Characterization of Two Taxol Photoaffinity Analogues Bearing Azide and Benzophenone-Related Photoreactive Substituents in the A-Ring Side Chain

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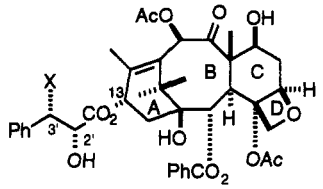
Taxol is a structurally novel and clinically effective antitumor drug, which, unlike other antimitotic agents, induces the assembly of tubulin into microtubules. To characterize the binding site(s) of taxol on the microtubule, taxol-based photoaffinity reagents 1 and 2 bearing photoreactive groups on the A-ring side chain were prepared and evaluated. Taxol analogue 1 exhibits better microtubule assembly activity, greater cytotoxicity toward J774.2 cells, and more specific and efficient photolabeling of the β-subunit of tubulin than does analogue 2. Therefore, it would appear that 1 is the better candidate for further studies aimed at the characterization of the taxol binding site on the microtubule.

Introduction

Taxol,[1a] a novel diterpenoid isolated from the bark of the western yew, *Taxus brevifolia*, is a promising anticancer drug that demonstrates a broad spectrum of antitumor activity in a variety of human tumors.[2] Recently, taxol was approved by the FDA for the treatment of drug-refractory ovarian carcinoma.



TAXOL	X = PhCONH
1	X = 4-(N_3)-C_6H_4CONH
2	X = 4-(PhCO)-C_6H_4CONH
3	X = NH_2

Microtubules are formed through the polymerization of tubulin α,β-heterodimers. Taxol interferes with the normal function of microtubules, but unlike other plant-derived antimitotic agents (e.g., colchicine, podophyllotoxin, and the vinca alkaloids) that inhibit microtubule assembly, taxol promotes the polymerization of tubulin even under conditions normally unfavorable for this process.[3] Taxol binds to the microtubule polymer[4] and stabilizes it against depolymerization by Ca ion[5] and podophyllotoxin.[6] To understand the interaction between taxol and microtubules, our efforts have been directed toward determining the structure of the binding site(s) of the drug on microtubules.

Direct photolabeling has proven to be an excellent tool for elucidating ligand binding locations on tubulin. Photolabeling experiments have demonstrated that GTP[7] and colchicine[8] bind preferentially to the β-subunit of tubulin. Experiments done with taxol indicate that it labels β-tubulin specifically.[9] However, the efficiency of the direct photolabeling of tubulin by taxol is too low to allow further structural charcterization of the taxol binding site(s). 7-Substituted photoaffinity analogues of taxol also have been synthesized and evaluated.[10]

We report here on the properties of two new photoaffinity taxol analogues that bear azide and benzophenone-related[11] photoreactive moieties in the A-ring side chain of taxol. The A-ring side chain is critical to the interaction of taxol with microtubules,[1] and therefore should be a particularly revealing location for photoreactive functionality.

Results and Discussion

Preparation of Taxol Analogues 1 and 2. Analogues 1 and 2 were prepared by the selective *N*-acylation of 3[12] with 4-azidobenzoic acid *N*-hydroxysuccinimide ester and 4-(benzoyl)benzoyl chloride, respectively. The tritiated versions, [^{3}H]-1 and [^{3}H]-2, carrying tritium on the aromatic rings of the *N*-acyl substituents, were prepared similarly from the corresponding titiated acylating agents.

Effects of Taxol and Analogues 1 and 2 on Microtubule Assembly and Cell Growth. The effects of taxol and taxol analogues 1 and 2 on microtubule assembly are presented in Figure 1 and summarized in Table 1. Both 1 and 2 induced the assembly of Ca ion-stable microtubules in the absence of GTP. These features are characteristic of the assembly of microtubules by taxol. Competition studies using tritiated taxol and unlabeled analogues 1 and 2 demonstrated that the analogues share with taxol the same binding site(s) on microtubules (Figure 2).

