A Receptor Protein−Based Bioassay for Quantitative Determination of Paclitaxel

ARTICLE *in* ANALYTICAL CHEMISTRY · OCTOBER 1997

Impact Factor: 5.64 · DOI: 10.1021/ac970233h · Source: PubMed

3 AUTHORS, INCLUDING:

Shin-ichiro Suye
University of Fukui
87 PUBLICATIONS **687** CITATIONS

Anal. Chem. **1997,** *69,* 3633−3635

A Receptor Protein-Based Bioassay for Quantitative Determination of Paclitaxel

Shin-Ichiro Suye,† Sagun Tandel, and Ashok Mulchandani*

Chemical Engineering Department, University of California, Riverside, California 92521

A novel receptor-based bioassay for the quantitative measurement of Taxol was developed. The assay was based on the well-investigated and established finding that Taxol, its active analogs, and active metabolites bind reversibly to the receptor protein tubulin, a process similar to antibody and antigen interaction. The assay was performed in a competitive format by allowing a mixture of horseradish peroxidase-labeled Taxol and Taxol in the analyte sample to compete for the Taxol binding site of a polystyrene microtiter plate wall coated with purified tubulin and subsequently measuring the tubulin−Taxol complex by determining the activity of the horseradish peroxidase label. Using this method, Taxol was measured very sensitively, linear range of 0.0001−1 nM, and selectively, without interference from non-tumor-active compounds such as baccatin III, cephalomaninne, and 10-deacetyl taxol. The method was applied for the determination of picomolar concentrations of Taxol in human plasma.

Taxol (Taxol is a registered trademark of Bristol-Meyers Squibb Co. The approved generic name for the drug is paclitaxel. In this paper, the name Taxol is used to refer to the chemical compound.), a complex diterpenoid first isolated from the bark of pacific yew tree, *Taxus brevifolia*[1] , has antineoplastic activity against several murine tumors. It has received FDA approval for the treatment of ovarian cancer[2] and is currently undergoing phase II trials for the treatment of non-small-cell lung cancer.[3,4] Clinical studies for treatment of various cancers has shown that Taxol has a very narrow therapeutic range, and there is a wide variation in the elimination half-life (1.3−8.6 h) of Taxol between patients.[5] Patients undergoing Taxol therapy suffer from neuropenia, neuropathy, hypersensitivity, and mucositis.[3−6] In addition, recent studies have shown that some of its metabolites also have anticancer activity.[7,8] All this makes it necessary to have a sensitive and rapid analytical technique to monitor not only Taxol but also its active metabolites in biological samples for better patient management.[5]

Various methods have been reported for the determination of Taxol, its analogs, and its metabolites in tissue and body fluids as well as in yew trees, including tandem mass spectrometry,[9] HPLC,[10−18] and enzyme-linked immunosorbent assay (ELISA) methods.[19−21] HPLC methods suffer from the need to pretreat samples and their tedious procedures. Immunoassays based on polyclonal and monoclonal antibodies have cross-reactivity with inactive analogs of Taxol, such as baccatin III, cephalomannine, and 10-deacetylbaccatin III. Tandem mass spectrometry has been found unreliable due to large standard deviation. Furthermore, none of these methods measure Taxol concentration at the receptor level, which is desirable for better patient management.

Taxol binds tubulin specifically and stimulates polymerization of tubulin in the absence of microtubule-associated proteins (MAPs) or organic buffers.[22,23] The binding of the target drug Taxol to tubulin is reversible, a process similar to that of an antigen and an antibody. Therefore, like many immunoassays presently being used for determination of antigens, binding of Taxol to tubulin can be exploited for the development of a specific bioassay for determination of the Taxol concentrations. This paper describe a novel receptor (tubulin) protein-based bioassay method for the determination of the Taxol and its application for the determination of Taxol level in human plasma.

* Address correspondence to this author at (phone) 909-787-5190, (Fax) 909-787-3188, or (E-mail) ashok_mulchandani@qmail.ucr.edu.

† On leave from the Department of Applied Chemistry and Biotechnology, Fukui University, Bunkyo, Fukui 910, Japan.

