Predominant Labeling of β- over α-Tubulin from Porcine Brain by a Photoactivatable Taxoid Derivative†

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ABSTRACT: An [(azidophenyl)ureido]taxoid (TaxAPU) was synthesized in a radiolabeled form by coupling an aminotaxoid to tritiated *N*-methyl-*N*-(chloroformyl)-*p*-azidoaniline. TaxAPU was used to photolabel polymerized porcine brain tubulin. This newly synthesized probe possesses taxoid properties as demonstrated by its effect, in the absence of light, on the kinetics of tubulin assembly and microtubule disassembly and on the critical concentration of tubulin. TaxAPU apparently competes with Taxol for the same binding site with an equilibrium dissociation constant of 6 μM. The photoactivation of 266 nm of the radiolabeled probe in the presence of microtubules led to a covalent incorporation of radioactivity. Analysis of the radiolabeled polypeptides by electrophoresis under denaturing conditions revealed a specific incorporation of tritium in both the α- and β-subunits of tubulin. A dependence on probe concentration was observed for the irreversible radioactivity incorporated into both subunits and maintained essentially a ratio of 2.5:1 between β/α. Therefore, TaxAPU constitutes a true photoaffinity probe for the taxoid binding site on microtubules. Our results complement those reported by Rao *et al.* (1992) of photo-cross-linking experiments with unmodified Taxol.

Microtubules are noncovalent polymers of tubulin, a globular αβ heterodimeric protein of 2 × 50 000 Da. They are involved in many cellular functions including maintenance of cell shape, cellular motility, and intracellular organelle transport (Hyams & Lloyd, 1993). Moreover, microtubules are essential for chromosome segregation in dividiing cells, a dynamic array of microtubules being required for spindle formation and function (Wadsworth, 1993).

By their implication in mitosis, microtubules constitute the target of antimitotic drugs (Iwasaki, 1993; Hamel, 1992; Rowinsky & Donehower, 1991). Among several drugs, taxoids exhibit remarkably high antitumor activities (Rowinsky & Donehower, 1991; Lavelle, 1993) and appear to be the most exciting antitumor agents recently developed.

Taxol, isolated from *Taxus brevifolia* (Wani *et al.*, 1971), has been shown to have the unique property of inducing the assembly of tubulin both *in vitro* (Schiff *et al.*, 1979) and in living cells (Schiff & Horwitz, 1980). *In vitro*, Taxol interacts reversibly with microtubules and enhances both the rate and extent of tubulin assembly, and decreases the critical concentration of tubulin; *i.e.*, a shift is observed in the tubulin–microtubule equilibrium (Parness & Horwitz, 1981). The promotion of tubulin assembly occurs even in the absence of GTP, which is normally required (Schiff & Horwitz, 1981; Carlier & Pantaloni, 1983). These events are associated with a greater stabilization of the polymers in the presence of Taxol (Wilson *et al.*, 1985) which become resistant to cold-induced depolymerization (Schiff *et al.*, 1979). Since the dynamic instability of microtubules is crucial for their biological activities, stabilization of microtubules by Taxol is thought to be the basis for its cytotoxicity (Jordan *et al.*, 1993).

Taxotere is a new, semisynthetic drug related to Taxol (Mangatal *et al.*, 1989; Guéritte-Voegelein *et al.*, 1991). Like Taxol, Taxotere induces microtubule assembly and stabilizes the polymers against cold-induced depolymerization (Ringel & Horwitz, 1991). At the cellular level, bundles of microtubules are observed and the mitosis is effectively stopped (Ringle & Horwitz, 1991). This new compound is undergoing very promising clinical trials (Lavelle, 1993; Rothenberg, 1993). Both taxoids compete for the same binding site on microtubules with a 2-fold higher affinity for Taxotere (Diaz & Andreu, 1993). Assembly of tubulin is coupled to the binding of one taxoid per αβ subunit, whereas no binding on unassembled tubulin is detected (Diaz *et al.*, 1993), indicating a tight linkage between taxoid binding to tubulin polymerization.

However, the mechanism of action of taxoids remains controversial: they could either strengthen the weak lateral interactions between tubulin subunits (Howard & Timasheff, 1988; Andreu *et al.*, 1992) or improve the longitudinal bonds of microtubules as proposed by Dye *et al.* (1993).

The precise location of the taxoids' binding site may help not only to provide a better insight into their mechanism of action at the molecular level but also in the design of new,

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[1] Abbreviations: TaxAPU, [(azidophenyl)ureido]taxane derivative; Taxol (paclitaxel), 4,10-diacetoxy-2α-(benzoyloxy)-5β,20-epoxy-1,7β-dihydroxy-9-oxotax-11-en-13α-yl (2*R*,3*S*)-3-[(phenylcarbonyl)amino]-2-hydroxy-3-phenylpropionate; Taxotere (docetaxel, trademark of Rhône-Poulenc Rorer RP56976), 4-acetoxy-2α-(benzoyloxy)-5β,20-epoxy-1,7β,10β-trihydroxy-9-oxotax-11-en-13α-yl (2*R*,3*S*)-3-[(*tert*-butoxycarbonyl)amino]-2-hydroxy-3-phenylpropionate; MES, 2-(*N*-morpholino)ethanesulfonic acid; EGTA, ethylenebis(oxyethylenenitrilo)tetraacetic acid; SDS, sodium dodecyl sulfate; GTP, guanosine 5′-triphosphate; NMR, nuclear magnetic resonance; MS, mass spectrometry; IR, infrared.

