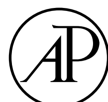
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A tubulin-based fluorescent polarization assay for paclitaxel

Sergi Morais,[a] Sean O'Malley,[b] Wilfred Chen,[a] and Ashok Mulchandani[a,*]

[a] *Department of Chemical and Environmental Engineering, University of California, Riverside, CA 92521-0444, USA*
[b] *Hawaii Biotech, Inc., Aiea, HI 96701, USA*

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Abstract

This paper reports the development, characterization, and application of a separation-free, homogeneous, and simple to perform fluorescence polarization bioassay for paclitaxel. The bioassay is based on the binding interaction of paclitaxel to tubulin, the receptor protein on which this drug acts. The bioassay was carried out in a competitive format where the paclitaxel and a synthetic fluorescent-labeled paclitaxel (Rh-Tx) competed for tubulin binding, causing a change in fluorescence polarization, which was an inverse function of the paclitaxel concentration. The bioassay had a dynamic range from 0.03 to 0.35 μM, which falls in the therapeutic range (0.01–10 μM), and a limit of detection of 23 nM. The bioassay was selective against other naturally occurring taxanes such as baccatin III and 10-deacetylbaccatin III. However, there was interference from cephalomannine. The excellent sensitivity, accuracy, reproducibility, and simplicity make this analytical technique suitable for routine and high-throughput analyses and might be helpful in providing better care for patients. The bioassay was successfully applied to the determination of paclitaxel in human plasma.
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Keywords: Paclitaxel; Tubulin; Bioassay; Fluorescence polarization; Plasma; Cancer

Paclitaxel, better known by its trade name Taxol, has demonstrated anticancer activity against several classically refractory tumors. The Food and Drug Administration approves the use of paclitaxel for the treatment of breast, ovarian, and non-small-cell lung cancers and AIDS-related Kaposi's sarcoma [1]. The mechanism of action of paclitaxel is based on the inhibition of human tumor cell replication by arresting the division of microtubules, which are minute cellular organelles vital to cell division that are the result of tubulin polymerization [2]. Despite its success, paclitaxel has problems. It has a narrow therapeutic range, a broad elimination half time in patients, and many side effects that have severe implications for patients undergoing treatment. From the research point of view, the fully synthetic and renewable sources for paclitaxel demand alternative methods to measure the antitumor activity of new drugs and their metabolites. To respond to both clinical and research demands, there is a need to determine paclitaxel in a rapid, sensitive, and selective way.

The analysis of paclitaxel and other taxanes is performed mainly by reversed-phase high-performance liquid chromatography (HPLC) [3,4], although other methods such as multimodal thin-layer chromatography [5], micellar electrokinetic chromatography [6], tandem mass spectrometry [7], and gas chromatography have been reported. Chromatography-based methods, however, are time consuming, tedious (requiring pretreatment and concentration), and relatively insensitive [8]. Therefore, there is a great need for sensing techniques that would enable more sensitive and rapid measurement. Immunological methods for determination of paclitaxel in human plasma and tissue of *Taxus* species have also been reported and commercialized [9–15]. Although very sensitive and in general selective, these methods are laborious, require excessive handling, and are time consuming because of the slow reaction rate due to limited mass transport and diffusional resistance across the solid and liquid phases involved in the process. Separation-free homogeneous methods offer

* Corresponding author. Fax: +1-909-787-5696.
E-mail address: adani@engr.ucr.edu (A. Mulchandani).

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several advantages over heterogeneous methods. Since no separation is involved, the number of procedural steps is drastically reduced, thus, decreasing the time and potential sample loss within the physical transfer step. Furthermore, the reaction rate is not limited by the mass transport. More importantly, none of the current methods measure the concentration of this drug at the receptor level.

