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Characterization of Polyclonal and Monoclonal Anti-Taxol Antibodies and Measurement of Taxol in Serum[1]

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ABSTRACT

Anti-taxol antibodies were generated in the rabbit using a taxol-bovine serum albumin conjugate prepared from 2′-succinyltaxol using a mixed anhydride procedure. Immunization with 2′-succinyltaxol-bovine serum albumin gave rise to polyclonal anti-taxol antibodies. By a radioimmunoassay using [^{3}H]taxol, a standard curve gave a 50% inhibitory concentration of 1.0 nM. Taxol levels in human serum could be measured, with the lower limit of detection and measurement being 0.1 nM or 0.085 ng/ml.

Two mouse monoclonal anti-taxol antibodies were isolated by immunizing BALB/c mice with the same antigen. One was an immunoglobulin G_1 (69E4A8E) and the other was immunoglobulin M (29B7B3C). The specificity of these antibodies was determined by a competitive enzyme-linked immunosorbent assay with taxol and 10 different related derivatives and analogues. 29B7B3C had higher binding affinities for biologically active derivatives and markedly lower affinities for inactive derivatives; *i.e.*, the specificity was consistent with the results of tubulin disassembly and cytotoxicity studies using the same taxol derivatives, making it suitable for screening for taxol or taxol-like compounds in extracts of natural products. 69E4A8E recognized the benzamidocarbamyl group at the C-3′ position of taxol and had a lower affinity for other active compounds with different substitutions. Taxol levels in human serum could be detected and measured by 69E4A8E using a competitive enzyme-linked immunosorbent assay. The lower limit of measurement was about 50 nM or approximately 42 ng/ml. Similar measurements could be made by radioimmunoassay.

INTRODUCTION

Taxol, a compound extracted from the western yew, *Taxus brevifolia*, was shown to have antitumor activity (1). Its underlying mechanism is to promote and stabilize microtubule assembly and inhibit disassembly to tubulin (2). The binding site of taxol in microtubules differs from that of other antitubulin drugs, such as colchicine, podophyllotoxin, and vinblastine, which inhibit tubulin polymerization (3, 4).

In clinical trials, taxol was found to be effective in the treatment of ovarian (5, 6) and breast cancers (7) and melanoma (8). As with all anticancer agents, there are side effects; in this case neutropenia, hypersensitivity reactions, mucositis, and neurological and possible cardiac toxicity were reported during clinical trials (9). It would be useful, therefore, to be able to measure taxol levels in patients under treatment in order to optimize treatment. A sensitive assay for measuring taxol levels is by HPLC[3] (9, 10). In this article we report a polyclonal and two monoclonal antibodies that can be used to measure taxol levels in human serum with high sensitivity and are more amenable for the measurement of large numbers of samples. The monoclonal antibodies also have the potential to be used to screen for taxol or taxol-like compounds in extracts of natural products.

MATERIALS AND METHODS

Reagents

Taxol (NSC 125973), cephalomannine (NSC 318735), baccatin III (NSC 330753), and [^{3}H]taxol (23 Ci/mmol) (NSC 125973) were obtained from the National Cancer Institute. The following taxol derivatives were a generous gift from Dr. D. G. I. Kingston (Virginia Polytechnic Institute and State University, Blackburg, VA): 2′-(triethylsilyl)taxol; 7-epitaxol; 2-debenzoylisotaxol; 2′-(*N*-benzyloxycarbamyl)-β-alanyl)-7-oxo-5,6-dehydro-5-*O*-secotaxol; 20-acetoxy-4-deacetyl-5-epi-20,*O*-secotaxol; 10-deacetylbaccatin III; and 7-(triethylsilyl)-baccatin III. We thank Dr. P. Potier of the Institut de Chimie des Substances Naturelles, Gif-Sur-Yvette, France, and Dr. J-L. Fabre of Rhone-Poulenc Rorer, France, for the sample of taxotere.