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more efficient drugs. We have undertaken to define the taxoid binding site by photoaffinity labeling, an excellent tool for the identification and characterization of ligand-specific mediators of many biological and pharmacological phenomena (Bayley & Staros, 1984; Schuster *et al.*, 1989). This technique has been applied to identify the binding site of several drugs and ligands of tubulin such as vinblastine (Safa *et al.*, 1987), colchicine (Williams *et al.*, 1985; Hahn *et al.*, 1992), rhizoxin (Sawada *et al.*, 1993), and GTP (Hesse *et al.*, 1987; Linse & Mandelkow, 1988). One study has been performed using direct photoaffinity labeling of tubulin by Taxol which indicated selective binding of Taxol to β-tubulin (Rao *et al.*, 1992). Many workers in the field have been interested in designing Taxol-derived photoaffinity probes, modified on the C-7 position (Georg *et al.*, 1992; Carboni *et al.*, 1993; Rimoldi *et al.*, 1993), but no successful irradiation experiment has been reported so far.

In the present study, we describe the synthesis and the biological evaluation of a new photoactivatable taxoid, modified on the 3′-amino side chain as well as the specific light-induced irreversible binding of the photoaffinity probe to both the α- and β-tubulin subunits.

MATERIALS AND METHODS

Materials

MES was purchased from Calbiochem. EGTA, SDS were from Sigma. GTP came from Boehringer. Acrylamide (30%)/bisacrylamide (0.8%) stock solutions were from Severn Biotech Ltd. Ammonium persulfate was from Biorad. All other chemicals were of analytical grade from Prolabo and Merck.

Drugs

Taxotere (docetaxel, RP 56976) and Taxol (paclitaxel, RP 48725) were from Rhône-Poulenc Rorer. Stock solutions were made at 10 mg/mL in absolute ethanol and stored at −20 °C. Further dilutions were made in absolute ethanol. The concentrations of Taxol and Taxotere were measured spectrophotometrically in CH_3OH using extinction coefficients ϵ_{227} of 29 800 (Wani *et al.*, 1971) and ϵ_{228} of 15 460 (Mangatal *et al.*, 1989), respectively. [^{3}H]Taxol (specific activity 23 Ci/mmol) in absolute ethanol was from Moravek Biochemicals Inc.

Synthesis of TaxAPU and [^{3}H]TaxAPU

Synthesis of TaxAPU **6** [[(*Azidophenyl*)*ureido*]*taxoid*]. *N-Methyl-N-(chloroformyl)-p-azidoaniline* (**4**). This compound was prepared from *N*-methyl-*N*-(chloroformyl)-*N*′-(*tert*-butyloxycarbonyl)-*p*-phenylenediamine derivative **3** by subsequent deprotection, diazotization, and azidation procedures (Kessler *et al.*, 1990). The resulting bifunctional reagent **4** was purified by silica gel chromatography (elution with ethyl acetate/hexane, 2/8) and used thereafter for the coupling reaction with aminotaxoid **5** (Guéritte-Voegelein *et al.*, 1991).

Coupling of Reagent **4** *with Aminotaxoid* **5**. A solution of aminotaxoid **5** (353 mg) and *N*-methyl-*N*-(chloroformyl)-*p*-azidoaniline (**4**) in pyridine (1 mL) was stirred under nitrogen for 4 days at room temperature before being quenched with water (25 mL). The aqueous phase was extracted with three portions of ethyl acetate (3 × 15 mL). The organic layer was concentrated in vacuo to give 0.5 g of crude material. Purification of silica gel chromatography (eluent: dichloromethane/methanol, 97/3) afforded 117 mg of pure compound **6**. This compound exhibited IR, ^{1}H NMR, and MS spectra in agreement with the indicated structure: ^{1}H-NMR ($CDCl_3$, 400 MHz) δ 1.14 (s, 3H, CH_3), 1.26 (s, 3H, CH_3), 1.77 (s, 3H, CH_3), 1.80 (s, 3H, CH_3), 1.84 and 2.58 (2 m, 1H each, CH_2 6), 2.30 (d, J = 9 Hz, 2H, CH_2 14), 2.37 (s, 3H, $COCH_3$), 3.16 (s, 3H, NCH_3), 3.50 (bs, 1H, OH 2′), 3.90 (d, J = 7 Hz, 1H, H 3), 4.15–4.35 (m, 2H, H 7 and OH 10), 4.20 and 4.32 (2 d, J = 9 Hz, 1H each, CH_2 20), 4.60 (m, 1H, H 2′), 4.93 (dd, J = 9.5 and 1.5 Hz, 1H, H 5), 5.20 (s, 1H, H 10), 5.21 (d, J = 10 Hz, 1H, CONH), 5.42 (dd, J = 10 and 3 Hz, 1H, H 3′), 5.69 (d, J = 7 Hz, 1H, H 2), 6.19 (bt, J = 9 Hz, 1H, H 13), 7.08 and 7.25 (2 d, J = 8.5 Hz, 2H each, H aromatics in para substituted), 7.20–7.40 (m, 5H, C_6H_5 3′), 7.52 (t, J = 7.5 Hz, 2H, $OCOC_6H_5$ H meta), 7.62 (t, J = 7.5 Hz, 1H, $OCOC_6H_5$ H para), 8.12 (d, J = 7.5 Hz, 2H, $OCOC_6H_5$ H ortho); MS (FAB) m/z 882 (MH^+), 853 ($M - N{=}N)^+$, 356 (azido side chain H^+);IR (CH_2Cl_2) 3575, 3430, 2980, 2935, 2900, 2120, 1730, 1665, 1605, 1585, 1505, 1500, 1450, 1250, 1125, 1050, 987 cm^{-1}.