The specific interaction of fluorescent paclitaxel analogs with dimeric tubulin has been reported [16]. Therefore, like many receptor-based assays, the binding of paclitaxel to tubulin can be exploited for the development of a specific and sensitive bioassay for determining paclitaxel at the receptor level. Several methods that provide a means for measuring the binding of a ligand to its receptor exist. Among these methods, fluorescence polarization (FP)[1] combines the advantages of the homogeneous assays with the use of nonhazardous radioactive material or expensive components such as the fluorescent probes. Furthermore, FP is a powerful and sensitive technology that provides valuable information on the interactions of small molecules with their receptors. It has been used for the study of protein–protein, DNA–protein, and taxane–tubulin molecular interactions [17–19] and for immunoanalysis of toxins [20], drugs [12], and herbicides [21]. Since FP is most readily applicable to the analysis of the binding interaction between a small-molecular-mass compound and a receptor molecule, one can consider applying this technique to the analysis of paclitaxel by investigating its interaction with tubulin. In practice, low-molecular-weight fluorophores are very flexible and rotate rapidly in solution, depolarizing the plane-polarized light. On the other hand, large fluorescently labeled molecules tumble more slowly; thus, the polarization of the light remains relatively constant between excitation and emission states. Therefore, low-molecular-mass compounds have low polarization values while high-molecular-weight compounds show greater polarization values. This intrinsic property of the fluorescent probe can be utilized in a binding assay for paclitaxel analysis using tubulin as the receptor.

The objective of this work was to develop a fluorescence polarization-based bioassay for analysis of paclitaxel using the binding interaction between the drug and the tubulin. The bioassay was performed in a competitive format involving a competition between fluorescent-labeled paclitaxel and native paclitaxel for tubulin binding. Essentially, paclitaxel and the fluorescent probe competed for tubulin binding, causing a change in fluorescence polarization, which was an inverse function of the paclitaxel concentration. The practical application of the proposed bioassay was assessed by measuring spiked human plasma. This bioassay is simple to set up, highly sensitive, reproducible, and well-suited to high-throughput analysis.

[1] *Abbreviations used:* FP, fluorescence polarization; DMSO, dimethyl sulfoxide; MES, 4-morpholine ethanesulfonic acid; BSA, bovine serum albumin; Rh-Tx, tetramethylrhodamine cadaverine-labeled paclitaxel; ELISA, enzyme-linked immunosorbent assay; EDTA, ethylenediaminetetraacetic acid; GTP, guanosine 5′-triphosphate.

Materials and methods

Reagents

Paclitaxel was purchased from ICN Pharmaceuticals (Costa Mesa, CA) and used as supplied. A 3.3 mM paclitaxel solution in dimethyl sulfoxide (DMSO) was used as the stock solution. Cephalomannine, baccatin III, and 10-deacetylbaccatin III were gifts from the National Cancer Institute; 1 mM taxane analog solutions in DMSO were used as stock solutions. Tetramethylrhodamine cadaverine was purchased from Molecular Probes, Inc. (Eugene, OR). 7-Succinylpaclitaxel was prepared according to literature procedures [9]. Cow brains were obtained from Hallmark Meat Packing (Chino, CA). Phosphocellulose P-11 was acquired from Whatman Co. (Kent, UK). MES, EDTA, GTP, bovine serum albumin (BSA) and human plasma were purchased from Sigma Chemical Co. (St. Louis, MO). All other chemicals were analytical grade.

Preparation of purified tubulin

Cow brain tubulin was purified by two cycles of temperature-dependent assembly–disassembly followed by phosphocellulose column chromatography [22]. The purified tubulin was 95% pure, as determined by gel-electrophoresis on a 12% SDS polyacrylamide gel followed by Coomassie blue staining. Because the drug-binding capabilities of the receptor were highly sensitive to aging, the purified tubulin was stored at −70 °C until use.

Synthesis of tetramethylrhodamine cadaverine-labeled paclitaxel (Rh-Tx)

To a 500-μl solution of 7-succinylpaclitaxel (2.5 mg, 2.6 μmol) in anhydrous methylene chloride, 2.2 mg of tetramethylrhodamine cadaverine (4.1 μmol) and 2.1 mg of *N*,*N*′-dicyclohexylcarbodiimide (10 μmol) were added. The 800 μl of distilled methlylene chloride was added and the reaction mixture stirred for 20 h at room temperature under nitrogen stream. The reaction product was concentrated by micro rotary evaporation, redissolved in methanol, and purified by HPLC using 80% 20 mM ammonium acetate in methanol to 100% methanol gradient over 30 min. A UV–Vis detector followed the HPLC purification and the exact molecular weight

of the product was confirmed by matrix-assisted laser desorption ionization time-of-flight analysis.

