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Relationships between the structures of taxol and baccatine III derivatives and their *in vitro* action on the disassembly of mammalian brain and *Physarum* amoebal microtubules

***(Physarum* tubulin/brain tubulin/*Plasmodium*)**

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ABSTRACT **The *in vitro* disassembly of microtubules from mammalian brain and *Physarum* is inhibited by various derivatives of taxol and baccatine III. Structure–activity relationships of the taxol derivatives were identical for both mammalian brain and *Physarum* microtubules. This observation suggests that the site of action of taxol has been preserved during the evolution of these two different eukaryotic lines. The substituent at C-13 of taxol was required to prevent disassembly of brain microtubules with or without microtubule-associated proteins. In contrast, both taxol and baccatine III prevented the disassembly of *Physarum* microtubules to the same extent, showing that the substituent at C-13 was not required in the interaction with *Physarum* tubulin. The different effects of baccatine III and taxol derivatives indicate that measuring the disassembly of microtubules from different organisms could be a useful parameter in the search for derivatives exhibiting antiparasitic activity.**

Assembly properties of tubulin are highly preserved along the various evolutionary pathways of eukaryotic cells as illustrated by the *in vitro* coassembly of tubulin from fungi (1–3), ciliates (4), myxomycetes (5), algae (6, 7), and higher plants (8) with mammalian brain tubulin. In contrast, the sensitivity of eukaryotic cells towards microtubule poisons depends on a selective pharmacological specificity of their tubulin (9–11), allowing the use of spindle or microtubular poisons such as griseofulvin (12–14) and methyl 2-benzimidazolecarbamate derivatives (10, 15) as antifungic and/or antihelmintic drugs. Functional tubulin capable of self-assembly can be obtained from animal sources (16–21) and a limited number of eukaryotic cells that do not belong to the animal kingdom (5, 22–24). The action of microtubular poisons on animal tubulin has been studied extensively, but a comparison of their action on tubulin purified from different organisms has not been carried out. Among the numerous microtubular poisons, taxol (25, 26) possesses the unique property of stabilizing microtubules both *in vivo* (27, 28) and *in vitro* (29). The effects of taxol are widespread among eukaryotic cells, including mammalian cells (27, 28, 30, 31), *Xenopus* eggs (32), sea urchin eggs (33), *Haemanthus* endosperm (34), *Trypanosoma* (35), *Plasmodium* (unpublished results), and *Physarum* amoebae (36, 37). In order to compare the interaction of taxol on tubulin from two distinct evolutionary lines of eukaryotic cells, we have studied the effects of various taxol derivatives, in particular baccatine III (Fig. 1), on microtubules assembled *in vitro* from mammalian brain (17) and *Physarum* amoebal tubulin (23).

In vitro, taxol binds directly to tubulin and microtubules (38); induces the assembly of tubulin in the absence of exogenous GTP (39), in the presence of calcium ions (29), or at low temperatures (40); and prevents microtubule disassembly at 4°C or in the presence of calcium ions (29). The activity of taxol derivatives on tubulin has been quantified by inhibition of binding of tritiated taxol to microtubules (41), by tubulin assembly in the absence of GTP (41), and by inhibition of tubulin disassembly at 4°C (42). The latter method seemed the most suitable one for comparing the activity of taxol derivatives on *Physarum* and mammalian tubulin because it can be applied easily to the small quantities of *Physarum* tubulin that are accessible (23, 43), and it is sensitive enough to distinguish between compounds showing a 2-fold difference of activity.

FIG. 1. Structures of taxol (*Upper*) and 10-deacetylbaccatine III (*Lower*).

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Abbreviations: Me_2SO, dimethyl sulfoxide; MAPs, microtubule-associated proteins.