HPLC analysis of the pellets of the steady-state microtubules revealed the presence of the analogues on the microtubule polymers. Negatively stained electron micrographs (not shown) of the steady-state polymers induced by 1 demonstrated the existence of normal microtubules plus the hoops and ribbons seen in micro-

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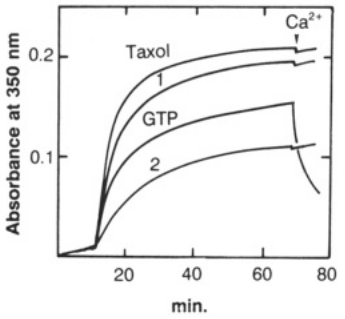

Figure 1. Assembly of microtubule protein (MTP) in the presence of GTP, taxol, **1**, or **2**. MTP (1.5 mg-mL^{-1}) was incubated at 35 °C with either 1 mM GTP or 20 μM taxol, **1**, or **2**. The assembly reaction was followed turbidimetrically. At the time denoted, 4 mM $CaCl_2$ was added to each experimental sample.

Table 1. Effect of Taxol, **1**, and **2** on Assembly Properties[a] of Microtubule Protein (MTP) and Cytotoxicity for J774.2 Cells

compd	% assembly	rel initial slope	ED_{50},[b] μM
taxol	100	100	0.09
1	90	84	0.28
2	39	15	1.5
GTP	70	65	–

[a] 1.5 mg/mL MTP + 15 μM compound. [b] ED_{50}: compound concentration that inhibits cell division by 50% after 72 h.

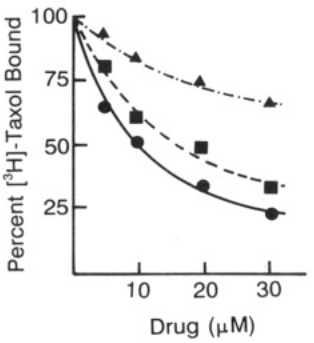

Figure 2. Competition between taxol and **1** or **2** for the binding site(s) on microtubules. MTP (1.5 mg-mL^{-1}) was assembled in the presence of 10 μM [^{3}H]taxol (this concentration was stoichiometric with that of tubulin) and various concentrations of unlabeled taxol (●, —), **1** (■, - - -), or **2** (▲, - • -). [^{3}H]Taxol and the competing compounds were added simultaneously. The microtubule polymer was centrifuged through a 50% sucrose layer, and the pellet was analyzed for the taxol analogue/tubulin ratio. Binding obtained in the presence of [^{3}H]taxol alone represents the 100% value (counts-min^{-1}-μg of protein^{-1}).

tubules assembled by taxol.[4] However, **2** led only to the formation of normal microtubules. This difference apparently is related to the rate of microtubule assembly.[13]

The ED_{50} values (compound concentration that inhibits cell division by 50% after 72 h) for J774.2 cells exhibited by **1** and **2** were determined with a range of taxol analogue concentrations. From Table 1, it can be seen that **1** and **2** are approximately 3- and 17-fold less cytotoxic than taxol, respectively. This follows the behavior of these analogues in the tubulin assembly studies and parallels their structual deviation from taxol. The modestly altered azido analogue **1** exhibited biological properties very similar to those observed for taxol. However, analogue **2**, which incorporates the large benzoyl substituent, is significantly less active.

The incubation of cells in the presence of taxol results in the formation of bundles of highly ordered arrays of microtubules.[14] To determine the effects of **1** and **2** on cellular microtubules, CHO (Chinese hamster ovary) cells were grown on coverslips for 24 h at which time the medium was replaced with fresh medium containing 10 μM taxol analogue. The cells were incubated with a taxol analogue for 4 h prior to fixation with formaldehyde. The effects of the taxol analogues on the microtubule cytoskeleton were examined by immunofluorescence microscopy using tubulin antibodies. Taxol and **1** induced the formation of microtubule bundles, whereas **2** failed to do so (data not shown) under the conditions of our experiments.