Synthesis of [^{3}H]*TaxAPU* **6**: [[([*methyl-*^{3}*H*]*Azidophenyl*)*ureido*]*taxoid*]. [*methyl-*^{3}*H*]-*N-Methyl-N*′-(*tert-butoxycarbonyl*)-*p-phenylenediamine* (**2**). *N*-(*tert*-Butoxycarbonyl)-*p*-phenylenediamine (**1**) (64.8 mg, 0.3 mmol) was dissolved in ethyl acetate (10 mL). Formaldehyde (19 μL of a 37% aqueous solution) and Pd/C (10%, 150 mg) were added. The mixture was tritiated (20 Ci, T_2 gas) at atmospheric pressure for 4 h. After filtration of the catalyst and evaporation of the solvent, the residue was purified by reversed-phase (C_{18}) medium-pressure chromatography (eluent $MeOH/H_2O$, 65/35). A specific radioactivity of 7 Ci/mmol was determined by mass spectrometry.

[*methyl-*^{3}*H*]-*N-Methyl-N-(chloroformyl)-N*′-(*tert-butoxycarbonyl*)-*p-phenylenediamine* (**3**). A solution of **2** (735 mCi, 0.105 mmol) and triethylamine (30 μL, 0.22 mmol) in 1 mL of dry toluene was added dropwise to a chilled solution of phosgene in toluene (44%, 204 μL, 0.8 mmol). After the addition, the mixture was stirred for 3 h at 25 °C. The solvent was removed under reduced pressure, and the residue was taken up in ethyl acetate (4 mL). The organic layer was then washed with water, dried, and concentrated under vacuum to afford **3** which was of sufficient purity for further reactions.

[*methyl-*^{3}*H*]-*N-Methyl-N-(chloroformyl)-p-azidoaniline* (**4**). The previously obtained crude compound **3** was stirred in trifluoroacetic acid (0.12 mL) at 20 °C for 1 h. The reaction mixture was then cooled to 0 °C and set in the dark before adding an aqueous solution of $NaNO_2$ (7.5 mg in 0.15 mL). The mixture was stirred for 2 h before adding an aqueous solution of NaN_3 (7.1 mg in 0.15 mL). After additional stirring for 15 min at room temperature, the compound was extracted with ethyl acetate and the organic phase was dried and concentrated under vacuum. The obtained residue was purified by chromatography (silica gel, dichloromethane as eluent) to provide 650 mCi of pure compound **4**.

[^{3}*H*]*TaxAPU* (**6**). A solution containing 650 mCi of **4** and 54 mg of aminotaxoid **5** in 0.75 mL of pyridine was stirred for 3 days at room temperature before being quenched with water (5 mL). After extraction with ethyl acetate, the solvent was evaporated and the residual mixture was purified by silica gel chromatography (eluent: dichloromethane/methanol, 97/3) to afford compound **6** with a 85% radiochemical purity. The arylazido compound was finally purified by HPLC (silica gel; hexane/*tert*-butyl methyl ether/ethanol, 80/12/8) to give 80 mCi of pure **6** (specific radioactivity 7 Ci/mmol). The radioactive purity was checked by thin-layer chromatography on silica gel (dichloromethane/methanol, 9/1; R_f = 0.5) by

HPLC Sil zorbax 4.6 × 250 mm, eluent hexane/*tert*-butyl methyl ether/ethanol, 80/12/8, flow rate 1 mL/min, retention time 19.6 min. Tritium NMR spectroscopy (320 MHz, $CDCl_3$) gave a unique singlet at δ = 3.1 ppm. Comigration with an unlabeled reference was observed in all chromatographic systems.

Preparation of Pure Tubulin

Porcine brain tubulin was prepared by three cycles of polymerization–depolymerization (Shelanski *et al.*, 1973) followed by chromatography on phosphocellulose P11 (Whatman) (Weingarten *et al.*, 1975). The eluted tubulin, depleted of Maps, was concentrated by ultrafiltration, adjusted to 0.05 M MES, pH 6.8, 0.25 mM $MgCl_2$, 0.5 mM EGTA, 3.4 M glycerol, and 0.2 mM GTP, and stored at –80 °C at a concentration of 5–10 mg/mL.

Microtubule Assembly and Disassembly

Tubulin, at 0–2 °C, was supplemented with 6 mM $MgCl_2$ and 1 mM GTP (assembly buffer) and used within a concentration range of 10–15 μM (1–1.5 mg/mL). Polymerization was initiated by a temperature shift from 6 °C to 37 °C in a thermostated 1-cm light path cell and was monitored turbidimetrically at 350 nm (Gaskin *et al.*, 1974) with a UVIKON 931 spectrophotometer (KONTRON) equipped with a thermostatically controlled cell holder. Cold reversibility was evaluated by shifting the temperature at 6 °C until the drop in turbidity was completed. Taxoids in ethanol were added to the tubulin solution (typically less than 1%) just prior to assembly at 0–2 °C. Alternatively, microtubule assembly was measured at polymerization steady state by a sedimentation assay. Tubulin was first cycled (*i.e.*, polymerized at 37 °C for 15 min, sedimented for 15 min at 340000*g*), and then the pellet was resuspended in 100 mM phosphate buffer, pH 7, containing 1 mM EGTA and 1 mM $MgCl_2$ (PEM buffer) at 0–2 °C. This solution was clarified by a sedimentation for 10 min at 340000*g*. Tubulin samples in PEM buffer (0.1 mL) were supplemented with 6 mM $MgCl_2$, 1 mM GTP, and the desired amount of drug and warmed at 37 °C for 1 h. The resultant polymers were sedimented at 90 000 rpm for 5 min in the TLA100.1 rotor of a TL100 ultracentrifuge (Beckman). Tubulin concentrations in supernatants were measured by a Bradford assay (Biorad protein assay). The critical concentration for polymerization corresponds to the concentration of tubulin in supernatants. The critical concentration was expressed as a function of taxoid concentration.

