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Communications to the Editor

A New Paclitaxel Photoaffinity Analog with a 3-(4-Benzoylphenyl)propanoyl Probe for Characterization of Drug-Binding Sites on Tubulin and P-Glycoprotein

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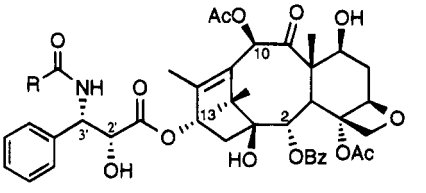
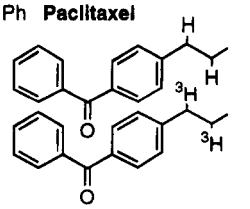
Paclitaxel (Taxol, **1**), a complex diterpenoid isolated from the bark of the western yew *Taxus brevifolia*, is currently one of the most exciting leads in cancer chemotherapy.[1] The drug has been approved by the FDA for the treatment of advanced ovarian and breast carcinomas and is in clinical trials for various other neoplasms.[2] Paclitaxel, a potent inhibitor of cell replication, enhances the polymerization of tubulin into stable bundles of microtubules.[3,4] The drug has a binding site on the microtubule polymer,[5] in contrast to other antimitotic agents such as vinblastine and colchicine whose major binding site is the tubulin heterodimer. Paclitaxel interacts with the β-tubulin subunit, and recent studies utilizing 3′-(4-azidobenzamido)paclitaxel, a photoaffinity analog of paclitaxel, have indicated that the N-terminal 31 amino acids of β-tubulin represent one domain of the binding site for paclitaxel in β-tubulin.[6-8] In order to further characterize the paclitaxel-binding site(s) on microtubules, development of new photoaffinity paclitaxel analogs that can be radiolabeled to high specific activity is desirable.

The development of drug resistance in human tumors represents a serious clinical problem. When mammalian cells are treated with paclitaxel or other natural product hydrophobic antineoplastic agents, they often display the multidrug resistance (MDR) phenotype that is characterized by overproduction of P-glycoprotein. The latter is an integral membrane glycoprotein that acts as a drug-efflux pump to maintain the intracellular concentration of drugs below cytotoxic levels.[9,10] Two photoaffinity drug-binding domains have been mapped in P-glycoprotein,[11-13] one in each half of P-glycoprotein. These studies have not been done with photoaffinity analogs of antitumor drugs such as paclitaxel but, rather, with photolabeled calcium channel blockers. Therefore, a search for efficient photoaffinity paclitaxel analogs for the characterization of antitumor drug-binding site(s) on P-glycoprotein is an important endeavor.

In the present report, 3′-*N*-BzDC-3′-*N*-debenzoylpaclitaxel (**2**) (BzDC = *p*-benzoyldihydrocinnamoyl or 3-(4-benzoylphenyl)propanoyl), a new photoreactive analog of paclitaxel, and its ditritiated derivative ([^{3}H]-**2**) have been synthesized and evaluated for their ability tophotolabel tubulin and P-glycoprotein. The radiolabeled photoreactive moiety, i.e., 2,3-ditritio-3-(4-benzoylphenyl)propanoyl group, can be prepared at a high specific activity and has proven to be an excellent tool in a variety of systems.[14]



1: R = Ph **Paclitaxel**



2: R =

[^{3}H]-**2**: R = ^{3}H

Synthesis of BzDC-paclitaxel 2 and [^{3}H]BzDC-paclitaxel [^{3}H]-2. 3′-*N*-BzDC-3′-*N*-debenzoylpaclitaxel (**2**) was synthesized in good yield (62% isolated yield; 82% conversion yield for two steps) by reacting 3′-*N*-

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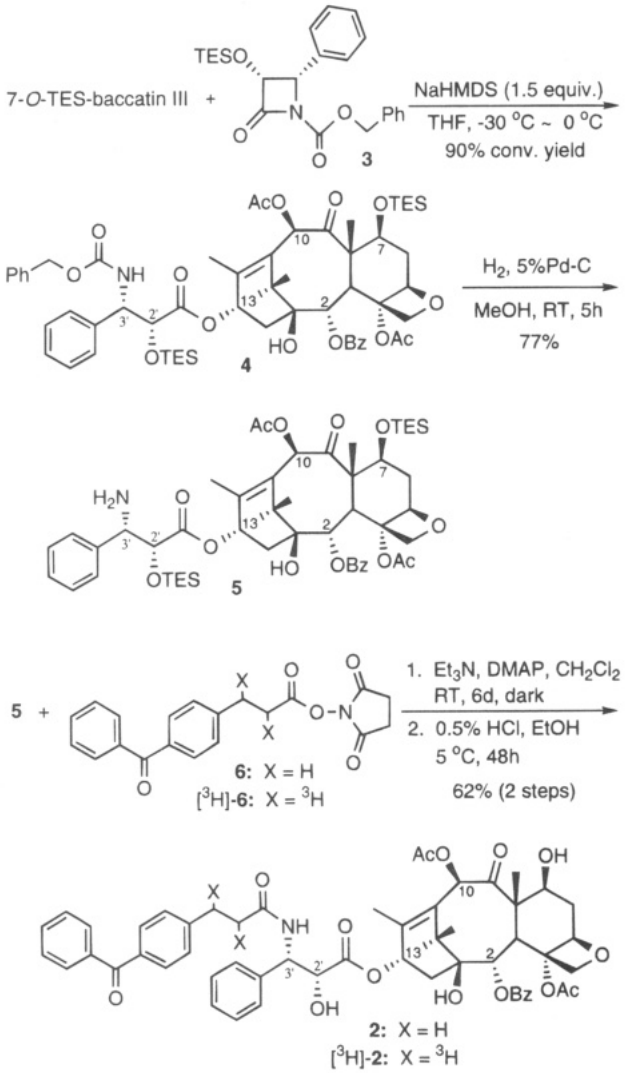
Scheme 1