BSA, RSA, charcoal, PVP, and succinic anhydride were purchased from Sigma Chemical Co. (St. Louis, MO). Isobutylchloroformate and *n*-tributylamine were from Eastman Kodak Corp. (Rochester, NY). Dextran T70 was purchased from Pharmacia LKB Biotechnology (Uppsala, Sweden). Fetal calf serum was from Hyclone (Logan, UT). Peroxidase-conjugated goat anti-mouse IgG + IgM was purchased from TAGO (Burlingame, CA). The isotyping kit was from Zymed (San Francisco, CA).

Synthesis of 2′-Hemisuccinyltaxol

The method of Deutsch *et al.* (11) was used with some modifications. Taxol (20 mg) and succinic anhydride (36 mg) were dried for 4 h at room temperature under vacuum over P_2O_5 and dissolved in 480 μl of dry pyridine. After standing at room temperature overnight, the pyridine was removed under vacuum and the residue was washed once with 2 ml of distilled water. Acetone (1 ml) was added, and distilled water was added dropwise to the acetone solution until a few crystals (2′-hemisuccinyltaxol) appeared. The mixture was kept at 4°C for 3 h and the crystals were recovered by filtration and dried under vacuum. The product was obtained in 70% yield.

Synthesis of 2′-Hemisuccinyltaxol-Protein Conjugates

A modification of the procedures developed by Jaziri *et al.* (12) was used. 2′-Hemisuccinyltaxol (10 mg) was dissolved in 1 ml DMSO and 300 μl acetonitrile, and 50 μl (35 mg; 0.19 mmol) of *n*-tributylamine were added. The mixture was cooled to 4°C in an ice bath, and 25 μl (25 mg; 0.18 mmol) of isobutylchloroformate were added to the mixture which was kept in the ice bath for 30 min.

The solution was added dropwise into a BSA or RSA solution [25 mg, (3.73×10^{-4} mmol) in 3 ml distilled H_2O, pH 9.5, at 4°C]. The pH was adjusted immediately to 7.5 with 1 N HCl and the mixture was kept at 4°C overnight and dialyzed against PBS at 4°C overnight.

Rabbit Antibodies

A female New Zealand White rabbit was immunized i.d. along the back, with a 1/1 (v/v) mixture of 1 mg of 2′-hemisuccinyltaxol-BSA conjugate (taxol-BSA) in PBS and complete Freund's adjuvant. The rabbit was boosted with 0.5 mg of taxol-BSA in IFA at 3–4-week intervals and bled weekly following each boost.

MAb

BALB/c mice (Charles River) were immunized i.p. with 0.5 mg taxol-BSA emulsified in complete Freund's adjuvant. Mice were boosted twice at 2- or

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[3] The abbreviations used are: HPLC, high-performance liquid chromatography; MAb, monoclonal antibodies; DMSO, dimethyl sulfoxide; RIA, radioimmunoassay(s); IFA, incomplete Freund's adjuvant; BSA, bovine serum albumin; RSA, rabbit serum albumin; PVP, polyvinylpyrrolidone; PBS, phosphate-buffered saline; ELISA, enzyme-linked immunosorbent assay; PBS-T-20, PBS containing 0.1% Tween 20; i.d., intradermal.

3-week intervals with 0.25 mg of taxol-BSA emulsified in IFA. Five days before the fusion, the mice were given i.p. injections with 0.25 mg of taxol-BSA in PBS. Spleen cells were fused with nonsecreting myeloma cells P3x63-Ag8.653 (13), according to the method of Sharon *et al.* (14). Three weeks later, the hybridoma supernatant was assayed for the presence of anti-taxol antibodies by ELISA (see below). The positive clones were confirmed for taxol binding by a competitive ELISA (see below). Clones positive by competitive ELISA were subcloned twice by limiting dilution. Ascites were obtained by injecting 10^6 to 10^7 cells i.p. into BALB/c mice that had been primed with IFA i.p. 5 days before.