Fluorescence instrumentation

Fluorescence polarization measurements were performed using a Shimadzu RF-551 spectrofluorometer, fitted with a 150-W xenon lamp and a R452-01 photomultiplier tube. Polaroid polarizing filters (Polaroid Corp., Cambridge, MA) were placed in the path of the excitation source and between the sample cell and the photomultiplier tube. Readings were taken using the 90° geometry, with both excitation and emission slit widths set at 5 nm. Fluorescence polarization (*P*) was calculated by the equation, $P = (I_{VV} - GI_{VH})/(I_{VV} + GI_{VH})$, where the subscripts to the fluorescence intensity values (*I*) refer to orientations of the excitation and emission polarizers (V, vertical; H, Horizontal) and are reported in "minipolarization" units (mP). The correction factor *G* represents the ratio of the outputs from the photomultiplier tube (I_{HV}/I_{HH}) and is experimentally determined along with I_{VV} and I_{VH}. Polarized light is monitored by setting the excitation and emission wavelength at 545 and 580 nm, respectively.

Bioassay procedure

All experiments were performed in MESG buffer (100 mM MES buffer containing 1 mM GTP, pH 6.2). Ten microliters of 500 nM Rh-Tx was added to 180 μl of working buffer containing known concentrations of paclitaxel/taxane in a 500-μl microcentrifuge Eppendorf tube and mixed gently, and the polarization was measured (each vial serving as its own blank). Subsequently, 10 μl of freshly thawed tubulin (25 μM) was added, mixed gently, and incubated at room temperature in darkness for 10 min, and the polarization was measured again.

Analysis of paclitaxel in human plasma

Human plasma, prepared by reconstituting lyophilized powder with 5 ml of deionized water, was filtered through a 0.22-μm filter and diluted with an equal volume of working buffer prior to use to avoid matrix interference. The prepared plasma was spiked with known amounts of paclitaxel and analyzed by the above-described procedure.

Results and discussion

Optimization of assay conditions

The intrinsic limitations of using two filters for FP attenuated the real fluorescence signal of the probe. The optimal concentration of Rh-Tx for FP studies was 25 nM. This was the lowest concentration of the probe that yielded a practical fluorescent intensity and generated a stable polarization value. At this concentration, an average value of 70 mP was achieved.

A pure receptor is desirable to reduce background caused by nonspecific binding of proteins when devising an FP-based binding assay. The nonspecific binding was tested by the addition of BSA, keeping the probe concentration constant at 25 nM. As is shown in Fig. 1, polarization values barely increased to 75 mP, indicating a maximum of 5 mP background signal even for high protein concentration (5 μM BSA). On the other hand, as is depicted in Fig. 1, the addition of tubulin to the probe solution increased the polarization value from 70 mP to as high as 175 mP, reaching a plateau when the total tubulin concentration was above 1 μM; 1.25 μM tubulin, which was 50-fold more than the probe concentration, was used for the following experiments. These results confirmed the specific binding that occurred between the probe Rh-Tx and the tubulin. Under the optimal conditions (25 nM probe and 1.25 μM tubulin) a working signal window of 105 mP (70–175 mP) was reached.

The binding kinetics of Rh-Tx to tubulin and the stability of the binding complex were investigated at room temperature also. The rate of binding between tubulin and paclitaxel is a critical factor in determining the time necessary for each assay. As shown in Fig. 2, the kinetics of the interaction between the receptor and the target is fast and the equilibrium is attained within 10 min, which was selected as the incubation time for subsequent analyses. The stability of the signal over a 1-h period at room temperature was especially promising for high-throughput analysis.