(1) Wani, M. C.; Taylor, L.; Wall, M. E.; Coggon, P.; McPhail, A. T. *J. Am. Chem. Soc.* **1971**, *93*, 2325−7.
(2) Joyce, C. *Bioscience* **1993**, *43*, 133−6.
(3) Murphy, W. K.; Fossella, F. V.; Winn, R. J.; Shin, D. M.; Hynes, H. E.; Gross, H. M.; Davilla, E., Leimert; J., Dhingra, H.; Raber, M. N.; Krakoff, I. H.; Hong, W. K. *J. Natl.Cancer Inst.* **1993**, *85*, 384−8.
(4) Chang, A. Y.; Kim, K.; Glick, J.; Anderson, T.; Karp, D.; Johnson, D. *J. Natl.Cancer Inst.* **1993**, *85*, 382−94.
(5) Rowinsky, E. K.; Cazenave, L. A.; Donehower, R. C. *J. Natl.Cancer Inst.* **1990**, *82*, 1247−59.
(6) Spencer, C. M.; Faulds, D. *Drugs* **1994**, *48*, 794−847.
(7) Kumar, G.; Ray, S.; Walle, T.; Huang, Y.; Willingham, M.; Self, S.; Bhalla, K. *Cancer Chemother. Pharmacol.* **1995**, *36*, 129−35.
(8) Sparreboom, A.; Huizing, M. T.; Boesen, J. J.; Nooijen, W. J.; van Tellingen, O.; Beijnen, J. H. *Cancer Chemother. Pharmacol.* **1995**, *36*, 299−304.
(9) Hoke, S. H., II; Wood, J. M.; Cooks, R. G.; Li, X.-H.; Chang, C. *Anal. Chem.* **1992**, *64*, 2313−5.
(10) Willey, T. A.; Bekos, E. J.; Gaver, R. C.; Duncan, G. F.; Tay, L. K.; Beijnen, J.H.; Farmen, R. H. *J. Chromatogr.* **1993**, *621*, 231−8.
(11) Sharma, A.; Conway, W. D.; Straubinger, R. M. *J. Chromatogr., B* **1994**, *655*, 315−9.
(12) Richheimer, S. L.; Tinnermeier, D. M.; Timmons, D. W. *Anal. Chem.* **1992**, *64*, 2323−6.
(13) Rizzo, J.; Riley, C.; Von Hoff, D.; Kuhn, J.; Phillips, J.; Brown, T. *J. Pharm. Biomed. Anal.* **1990**, *8*, 159−64.
(14) Huizing, M. T.; Rosing, H.; Koopman, F.; Keung, A. C. F.; Pinedo, H. M.; Beijnen, J. H. *J. Chromatogr.* **1995**, *664*, 373−82.
(15) Huizing, M. T.; Keung, A. C.; Rosing, H.; van der Kuij, V.; ten Bokkel Huinink, W. W.; Mandjes, I. M.; Dubbelman, A. C.; Pinedo, H. M.; Beijnen, J. H. *J. Clin. Oncol.* **1993**, *11*, 2127−35.
(16) Castor, T. P.; Tyler, T. A. *J. Liq. Chromatogr.* **1993**, *16*, 723−31.
(17) Fang, W.; Wu, Y.; Zhou, J.; Chen, W.; Fang, Q. *Phytochem. Anal.* **1993**, *4*, 115−9.
(18) Choi, M.-S.; Kwak, S.-S.; Liu, J. R.; Park, Y.-G.; Lee, M.-K; An, N.-H. *Planta Med.* **1995**, *61*, 264−6.
(19) Grothaus, P. G.; Raybould, T. J. G.; Bignami, G. S.; Lazo, C. B.; Byrnes, J. B. *J. Immunol. Methods* **1993**, *158*, 5−15.
(20) Leu, J.-G.; Chen, B.-X.; Schiff, P. B.; Erlanger, B. F. *Cancer Res.* **1993**, *53*, 1388−91.
(21) Leu, J.-G.; Jech, K. S.; Wheeler, N. C.; Chen, B.-X.; Erlanger, B. F. *Life Sci.* **1993**, *53*, PL183−7.
(22) Kumar, N. *J. Biol. Chem.* **1981** *256*, 10435−41.
(23) Schiff, P. B.; Horwitz, S. B. *Biochemistry* **1981** *20*, 3247−52.

S0003-2700(97)00233-3 CCC: $14.00

EXPERIMENTAL METHODS

Materials. Taxol, cephalomannine, baccatin III, 10-deacetyltaxol, 7-epi-10-deacetyltaxol, and 10-deacetylbaccatin III were gifts from the National Cancer Institute. Taxol was also purchased from ICN Pharmaceuticals (Costa Mesa, CA). Cow brains were obtained from Hallmark Meat Packing (Chino, CA). Sephadex G-150 was purchased from Pharmacia Biotech Co. (Uppsala, Sweden). Cellulosephosphate P-11 was obtained from Whatman Co. (Kent, UK). Human plasma, EGTA, bovine serum albumin (BSA), and horseradish peroxidase (HRP) were purchased from Sigma Chemical Co. (St. Louis, MO). All other chemicals were analytical grade.