ELISA for Anti-Taxol MAb Screening

Polystyrene microplates (Corning 25855) were coated with 100 μl of taxol-RSA (250 ng/ml) in 0.1 M sodium bicarbonate, pH 9.3, overnight at 4°C. The plates were washed with PBS-T-20 3 times, and 100 μl of culture supernatants were incubated in the wells for 2 h at 37°C. The plates were washed three times with PBS-T-20, and 100 μl of a 1/3000 dilution of horseradish peroxidase-labeled goat anti-mouse IgG + IgM in PBS-Tween-20 were added to each well and incubated at 37°C for 1 h. After the plates were washed three times with PBS-Tween-20, 100 μl of substrate (7 mg *o*-phenylenediamine dihydrochloride in 10 ml of 0.1 M citrate-phosphate buffer, pH 5, containing 5 μl of 30% H_2O_2) were added to each well. The reaction was stopped after 10 min by the addition of 40 μl of 8 N H_2SO_4, and the absorbance of each well was measured at 490 nm on a Dynatech Microplate reader.

Competitive ELISA

Polystyrene microplates were coated with 100 μl of taxol-RSA (250 ng/ml) in 0.1 M sodium bicarbonate, pH 9.3, overnight at 4°C. The wells were washed with PBS-T-20 3 times and blocked with 200 μl of PBS, containing 1% fetal calf serum, for 1 h at 37°C. Culture supernatant (100 μl) was added to the coated plate either in the presence or in the absence of 50 μM taxol in PBS-T-20 (from a 10 mM taxol stock solution in dimethyl sulfoxide), followed by incubation at room temperature for 90 min. After four washings with PBS-T-20, bound antibodies were detected with 100 μl of 1/3000 dilution of peroxidase-labeled goat anti-mouse IgG + IgM in PBS-T-20 for 1 h at 37°C. Color was developed and absorbance was measured as described above.

For the dose-dependent inhibition of binding of anti-taxol to taxol-RSA, 100 μl of diluted MAb IgM (29B7B3C) or MAb IgG (69E4A8E) ascites were added to the coated well with serial dilutions of taxol or its derivatives, from 0.1 mM to 0.24 nM (all derivatives were from a 10^{-2} M stock solution in DMSO), in PBS-T-20 + 2.5% fetal calf serum + 3.5% PVP + 1% DMSO.

Determination of Taxol in Human Serum

ELISA. A standard curve was determined by adding a mixture of 50 μl of 1/8000 dilution of 69E4A8E ascites in PBS-T-20 and 50 μl of serial 5-fold dilutions of taxol (from 0.1 mM to 0.24 nM) in PBS-T-20 into the taxol-BSA-coated wells.

To measure taxol levels in human serum, different amounts of taxol in DMSO were added to human serum; the final concentration of DMSO was, in all cases, 0.5%. A mixture of 50 μl of 1/8000 dilution of 69E4A8E ascites and 50 μl of a 1/5 dilution of serum in PBS-T-20 was added to taxol-RSA-coated plates, followed by incubation at room temperature for 90 min. Bound antibodies were detected as described above.

Radioimmunoassay. For a standard curve of anti-taxol antibody binding to [^{3}H]taxol, 100 μl of diluted 29B7B3C or 69E4A8E ascites or rabbit antiserum in RIA buffer (PBS + 0.1% Tween 20 + 0.1% gelatin + 0.1% NaN_3) were incubated for 2 h at room temperature with 100 μl of [^{3}H]taxol (~10,000 cpm) in RIA buffer, in the presence of 100 μl of serially diluted taxol solutions in RIA buffer. Bound ligand was separated from free ligand by the addition of 100 μl of a 2.5% dextran-coated charcoal solution in RIA buffer, incubation for 3 min at 4°C, and centrifugation in an Eppendorf centrifuge for 2 min. The supernatant, containing bound [^{3}H]taxol, was counted for radioactivity. To characterize the antiserum, taxol derivatives were incubated at room temperature for 2 h with the rabbit antiserum and [^{3}H]taxol. Bound [^{3}H]taxol was determined as described above.

To measure taxol levels in human serum by RIA, 100 μl of 1/150 dilution of rabbit anti-taxol antiserum or 1/150 dilution of 69E4A8E ascites were added to 100 μl of [^{3}H]taxol in RIA buffer and 100 μl of undiluted to 1/100 dilution of human serum samples originally containing concentrations of taxol from 0.005 to 5 μM. For the higher concentrations, the serum was diluted with RIA buffer to bring the concentrations within the working range of the RIA (0.03–10 nM). After incubating for 2 h at room temperature, bound [^{3}H]taxol was determined as described above.

