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TAXANE-SPECIFIC MONOCLONAL ANTIBODIES: MEASUREMENT OF TAXOL, BACCATIN III, AND "TOTAL TAXANES" IN *TAXUS BREVIFOLIA* EXTRACTS BY ENZYME IMMUNOASSAY[1]

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ABSTRACT.—Three monoclonal antibodies with either specificity to taxol or baccatin III, or cross-reactivity with several common taxanes have been prepared and used to develop sensitive competitive-inhibition enzyme immunoassays. The hybridomas producing these monoclonal antibodies were obtained by fusing P3X63Ag8.653 plasmacytoma cells and splenocytes from mice hyperimmunized with keyhole limpet hemocyanin-7-succinyltaxol or -7-succinylbaccatin III conjugates. Direct and indirect competitive inhibition enzyme immunoassays were developed with these monoclonal antibodies and microtiter plates coated with bovine serum albumin conjugates of the complementary hapten. Detection limits for the direct competitive inhibition enzyme immunoassays, conducted in buffer containing 10% MeOH, were 0.6 nM taxol for 3C6 (anti-taxol); 1.1 nM baccatin III for 3H5 (anti-baccatin III); and 0.6 nM taxol or baccatin III for 8A10 (anti-taxane). The immunoassays accurately detected taxol, baccatin III, and "total taxanes" in crude MeOH extracts of *Taxus brevifolia* bark and in hplc fractions of these extracts.

The anticancer agent taxol (paclitaxel) is one of several structurally related diterpene natural products isolated from bark of the Pacific yew (*Taxus brevifolia* Nutt.) (Taxaceae). In clinical trials, taxol has shown activity against advanced cancer of the ovary, lung, breast, and malignant melanoma (1–4). An expanding clinical trial effort and recent U.S. Food and Drug administration authorization of taxol for treatment of refractory ovarian cancer and metastatic breast cancer has increased demand for this drug. Taxol and other natural taxane congeners have been isolated from several renewable sources, including *T. brevifolia* needles (5–8), other *Taxus* spp. (5, 7, 9–25), *Taxus* spp. tissue culture (26–36), and most recently, *Taxomyces* fungal cultures (37, 38). Natural taxane congeners are utilized as starting materials for the semi-synthesis of taxol and other analogues, such as Taxotere® (docetaxel), which may have improved activity or formulation characteristics (39–44). The optimization of taxol and taxane production by tissue culture or semi-synthesis requires rapid and accurate screening methods for the identification of high-yielding natural sources of taxol or other taxanes.

Antibody-based immunoassays provide a cost-effective alternative to instrumental methods of analysis for large-scale natural product screening efforts, since they frequently require less time-consuming sample preparation and have high sample throughput. There have been four previous reports of anti-taxane antibodies: Jaziri *et al.* (45) and our group (46) independently reported the development of competitive inhibition enzyme immunoassays (CIEIA) for the semi-quantitative determination of taxanes in *Taxus* tissues using rabbit antisera elicited by immunization with a 2′-succinyltaxol-bovine serum albumin (BSA) conjugate and a 7-succinyltaxol-keyhole limpet hemocya-

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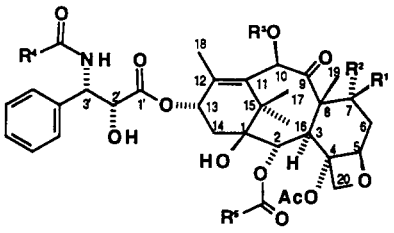
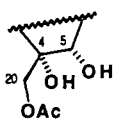
[2]Deceased.

nin (KLH) conjugate, respectively; Leu *et al.* (47) developed radioimmunoassays (RIA) and CIEIA using rabbit polyclonal antibodies and two monoclonal antibodies (mAb) elicited against 2′-succinyltaxol-BSA; and Concetti *et al.* have reported polyclonal chicken egg yolk antibodies elicited against 10-succinyl-10-deacetylbaccatin III-BSA (48).

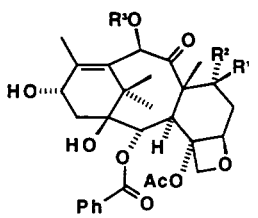
Our antiserum cross-reacts with taxol and cephalomannine above a detection limit of 0.25–0.35 nM by indirect CIEIA. Baccatin III and 10-deacetylbaccatin III were not recognized by these rabbit antibodies. When used to quantitate taxanes in crude extracts of Pacific yew bark, we observed that the CIEIA generally overestimated the amount of taxol and underestimated the total taxane content in the crude extracts (compared to an hplc assay). This result suggested that the polyclonal antibodies recognize epitopes



	R^1	R^2	R^3	R^4	R^5
Taxol (paclitaxel; Taxol®)	OH	H	Ac	Ph	Ph
Taxotere®[a]	OH	H	H	*t*-BuO	Ph
10-Deacetyltaxol	OH	H	H	Ph	Ph
7-*epi*-10-Deacetyltaxol	H	OH	H	Ph	Ph
7-Xylosyl-10-deacetyltaxol	O-β-xylose	H	H	Ph	Ph
7-*epi*-Taxol	H	OH	Ac	Ph	Ph
Cephalomannine	OH	H	Ac	(*Z*) $CH_3CH{=}C(CH_3)$—	Ph
2-Debenzoyl-2-(*p*-trifluoro-methylbenzoyl)taxol[a]	OH	H	Ac	Ph	*p*-$CF_3C_6H_4$



	R^1	R^2	R^3	R^4	R^5
20-Acetoxy-4-deacetyl-5-*epi*-20,*O*-secotaxol[a]	OH	H	Ac	Ph	Ph