Table 1. ID_{50} of taxol and taxol derivatives for microtubule disassembly

Compound	Radical substitutions				ID_{50} for disassembly of microtubules, μM		R*
	C-7	C-10	C-2′	C-5′	Mammalian	*Physarum*	
A Taxol	OH	$OCOCH_3$	OH		0.5	0.9	0.6
B 7-Xylosyltaxol	O-xylose	$OCOCH_3$	OH		0.2	0.4	0.5
C 10-Deacetyl-7-xylosyltaxol	O-xylose	OH	OH		0.3	0.5	0.6
D 7-Xylosylcephalomanine	O-xylose	$OCOCH_3$	OH	H, CH_3, CH_3	0.25	0.2	1.25
E 7-Acetyltaxol	$OCOCH_3$	$OCOCH_3$	OH		1.0	1.0	1.0
F 2′-Acetyltaxol	OH	$OCOCH_3$	$OCOCH_3$		15.0	8.4	1.8

*Ratio of mammalian tubulin/*Physarum* tubulin ID_{50} values.

MATERIALS AND METHODS

Taxol Derivatives. Products A–D in Table 1 and G and O in Table 2 were extracted from the trunk bark of *Taxus baccata* L. while compound H (Table 2) was extracted from the leaves of the same species (44). Product E (Table 1) was obtained from taxol (product A) after protection of the C′-2 hydroxyl (trichloro-2,2,2-ethylchloroformate/pyridine for 15 min at 20°C), acetylation of the C-7 hydroxyl (acetic anhydride/pyridine for 15 hr at 20°C), and deprotection of the C′-2 hydroxyl (zinc/acetic acid for 4 hr at 40°C). Product F (Table 1) was obtained from product A (acetic anhydride/pyridine for 15 min at 20°C). Products I–N and P (Table 2) were prepared from product H as follows: I, acetic anhydride/pyridine for 15 hr at 20°C; J, 1% sodium bicarbonate in methanol/water, 3:1 (vol/vol), for 24 hr at 20°C with an argon atmosphere; K, chromic anhydride/pyridine for 3 hr at 20°C; L, 10 equivalents of sodium hypochlorite/acetic acid for 1 hr at 20°C; M, 0.18 M hydrochloric acid/methanol for 1 hr at 80°C; N, acetic anhydride/pyridine for 24 hr at 80°C; and P, manganous dioxide/acetone for 24 hr at 20°C. These products were purified by preparative TLC on silica. Their structures were determined by NMR spectroscopy (Bruker, 400 MHz) and mass spectroscopy (AEI MS9, chemical ionization in isobutane). A detailed description of experimental procedures and products spectra has been published (44). All products were dissolved in dimethyl sulfoxide (Me_2SO) in order to study their action on tubulin assembly/disassembly.

Preparation of Microtubule Protein: Microtubule Assembly/Disassembly. Mammalian microtubule proteins were purified from fresh pig brain tubulin by two cycles of assembly at 37°C in the presence of 4 M glycerol and disassembly at 0°C (45). Aliquots of the disassembled material were kept in 0.1 M 2-(*N*-morpholino)ethanesulfonic acid (Mes), pH 6.6/1 mM EGTA/0.5 mM $MgCl_2$/1 mM GTP/8 M glycerol at −20°C. Immediately before use, a cycle of assembly/disas-

Table 2. ID_{50} of baccatine III and baccatine III derivatives for microtubule disassembly

Compound	Radical substitutions					ID_{50} for disassembly of microtubules, μM		R*
	C-7	C-9	C-10	C-11, C-12	C-13	Mammalian	*Physarum*	
G Baccatine III	OH	= O	$OCOCH_3$		OH	26	1.2	22
H 10-Deacetylbaccatine III	OH	= O	OH		OH	23	0.9	25
I 7-Acetyl-10-deacetylbaccatine III	$OCOCH_3$	= O	OH		OH	41	0.8	51
J 10-Deacetylbaccatine V	OH epimerized	= O	OH		OH	125	3.0	41
K 10-Deacetyl-13-oxobaccatine III	OH	= O	OH		= O	175	4.8	36
L 10-Deacetyl-11-hydroxy-13-oxobaccatine III	OH	= O	OH	OH	= O	340	9.5	36
M 10-Deacetyl-8′-epibaccatine III†	OH	= O	OH		OH	100	4	25
N 7,13-Diacetylbaccatine III	$OCOCH_3$	= O	$OCOCH_3$		$OCOCH_3$	335	5	67
O Baccatine VI	$OCOCH_3$	$OCOCH_3$	$OCOCH_3$		$OCOCH_3$	70	100	0.7
P 10-Deacetyl-10-oxobaccatine III	OH	= O	= O		OH	19	3	6