RESULTS

Characterization of Antibodies. Antibodies generated in a rabbit using a taxol-BSA conjugate were assayed for specificity by RIA (Fig. 1). The antibodies bound taxol and cephalomannine with almost equal affinity. Two inactive derivatives, baccatin III and 20, *O*-secotaxol, were bound with affinities about 3 orders of magnitude lower than taxol. Taxotere, a biologically active compound (15, 16), was bound with 100-fold lower affinity than taxol.

Two mouse monoclonal anti-taxol antibodies were isolated, 29B7B3C (IgM) and 69E4A8E (IgG_1). Taxol inhibited the binding of both antibodies to taxol-RSA, as shown by ELISA, with a 50% inhibitory concentration for taxol of about 0.1 μM. The specificities of the antibodies were determined by a competitive ELISA with taxol and 10 related derivatives. (Figs. 2 and 3). Because many of the derivatives were not soluble in 1% DMSO at their higher concentrations, PVP (3.5%) was introduced into the solution. Yonish-Rouach *et al.* (17) found that cyclosporin A, which is not soluble in water, could be solubilized in an aqueous solution containing 3.5% PVP (pH 7.4) without affecting immunological assays of cyclosporin A. We found that taxol and its derivatives were also more soluble in the presence of 3.5% PVP without any deleterious effect on the immunoassays (data not shown).

The 50% inhibitory concentration of each derivative, as determined by the ELISA inhibition assays (Figs. 2 and 3), are shown in Table 1. Both monoclonal antibodies had higher binding affinities for biologically active derivatives (taxol, cephalomannine, and 7-epitaxol) than for inactive derivatives (baccatin III derivatives and derivatives with an open oxetane ring). Specificity was consistent with the results of tubulin disassembly assays and cytotoxicity studies using the same taxol derivatives (10). An exception was the biologically active derivative taxotere which was recognized poorly by 69E4A8E. This had also been the case with the rabbit antiserum (see above).

Measurement of Taxol Levels in Human Serum by ELISA and RIA. For these experiments, known amounts of taxol were dissolved in human serum.

Taxol levels in human serum were measured by RIA using the rabbit antiserum. The results are in Table 2. The lowest concentration of taxol detected was 5 nM. However, the lower limit of measurement, as determined from the standard inhibition curve, was 0.1 nM (0.085

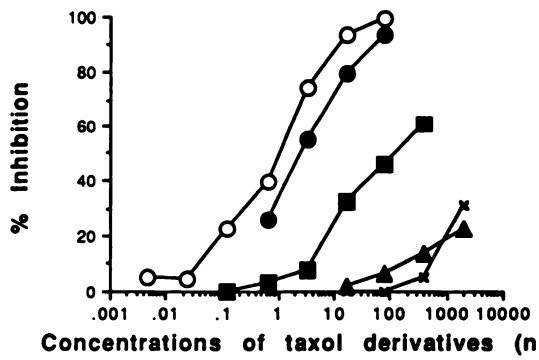

Fig. 1. Inhibition of the binding of rabbit anti-taxol antiserum to [^{3}H]taxol by taxol derivatives and analogues. ○, taxol; ●, cephalomannine; ■, taxotere; ▲, baccatin III; ×, 20-acetoxy-4-deacetyl-5-epi-20,*O*-secotaxol. The results are means of duplicate points and are expressed as the percentage of inhibition relative to the specific [^{3}H]taxol binding measured in the absence of inhibitors in RIA.

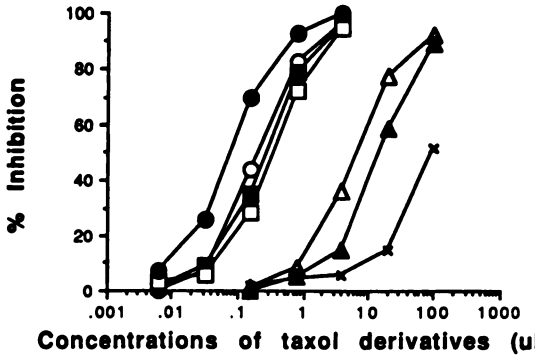

Fig. 2. Inhibition of the binding of 29B7B3C to taxol-RSA-coated wells by taxol derivatives and analogues. ○, taxol; ●, cephalomannine; □, 7-epitaxol; ■, taxotere; △, 2′-(triethylsilyl)taxol; ▲, baccatin III; ×, 20-acetoxy-4-deacetyl-5-epi-20,*O*-secotaxol. The results are means of duplicate points and are expressed as the percentage of inhibition relative to the absorbance at 490 nm measured in the absence of inhibitors in the ELISA.