	R^1	R^2	R^3
Baccatin III	OH	H	Ac
Baccatin V	H	OH	Ac
10-Deacetylbaccatin III	OH	H	H
7-*epi*-10-Deacetylbaccatin III	H	OH	H

[a]Synthetic analogues.

restricted to the taxane C-13 side-chain, a moiety not present in the baccatins. The specificity of the polyclonal antibody-based immunoassays of Jaziri *et al.* (45) and Leu *et al.* (47) are similar to ours with detection limits of 23.5 nM by CIEIA and 0.1 nM by RIA, respectively. In contrast, the polyclonal chicken antibodies of Concetti *et al.* (48) recognize the baccatins and other taxanes that lack the C-13 side-chain. The detection limit of CIEIA employing these egg antibodies with 10-deacetylbaccatin III is 13.7 nM.

Monoclonal antibodies specific for individual taxane congeners would permit the rapid differential measurement of these materials in crude sample extracts, thus increasing the accuracy and utility of immunoassay methods. Leu *et al.* have characterized two taxane-reactive mAbs (47): mAb 69E4A8E is highly specific for taxol, whereas the specificity of mAb 29B7B3C is similar to the rabbit antisera discussed earlier. In this paper, we report the production of three new high affinity mAbs with specificity to taxol (3C6), baccatin III (3H5), and the taxane diterpene skeleton (8A10), and the development of sensitive CIEIA employing them.

The objectives of the present study were: (a) to produce taxane congener-specific mAbs and a mAb which cross-reacts with most natural taxanes, and (b) use these mAbs to develop CIEIA for the selective determination of individual and total taxanes in unpurified extracts of *Taxus* biomass and cell cultures. The detection limits and specificity of each CIEIA were examined and a study was performed to compare the quantitation of taxanes in crude *T. brevifolia* bark extracts by CIEIA and hplc.

RESULTS AND DISCUSSION

Three mAbs were isolated which selectively bound either taxol, baccatin III, or an epitope shared by most natural and semi-synthetic taxanes. These mAbs were designated 3C6, 3H5, and 8A10, respectively. 3C6 (IgG_{2a}) resulted from fusion of splenocytes harvested from a BALB/c mouse immunized with the previously described 7-succinyltaxol-KLH immunogen (53), while 3H5 (IgG_1) and 8A10 (IgG_{2a}) were produced from Swiss-Webster mice immunized with 7-succinylbaccatin III-KLH. Growth of 3H5 and 8A10 as ascitic hybridoma tumors in BALB/c mice required pretreatment with an immunosuppressive anti-mouse lymphocyte serum.

The sensitivity of direct CIEIA systems developed with these antibodies was assessed in buffer containing 10% MeOH to compensate for the limited aqueous solubility of the taxanes. Binding of alkaline phosphatase-labeled 3C6 or 8A10 antibody to solid-phase BSA-conjugated antigen was inhibited 50% (IC_{50}) by 2–3 nM taxol, while enzyme-labeled 3H5 binding was inhibited 50% by 4–5 nM baccatin III (Figure 1). By comparison, the affinities of these mAbs are almost an order of magnitude higher than those described by Leu *et al.* (IC_{50}: mAb 29B7B3C–70 nM, mAb 69E4A8E–50 nM) (47). Detection limits for the direct CIEIA, conducted in buffer containing 10% methanol, were 0.6 nM taxol for 3C6 (anti-taxol); 1.1 nM baccatin III for 3H5 (anti-baccatin III); and 0.6 nM taxol or baccatin III for 8A10 (anti-taxane). Indirect CIEIA systems employing these mAbs were slightly less sensitive than the direct systems (Figure 1).

The cross-reactivity profiles of these mAbs were determined by indirect CIEIA with a panel of 10 natural and 3 synthetic taxane derivatives (Table 1). Antibody 3C6 binds taxol, 7-*epi*-taxol, 10-deacetyltaxol, 7-*epi*-10-deacetyltaxol, and 7-xylosyl-10-deacetyltaxol, while its affinity for cephalomannine is 22-fold lower than for taxol. MAb 3C6 does not bind any of the baccatin derivatives, which lack a C-13 side-chain. This degree of selectivity allows for the accurate determination of taxol in a 1:1 mixture of taxol and cephalomannine (data not shown). Taxotere®, a semi-synthetic taxoid that differs from taxol in that it lacks the C-10 acetyl group and has a *tert*-butyloxycarbamido group in place of the benzamido C-3′ substituent, is 31-fold less reactive than taxol with