Interaction of TaxAPU with Microtubules

Binding of TaxAPU to microtubules was measured by a sedimentation assay using ^{3}H-labeled TaxAPU. Tubulin (8 μM) in assembly buffer at 0–2 °C was divided into several samples of 0.2 mL containing different amounts of [^{3}H]-TaxAPU. The samples were incubated for 1 h at 37 °C and centrifuged for 4 min at 37 °C and 390000*g* in a TL100 ultracentrifuge. The amount of bound [^{3}H]TaxAPU was determined by measuring the radioactivity of total tritium and tritium present in the supernatant in a LS 3801 liquid scintillation analyzer (Beckman). Since taxoids bind essentially to microtubules and not to dimeric tubulin (Diaz *et al.*, 1993), the amount of bound [^{3}H]TaxAPU corresponds to the difference between total tritium and tritium in the supernatant. The concentration of microtubules is the difference between the total tubulin and the tubulin in supernatant, *i.e.* the critical concentration, evaluated by a Bradford assay. In this experiment, the critical concentration was not constant due to the promotion of tubulin assembly in presence of taxoids. Thus, the microtubule concentration must be measured for each taxoid concentration. The sedimentation assay was also used for competition experiments between TaxAPU and [^{3}H]Taxol.

Photoaffinity Labeling

Irreversible Binding. Tubulin (10 μM) was polymerized in assembly buffer for 30 min at 37 °C in the presence of increasing concentrations of [^{3}H]TaxAPU (2–20 μM). The GTP concentration was reduced to 30 μM instead of 1 mM used in standard conditions in order to avoid any screening effect due to excess GTP at the irradiation wavelength used. Samples of 1.3 mL of microtubule solution were transfered to a 1-cm light pathlength quartz cell and irradiated at 37 °C for 10 min at 266 nm using a monochromatic light beam. The light intensity was measured with a thermopile and adjusted to an energy of 200 μV.

SDS–PAGE. After irradiation, samples of [^{3}H]TaxAPU labeled microtubule were boiled with denaturing solution (v/v 0.125 M Tris-HCl pH 6.8, 4% SDS, 10% glycerol, 4% β-mercaptoethanol, and 0.02% bromophenol blue) for 5 min. Aliquots (20 μg of protein) were loaded on 5–15% acrylamide slab gels (Laemmli, 1970). Electrophoresis was conducted for 18 h at 50 V. Gels were stained with Coomassie blue R.

Measurement of ^{3}H Radioactivity Associated with Polypeptides Present in Polyacrylamide Gel Electrophoresis. The method of Tao and Lamkin (1984) was used. The gel was cut into 2-mm slices which were digested in scintillation vials containing 200 μL of 30% hydrogen peroxide; the tightly capped vials were heated overnight to 65 °C before adding 100 μL of acetic acid and 3 mL of scintillation cocktail (Aquasol 2 from Dupont NEN). After being shaken, the samples were counted.

Gel Autoradiography. Coomassie blue stained gels were treated with Entensify (NEN). The slabs were laid onto 3-mm Whatman paper and dried under vacuum at 80 °C. The dried gels were then autoradiographed for 11 days at –80 °C using hyperfilm-MP (Amersham).

RESULTS

Synthesis of TaxAPU and [^{3}H]TaxAPU

The synthesis of the [(azidophenyl)ureido]taxoid is outlined in Figure 1 and is described under Methods. This synthesis takes advantage of the availability of the aminotaxoid **5** (Guéritte-Voegelein *et al.*, 1991) which can be directly modified by an electrophilic bifunctional photoactivatable reagent (Bayley, 1983). We selected *N*-methyl-*N*-(chloroformyl)-*p*-azidoaniline, a bifunctional reagent which directly derives from its aryldiazonium precursors (Kessler *et al.*, 1990) and for which a radiolabeled synthesis has been achieved (Klotz *et al.*, 1991). Similar chemical modification of naltrexamine has already been used for a successful labeling of μ- and κ-opioid receptors (Méjean *et al.*, 1992). The synthesis of [^{3}H]TaxAPU followed identical procedures (see Methods) and gave access to the derivative with a specific radioactivity of 7 Ci/mmol). This chemical was isotopically diluted (5 times) for the photoaffinity labeling experiments.

Reversible Interaction of TaxAPU with Tubulin

Effect of TaxAPU on Tubulin Assembly. Figure 2A shows a comparative effect of three taxoids used at 2 μM concentra-

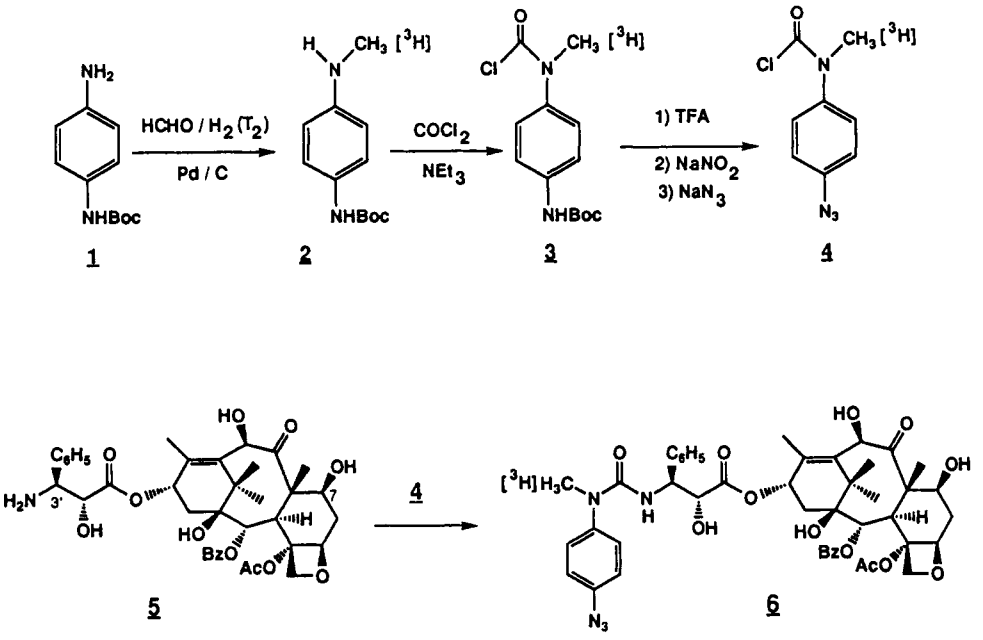

FIGURE 1: Synthesis of TaxAPU and [^{3}H]TaxAPU.