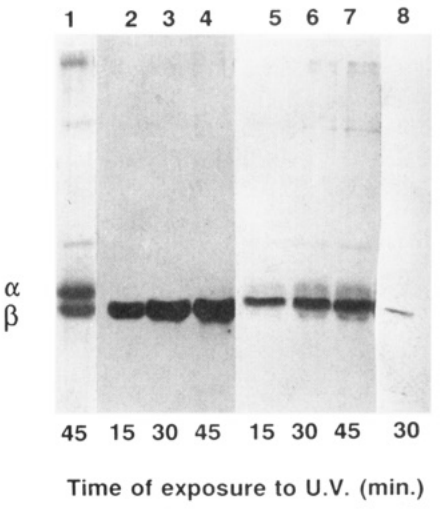

Figure 3. The photoaffinity efficiency and binding location on tubulin of taxol, **1**, and **2**. 10 μM [^{3}H]-**1** (lanes 2–4), 10 μM [^{3}H]-**2** (lanes 5–7), or 10 μM [^{3}H]taxol (lane 8) were added to 10 μM MTP, followed by incubation at 37 °C for 30 min. Samples were exposed to UV irradiation for the times indicated. At the end of each incubation, aliquots were processed for SDS–PAGE analysis and autoradiography. Lane 1 represents a typical Coomassie stained and dried SDS–PAGE gel of MTP. Lanes 2–8 are an autoradiograph of the same gel.

Photoaffinity Labeling of Microtubules by 1 and 2. The results of the tubulin photoaffinity labeling experiments with taxol analogues [^{3}H]-**1** and [^{3}H]-**2** are presented in Figure 3. Both photoaffinity reagents labeled the tubulin β-chain. However, [^{3}H]-**1** labeled the tubulin β-subunit with excellent specificity (Figure 3, lanes 2–4), whereas [^{3}H]-**2** was less discriminate (Figure 3, lanes 5–7). These observations corroborate the earlier finding[9] that taxol itself labels the β-subunit in a direct photoaffinity labeling experiment. Furthermore, [^{3}H]-**1** labeled the tubulin β-subunit with greater efficiency (per unit irradiation time) than did [^{3}H]-**2** despite the latter's higher specific activity ([^{3}H]-**1**: 2.75 Ci-mmol^{-1}; [^{3}H]-**2**: 12 Ci-mmol^{-1}). [^{3}H]-**1** (2.75 Ci-mmol^{-1}) also photolabeled the tubulin β-subunit with at least a 2 order of magnitude better efficiency than did [^{3}H]taxol (19.3 Ci-mmol^{-1}; Figure 3, lane 8). These results were obtained both with MTP and PC-tubulin (tubulin free of MAPs). Tubulin photodegradation products were not detected electrophoretically during the irradiation experiments (Figure 3, lane 1). Experiments have been reported indicating that tubulin loses its assembly competency during UV irradiation in proportion to the time and intensity of exposure.[15]

To confirm that taxol and [^{3}H]-**1** competed in the photoaffinity labeling experiments for the same site(s) on the tubulin β-subunit, an excess amount of cold taxol or cold **1** was added before UV irradiation (Figure 4). The results indicate that cold taxol and cold **1** suppress the photoaffinity labeling of tubulin by [^{3}H]-**1** and that **1** is binding the same site(s) as taxol on β-tubulin. Therefore, it is highly likely that the labeling of the tubulin β-subunit