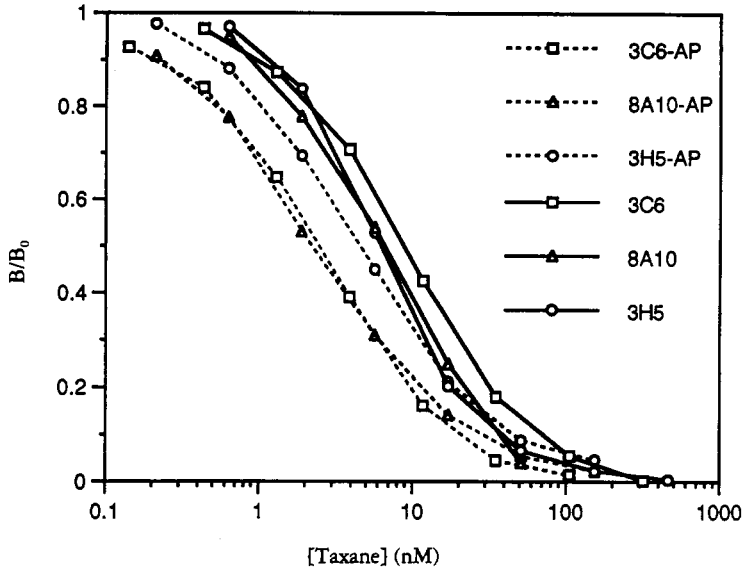


FIGURE 1. Standard curves for direct (----) and indirect (—) taxane CIEIAs in PBS containing 0.25% BSA, 0.05% Tween-20, pH 7.0, and 10% MeOH. The 3C6 (taxol-specific) standard curve was generated with taxol as analyte, while the 3H5 (baccatin III-specific) and 8A10 ("generic" taxane-specific) standard curves employed baccatin III. The B/B_0 values for each dilution were calculated by dividing the mean OD_{405} of a given set of replicates containing taxane inhibitor (B) by the mean OD_{405} of all the wells containing no inhibitor (B_0). Data points represent the mean of triplicate determinations from representative experiments.

mAb 3C6. The inability of mAb 3C6 to discriminate among C-7 substituted taxanes was expected, because it was elicited in response to a C-7 derived hapten. Anti-hapten antibodies are typically elicited to molecular determinants distal to the site of conjugation to the carrier protein (49). This reactivity pattern suggests that the epitope recognized by 3C6 likely resides, at least in part, in the distal portion of the C-13 sidechain. Antibody 3C6 also does not bind 2-debenzoyl-2-(*p*-trifluoromethylbenzoyl)taxol, suggesting that the 3C6 epitope also encompasses the C-2 benzoyl substituent. The cross-reactivity pattern of mAb 3C6 is similar in many respects to mAb 69E4A8E described by Leu *et al.* (47).

Antibody 3H5 binds with equal affinity to baccatin III and its 7-epi isomer, baccatin V. This mAb exhibits a 23- and 15-fold reduction in binding to 10-deacetylbaccatin III and its 7-epi- isomer, respectively, and a lack of reactivity towards taxanes bearing a C-13 substituent. This suggests that the 3H5 epitope encompasses the C-10 through C-13 region of the molecule.

Antibody 8A10 was elicited with the same immunogen as 3H5, however, this mAb exhibits nearly equal reactivity with all natural taxanes tested to date, as well as Taxotere®. MAb 8A10 also binds, to a lesser extent, with 2-debenzoyl-2-(*p*-trifluoromethylbenzoyl)taxol. This reactivity pattern suggests that the epitope recognized by 8A10 includes the C-6 through C-2 region of the structure. The dominant structural features of this region are the oxetane ring and the C-2 benzoyl group.

As would be predicted by the proposed C-6 to C-2 epitope of 8A10, this mAb was unreactive with 20-acetoxy-4-deacetyl-5-*epi*-20,*O*-secotaxol, a taxol derivative with an opened oxetane ring. MAb 3C6 did not exhibit cross-reactivity with this taxane either,

TABLE 1. Cross-Reactivity of Taxol- (3C6), Baccatin III- (3H5), or "Generic Taxane"-Specific (8A10) Monoclonal Antibodies Determined by Indirect CIEIA.

Taxane	IC_{50}[a] (nM)		
	3C6	3H5	8A10
Taxol (paclitaxel; Taxol®)	10	>316	7
10-Deacetyltaxol	15	>333	10
7-*epi*-10-Deacetyltaxol	25	>333	15
7-Xylosyl-10-deacetyltaxol	30	>286	17
7-*epi*-Taxol	80	>316	50
Cephalomannine	220	>325	8
Baccatin III	>511	10	12
Baccatin V	>460	10	10
10-Deacetylbaccatin III	>551	230	21
7-*epi*-10-Deacetylbaccatin III	>496	150	27
Taxotere® (docetaxel)	>318	>318	10
2-Debenzoyl-2-(*p*-trifluoromethylbenzoyl)taxol	>293	>293	>293[b]
20-Acetoxy-4-deacetyl-5-*epi*-20,*O*-secotaxol (Meerwein product)	>310	>310	>293

[a]Cross-reactivity is expressed as the concentration of analyte required to inhibit antibody binding to solid phase antigen by 50% (IC_{50}) in the indicated CIEIA. Values expressed as >X represent the highest concentration tested (either 270 or 300 ng/ml).

[b]Inhibited 40% at 293 nM.

although its epitope is not thought to include the oxetane moiety. Opening of the oxetane ring induces large conformational changes in both the eight-membered B- and six-membered C-rings (50), causing a radical change in the molecular topology, which may be responsible for the observed reduction of binding of mAb 3C6 and possibly 8A10. This hypothesis is supported by the observation of Concetti *et al.* that their chicken antibodies do not react with taxane derivatives lacking the oxetane ring except for two open oxetane analogues which also have an intramolecular bond between C-3 and C-11. This additional bond is known to stabilize the "normal" taxane ring conformation (48).

In order to determine the utility of CIEIA incorporating mAbs 3C6, 3H5, and 8A10 for the analysis of natural taxane-containing samples, semi-purified extracts of three samples of *T. brevifolia* bark and three samples of *T. brevifolia* needles were prepared according to the procedure of Wheeler *et al.* (5). The extracts were assayed for baccatin III, taxol, and cephalomannine by hplc and for baccatin III, taxol, and total taxanes by CIEIA (Table 2). The values for baccatin III content determined by CIEIA were almost identical with the concentrations found by hplc for all six samples. Similarly, the values for the taxol content in the needle samples determined by both methods were in close agreement. In contrast, the taxol content of the bark samples determined by CIEIA was 1.5–3 times greater than the hplc value.