tion on tubulin polymerization and cold-induced depolymerization. TaxAPU, Taxol, and Taxotere have qualitatively the same effects on tubulin assembly and cold-induced depolymerization. TaxAPU reduces the lag period for tubulin assembly as well as the depolymerization rate. TaxAPU behaves like a taxoid but clearly is less efficient in affecting the tubulin assembly than the two leading taxoids: Taxol or Taxotere. A concentration-dependence study for TaxAPU on microtubule depolymerization (Figure 2B) shows the existence of a relationship between microtubule stabilization and probe concentration. A quantitative comparison of TaxAPU and Taxol, displayed in the inset, was based on the stabilization effect at low temperature, since the effect on depolymerization at low temperature seems to be the best method for quantitative comparison of new taxoids with Taxol (Lataste *et al.*, 1984). IC_{50} values for the concentration of drug which reduced the rate of depolymerization by 50% were obtained for Taxol and TaxAPU. Under these conditions, an IC_{50} value of 0.4 μM was obtained for Taxol, which compares well with the literature values (Lataste *et al.*, 1984). The IC_{50} for TaxAPU was 1.8 times higher (0.72 μM). Nevertheless, the substoichiometric IC_{50} value indicates that TaxAPU interacts with microtubules in a similar way to Taxol.

Effect of TaxAPU on the Critical Concentration of Tubulin Assembly. Critical concentration is defined as the minimum concentration of tubulin required for its polymerization. This value depends on solution parameters and is known to be lowered by the presence of taxoids (Schiff *et al.*, 1979). The effect of TaxAPU and the two reference taxoids on the critical concentration is shown in Figure 3. This assay was used as a test for the taxoid-like effect of TaxAPU. Comparable decreases on the critical concentration of tubulin are observed with all three derivatives, with Taxotere being more efficient and TaxAPU less efficient than Taxol in decreasing the critical concentration, again suggesting that TaxAPU acts like a taxoid on microtubules.

Binding Studies of TaxAPU and [^{3}H]TaxAPU on Microtubules. In order to ascertain whether or not TaxAPU and Taxol share common binding sites, both ligands were allowed to interact simultaneously with a microtubule solution. The binding of [^{3}H]Taxol to microtubules was assayed by sedimentation as described under Materials and Methods. The data, shown in Figure 4A, were analyzed with the nonlinear regression data analysis program Enzfitter from Biosoft. Saturation curves of Taxol binding with increased apparent equilibrium dissociation constants were observed in the presence of increasing concentrations of TaxAPU. The data fit a simple model in which TaxAPU binds to the same site as Taxol, as shown by the linear variations of the apparent K_Ds with TaxAPU concentrations (inset Figure 4A). Taxol and TaxAPU equilibrium dissociation constants were respectively of 0.7 and 6 μM, the stoichiometry of taxoid binding being 1.07 per mole of polymerized tubulin which is in accordance with the known values (Parness & Horwitz, 1981). The interactions of TaxAPU and Taxotere with microtubules were also found to be mutually exclusive (data not shown).

Direct binding of TaxAPU to microtubules was further assayed by sedimentation using [^{3}H]TaxAPU. The data, in Figure 4B, demonstrate that TaxAPU binds to microtubules in a saturable manner with an equilibrium dissociation constant of 3 μM and a stoichiometry of 0.9 mol of TaxAPU/mol of polymerized tubulin subunits. The K_D value is of the same order of magnitude as that calculated in the preceding indirect binding measurement.

Photoaffinity Labeling of Tubulin with [^{3}H]TaxAPU

Irradiation Conditions. Different aspects had to be taken into account to choose the irradiation conditions. First, due to the partial overlap of the absorption spectra of GTP and TaxAPU, the GTP concentration needed to be reduced from 1 mM to 30 μM. This reduction of GTP concentration does not affect the tubulin polymerization in the presence of taxoids (Schiff & Horwitz, 1981; Carlier & Pantaloni, 1983). The selection of the irradiation wavelength (266 nm) and energy (200 μV) resulted from different considerations: (i) its corresponds to a satisfactory extinction coefficient ratio between TaxAPU and GTP, (ii) it permits irradiation at sufficient energy from a 1000-W Xe/Hg light source connected to a monochromator, and finally (iii) it is slightly destructive for tubulin only moderately affecting its polymerization (data not shown). Irradiation, under those conditions for 10 min, allowed a substantial photodecomposition of TaxAPU and was thus selected as optimal irradiation conditions for the photoaffinity labeling experiments.