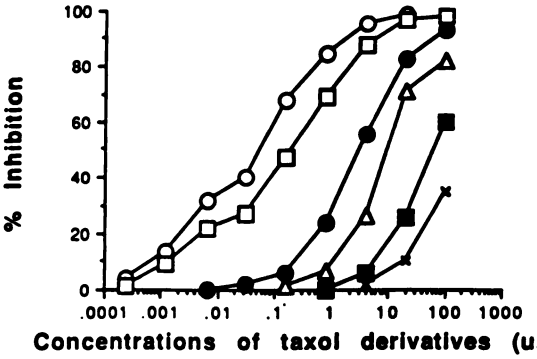

Fig. 3. Inhibition of the binding of 69E4A8E to taxol-RSA-coated wells by taxol derivatives and analogues. ○, taxol; ●, cephalomannine; □, 7-epitaxol; ■, taxotere; △, 2′-(triethylsilyl)taxol; ×, 20-acetoxy-4-deacetyl-5-epi-20,*O*-secotaxol. The results are means of duplicate points and are expressed as the percentage of inhibition relative to the absorbance at 490 nm measured in the absence of inhibitors in the ELISA assay.

ng/ml). The MAb could also be used to measure taxol levels by RIA, but the lower limit of measurement was 50 nM (data not shown).

Taxol levels in human serum could be also measured by ELISA using 69E4A8E (Table 3). In preliminary experiments, we found that undiluted human serum partially blocked the binding of antibody to taxol-RSA as measured by ELISA. Interference of binding was minimized by a 1:5 dilution of the serum with PBS. The lower limit of measurement was about 50 nM or 42 ng/ml.

DISCUSSION

Taxol is a diterpenoid, which has a 20-carbon skeleton, with a complex ester side chain at C-13 and an oxetane ring. The three-dimensional structure of taxol has an inverted cup-like shape. Guerritte-Voegelein *et al.* (16, 18) determined the three-dimensional structure of taxotere, a semisynthetic biologically active taxol analogue, by X-ray analysis; it also has an inverted cup shape and the same skeleton as taxol. The taxotere molecule is stabilized by intramolecular hydrogen bonds between C-3′H and the C-4 acetyl group and between C-2′H and C-18H_3, as well as a repulsive interaction between the substituents at C-2′, C-3′, and the taxane skeleton (16).

Structure-activity studies have revealed that the C-13 ester side chain (19, 20) and a closed oxetane ring (10, 21) are crucial to the activity of taxol derivatives. Opening of the oxetane ring results in a considerable conformational change of the molecule (10, 21). Modification of substituents at C-10 and/or C-17 can alter activity but not markedly (10, 19, 20).

We have prepared three antibodies specific for taxol: one rabbit antiserum and two monoclonal antibodies. With respect to the monoclonal antibodies, one is an IgG (69E4A8E) and the other is an IgM (29B7B3C). All of them bind taxol and active derivatives well and can be used to measure taxol levels in human serum (see below).

All of the antibodies are sensitive to the presence of the side chain ester at C-13 and an intact oxetane ring. In other words, biologically active compounds are bound well and inactive derivatives are bound poorly. An exception is the inability of the rabbit serum and 69E4A8E to recognize taxotere, a semisynthetic biologically active taxol analogue. The differences between taxol and taxotere are that taxotere lacks an C-10 acetyl group and has a *tert*-butyloxycarbamido group rather than a benzamido group at the C-3′ position. It is likely to be the latter that is the significant difference because the C-10 acetyl group is not necessary for activity. Moreover, cephalomannine, which is acetylated at C-10, is more poorly recognized by 69E4A8E. Apparently, the phenyl ring of taxol is an important determinant group for binding to the rabbit antibodies and to 69E4A8E.