The total taxane content determined by 8A10 CIEIA is higher than the sum of taxol, baccatin III, and cephalomannine hplc determinations, especially for the bark samples. This likely reflects the contribution of other taxanes which were not directly determined in the hplc analysis. Bark extract T1428, which had a tenfold discrepancy in the total taxane content and a twofold discrepancy in the taxol content determined by CIEIA and hplc, was chosen to further investigate this question. This extract was fractionated by hplc with photodiode array detection and the immunoreactivity of each fraction determined by CIEIA with all three antibodies. Tentative identification of several components was made by comparison of retention times and uv spectra with that of eight taxane standards.

TABLE 2. Taxol, Baccatin III, and Total Taxane Content of *T. brevifolia* Extracts by Hplc and CIEIA.

Sample (bark/needles)	% Taxol		% Baccatin III		% Total Taxane	
	hplc	3C6	hplc	3H5	hplc[a]	8A10
T1426 (b)	0.022	0.037	0.002	0.001	0.031	0.343
T1428 (b)	0.045	0.098	0.020	0.032	0.089	0.882
T1430 (b)	0.035	0.111	0.016	0.015	0.064	0.428
T1427 (n)	0.003	0.003	0.022	0.021	0.025	0.090
T1429 (n)	0.003	0.003	0.022	0.018	0.027	0.040
T1431 (n)	0.001	0.002	0.007	0.006	0.009	0.051

[a]Sum of taxol, baccatin III, and cephalomannine determined by hplc.

The specificity of antibodies 3H5 and 3C6 is well illustrated by the immunochromatogram data (Figure 2). Only fraction 4 exhibited substantial immunoreactivity with mAb 3H5. Comparison of retention times with standards indicates that the major component of fraction 4 is baccatin III. Likewise, mAb 3C6 reacted

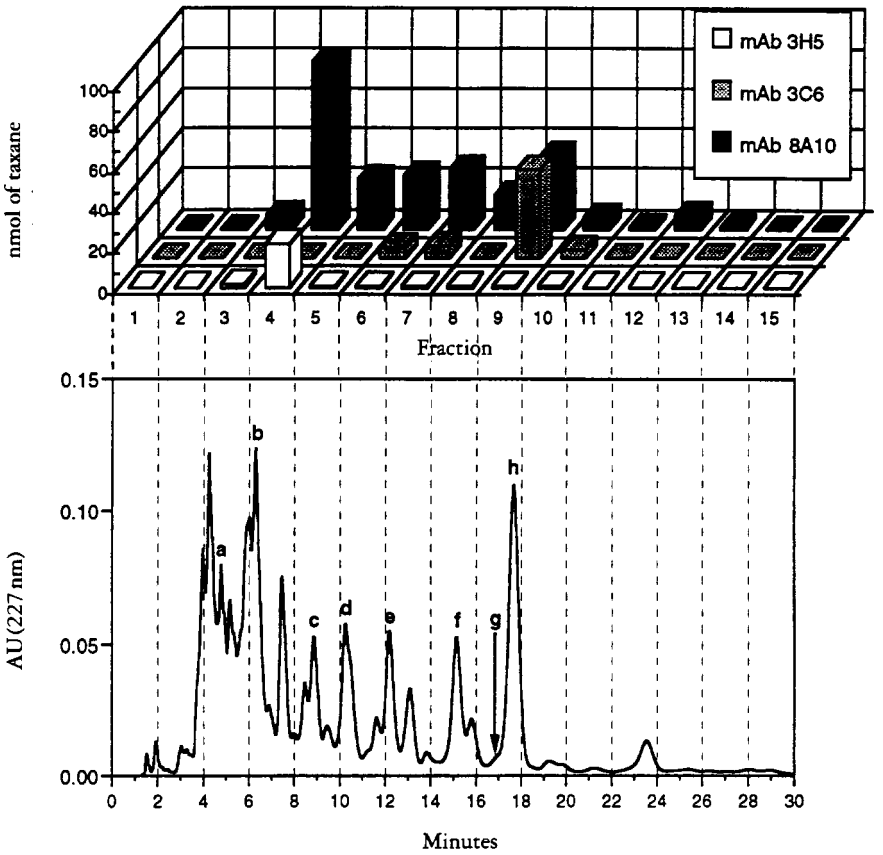


FIGURE 2. Detection of taxanes in hplc fractionated *T. brevifolia* extract T1428. The extract was prepared as described in the Experimental. Hplc fractions were collected over 2-min intervals and assayed with each of the three taxane CIEIA. Labeled peaks have the same retention time as authentic taxane standards: (a) 10-deacetylbaccatin III, (b) baccatin III, (c) baccatin V, (d) 7-xylosyl-10-deacetyltaxol, (e) 10-deacetyltaxol, (f) cephalomannine, (g) 7-*epi*-10-deacetyltaxol, (h) taxol.

strongly with fraction 9 and, to a much lesser extent, with fractions 6, 7, and 10. Comparison of the hplc data with authentic taxane standards indicates that fractions 9, 6, and 7 contain taxol, 7-xylosyl-10-deacetyltaxol, and 10-deacetyltaxol, respectively.