*Ratio of mammalian tubulin/*Physarum* tubulin ID_{50} values.
†Epimerized at position C-8.

sembly was performed. The last pellet obtained, resuspended in the above buffer without glycerol at a protein concentration of 2 mg per ml, consisted of 80% pure tubulin and was used in all experiments unless otherwise stated. *Physarum* microtubule proteins were purified from 12–18 liters of exponentially growing axenic amoebae of *Physarum polycephalum* (strain Cld axe) (46) by the procedure of Roobol *et al.* (23). After one cycle of assembly/disassembly, the disassembled material was centrifuged 1 hr at 183,000 × *g* (Beckman rotor TI50, 4°C, 45,000 rpm) and kept in liquid nitrogen. The material obtained from three or four preparations was mixed and submitted to an additional cycle of assembly/disassembly. The final supernatant [in 0.1 M Pipes, pH 6.9/2 mM EGTA/0.1 mM EDTA/1 mM magnesium sulfate containing leupeptine (50 μg/ml; Peptide Institute, Osaka, Japan), GTP (1 mM), pancreatic DNase (10 μg/ml), and pancreatic RNase (20 μg/ml)] was adjusted to 4–5 mg of protein per ml and consisted of 30% pure tubulin. Monitoring of microtubule assembly and disassembly was performed at 350 nm at 37°C and 4°C, respectively, for mammalian microtubules and at 400 nm at 30°C and 4°C, respectively, for *Physarum* microtubules (47). The final concentration of Me_2SO introduced in the reaction mixtures with taxol derivatives varied from 0.5% to 3% (vol/vol) for mammalian tubulin and from 0.5% to 1.5% (vol/vol) for *Physarum* tubulin. The appropriate controls were run in the presence of the same amounts of Me_2SO.

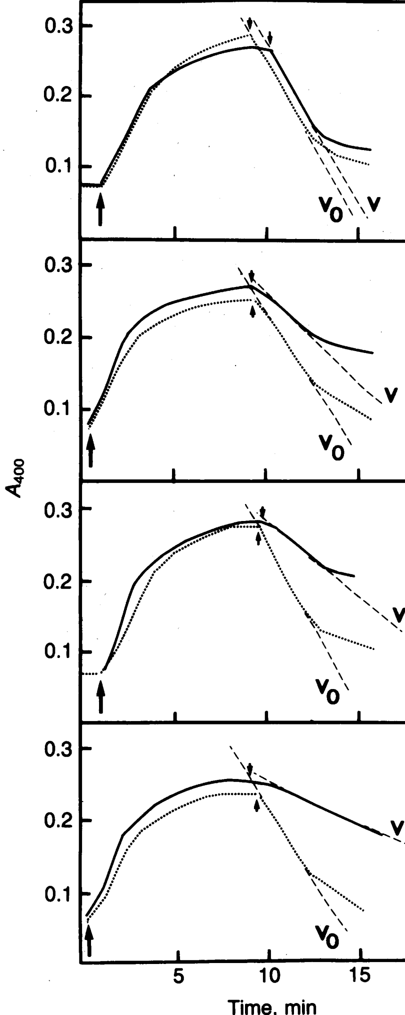

FIG. 2. Action of 7-xylosyltaxol on the disassembly of *Physarum* microtubules. In each panel the solid line indicates microtubule assembly/disassembly followed by the variation of absorbance at 400 nm in the presence of various amounts of 7-xylosyltaxol (compound B in Table 1) dissolved in Me_2SO at 0.85, 2.55, 4.25, and 8.5 μM, respectively, from top to bottom. The dotted line indicates microtubule assembly/disassembly in the presence of Me_2SO alone at 0.5%, 1.5%, 0.5%, and 1% (vol/vol) Me_2SO, respectively, from top to bottom. Large arrows show temperature shifts from 4°C to 30°C (microtubule assembly), while small arrows show temperature shifts from 30°C to 4°C (microtubule disassembly). The dashed lines indicate the slopes V and V_0 of microtubule disassembly in the presence and in the absence of 7-xylosyltaxol.