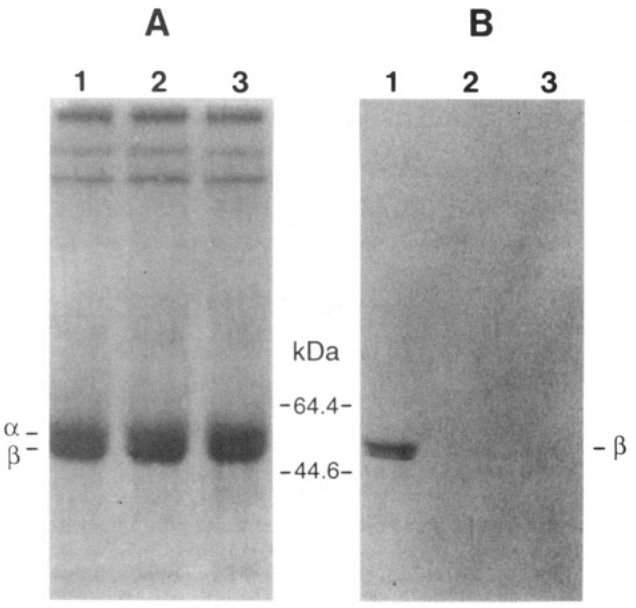

Figure 4. Effect of unlabeled taxol and **1** on the binding of [^{3}H]-**1**. [^{3}H]-**1** (10 μM) was added to MTP (11 μM tubulin) and the sample incubated for 30 min at 37 °C prior to irradiation with UV light for 15 min before (lane 1) or after the addition of unlabeled **1** (100 μM, lane 2) or unlabeled taxol (100 μM, lane 3). The samples (20 μL) were diluted with an equal volume of 2X SDS sample buffer and analyzed by SDS–PAGE and autoradiography. (A) Coomassie stained and dried gel. (B) Autoradiograph of the same gel.

by [^{3}H]-**1** is the result of specific binding and not the result of nonspecific association with the β-subunit.

Conclusions

Two effective taxol analogues (**1** and **2**) that carry photoreactive functionality in the A-ring side chain have been evaluated. Both analogues photoincorporate into β-tubulin, but analogue **1** exhibits the greater specificity for β-tubulin. This specificity of **1** together with its better labeling efficiency makes it the more attractive candidate for the further characterization of the taxol binding site(s) on microtubules. Indeed, we have found that **1** labels the *N*-terminal domain of β-tubulin.[16]

Experimental Section

Taxol was obtained from the National Cancer Institute, and [^{3}H]taxol, generally labeled with tritium (6.2 and 19.3 Ci-mmol^{-1}), was prepared by Rotem Industrials, Ltd. (Beer Sheva, Israel) and was kept in methanol at −70 °C. [^{3}H]-4-azidobenzoic acid *N*-hydroxysuccinimide ester was obtained from NEN-DuPont and diluted with cold material, as appropriate. [^{3}H]-4-Benzoylbenzoic, from Rotem Industrials, Ltd., was diluted with unlabled acid, as appropriate, and converted to the acid chloride by treatment with thionyl chloride. For the biological studies, taxol and the taxol analogues were dissolved in DMSO. The maximum final concentration of DMSO was 1% in experiments involving tubulin assembly and 0.2% in assays determining cell cytotoxicity. These concentrations had no significant effect on tubulin polymerization, microtubule structure, or the replication of cells.

^{1}H and ^{13}C NMR spectra of $CDCl_3$ solutions were recorded at 300 and 75 MHz, respectively, using tetramethylsilane (^{1}H δ = 0 ppm), residual chloroform (^{1}H δ = 7.27 ppm), or $CDCl_3$ (^{13}C δ = 77 ppm) as the internal standard. For ^{13}C NMR spectra, degrees of proton substitution were assigned with the aid of 1D DEPT experiments. High-resolution mass spectral determinations were performed at the Mass Spectrometry Laboratory, Department of Chemistry, University of Pennsylvania, Philadelphia, PA.

Preparation of Taxol Analogues 1 and 2. To 0.023 mmol of **3** and 2 mg 4-(dimethylamino)pyridine in 1.3 mL of pyridine at ambient temperature was added dropwise and with stirring 0.028 mmol of the acylating agent. After 30 min, an additional 0.0184 mmol of the acylating agent was added if necessary, and stirring was maintained another 30 min. The mixture was then diluted with methylene chloride and washed with 1 N hydrochloric acid, water, and the organic phase was dried (sodium sulfate). Concentration and purification of the residue by column chromatography (silica gel), eluting with ethyl acetate–hexanes, provided a product that was chromatographically and spectroscopically pure. [^{3}H]-**1** (1.69 and 2.75 Ci-mmol^{-1}) and [^{3}H]-**2** (12 Ci-mmol^{-1}) were prepared in similar fashion from the corresponding tritiated acylating agents.