The presence of unidentified taxanes is clearly shown in the 8A10 immunochromatogram data where immunoreactivity was observed for fractions 3–10 and 12. Fractions 3–9 all contain peaks tentatively identified as taxanes by comparison of retention times and uv spectra with authentic taxane samples (Figure 2). A striking feature of this data is the fourfold differential in the immunoreactivity of fraction 4 with mAb 8A10 as compared with 3H5. Inspection of the uv chromatogram indicates that peaks in this region were poorly resolved and that fraction 4 contains at least two major peaks in addition to that assigned as baccatin III. Both of these unidentified peaks have taxane-like uv spectra. The retention time and uv spectrum of the major component of fraction 5 match those of our baccatin V standard and this fraction is immunoreactive with mAb 8A10. However, the lack of reactivity with mAb 3H5 confirms that this peak is not baccatin V and suggests that it is an unidentified taxane that co-migrates with baccatin V under our hplc conditions. The immunoreactivity observed for fractions 9 and 10 was essentially equal for mAbs 3C6 and 8A10, indicating the homogeneity of these fractions. In contrast, fractions 6 and 7 exhibited fivefold greater reactivity with mAb 8A10 than with 3C6. The hplc data for these fractions indicates the presence of at least one additional unidentified peak in each of these fractions with a taxane-like uv spectrum. None of our standards matched the retention times of fractions 10 and 12 although the uv spectra of these peaks also suggested that they may be taxanes. Thus, mAb 8A10 could be used to guide the isolation of additional taxanes that may not be found by conventional bioactivity-guided fractionation. Such taxanes may be valuable as starting materials for the semi-synthesis of taxol and other novel taxoids.

Samples can often be analyzed by immunoassay without the labor-intensive sample workup usually required for hplc analysis of trace components in complex biological sample matrices. Biomass samples corresponding to the above semi-purified extracts were subjected to a simple methanolic extraction and assayed for baccatin III, taxol, and total taxanes by CIEIA (Table 3). The observation that the values obtained for the crude extracts were generally higher than those obtained for the semi-purified extracts (Table 2) raises the possibility of matrix interferences; consistency in answers obtained from different dilutions of each extract (data not shown), however, suggests that matrix interference is negligible (51). Alternatively, the higher values observed for the crude extracts could reflect poor recovery of known taxanes and/or unidentified immunoreactive polar taxanes and taxane precursors. Unfortunately, crude extracts cannot be analyzed by hplc to confirm this possibility. In addition, while the biomass used for preparation of the crude MeOH extracts was from the same bulk sample used to prepare the semi-purified extracts, it was not the identical sample. This may account for some of the observed variability between these extracts.

TABLE 3. Taxol Content of Crude MeOH Extracts by CIEIA.

Sample (bark/needles)	% Taxol (3C6)	% Baccatin III (3H5)	% Total Taxanes (8A10)
T1426 (b)	0.054	0.012	0.377
T1428 (b)	0.116	0.043	0.853
T1430 (b)	0.216	0.022	0.470
T1427 (n)	0.006	0.040	0.120
T1429 (n)	0.015	0.030	0.115
T1431 (n)	0.007	0.012	0.046

Both hplc and CIEIA are subject to possible errors from co-eluting or cross-reacting components in the extracts. Partial purification can eliminate many of these interferences but may result in loss of analyte due to poor extraction efficiency. However, either method can provide reliable estimates of relative taxane content between samples. Omission of tedious sample preparation is a major advantage of CIEIA: thus, when only "taxol-like," "baccatin-like," or "taxane-like" structural information is required, these assays are well suited for the rapid screening of *Taxus* biomass and cell cultures. In addition, CIEIA can be adapted to various portable non-instrumental formats suitable for field work.

The three assays reported here provide a powerful set of tools for the identification of new sources of known taxanes and the discovery of novel taxane congeners. Indeed, they have been used to facilitate identification and development of a novel fungal source of taxol (38) and monitor taxol production in transgenic *Taxus* callus cultures (30). These assays may also be useful for the determination of taxol, Taxotere®, or other taxoid drugs and their metabolites in patient tissues during current and future clinical trials of these agents. Preliminary results for the determination of taxol in patient serum using the 3C6 assay will be reported elsewhere.

Currently, these high-affinity taxane mAbs are being investigated for use as immunoaffinity adsorbents. Potential applications include selective concentration of taxanes from complex extracts for instrumental or immunological analysis and scale-up for simplified purification of taxanes from plant biomass or cell culture.

EXPERIMENTAL

GENERAL EXPERIMENTAL PROCEDURES.—Taxol (paclitaxel; NSC-125973), cephalomannine (NSC-318735), 10-deacetylbaccatin III (NSC-251677), baccatin III (NSC-330753), 7-*epi*-10-deacetyltaxol (NSC-656177), baccatin V (NSC-656177), 10-deacetyltaxol (NSC-656201), and 7-*epi*-10-deacetybaccatin III (NSC-656178) standards were provided by Dr. K.M. Snader of the Drug Synthesis and Chemistry Branch, Developmental Therapeutics Program, Division of Cancer Treatment, National Cancer Institute, Bethesda, MD. 7-Xylosyl-10-deacetyltaxol and 7-*epi*-taxol standards were provided by Dr. B. Roach of Phyton Catalytic Inc., Ithaca, NY. 2-Debenzoyl-2-(*p*-trifluoromethylbenzoyl)taxol and 20-acetoxy-4-deacetyl-5-*epi*-20,*O*-secotaxol (Meerwein product) standards were provided by Dr. D.G.I. Kingston of Virginia Polytechnic Institute and State University, Blacksburg, VA. Taxotere® standard was provided by Dr. J.L. Fabre of Rhône-Poulenc Rorer S.A., France. Larger quantities of taxol for hapten synthesis were obtained from NaPro Biopharmaceuticals, Boulder, CO, and used to prepare baccatin III according to the method of Magri *et al.* (52). Immulon 2™ microtiter plates were purchased from Dynatech Laboratories, Inc., Chantilly, VA. Calf intestine alkaline phosphatase (AP) was purchased from Biozyme Laboratories International, Ltd., San Diego, CA. AP-labeled goat anti-mouse IgG and IgM conjugate was purchased from Caltag Laboratories, Inc., San Francisco, CA. KLH, BSA, and *p*-nitrophenylphosphate (pNPP) were purchased from Sigma Chemical Co., St. Louis, MO. SulfoSMCC was from Pierce, Rockford, IL. All other chemicals were purchased from Sigma Chemical Co., St. Louis, MO or Aldrich Chemical Co., Milwaukee, WI.