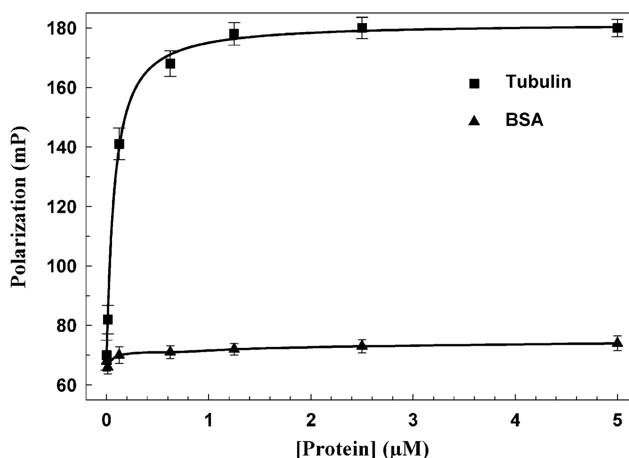

Fig. 1. Effect of tubulin concentration on the fluorescence polarization response. Operating conditions: 25 nM of Rh-Tx in 190 μl of MESG buffer mixed with 10 μl of appropriate concentration tubulin and incubated at room temperature in dark for 10 min. Data points are average of three determinations ± 1 SD.

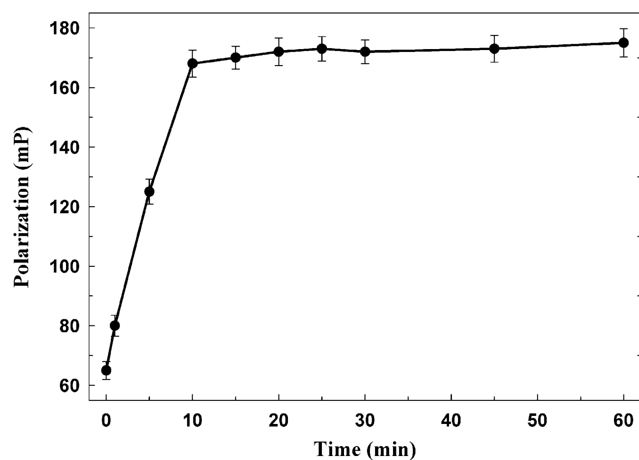

Fig. 2. Kinetics of Rh-Tx binding to tubulin. Operating conditions: 25 nM of Rh-Tx in 190 µl of MESG buffer mixed with 10 µl of 25 µM tubulin and incubated at room temperature in dark. Data points are average of three determinations ± 1 SD.

Analytical performance of the assay

The ultimate purpose in establishing a homogeneous tubulin receptor-based FP assay was to have the ability to quickly analyze paclitaxel levels for both clinical and research purposes. Thus, the bioassay was characterized with regard to sensitivity, lower and upper detection limits, selectivity, and precision. In this sense, Fig. 3 shows a plot of the normalized fluorescence polarization (F/F_0) vs paclitaxel concentration. As the results show, the assay has a very broad dynamic concentration, ranging from 30 nM (corresponding to 20% inhibition) to 350 nM (corresponding to 80% inhibition). The sensitivity, defined as 50% inhibition, is 82 nM while the lower detection limit, corresponding to 10% inhibition,

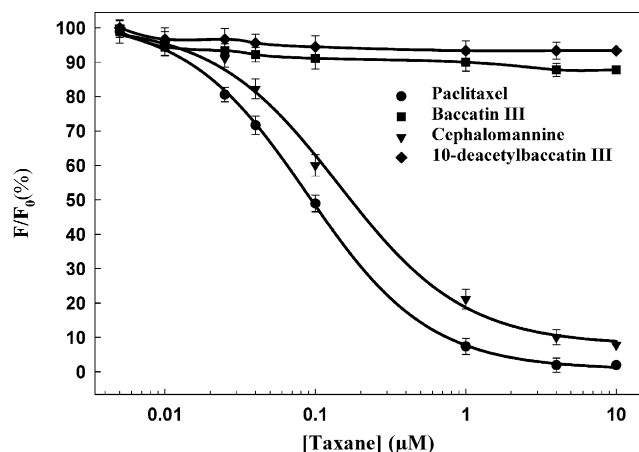

Fig. 3. Calibration plots for paclitaxel and its related compounds. Operating conditions: 25 nM Rh-Tx plus appropriate concentration taxane in 190 µl of MESG buffer mixed with 10 µl of 25 µM tubulin and incubated at room temperature in dark for 10 min. Data points are average of three determinations ± 1 SD.

