] A. Tardieu, V. Luzzati, F.C. Reman, J. Mol. Biol. 75 (1973) 711–733.
[71] L.D. Altstiel, F.R. Landsberger, Nature 269 (1977) 70–72.
[72] J.P. Famaye, Clin. Exp. Rheumatol. 6 (1988) 305–317.
[73] F.D. Malkinson, Arch. Dermatol. 118 (1982) 453–457.
[74] M. Levy, M. Spino, S.E. Read, Pharmacotherapy 11 (1991) 196–211.
[75] M. Schlesinger, M. Ilfeld, Z.T. Handzel, Clin. Exp. Immunol. 54 (1991) 73–79.
[76] D. Ilfeld, S. Weil, O. Kuperman, Clin. Exp. Immunol. 46 (1981) 77–81.
[77] D. Ilfeld, O. Kuperman, Clin. Exp. Immunol. 50 (1982) 438–442.
[78] D. Ilfeld, E. Theodor, G. Delpire, O. Kuperman, Clin. Exp. Immunol. 57 (1984) 438–442.
[79] D. Ostermann, N. Perico, O. Imberti, C. Barbui, M. Bontempelli, G. Remuzzi, J. Am. Soc. Nephrol. 4 (1993) 1294–1299.
[80] L.S. Milner, D. Lotan, M. Mills, P.R. Goodyear, J.S. Fong, B.S. Kaplan, Nephron 46 (1987) 11–17.
[81] M.J. Lyons, R. Amador, C. Petito, K. Nagashima, H. Weinreb, J.B. Zabriskie, J. Exp. Med. 164 (1986) 1803–1808.
[82] J.M. Zamora, H.L. Pearce, W.T. Beck, Mol. Pharmacol. 33 (1988) 454–462.
[83] S. de Petris, Nature 250 (1974) 54–56.
[84] R.D. Berlin, J.M. Oliver, T.E. Ukena, H. Yin, Nature 247 (1974) 45–46.
[85] R.D. Berlin, J.P. Fera, Proc. Natl. Acad. Sci. USA 74 (1977) 1072–1076.
[86] F. Wunderlich, R. Müller, V. Speth, Science 182 (1973) 1136–1138.
[87] M. Minetti, A.M.M. Di Stasi, Biochemistry 26 (1987) 8133–8137.
[88] M. Korkmaz, E. Aksöz, Z. Naturforsch. 52c (1997) 522–529.
[89] A. Edstrom, L.J. Erkell, H.A. Hansson, Virchows Arch. B Cell Pathol. 19 (1975) 101–113.

[90] R.W. Hahnenberger, Acta Physiol. Scand. 98 (1976) 425–432.
[91] G. Hellekant, V. Gopal, Y. Ninomiya, Acta Physiol. Scand. 105 (1979) 52–57.
[92] J.H. Eichhorn, B. Peterkofsky, J. Cell Biol. 81 (1979) 26–42.
[93] D. Papahadjopoulos, K. Jacobson, G. Poste, G. Shepherd, Biochim. Biophys. Acta 394 (1975) 504–519.
[94] H.J. Galla, J.R. Trudell, Biochim. Biophys. Acta 599 (1980) 336–340.
[95] D.B. Goldstein, Annu. Rev. Pharmacol. Toxicol. 24 (1984) 43–64.
[96] I. Ueda, M. Hirikawa, K. Arakawa, H. Kamaya, Anesthesiology 64 (1986) 67–71.
[97] H. Tanii, X.P. Zhang, R. Oka, K. Saijoh, Arch. Toxicol. 71 (1997) 622–626.
[98] Y. Engelborghs, J. Biol. Chem. 256 (1981) 3276–3278.
[99] R.M. Chabin, F. Feliciano, S. Bane Hastie, Biochemistry 29 (1990) 1869–1875.
[100] L.S. Goodman, A. Gilman, in: L.S. Goodman, A. Gilman (Eds.), The Pharmacological Basis of Therapeutics, 6th edn., McMillan, New York, 1980, pp. 717–722.
[101] C. Le Hello, Ann. Med. Interne Paris 147 (1996) 185–211.
[102] L.S. Bain, D.B. Galloway, J.C. Petrie, R.A. Wood, Br. Med. J. 1 (1974) 446–448.
[103] S.B. Kulkarni, M. Singh, G.V. Betageri, J. Pharm. Pharmacol. 49 (1997) 491–495.