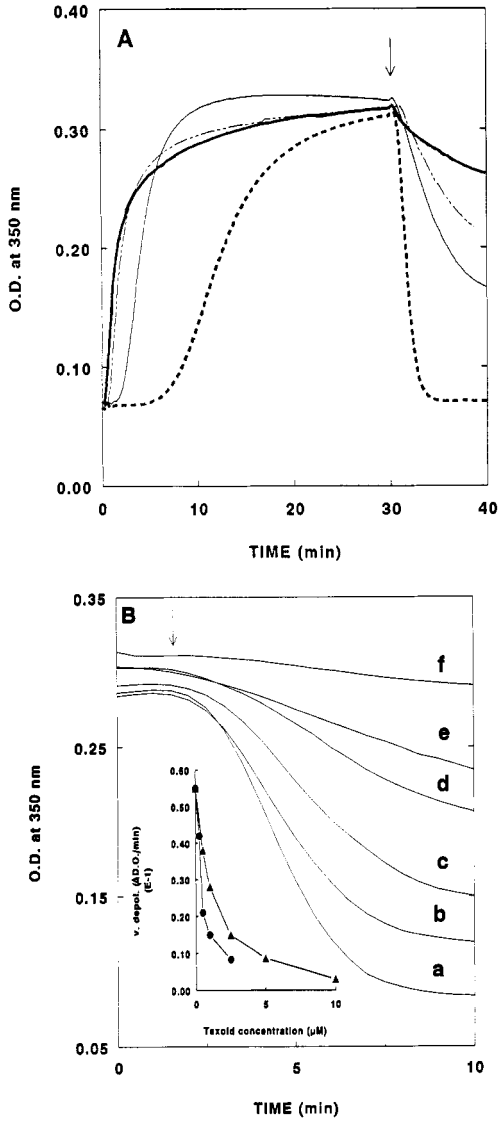

FIGURE 2: Promotion of tubulin assembly and stabilization of microtubules by TaxAPU. (A) Pure tubulin (9 μM) was polymerized at 37 °C in assembly buffer in the presence of 2 μM TaxAPU (—), 2 μM Taxol (- - —), 2 μM Taxotere (▬). At the time indicated by the arrow, the temperature was shifted to 6 °C. Control without taxoid (- - -). (B) A solution of tubulin (9 μM) was polymerized for 30 min and 37 °C in assembly buffer with or without (a) TaxAPU 0.5 μM (b); 1 μM (c); 2.5 μM (d); 5 μM (e); or 10 μM (f). At the time indicated by the arrow, the solution was cooled at 6 °C. Inset: The microtubule depolymerization rates are calculated for each TaxAPU concentration (▲) and compared to those obtained with Taxol (●).

Photoaffinity Labeling of Tubulin with [^{3}H]TaxAPU. After irradiation of the [^{3}H]TaxAPU–tubulin complex in the described conditions, a covalent incorporation of the radiolabeled probe was observed. The incorporation of radioactivity into the α- and β-subunits of tubulin was analyzed after SDS polyacrylamide gel electrophoresis both by counting the radioactivity in gel slices and by autoradiography. Figure 5 shows radioactivity incorporation along the gel after photolysis of tubulin in the presence of 4 μM [^{3}H]TaxAPU. A protected labeling experiment, for which identical experimental conditions were used, except the additional presence of an excess unlabeled Taxotere (300 μM), is also shown. The concentration of Taxotere used corresponds to the upper solubility limit. When concentration of Taxotere was increased to 300 μM, the binding of [^{3}H]TaxAPU to microtubules was greatly reduced but not totally abolished. The specific labeling is defined by the difference of incorporation of radioactivity between the two experiments. The α- and β-subunits of tubulin were identified by Coomassie blue staining (lower part of the figure). Following SDS polyacrylamide gel electrophoresis, the incorporation of radioactivity in the α- and β-subunits represents over 80% of the total radioactivity, partially protected by Taxotere (61% and 64%, respectively). Table 1 gives the specific radioactivity incorporation in both α- and β-subunits after SDS polyacrylamide gel electrophoresis of samples containing 20 μg of labeled tubulin for five different concentrations of the radiolabeled probe ranging from 2 to 20 μM. The variation of the specific radioactivity as a function of the probe concentration, for both subunits, is shown in Figure 6.

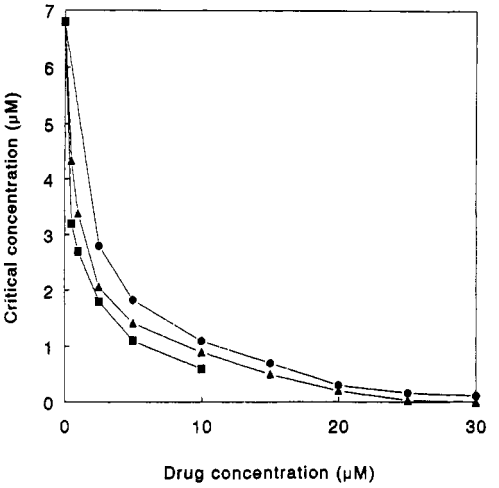

FIGURE 3: Effect of TaxAPU on the critical concentration of tubulin. A tubulin solution (15 μM) was incubated in PEM buffer supplemented with 6 mM $MgCl_2$ and 1 mM GTP at 37 °C with increasing concentrations of Taxotere (■), Taxol (▲), or TaxAPU (●). After 1 h, the samples were sedimented and the critical concentration measured as described under Materials and Methods.

DISCUSSION

To understand the taxoids–tubulin interaction at a molecular level, it is necessary to identify and characterize the ligand binding site on the protein. Several methods can be envisioned to undertake such studies which includes irreversible labeling techiques such as photoaffinity labeling. Several attempts to covalently label the taxoids binding site on tubulin have been reported in the literature. A series of photosensitive 7-acyl-Taxol derivatives (Georg *et al.*, 1992; Carboni *et al.*, 1993; Rimoldi *et al.*, 1993) have been synthesized as potential labels for the taxoid binding site on polymerized tubulin. However, the probes which could be synthesized in a radiolabeled form failed to covalently label, in a specific manner, polymerized tubulin. It was concluded that this position was not suitable for photoaffinity labeling studies (Rimoldi *et al.*, 1993). Radiolabeled Taxol could be directly cross-linked to tubulin by UV irradiation (Rao *et al.*, 1992) leading to specific radioactivity incorporation on the β-subunit of tubulin. Photochemical cross-linking reactions offer the advantage of using a nonmodified ligand for which the biological properties are well-defined, but suffer often from low coupling efficiencies. In the present case, the precise localization of the taxoids' binding site on tubulin, either on one subunit or both subunits, requires the use of a highly reactive probe which is able to label its entire surrounding (Kotzyba-Hibert *et al.*, 1994). In the absence of such probes, the precise location of a binding site on a protein remains difficult.