SYNTHESIS OF 7-SUCCINYL TAXOL.—The hapten, 7-succinyltaxol, was synthesized and conjugated to BSA or KLH as previously described (46).

SYNTHESIS OF 7-SUCCINYL BACCATIN III.—To a solution of baccatin III (56.5 mg, 9.63×10^{-5} mol) in anhydrous *N,N*-dimethylformamide (DMF) (3.0 ml) was added succinic anhydride (57.8 mg, 5.78×10^{-4} mol, 6 equivalents) and *N,N*-dimethylaminopyridine (11.8 mg, 9.63×10^{-5} mol, 1 equivalent). After stirring at 85° for 48 h, the solvent was removed *in vacuo*. The residue was dissolved in CH_2Cl_2 (15 ml), and washed with 1 N aqueous HCl (2×10 ml) and brine (1×10 ml). The organic fraction was dried over Na_2SO_4 and the solvent evaporated. Purification was effected by chromatography (Chromatotron, 1 mm silica plate) with 10% MeOH in CH_2Cl_2. This provided 39 mg (55% yield) of the desired compound. ^{1}H nmr (500 MHz, $CDCl_3$) δ 8.11–8.09 (2H, m, Ph), 7.63–7.60 (1H, m, Ph), 7.50–7.47 (2H, m, Ph), 6.23 (1H, s, H-10), 5.63 (1H, dd, J=7.0 and 10.75 Hz, H-7), 5.62 (1H, d, J=7.0 Hz, H-2), 4.96 (1H, dd, J=9.4 and 1.5 Hz, H-5), 4.86 (1H, br t, J=8.25 Hz, H-13), 4.32 (1H, d, J=8.5 Hz, H-20), 4.14 (1H, d, J=9.0 Hz, H-20), 3.99 (1H, d, J=7.0 Hz, H-3), 2.7–2.6 (m, succinyl, H-14, H-6), 2.29 (3H, s, acetate), 2.16 (3H, s, acetate), 2.10 (3H, s, H-18), 1.79 (3H, s, H-19), 1.12 and 1.08 (H-16 and H-17, both s).

SYNTHESIS OF 7-SUCCINYLBACCATIN III–KLH.—To a solution of 7-succinylbaccatin III (1.1 mg, 1.50×10^{-6} mol) in anhydrous DMF (670 μl) were added solutions of *N*-hydroxysuccinimide (NHS, 688 μg, 5.98×10^{-6} mol, 4 equivalents) in anhydrous DMF (69 μl), and 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (EDC, 314 μg, 1.64×10^{-6} mol, 1.1 equivalent) in anhydrous DMF (31 μl). The solution was stirred at room temperature for 1 h and then to it was added a solution of KLH (10.5 mg, 1.05×10^{-7} mol, 0.07 equivalent) in sodium phosphate buffer (50 mM, pH 7, 2.31 ml).

After stirring for 12 h at room temperature, the reaction mixture was concentrated by centrifugal ultrafiltration using a Centricon 30 microconcentrator (Amicon, Beverly, MA). The insoluble conjugate was separated by centrifugation and washed repeatedly with 50 mM sodium phosphate, pH 7.0, containing 0.15 M NaCl.

SYNTHESIS OF 7-SUCCINYLBACCATIN III–BSA.—To a solution of 7-succinylbaccatin III (0.81 mg, 1.1×10^{-6} mol) in anhydrous DMF (494 μl) were added solutions of NHS (510 μg, 4.4×10^{-6} mol, 4 equivalents) in anhydrous DMF (51 μl) and EDC (230 μg, 1.2×10^{-6} mol, 1.1 equivalents) in anhydrous DMF (23 μl). The solution was stirred at room temperature for 1 h and then to it was added a solution of BSA (4.96 mg, 2.2×10^{-7} mol, 0.2 equivalent) in sodium phosphate buffer (50 mM, pH 7, 1.73 ml).

After stirring for 12 h at 25°, the solution was concentrated using a Centricon 30. Insoluble material was removed by centrifugation and the soluble fraction exchanged into 50 mM sodium phosphate, pH 7.0, containing 0.15 M NaCl and 0.02% sodium azide (PBS). Quantitation by uv spectroscopy indicated that 9.74 mg of soluble protein conjugate were obtained.

IMMUNIZATION OF ANIMALS.—Female BALB/c and Swiss-Webster mice (Simonsen Laboratories, Gilroy, CA) received 100 μg 7-succinyltaxol-KLH or 7-succinylbaccatin III–KLH in 0.3 ml complete Freund's adjuvant, followed at two-week intervals with 50 μg of immunogen in 0.3 ml incomplete Freund's adjuvant. All injections were administered i.p. Mice were bled from the tail vein 7 days after each booster injection, to obtain sera for monitoring antibody titer against hapten-BSA conjugates. Mice that were to be used as spleen cell donors for hybridoma production were given 50 μg of immunogen i.v. in sterile PBS without sodium azide, four days prior to fusion.