Preparation of Purified Tubulin. Cow brain tubulin protein was purified by two cycles of temperature-dependent assembly−disassembly followed by phosphocellulose column chromatography.[24]

Preparation of Horseradish Peroxidase-Labeled Taxol (TX−HRP). The preparation of HRP-labeled Taxol involved three steps. In the first step, Taxol was converted to 2′-*O*-*tert*-butyldimethylsilyloxy-Taxol (**I**) by reacting Taxol (10 mg) with 50 μL (5 equiv) of silylating solution consisting of *tert*-butyldimethylsilyl chloride (10.2 g) and imidazole (9.3 g) dissolved in dry dimethylformamide (18 mL) for 12 h at room temperature. The reaction mixture was then diluted with excess dichloromethane and washed with 1 N HCl, followed by 10% sodium bicarbonate solution, and finally brine. The reaction mixture was then dried over sodium sulfate and the solvent removed by evaporation. The product was isolated by preparative HPLC using methanol−water (85:15) carrier and the structure characterized using NMR. The final yield was 50−60%. Subsequently, 10 mg of pure **I** was reacted with excess carbonyldiimidazole (30 mg) in anhydrous dichloromethane at room temperature for 18 h to synthesize 7-imidazoyl-Taxol (**II**). At the end of the reaction, monitored by TLC, the solution was diluted with excess dichloromethane and washed with 1 N HCl (2 × 1 mL) and brine (1 × 1 mL). The dichloromethane layer was dried on sodium sulfate and the solvent evaporated. The residue was dissolved in tetrahydrofuran (THF) and treated with 2 M triethylammonium hydrogen fluoride/THF (5.5 μL, 11 μmol, 1.1 equiv) for 6 h. The solvent was evaporated and residue purified by column chromatography on silica gel using ethyl acetate-dichloromethane (50:50) as the mobile phase. The yield of the reaction was 92%, and the structure was confirmed by NMR. Finally, 1.1 μmol of **II** dissolved in anhydrous dimethylformamide was added to a 600 μL solution of HRP (8.8 mg, 0.22 μmol) in 0.1 M pH 8.5 borate buffer and the resultant mixture reacted for 12 h at room temperature. The reaction mixture was then dialyzed overnight against phosphate buffer saline at 4 °C and the insoluble material removed by centrifugation (10000*g* for 10 min). A 3.5 mg sample of conjugate, obtained after dialysis, was used without any purification.

Determination of Taxol. Polystyrene microplates (Corning Costar, Cambridge, MA) were coated with phosphocellulose-purified tubulin by incubating 100 μL/well of 100 μg/mL tubulin in PEM buffer (0.1 M PIPES−NaOH, pH 6.9, containing 1 mM EGTA and 1 mM $MgSO_4$) overnight at 4 °C. Plates were then washed three times with PBS containing 0.01% Tween 20 (PBST) and blocked by incubating 200 μL/well of 1.0% BSA in PBST for 1 h at 25 °C. After blocking, wells were washed three times with PBST and used for assays.

(24) Lee, J. C.; Timasheff, S. N. *Biochemistry* **1975**. *14*, 5183−7.

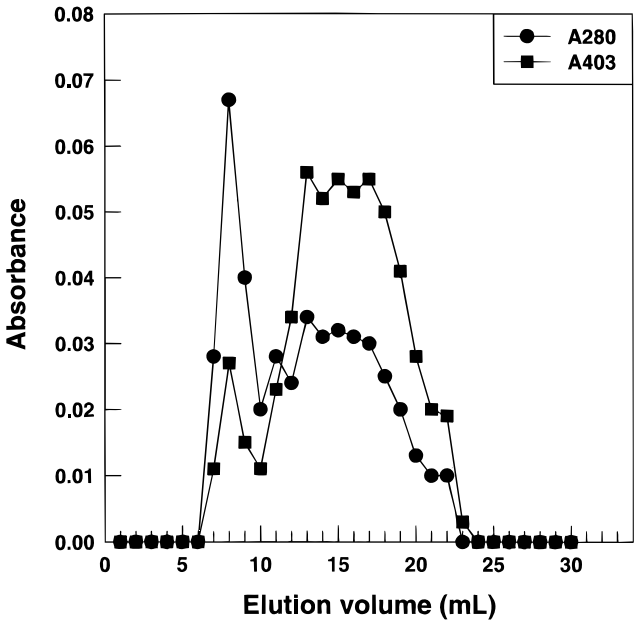

Figure 1. Elution profile of the reaction mixture of TX−HRP and tubulin on a G-150 column.