debenzoyl-2′,7-bis(*O*-TES)paclitaxel (**5**)[15] (TES = triethylsilyl) with *N*-[[3-(4-benzoylphenyl)propanoyl]oxy]succinimide (**6**)[14a] (2 equiv) in the presence of triethylamine and a catalytic amount of DMAP in CH_2Cl_2 at room temperature in the dark for 6 days followed by removal of TES protection with 0.5% HCl in ethanol at 4 °C for 2 days and purification on a silica gel column (Scheme 1). In a similar manner, [^{3}H]-**2** was synthesized by *N*-acylation of **5** with *N*-[[2,3-ditritio-3-(4-benzoylphenyl)-propanoyl]oxy]succinimide[14a] ([^{3}H]-**6**) followed by purification on a reversed phase semipreparative HPLC C-18 column. The radiochemical purity of [^{3}H]-**6**, thus obtained, was determined to be >99.9% based on TLC radioscanning analysis, and [^{3}H]-**6** was found to possess a high specific activity (34 Ci/mmol).

3′-*N*-Debenzoyl-2′,7-bis(*O*-TES)paclitaxel (**5**)[15] was readily obtained through coupling of 7-*O*-TES-baccatin III with *N*-Cbz-β-lactam **3**, following the standard procedure developed in these laboratories,[16,17] followed by hydrogenolysis of the Cbz protecting group and purification on a silica gel column as shown in Scheme 1.

Microtubule Assembly. Both paclitaxel (**1**) and the photoaffinity analog BzDC-paclitaxel **2** promoted the assembly of tubulin into microtubules in the absence of GTP (Figure 1). Addition of Ca^{2+} resulted in the depolymerization of the microtubules polymerized by

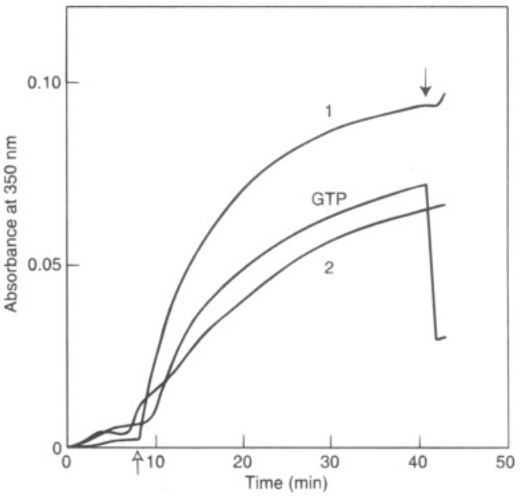


Figure 1. Assembly of MTP in the presence of GTP, **1**, or **2**. MTP (8.5 μM tubulin) was incubated at 35 °C with 1 mM GTP or 10 μM **1** or **2**. Additions were made at the time indicated by an open arrow; 4 mM $CaCl_2$ was added to each sample at the time denoted by a filled arrow.

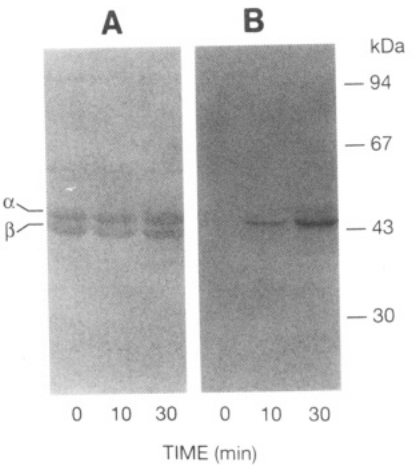


Figure 2. Effect of ultraviolet radiation on the binding of [^{3}H]-**2** to tubulin. [^{3}H]-**2** (5 μM) was added to MTP (5 μM tubulin), incubated for 30 min at 37 °C, and exposed to ultraviolet light (350 nm) for the indicated time. At the end of the incubation, a 40 μL aliquot was withdrawn, mixed with an equal volume of 2 × SDS sample buffer, and analyzed by SDS−PAGE and fluorography: (A) Coomassie blue stained gel and (B) fluorograph of panel A. Fluorogram was exposed for 3 days.