PRODUCTION OF MONOCLONAL ANTIBODIES.—Hybridomas were prepared by polyethylene glycol-mediated fusion of splenocytes from hyperimmunized BALB/c or Swiss-Webster mice and the plasmacytoma cell line, P3X63Ag8.653 (ATCC CRL 1580), as described by Goding (53). Hybridomas secreting the desired mAbs were cloned three times by limiting dilution and cryopreserved in liquid N_2. MAb 3C6 (anti-taxol) was harvested from the ascitic fluid of BALB/c mice pretreated with 0.5 ml pristane (Sigma) then inoculated i.p. five days later with 2×10^6 hybridoma cells. MAb from hybridomas derived from Swiss-Webster B-lymphocytes (3H5 anti-baccatin III and 8A10 anti-taxane) was harvested from the ascitic fluid of BALB/c mice pretreated s.c. with 0.1 ml anti-mouse lymphocyte serum (Cedarline Laboratories, Hornby, Ontario, Canada), and i.p. with 0.5 ml incomplete Freund's adjuvant three days prior to injection of 2×10^6 hybridoma cells. The heavy and light chain isotype of mAbs was determined by ELISA, using a commercially available kit (Zymed, S. San Francisco, CA).

INDIRECT ELISA.—Indirect ELISA was used to determine serum anti-hapten antibody titer and to screen hybridoma culture supernatants. Immulon 2™ microtiter plate wells (Dynatech Laboratories, Chantilly, VA) were coated for 1 h at room temperature with 50 μl per well of 7-succinyltaxol-BSA or 7-succinylbaccatin III-BSA at an optimized concentration in PBS, pH 7.0. Optimal coating antigen concentrations were determined by checkerboard titration. Plates were washed three times with 0.01 M Tris, 0.15 M NaCl, 0.05% Tween-20, 0.02% NaN_3, pH 7.0 (TBS-T) blocked for 1 h at 22° with 200 μl per well of 1% BSA in PBS (PBS-B), then washed again three times with TBS-T. Fifty μl of serum or hybridoma culture supernatant, titrated in PBS containing 0.25% BSA, and 0.05% Tween-20, pH 7.0 (BPT) were added to each well, and the plates incubated at room temperature for 1 h. After washing three times in TBS-T, 50 μl per well of goat anti-mouse IgG and IgM AP conjugate, appropriately diluted in BPT, were added, and the plates incubated at room temperature for 1 h. After four final washes in TBS-T, 200 μl per well of 1 mg/ml pNPP in AP substrate buffer (25 mM Trizma base, pH 9.5; 0.15 M NaCl; 5 mM $MgCl_2$; 0.02% NaN_3) were added, and the absorbance of each well read after 60 min incubation at room temperature, on a Dynatech MR 5000 ELISA plate reader (Dynatech, Chantilly, VA; sample wavelength = 405 nm, reference wavelength = 690 nm).

ANTIBODY PURIFICATION.—MAbs were affinity purified from mouse ascitic fluids by discontinuous pH gradient elution from Protein A-Sepharose (54). Eluted peaks were concentrated and buffer salts exchanged into PBS using a Centricon 30.

PREPARATION OF MONOCLONAL ANTIBODY-ALKALINE PHOSPHATASE CONJUGATES.—Calf intestine AP was conjugated to purified mAbs by the method of Ishikawa *et al.* (55). The enzyme was dialyzed into borate

buffer (50 mM sodium borate, pH 7.6, containing 1 mM $MgCl_2$ and 0.1 mM $ZnCl_2$), then reacted with a 25-fold molar excess of sulfosuccinimidyl 4-(*N*-maleimidomethyl)-cyclohexane-1-carboxylate in borate buffer for 1 h at room temperature; 100-μl aliquots of the maleimido-modified AP (AP-MCC) were applied to 1-ml Sephadex G-25 (fine) spun columns (56) that had been equilibrated with 0.1 M Tris-HCl, pH 7.0, containing 1 mM $MgCl_2$ and 0.1 mM $ZnCl_2$, and centrifuged for 2 min. The AP-MCC filtrates were collected and combined.

Five mg of purified mAb were concentrated and buffer exchanged into 0.1 M sodium phosphate, pH 6.5, using a Centricon 30. MAb was thiolated with a 100-fold molar excess of *S*-acetylmercaptosuccinic anhydride in 0.1 M phosphate buffer, pH 6.5, for 30 min at room temperature, then 0.02 ml 0.1 M EDTA, pH 8.0, 0.1 ml 0.1 M Tris-HCl, pH 7.0, and 0.1 ml 1M hydroxylamine-HCl, pH 6.5, were added and the mixture incubated for 4 min at 30°. Thiolated mAb was applied under an Ar atmosphere to a Sephadex G-25 column which had been equilibrated with degassed 0.1 M sodium phosphate, pH 6.0, containing 2 mM EDTA. Thiolated mAb was eluted directly into a solution containing a fivefold molar excess of AP-MCC and the reaction was carried out for 18 h at 4°, under Ar. Conjugates were then concentrated in a Centricon 30 and purified by size-exclusion hplc on a Biosep-SEC3000 column (7.8×600 mm) (Phenomenex) using 10 mM Tris-HCl, pH 6.8, containing 0.1 M NaCl, 1 mM $MgCl_2$, 0.1 mM $ZnCl_2$, and 0.05% NaN_3 as eluent. The mAb-AP conjugates were concentrated in a Centricon 30, diluted with an equal volume of glycerol, and stored at −20°.