On the other hand, 29B7B3C binds taxotere as well as it does taxol and it does not bind inactive derivatives well. We suggest, therefore, that it should be possible to use this antibody to screen for taxol or taxol-like compounds in extracts of natural products. We have begun to investigate this possibility. Moreover, its interaction with active taxol-related compounds closely correlates with their effects on microtubulin disassembly, making 29B7B3C an excellent candidate for eliciting anti-idiotypic antibodies that mimic taxol (22).

Our antibodies can measure taxol levels in human serum to which known quantities of taxol were added. In clinical trials, HPLC has been used to measure taxol levels in serum, urine, and other biofluids,

Table 1 *Relative 50% inhibitory concentrations of taxol derivatives in competitive ELISA, tubulin disassembly, and cytotoxicity assays*

Taxol derivatives	Competitive ELISA		Tubulin disassembly[a]	Cytotoxicity[a] (KB cells)
	29B7B3C	69E4A8E		
Taxol	1	1	1	1
Cephalomannine	0.4	44.2	1.5	3.2
7-Epitaxol	1.7	3.1	3	3
Taxotere	1.2	1000	0.5	0.4[b]
2′-(Triethylsilyl)taxol	33.2	153.8		30,000[c]
2′-(*N*-Cbz-β-alanyl)-7-oxo-5,6-dehydro-5-*O*-secotaxol	>500	>1000		
20-Acetoxy-4-deacetyl-5-epi-20,*O*-secotaxol	473.7	>1000	>21	>100,000
2′-Debenzoylisotaxol	>500	>1000		
Baccatin III	63.2	>1000	52	1,700
10-Deacetylbaccatin III	63.2	>1000	46	400
7-(Triethylsilyl)baccatin III	>500	>1000	384[d]	

[a] All data are from Ref. 10.
[b] From experiments using J774.2 cells. No data for KB cells are available.
[c] From experiments using 2′-(*tert*-butyldimethylsilyl)taxol, which is similar in structure to 2′-(triethylsilyl)taxol.
[d] From experiments using 7-acetylbaccatin III.

Table 2 *Measurement of taxol levels in human serum by RIA using rabbit anti-taxol antiserum*

Actual taxol concentrations	Taxol concentrations found[a]
5 μM	5.87 ± 1.00[b] μM
500 nM	476 ± 4 nM
50 nM	32.7 ± 0.3 nM
10 nM	11.3 ± 0.5 nM
5 nM	5.67 ± 0.64 nM

[a] All samples were done in duplicate.
[b] Mean ± SD.

Table 3 *Measurement of taxol levels in human serum by ELISA using 69E4A8E*

Actual taxol concentrations	Taxol concentrations found[a]
5 μM	3.61 ± 0.35[b] μM
500 nM	600 ± 144 nM
50 nM	62.1 ± 9.8 nM

[a] Data were averaged from three duplicate experiments for each concentration.
[b] Mean ± SD.

with the lower limit of detection being 50 nM (9, 23). However, HPLC techniques are not as suitable as immunoassays for routine analyses of large numbers of samples of biological fluids. The only immunoassay reported thus far is that of Jaziri *et al.* (12). Their rabbit antiserum could detect as little as 23.5 nM or 20 ng/ml in plant extracts by ELISA. They did not examine human serum. Our monoclonal antibodies could measure taxol in a concentration range of about 10 nM to 1 μM in PBS. However, the presence of human serum interfered with the binding of the antibodies in ELISA assays, requiring a dilution step that decreased the sensitivity of the procedure to a lower limit of 50 nM. The interfering factor in human serum did not seem to be an endogenous taxol-mimicking substance, because, upon dilution, its inhibition curve was not similar to that of taxol. Moreover, serum did not interfere with the RIA. We will be investigating this further.

Rowinsky and Donehower (9) reviewed pharmacokinetic studies of taxol. In the doses recommended in phase II trials, *i.e.*, 200 to 250 mg/m^2 infusion over 24 h, the peak taxol levels in plasma were above 0.6 μM, well within the range detectable and measureable by our antibodies. We will be testing our antibodies in a planned clinical study.

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