RESULTS

Tubulin from pig brain or *Physarum* amoebae was assembled at 37°C and 30°C, respectively, in the presence of various concentrations of taxol derivatives and then induced to disassemble at 4°C (Fig. 2). The initial velocity of microtubule disassembly (V) was recorded using a turbidimetric method (47) (Fig. 2). The ratio V/V_0 (V and V_0 being the rates of microtubule disassembly in the presence and in the absence of drug, respectively) decreased quasi-exponentially with increasing drug concentration (Fig. 3). The concentration of drug leading to a 50% inhibition of the rate of microtubule disassembly (ID_{50}) was determined for each compound (Tables 1 and 2 and Fig. 2) and was used to quantify the effect of each drug.

Modification of the overall geometry of the molecule of the taxol series resulted in a loss of activity. Epimerization of the methyl at C-8 (compounds H and M in Table 2) reduced the activity by 75–80%, while the epimerization of the hydroxyl at C-7 (compounds H and J in Table 2) increased the ID_{50} 3- to 5-fold. Similarly, the absence of a double bond between C-11 and C-12 and/or the presence of a hydroxyl group at C-11 (compounds K and L in Table 2) reduced the activity by 50%. Comparison of taxol (compound A in Table 1) with 7-xylosyltaxol (compound B in Table 1) showed that a xylose substituent at C-7 increased the activity by a factor of 2. Likewise, the acetylation of the hydroxyl at C-7 (compounds A and E in Table 1 and compounds H and I in Table 2) slightly changed the activity. The presence of a ketone at C-10 (compounds G and P in Table 2) had limited effects, similar to those observed (41) when the acetyl was substituted by a hydroxyl (compounds B and C in Table 1 and compounds G and H in Table 2). However a ketone at position C-13 (compounds H and K in Table 2) reduced the activity 80–88%. It has been reported (41) that concomitant acetylation

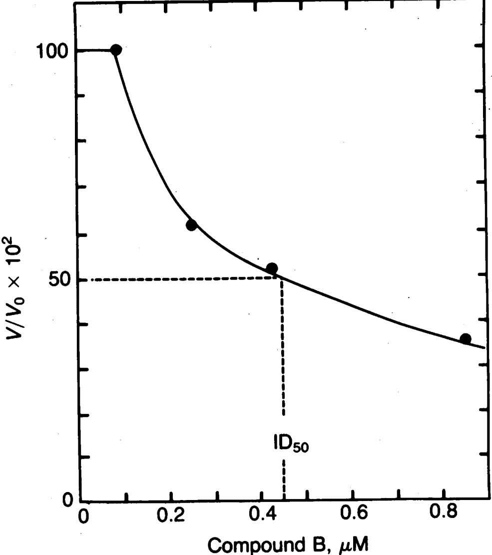

FIG. 3. Determination of the concentration of 7-xylosyltaxol leading to a reduction of 50% of the rate of *Physarum* microtubule disassembly (ID_{50}). V and V_0 are the rates of microtubule disassembly in the presence and in the absence of the drug, respectively.