Azidotaxol (1): prepared in 79% yield from 4-azidobenzoic acid *N*-hydroxysuccinimide ester; ^{1}H NMR δ 1.13 (3H, s C-17), 1.23 (3H, s, C-16), 1.67 (3H, s, C-19), 1.78 (3H, s C-18), 1.9–2.1 (3H, m, $^1/_2$ C-6, C-14), 2.22 (3H, s, OAc), 2.37 (3H, s OAc), 2.45–2.55 (1H, m, $^1/_2$ C-6), 3.79 (1H, d, J = 6.9, C-3), 4.18 (1H, $^1/_2$ AB q, J = 8.2, C-20), 4.28 (1H, $^1/_2$ AB q, J = 8.2, C-20), 4.38 (1H, dd, J = 6.8, 11.0, C-7), 4.75–4.8 (1H, m, C-2′), 4.92 (1H, d, J = 7.6, C-5), 5.66 (1H, d, J = 7.0, C-2), 5.76 (1H, dd, J = 2.4, 8.8, C-3′), 6.22 (1H, t, J = 8.2, C-13), 6.27 (1H, s, C-10), 6.98 (1H, app d, J = 8.8, NH), 7.01 (2H, app d, J = 8.6, N_3Ar), 7.3–7.5 (7H, m, Ph, OBz), 7.61 (1H, app t, J = 7.4, OBz), 7.73 (2H, d, J = 8.6, N_3Ar), 8.12 (2H, app d, J = 7.4, OBz); ^{13}C NMR δ 203.55, 172.76, 171.17, 170.36, 167.03, 165.96, 143.83, 141.87, 137.94, 133.20, 130.03, 129.15, 81.15, 79.04, 58.58, 43.16 (quaternaries), 133.70, 130.19, 129.00, 128.88, 128.70, 128.44, 128.35, 127.03, 119.07, 84.38, 75.54, 74.96, 73.18, 72.27, 72.14, 55.08, 45.67 (CH), 76.48, 35.63 (double signal) (CH_2), 26.84, 22.58, 21.77, 20.80, 14.79, 9.55 (CH_3); UV [MeOH, λ_{max} nm (ϵ)] 230 (23 000), 270 (20 000); HRFABMS calcd (M + Na) 917.3221, obsdvd 917.3236.

Benzoyltaxol (2): prepared in 66% yield from 4-benzoylbenzoyl chloride; ^{1}H NMR δ 1.14 (3H, s, C-16), 1.23 (3H, s, C-17), 1.68 (3H, s, C-19), 1.79 (3H, s, C-18), 2.23 (3H, s, OAc), 2.3–2.4 (2H, m, C-14), 2.38 (3H, s, OAc), 2.4–2.6 (2H, m, C-6), 3.67 (1H, d, J = 5.2, OH), 3.79 (1H, d, J = 7.0, C-3), 4.18 (1H, $^1/_2$ AB q, J = 8.4, C-20), 4.30 (1H, $^1/_2$ AB q, J = 8.4, C-20), 4.35–4.42 (1H, m, C-7), 4.80 (1H, dd, J = 5.2, 2.6, C-2′), 4.94 (1H, d, J = 7.8, C-5), 5.66 (1H, d, J = 7.0, C-2), 5.80 (1H, dd, J = 8.9, 2.6, C-3′), 6.24 (1H, t, J = 7.5, C-13), 6.27 (1H, s, C-10), 7.18 (1H, bd, J = 8.9, NH), 7.3–7.6 (5H, m, Ph), 7.61 (2H, t, J = 7.2, OBz), 7.7–7.9 (5H, m, OBz, Ar), 8.12 (2H, d, J = 7.2, OBz); ^{13}C NMR δ 203.56, 195.86, 172.65, 171.25, 170.35, 166.96, 166.20, 141.81, 140.44, 137.70, 136.77, 133.18, 81.12, 78.99, 77.42, 77.00, 58.53, 43.13 (quaternaries), 133.74, 132.99, 130.17, 130.13, 130.07, 129.05, 128.70, 128.44, 127.04, 127.02, 84.34, 77.20, 75.50, 74.85, 73.03, 72.32, 72.14, 55.06, 45.61 (CH), 76.57, 35.57 (double signal) (CH_2), 26.83, 22.61, 21.75, 20.85, 14.84, 9.54 (CH_3); UV [MeOH, λ_{max} nm (ϵ)] 232 (28 000), 258 (27 000); HRFABMS calcd (M + H) 958.3650, obsdvd 958.3591.