INDIRECT CIEIA FOR TAXOL.—Immulon 2™ microtiter plates were coated with 100 μl/well of 7-succinyltaxol-BSA or 7-succinylbaccatin III-BSA in PBS, blocked, and washed using the procedure described for the indirect ELISA. Standards (taxol for 3C6; baccatin III for 3H5 and 8A10) or the sample under test were serially diluted in BPT containing 20% MeOH (BPT-M) and triplicate 50-μl aliquots were combined with 50 μl mAb, diluted in BPT, and incubated at room temperature for 1 h. Microtiter plates were washed three times with TBS-T, then bound mAb was detected with 100 μl/well of AP-labeled goat anti-mouse IgG and IgM conjugate diluted in BPT. After incubation for 1 h at room temperature, plates were washed four times with TBS-T, then 200 μl/well of 1 mg/ml *p*-NPP, diluted in AP substrate buffer, was added. Plates were incubated for 1 hour at room temperature, then read as described above.

DIRECT CIEIA FOR TAXOL.—The methods for the direct and indirect CIEIAs were the same, except that the use of AP-labeled goat anti-mouse IgG and IgM conjugate was not required in the direct assay. Instead, taxol analyte or standard, serially diluted in BPT-M, was combined with mAb-AP conjugate, optimally diluted in BPT. After incubation for 1 h at room temperature, plates were washed four times with TBS-T, then 200 μl/well of 1 mg/ml *p*-NPP, diluted in AP substrate buffer was added, and the plates processed as described above.

PREPARATION OF *T. BREVIFOLIA* EXTRACTS.—*T. brevifolia* extracts were prepared at Program Resources Inc./DynCorp by a slight modification of the method of Wheeler *et al.* (5). Ground *T. brevifolia* bark or needles (10 g) were extracted in a separatory funnel with 50 ml of hexane for 24 h at room temperature. The hexane extract was discarded and the remaining plant material was extracted with 50 ml of CH_2Cl_2-MeOH (1:1) for 24 h at room temperature. The organic extract was evaporated to dryness at 35° under reduced pressure. The residue was dissolved in 10 ml of MeOH and partitioned between 50 ml each of CH_2Cl_2 and H_2O. The aqueous layer was discarded. The organic layer was further washed by pouring it through 50 ml of H_2O in a separatory funnel. The solvent was removed at 35° under reduced pressure in a tared flask and the residue mass was determined. Approximately 1 mg of extract residue and 1 g of the corresponding biomass samples were weighed into vials, coded, and shipped to Hawaii Biotechnology Group for CIEIA analysis.

HPLC ANALYSIS OF *T. BREVIFOLIA* EXTRACTS.—Extracts were assayed for taxol, cephalomannine, and baccatin III using the method of Wheeler *et al.* (5).

CIEIA ANALYSIS OF *T. BREVIFOLIA* EXTRACTS.—The samples of extract residue were dissolved in 1 ml of MeOH and assayed by the indirect CIEIA method described above. The fraction of extract residue was determined by dividing the weighed amount by the total mass of extract residue. The amount of taxane in the extract residue was then calculated by dividing the amount of taxane in the weighed amount by the fraction of extract residue. The percent of taxanes in the plant material was obtained by dividing the amount of taxane found in the residue by the dry wt of the plant material extracted and multiplying by 100.

CIEIA ANALYSIS OF *T. BREVIFOLIA* BIOMASS.—Ground biomass (needles or bark, 100 mg) was extracted in a glass vial with 1.0 ml of MeOH for 24 h at room temperature. The MeOH extract was filtered through a 0.2-μm filter and assayed by the indirect CIEIA method described above. The percent of taxanes in the plant material was obtained by dividing the amount of taxane found in the MeOH extract by the dry wt of the plant material extracted and multiplying by 100.

IMMUNOCHROMATOGRAM ANALYSIS OF *T. BREVIFOLIA* BARK EXTRACT.—Extract T1428 was fractionated by hplc on a Curosil G 6 μm phenyl-bonded Si gel column (4.6×150 mm) (Phenomenex) operated under isocratic conditions employing a mobile phase consisting of MeOH-MeCN-H_2O (10:40:50). The flow rate was 1 ml/min and the eluate was monitored with a photodiode array detector. The injection volume was 75 μl, and fifteen 2-ml fractions were collected. Of each fraction 1 ml was diluted with 2 ml of BPT-M and assayed for taxol, baccatin III, and total taxanes using the indirect CIEIA described above. CIEIA results were plotted as a histogram and compared with the uv chromatogram.

DATA ANALYSIS.—All samples tested by ELISA and CIEIA were run in triplicate and the mean result of each set of replicates was calculated. 7-Succinyltaxol-BSA- or 7-succinylbaccatin III-BSA-blocked wells treated with AP-conjugate and substrate were included on each plate to measure background color development, and the mean OD_{405} of these wells was subtracted from the mean OD_{405} of each set of control and test replicates before data analysis.

For CIEIAs, standard curves were constructed for each experiment using a set of taxol standard dilutions. B/B_0 values for each dilution were calculated by dividing the mean OD_{405} of a given set of replicates containing taxane inhibitor (B) by the mean OD_{405} of all the wells containing no inhibitor (B_0). The concentration of taxane inhibitor causing 50% inhibition (IC_{50}) or 20% inhibition (IC_{20}) were used to establish the sensitivity and cross-reactivity levels of the different CIEIA systems. Unknown taxane concentrations in samples under test were calculated from the OD_{405} of the sample dilution(s) that fell within the log-linear portion of the standard curve.

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