The present work describes the synthesis (Figure 1) of an [(azidophenyl)ureido]taxoid derivative (TaxAPU) by sub-

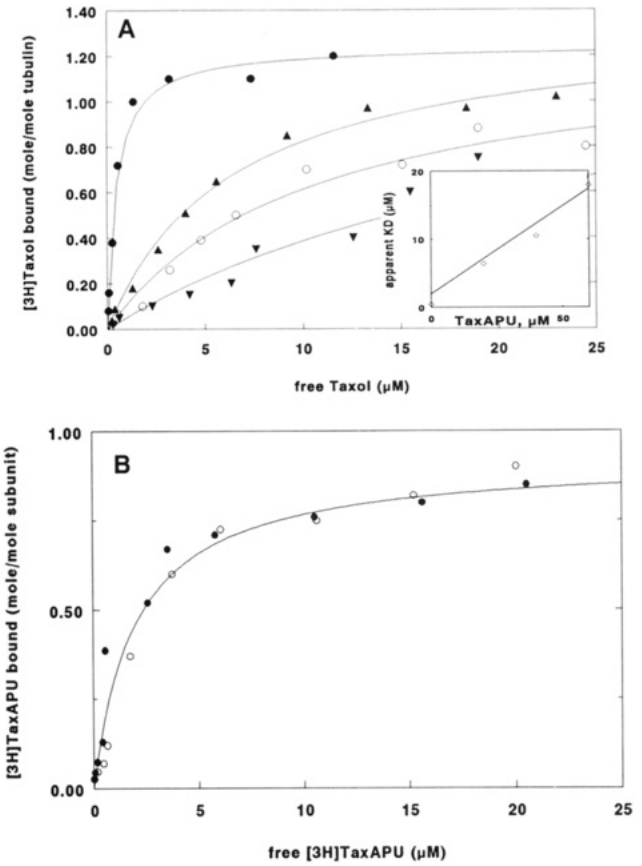

FIGURE 4: Reversible interaction of TaxAPU with microtubules. (A) Competition experiments between TaxAPU and Taxol. Increasing concentrations of [^{3}H]Taxol (0.5 to 30 μM) were incubated with a microtubule solution polymerized from a 6.5 μM tubulin solution. [^{3}H]Taxol bound (●) was determined after rapid sedimentation of microtubules as described under Materials and Methods. The same experiment was performed with 20 μM (▲), 40 μM (○), or 60 μM (▼) TaxAPU. Binding curves are calculated assuming a ligand binding to a single interaction site. Inset: Variations of apparent equilibrium dissociation constants with TaxAPU concentrations. (B) Binding isotherm of [^{3}H]TaxAPU to microtubules. The binding of [^{3}H]TaxAPU (0.07 Ci/mmol; 0.1–25 μM) was assayed by sedimentation using a microtubule solution assembled from a 6.5 μM tubulin solution. Open and filled circles represent two different experiments. The curve is calculated assuming a simple binding equilibrium to one site.

stituting the 3′-amino side chain with a *p*-azido-*N*-methyl-*N*-(chloroformyl)aniline, a bifunctional reagent which gives directly access to the photosensitive azidophenyl moiety without having to perform diazotization–azidation reactions on the sensitive taxoid molecule. This new photoactivatable taxoid retains taxoid properties according to a series of experiments including (i) its effect on tubulin assembly (Figure 2A,B), (ii) its effect on the critical concentration of tubulin assembly (Figure 3), and finally (iii) competition binding studies between TaxAPU and Taxol on microtubules (Figure 4). Clearly, TaxAPU is a taxoid derivative, although less potent than Taxol, which retains however sufficient affinity for the taxoid binding site (apparent $K_D \cong 6$ μM compared to 1 μM for Taxol) to be tested as a photoaffinity probe. Accordingly, it was important to synthesize the corresponding radiolabeled probe to evaluate its efficacy for labeling the taxoid binding site on tubulin. The synthesis of the tritiated probe took advantage of the availability of a radioactive heterobifunctional precursor (*N*-[^{3}H]-methyl-*N*-(chloroformyl)-*N*′-(*tert*-butoxycarbonyl)-*p*-phenylenediamine (Klotz *et al.*, 1991) and used a similar coupling procedure to aminotaxoid **5** as for the unlabeled chemical. The biological properties of

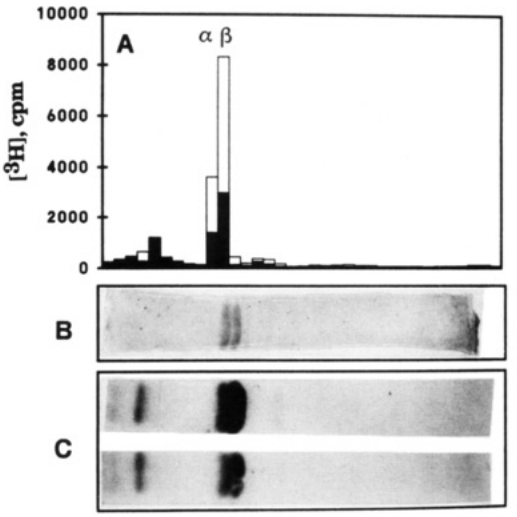

FIGURE 5: Covalent incorporation of [^{3}H]TaxAPU into microtubules upon UV irradiation. Tubulin (10 μM) was irradiated in the presence of [^{3}H]TaxAPU 4 μM as described under Materials and Methods. The sample was denatured and analysed by electrophoresis on a 5–15% acrylamide gel (total tubulin loaded, 20 μg). (A) The gel lane was cut into slices which were digested. The associated tritium radioactivity is reported for the experiment conducted in the absence (□) or presence (■) of 0.3 mM Taxotere. (B) Corresponding SDS–PAGE followed by Comassie blue staining. (C) Corresponding fluorographies with (lower panel) or without (upper panel) Taxotere.