Preparation of Microtubule Protein. Calf brain microtubule protein (MTP) was purified by two cycles of temperature-dependent assembly–disassembly by a procedure modified from Shelanski *et al.*,[17] which has been described.[18]

Pure tubulin preparations (PC-tubulin), depleted of MAPs, were prepared from a concentrated MTP batch that was eluted through a phosphocellulose column (PC-column, Whatman P-11). The column was prepared according to the procedure recommended by the supplier, and 2.5 mg of MTP per 1 mL of column volume was loaded and eluted with PC-buffer (0.02 M MES, 1 mM EGTA, 0.5 mM $MgCl_2$, pH 6.6) at a low flow rate (<10 mL-h^{-1}). The eluent was brought to the consistency of the assembly buffer, and aliquots were frozen in liquid nitrogen and stored at −70 °C until further use.

Microtubule Assembly and Taxol Analogue Binding. Microtubule assembly in the presence and absence of taxol and its analogues (assembly buffer: 0.1 M 2-(*N*-morpholino)ethanesulfonic acid [MES], 1 mM ethyleneglycol/*bis*(β-aminoethyl ether)–*N*,*N*,*N*′,*N*′-tetraacetic acid [EGTA], 0.5 Mm $MgCl_2$, and 3 M glycerol, pH 6.6) was monitored turbidimetrically at 35 °C and 350 nm.[19] Taxol analogue bound to microtubule polymers was assessed by HPLC analysis of the analogue–microtubule pellet. Microtubule polymers were centrifuged through a 50% sucrose cushion (Beckman 50 rotor, 45 000 rpm for 1 h), and the

pellet was resuspended in water and processed for HPLC analysis (see ref 18 for details) or, in the case of a radioactive taxol analogue, protein and taxol analogue concentrations were measured using the Lowry procedure.[20] and scintillation counting, respectively.

Samples for electron microscopy were placed on carbon-over-Parlodion-coated grids (300 mesh), negative stained with 2% uranyl acetate, and analyzed on a JEOL 100 CX electron microscope at 80 kV.

Photoaffinity Labeling of Microtubules with [^{3}H]Taxol, [^{3}H]-1, and [^{3}H]-2. The procedure employed has been described.[9] UV-induced cross-linking was carried out on preformed photoaffinity reagent–microtubule complexes in the presence of 1 mM GTP, 1 mM DTT, and assembly buffer. Substoichiometric concentrations of the photoaffinity reagents were used to avoid nonspecific labeling (although previous work[9] indicated that even with excess [^{3}H]taxol, no nonspecific photolabeling of microtubules was detected). Excess free photoaffinity reagent was removed by spinning the photoaffinity reagent–microtubule complexes through a 50% sucrose cushion (Beckman 50 rotor, 45 000 rpm for 1 h). The pellet was rinsed and resuspended in 300 μL of assembly buffer. Samples were placed on ice and irradiated (mineralight lamp, UVP Inc., Model R-52G, 0.9A, 254 nm) from a distance of 7 cm. Aliquots were taken at the times indicated, mixed with SDS–mercaptoethanol loading buffer, and immediately electrophoresed overnight on polyacrylamide gels (PAGE; 10 mA). The gels were stained, destained, enhanced with EN^{3}HANCE, washed with water, dried, and exposed to Kodak X-OMAT AR film at −70 °C.

Cell Culture and Immunofluorescence Studies. The murine macrophage-like cell line, J774.2, and Chinese hamster ovary (CHO) cells were maintained as described.[18] The effect of the compounds on cell growth (ED_{50}) was studied with J774.2 cells. Briefly, cells (3×10^5) in 60-mm tissue culture plates were incubated with a range of compound concentrations, and the number of viable cells in each plate was determined in a Coulter counter after 72 h. Immunofluorescence studies were performed on CHO cells using a mouse monoclonal antibody against tubulin as the first antibody and a rhodamine-conjugated rabbit anti-mouse antibody as the second antibody.[18]

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