GTP but not of those assembled by either **1** or **2.** Examination by electron microscopy of the microtubules formed in the presence of either **1** or **2** indicated that they were normal and similar in structure. These results indicated that **2** has the same characteristics as paclitaxel in the tubulin/microtubule system although it is not as potent as paclitaxel.

Photoaffinity Labeling of Microtubules by [^{3}H]-BzDC-paclitaxel [^{3}H]-2. The time course of photolabeling of tubulin with [^{3}H]-**2** is presented in Figure 2. The major radioactive band on the fluorogram was located between the α- and β-tubulin bands. It is very likely that [^{3}H]-**2** is labeling β-tubulin but that the photoderivatized β-subunit has an altered, i.e., retarded, mobility on the gel. This observation is consistent with a previous study in which a different benzophenone-containing paclitaxel analog appeared to retard β-tubulin migration during SDS−PAGE.[18] Previous photolabeling studies with microtubule protein and either

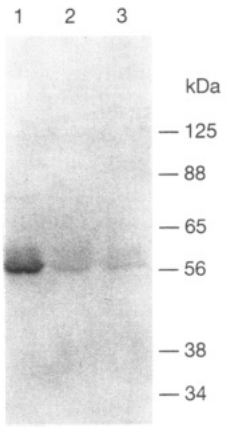


Figure 3. Competition for the [^{3}H]-**2**-labeled site on tubulin by **1** and **2**. The binding site on tubulin was labeled with 0.1 μM [^{3}H]-**2**; lane 1 was labeled in the absence of competitor, and lane 2 was competed with 50 μM cold **2** and lane 3 with 50 μM **1**. Samples were incubated for 30 min at 37 °C, irradiated at 350 nm for 1.5 h at 4 °C, and analyzed by SDS–PAGE and fluorography.

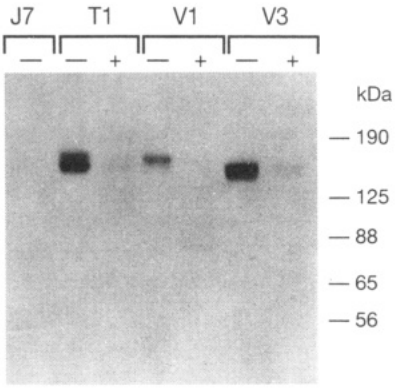


Figure 4. Photoaffinity labeling of P-glycoprotein. Membrane preparations from drug-sensitive (J7) and three different drug-resistant cell lines (T1, V1, V3) were labeled with 2 μM [^{3}H]-2 in the absence (−) or presence (+) of 50 μM 1. Samples were incubated at 25 °C for 1.25 h, irradiated at 350 nm for 1.5 h at 4 °C, and analyzed by 7% SDS–PAGE and fluorography.

[^{3}H]paclitaxel[6] or [^{3}H]-3′-(4-azidobenzamido)paclitaxel[7] indicated preferential labeling of β-tubulin. Studies with an azidophenyl(ureido)taxoid, a photoactivatable taxoid, demonstrated that ca. 70% of the label was found associated with β-tubulin.[19]

The specificity of the binding of [^{3}H]-**2** to tubulin was examined by a competition experiment in which a 500-fold molar excess[14c] of **2** or **1** was included in the assay. Both competitors significantly decreased the photoaffinity labeling of [^{3}H]-**2**, thereby indicating that they were indeed binding to the same or an overlapping site (Figure 3).

Photoaffinity Labeling of P-Glycoprotein by [^{3}H]-BzDC-paclitaxel [^{3}H]-2. [^{3}H]BzDC-paclitaxel [^{3}H]-**2** specifically photolabeled different isoforms of murine P-glycoprotein present in various drug-resistant cell lines[20] (Figure 4). Western blot analysis using a specific antibody to P-glycoprotein confirmed that the radiolabeled bands were indeed P-glycoprotein. The J7 parental drug-sensitive cells do not express P-glycoprotein in appreciable amounts. The cell line selected with paclitaxel, T1, expresses two isoforms of P-glycoprotein, whereas V1 and V3, selected with vinblastine, each express mainly a single P-glycoprotein isoform.[21] A 25-fold molar excess of paclitaxel (**1**) effectively displaced [^{3}H]-**2** from the P-glycoprotein binding site.

In conclusion, a new photoreactive analog of paclitaxel, [^{3}H]BzDC-paclitaxel [^{3}H]-**2**, has demonstrated an excellent ability to photolabel tubulin as well as P-glycoprotein. To the best of our knowledge, this is the first successful photoaffinity labeling of P-glycoprotein with a photoreactive analog of paclitaxel. Thus, [^{3}H]-**2** serves as an attractive agent for the characterization of the paclitaxel-binding site on tubulin as well as on P-glycoprotein. Further studies along this line are actively underway.

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Supporting Information Available: Preparations of microtubule protein and P-glycoprotein, Western blot analysis of P-glycoprotein, procedures for photoaffinity labeling of tubulin and P-glycopretein, general experimental procedures for the syntheses of **2** and **2a** from **3** and 7-*O*-TES-baccatin III, and the characterization data for new taxoids (5 pages). Ordering information is given on any current masthead page.

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