Table 1[a]

[^{3}H]TaxAPU concn, μM	% of specific labeling[b]	
	α-subunit	β-subunit
2	0.5	1.3
4	0.85	2.05
6	0.6	1.7
10	1.1	2.7
20	1.2	3.7

[a] A microtubule solution, from a 10 μM tubulin solution, was irradiated for 10 min at 266 nm in the presence of five different [^{3}H]TaxAPU concentrations with or without 0.3 mM Taxotere. The radioactivity associated to α- and β-tubulin was measured as described in Figure 5A. The difference of radioactivity in absence and presence of 0.3 mM Taxotere is the specific radioactivity incorporation. [b] The % refers to the theoretical amount that can be incorporated on each subunit.

[^{3}H]TaxAPU were identical to TaxAPU (not shown). Irreversible photolabeling of tubulin in the presence of [^{3}H]-TaxAPU was analyzed by SDS gel electrophoresis (Figure 5) and revealed that both α- and β-subunits were specifically labeled by this probe, with a predominant labeling of the β-subunit in all experiments. We observed the formation of higher molecular weight species during photolytic experiments of microtubule solutions whether TaxAPU was present or not, indicating possible cross-linking of tubulin. No specific incorporation of radioactivity was measured in these species when photochemical labeling reactions where done in the presence of [^{3}H]TaxAPU. It is possible that cross-linked species of tubulin are formed upon irradiation. The labeling of polymerized tubulin by [^{3}H]TaxAPU was studied as a function of the concentration of the probe and again analyzed on SDS gels (Table 1). As expected, the specificity of labeling, assessed by the protection experiment, diminished from 62% to 47% for the α-subunit and from 73% to 58% for the β-subunit, using increasing concentrations, from 2 to 20 μM, of the radiolabeled probe (not shown). It should be noticed that the incorporation of radioactivity on the two subunits is not fully protectable, due probably to the solubility limitation of using higher concentrations of protector during these experiments. The ratio of specific labeling on the two subunits

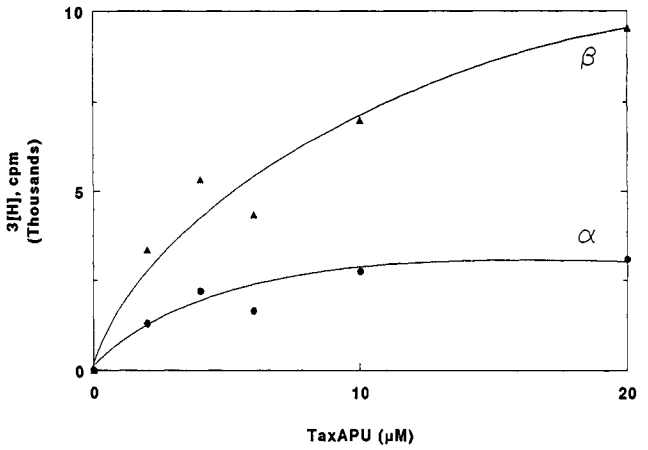

FIGURE 6: Specific incorporation of [^{3}H]TaxAPU to α- and β-subunits. The specific radioactivity incorporation in both α- and β-subunits were determined as the difference between the radioactivity incorporated when Taxotere is absent and present (0.3 mM) for five [^{3}H]TaxAPU concentrations: 2, 4, 6, 10, and 20 μM. The measurement of radioactivity is described in Figure 5A.

remained constant during these experiments: $\beta/\alpha \cong 2.5/1$, except for the highest probe concentration 20 μM, where a higher ratio (≥ 3) was observed. The variation of the percentage of specific labeling during these experimental is shown in Table 1 and indicates, as expected, an increase of the percent of labeling with an increase of the probe concentration, in both subunits, going from 0.5% to 1.2% for the α-subunit and from 1.3% to 3.7% for the β-subunit. The photoaffinity labeling results obtained with [^{3}H]TaxAPU demonstrate the involvement of both α- and β-tubulin subunits in the Taxol binding site, with a predominant contribution of the β-subunit and represent the first quantified photoaffinity labeling results on this interaction site. The experimental results clearly established the selectivity of the labeling by protection of the radioactivity incorporation in the presence of excess Taxotere.

Despite a moderate level of photochemical coupling to tubulin observed with this probe (less than 5% of total specific labeling using 20 μM ligand concentration), the labeling results were reproducible and showed the expected dependence with the probe concentration. Rao *et al.* described Taxol binding only to β-subunit. However, this is the first report of involvement of the α-subunit. Furthermore, affinity measured with our 3′-substituted taxoid indicates that this part of the taxoid side chain, rather than the "opposite" 7-OH position,[2] is in close interaction with tubulin. Work in progress aims to further characterize the site of interaction by identifying the amino acid residues on both subunits that are targeted by the labeled probe. Such information might lead to further understanding of the tubulin assembly–disassembly.

ADDED IN PROOF

While submitting the manuscript, the following publication appeared: Rao, S., Krauss, N. E., Heerding, J. M., Swindell, C. S., Ringel, I., Orr, G. A., & Horwitz, S. B. (1994) 3′-(*p*-Azidobenzamido) taxol Photolabels the N-terminal 31 Amino Acids of β-Tubulin. *J. Biol. Chem. 269*, 3132–3134.

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[2] Based on 3D structure on Taxotere (Guéritte-Voegelein *et al.*, 1990).

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