A standard calibration plot was prepared by adding a mixture of 40 μL of 1/50 dilution of 1 mg/mL TX−HRP conjugate in PBST and 10 μL sample into tubulin-coated wells. After incubation for 1 h at 37 °C, the plates were washed four times with PBST. Subsequently, 100 μL of substrate (10 mg of *o*-phenylenediamine in 10 mL of 0.1 M pH 4.6 citrate−phosphate buffer, containing 5 μL of 30% H_2O_2) was added to each well, and the plates were incubated for 1 h at 25 °C. The reaction was stopped by addition of 25 μL of 2.5 M HCl solution, and the absorbance of each well at 490 nm was read using a microplate reader (Model 3550-UV, Bio Rad, Richmond, CA). Several wells were used as reference wells where 100 μL of PBST solution replaces Taxol solution. These wells were used to calculate the 0% inhibition value. Taxol was dissolved in 100% dimethyl sulfoxide and stored at −20 °C. The final concentration of dimethyl sulfoxide was less than 1%.

To measure the Taxol level in human plasma, different amounts (0.1, 1, and 10 nM) of Taxol were added to 100 μL of human plasma. A mixture of 40 μL of a 1/50 dilution TX−HRP conjugate and 10 μL of a 1/5 dilution of plasma in PBST was added to the tubulin-coated plate followed by incubation at 37 °C for 1 h and washing. Bound conjugate were detected as described above.

RESULTS AND DISCUSSION

TX−HRP Conjugate. Initially, experiments were performed to investigate the microtubule assembly function and binding activity of TX−HRP conjugate. A tubulin polymerization assay was carried out by incubating excess of the TX−HRP conjugate (equimolar of 1.55 μmol/mL free Taxol) with tubulin preparation (1.1 mg/mL, 0.01 μmol/mL) in PEM buffer containing 1 mM GTP at 37 °C and following the increase of absorbance at 350 nm associated with the turbidity due to microtubule formation.[24] No increase in the absorbance at 350 nm was observed, indicating that tubulin polymerization was not caused by the TX−HRP conjugate. The reaction mixture was then applied on a Sephadex G-150 gel filtration chromatography column (350 mm long × 10 mm i.d.) and eluted with PEM buffer. The elution profile, shown in Figure 1, shows two major peaks. The first peak exhibited a

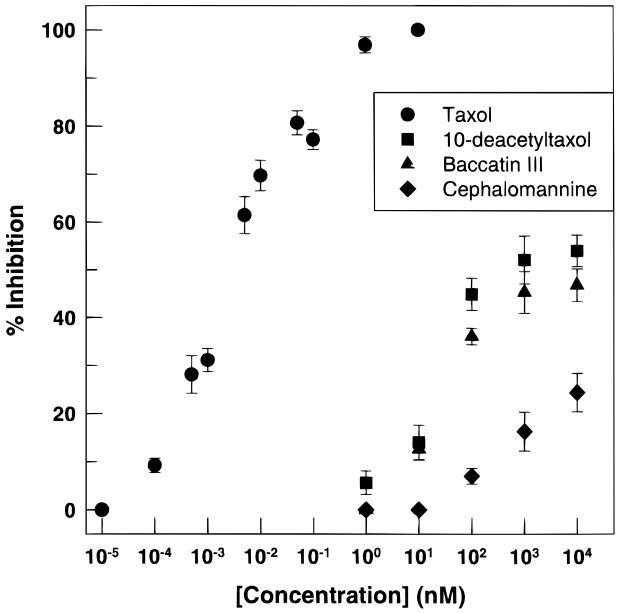

Figure 2. Calibration plot for Taxol and its related compounds (data points are mean ± SD from triplicates for each concentration).

ratio of absorbance at 403 and 280 nm in the range of 2.5, whereas for the second broad peak the ratio was in the range of 0.6−0.7. The first peak is for the tubulin associated/bound to the TX−HRP conjugate, while the second peak is for excess TX−HRP conjugate. The results suggested that TX−HRP conjugate does bind to tubulin, but incorporated TX−HRP conjugate no longer has the ability to promote microtubule assembly, probably due to steric hinderance by the HRP moiety on the conjugate.