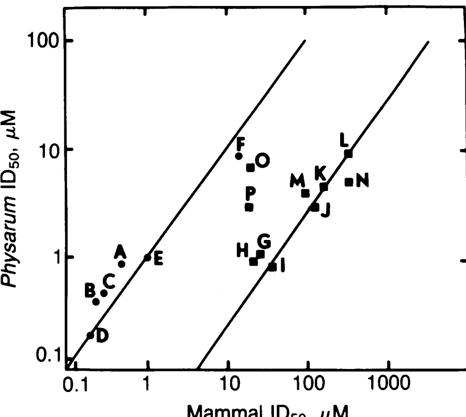

FIG. 4. Effect of various taxol and baccatine III derivatives on the disassembly of microtubules from mammals and *Physarum*. Each taxol derivative (●) and baccatine III derivative (■) is represented by the same letter as in Tables 1 and 2.

at positions C-7 and C-2′ abolished the activity. However, we found that the substitution of the hydroxyl at C-7 by an acetyl (compounds A and E in Table 1 and compounds H and I in Table 2) only slightly changed the activity.

Taxol and its five derivatives (compounds A–F in Table 1) are characterized by the esterification of the hydroxyl group at C-13 by a long substituent (Fig. 1 *Upper*). Substitution at C-5′ of the phenyl group by a tiglate (compounds B and D) did not affect the ID_{50}, but the acetylation of the hydroxyl group at C-2′ (compounds A and F) reduced the activity by 89–97%. In contrast with taxol, baccatine III and eight of its derivatives (compounds G–P in Table 2) are characterized by the absence of an acylating group at C-13 (Fig. 1 *Lower*). The ID_{50} of taxol and its derivatives varied from 0.2 to 15 μM for mammalian brain tubulin and from 0.2 to 8 μM for *Physarum* tubulin (Table 1). However, the relative efficiencies of these compounds to prevent disassembly were the same for mammalian brain and *Physarum* amoebal tubulin, and the ratio (*R*) of the ID_{50} values of a compound in the two systems varied from 0.4 to 1.8 (Table 1). Consequently, a plot of the logarithm of the ID_{50} values appears as a straight line with a slope of 1, which passes through the origin (Fig. 4). In contrast, except for compounds O and P, all derivatives of baccatine III were less active on mammalian microtubules than on *Physarum* microtubules, and the ratio (*R*) of their ID_{50} varied from 22 to 60 (Table 2). For example, taxol (compound A) and baccatine III (compound G) were equally active on *Physarum* microtubules (ID_{50}: 0.9 and 1.2 μM, respectively), whereas taxol was more potent than baccatine III on mammalian tubulin (ID_{50}: 0.5 and 26 μM, respectively). The plot of the logarithm of the ID_{50} of compounds G to M (Table 2) for mammalian and *Physarum* microtubules shows that the values generally fall on a straight line parallel to the plot of the ID_{50} of taxol derivatives and that they are 97.5% less active on mammalian microtubules than on *Physarum* microtubules (Fig. 4).

Neither the presence of leupeptin, pancreatic DNase, and pancreatic RNase (48) in the medium of assembly/disassembly of *Physarum* microtubules nor the lower temperature (30°C) used in order to induce microtubule assembly could account for the differences between the ID_{50} values of taxol and 10-deacetylbaccatine III derivatives. For example, the ID_{50} values of taxol and 10-deacetylbaccatine III towards sheep brain microtubule disassembly (0.54 and 26 μM, respectively) were not modified (0.68 and 25 μM, respectively) when the assembly/disassembly was performed in the conditions used for the assembly/disassembly of *Physarum* microtubules.

Preparations of tubulin from mammalian brain are known to contain microtubule-associated proteins (MAPs) (49, 50), which can be removed by chromatography on phosphocellulose (50). In contrast, the presence of MAPs in the preparations of *Physarum* tubulin has not been clearly established (23). The assembly and the disassembly of microtubules in the presence of taxol occurred without MAPs. For example, pig brain tubulin that had been purified on a phosphocellulose column was assembled [4 mg of protein per ml at 37°C in 0.05 M 2-(*N*-morpholino)ethanesulfonic acid, pH 6.6/12 mM $MgCl_2$/1 mM GTP/30% (vol/vol) glycerol]; taxol was 60 times more efficient in inhibiting subsequent disassembly than was 10-deacetylbaccatine III (ID_{50}: 1 and 60 μM, respectively). Similarly, when sheep brain tubulin, previously purified on a phosphocellulose column, was induced to assemble (3 mg of protein per ml at 37°C in 0.1 M Pipes, pH 6.5/0.5 mM $MgCl_2$/1 mM GTP/2 mM EGTA), taxol was 30- to 60-fold more potent than was 10-deacetylbaccatine III in promoting an equal microtubule assembly. Therefore, it is unlikely that MAPs account for the difference of sensitivity of mammalian tubulin towards taxol and baccatine III derivatives.