Measurement of Taxol Levels. A typical calibration curve for measurement of Taxol and Taxol-related compounds by the present method is shown in Figure 2. The results show that the binding of TX−HRP to tubulin was specifically inhibited by Taxol and extremely low Taxol concentrations in the range of 0.0001−1 nM could be measured. The lower detection limit of the present method for Taxol was significantly superior than the best reported values of 12 nM by HPLC,[10] 0.35 nM by ELISA[19] and 0.1 mM using tubulin polymerization bioassays.[25,26] The figure also shows the high specificity/selectivity of the assay for Taxol. Taxol-related compounds, baccatin III, 10-deacetyl-Taxol, and cephalomannine, showed no inhibitory activities at concentrations of less than 1−10 nM. These compounds inhibited the binding of TX−HRP conjugate to tubulin at a significantly lesser degree than Taxol. This is in agreement with the report that a 10-fold excess of baccatin III was required to reduce the binding of a photoaffinity analog of Taxol to tubulin.[27] Similarly, other Taxol-related compounds, such as 7-epi-10-deacetyl-Taxol and 10-deacetylbaccatin III, at concentrations as high as 10 μM, did not inhibit the binding of TX−HRP conjugate to tubulin (data not shown). This selectivity is far superior to that observed in the ELISA method based on a monoclonal anti-Taxol antibody, where cephalomannine was found to interfere.[19]

Table 1. Measurement of Taxol Levels in Human Plasma Using the Tubulin−Taxol Binding Assay

actual Taxol concn (pM)	measd Taxol concn[a] (pM)
2	2.56 ± 0.19[b]
20	23.5 ± 2.5
200	231 ± 2

[a] Data are average from triplicate experiments for each sample. [b] Mean ± SD.

The extremely high sensitivity of the tubulin binding-based assay for Taxol agrees well with the theoretical predictions based on the affinity constant and surface coverage of microtiter plate walls by tubulin.[28] According to the theoretical prediction, for a system with nanomolar affinity constant, 10 nM for Taxol−tubulin,[29] antigen (Taxol) determination down to picomolar should be possible.

Measurement of Taxol Levels in Human Plasma. The application of the tubulin binding method for the determination of Taxol in human plasma was evaluated. The results obtained were in good agreement with the value of the concentration of Taxol added to the plasma (Table 1). These results were obtained after diluting the plasma 5-fold to alleviate the interference due to plasma proteins in the Taxol−tubulin binding when the assay was performed in undiluted human plasma. The lowest concentration of Taxol measured in human serum, 2 pM, using the tubulin binding-based assay developed in this work is far superior to the 50 and 12 nM measured using ELISA[20] and HPLC,[10] respectively. The simple dilution step before measurement is a great advantage compared to the extensive sample preparation steps required for Taxol measurement by HPLC.[10,11] The 1 order of magnitude decrease in the assay sensitivity for plasma compared to buffer may be attributable to plasma components binding with Taxol and limiting the amount of Taxol available for binding with tubulin. Although somewhat less sensitive for plasma, the tubulin-binding assay technique is still very effective considering that the therapeutic range of Taxol is 100−10 000 nM.[5]

In summary, the above results clearly demonstrate the advantage of using a tubulin binding-based assay for the very sensitive and selective determination of Taxol and the application of this methodology for determination of Taxol in human plasma.

ACKNOWLEDGMENT

This research was financially supported by the National Science Foundation Grant BES-9415395.

Received for review March 3, 1997. Accepted June 16, 1997.[⊗]

AC970233H

[⊗] Abstract published in *Advance ACS Abstracts,* August 1, 1997.

(25) Hamel, E.; Lin, C. M.; Johns, D. G. *Cancer Treat. Rep.* **1982** *66*, 1381−6.
(26) Lin, C. M.; Jiang, Y. Q.; Chaudhary, A. G.; Rimoldi, J. M.; Kingston, D. G. I.; Hamel, E. *Cancer Chemother. Pharmacol.* **1996**, *38*, 136−40.
(27) Rao, S.; Krauss, N. E.; Heerding, J. M.; Swindell, C. S.; Ringel, I.; Orr, G. A.; Horwitz, S. B. *J. Biol. Chem.* **1994**, *269*, 3132−4.
(28) Eddowes, M. J. *Biosensors* **1987/88**, *3*, 1−15.
(29) Caplow, M.; Shanks, J.; Ruhlen, R. *J. Biol. Chem.* **1994**, *269*, 23399−402.