DISCUSSION

The *in vitro* stabilizing effects of the various taxol and baccatine III derivatives that we have investigated both on *Physarum* and mammalian microtubules allowed us to extend the previous structure–activity studies (41). When the substituent at C-13 of the molecule of taxol is replaced by a free hydroxyl, ketone, or acetyl group, as in the case of baccatine III or most of its derivatives (compounds G–N in Table 2), the activity on microtubules assembled from mammalian tubulin is 97.5% less than those on microtubules assembled from *Physarum* tubulin. We do not know what part of the substituent at C-13 is responsible for this differential effect because modification of its extremity did not alter the activity towards mammalian and *Physarum* microtubules, whereas substitution of the hydroxyl at C-2′ increased the ID_{50} for both types of microtubules. However, we do not know whether these structural alterations act by directly modifying the binding site on taxol or by indirectly changing its overall conformation.

Although it is necessary to consider that preparations of *Physarum* tubulin are less pure than the preparations of mammalian brain tubulin (23), neither the medium and the temperatures used for microtubule assembly/disassembly nor the presence or the absence of MAPs could account for the observed differences. These results suggest that baccatine III derivatives discriminate between the binding site on mammalian brain tubulin and the binding site on *Physarum* tubulin. The preferential *in vitro* action of baccatine III derivatives on *Physarum* microtubules suggests that these compounds might exhibit antiparasitic activity. However, 10 μM taxol inhibited amoebal growth in axenic liquid cultures while 10-deacetylbaccatine III was inactive at the maximum concentration used (200 μM). Similarly, 200 μM 10-deacetylbaccatine III led to only 11% of growth inhibition of *Plasmodium*, whereas an inhibition of 50% was obtained in the presence of 10 μM taxol (unpublished results). Thus, it will be necessary to investigate the cause of the *in vitro* inaccessibility of tubulin to 10-deacetylbaccatine III in order to use the differential *in vitro* sensitivity of microtubules towards taxol and baccatine III derivatives and to obtain new antiparasitic drugs.

In contrast with other microtubule poisons such as vinblastine and colchicine, which are unable to prevent the assembly of *Physarum* tubulin (23, 43), taxol is equally active in stabilizing microtubules assembled from mammalian brain tubulin and microtubules assembled from *Physarum* amoebal tubulin. Furthermore, the relative effects of all taxol derivatives studied so far (compounds B–F in Table 1) were the

same for both mammalian and *Physarum* microtubules, although the absolute extent of their stabilizing activity could differ (compare compounds A and E in Table 1). These results demonstrate that the site of action of taxol on tubulin has been preserved during the evolutionary processes that led to mammals and myxomycetes, although these two evolutionary lines most likely diverged 500–1000 Myr ago. This conclusion is in agreement with the reported action of taxol *in vivo* on mammalian cells (27, 28) and on *Physarum* amoebae (36, 37). Mutants of mammalian cells resistant to taxol have been isolated by Cabral *et al.* (51), who showed that resistance was due to a mutation in the gene coding for the α subunit of tubulin. If the site of action of taxol is located on the α subunit of tubulin, the equal sensitivity of mammalian brain and amoebal tubulin that we have observed is surprising because the α subunit appears to be less preserved than the β subunit (52–54). In order to account for the preservation of the site of action of taxol in the α subunit, an efficient selective pressure must be hypothesized. Hence, further investigation of the taxol binding site might reveal functional properties of the tubulin molecules or naturally occurring regulatory ligands.

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