is 23 nM. The limit of detection is comparable to the 12–20 nM reported for HPLC techniques [4,5] and better than the 50 nM reported for radiolabeled immunoassay [23] and the 100 nM for assays based on monitoring of paclitaxel-induced tubulin polymerization [24,25]. The limit of detection, is however, one to two orders of magnitude higher than that for antipalitaxel-based immunoassays (enzyme and fluorescence-labeled) and an earlier tubulin-based microtiter plate assay reported from this laboratory [26]. This tubulin-based FP bioassay, however, has the advantages of (1) short assay time (10 min compared to hours), (2) no handling once the assay is started as everything is performed in a single step compared to excessive handling required in ELISA, (3) low cost, and (4) environmental friendliness. Unlike ELISA the assay does not require expensive and environmentally unfriendly plastic microtiter plates. Although less sensitive, the lower detection limit of the developed assay is significantly lower than the useful therapeutic range of 100 to 10,000 nM [27]. Therefore making this analytical method an ideal technique to monitor levels in patients undergoing paclitaxel therapy.

To further verify the FP bioassay performance, several known naturally occurring taxanes with different biological activity were used to assess their competition profile with Rh-Tx. Generally, the tubulin-receptor-based FP assay had good specificity against baccatin III and 10-deacetylbaccatin III. However, cephalomannine did interfere with tubulin for binding Rh-Tx (IC_{50}, 0.24 nM; LDD, 0.029 nM) (Fig. 3). This interference is not relevant to the analysis of serum/plasma samples since a pharmaceutical preparation will contain paclitaxel alone. However, according to what is reported about paclitaxel structure–activity relationships, it seems likely that paclitaxel-related metabolites could show significant interference in this assay as does cephalomannine. Nevertheless, we believe that from a clinical and research point of view such cross-reactivity is beneficial since most compounds with the highest interference will be those showing antitumor activity. As the bioassay is based on the specific tubulin interaction, it could be used to screen and monitor at the receptor level the next generation of antimitotic drugs that are paclitaxel analogs and those paclitaxel metabolites with unknown antitumor activity.

The experimental specificity results obtained with this bioassay are in good agreement with the cross-reactivity data for tubulin-dependent and antibody-based assays and with the well-documented biological activity data (cephalomannine is 3-fold less and baccatin is 1000-fold less active than paclitaxel) [28].

Analysis of paclitaxel in human plasma

The practical application of the tubulin-based fluorescent polarization bioassay for the determination of

Table 1
Measurement of paclitaxel levels in human plasma

Paclitaxel added (nM)	Paclitaxel measured (nM)	
	Mean ± SD	Recovery (%)
0	<LDD	—
25	23.5 ± 0.7	94
40	36.2 ± 1.6	90
100	84.6 ± 6.8	85
400	352 ± 44.5	88
Mean		89

paclitaxel was assessed by spiking human plasma samples. As shown in Table 1, the results obtained (concentrations of paclitaxel in the analyzed samples were determined using the calibration curve in Fig. 3 fitted with a four-parameter logistic equation using the SigmaPlot (Jandel Scientific, Erkrath, Germany) software package were in good agreement (between 85 and 94%) with the amount spiked, demonstrating the validity of the newly developed assay to a practical problem. Additionally, the bioassay had good precision as demonstrated by the low coefficient of variation (3–12%) between replicate measurements of paclitaxel-spiked human plasma samples. The simple pretreatment of filtration followed by dilution implemented in this work to alleviate the matrix interference (light scattering) is a significant benefit over the extensive sample preparation steps required for paclitaxel determination by HPLC.

Conclusions

In conclusion, a simple homogeneous-phase, rapid, low cost, selective, and sensitive tubulin-based fluorescent polarization bioassay has been developed and successfully applied to measure paclitaxel in human plasma. Since the bioassay utilizes tubulin, the receptor protein that is the target of the drug paclitaxel, as the biological recognition element, it is expected that the analytical information obtained by employing this technique will help in controlling administration of the drug in the proper therapeutic range and thereby improve patient care. The simplicity coupled with very good detection limit and selectivity of the reported homogeneous assay using a spectrofluorometer equipped with polarizers or a multiwell fluorescence polarization plate reader, fast becoming standard instrumentation in clinical and research laboratories, will make it possible to easily adapt the proposed analytical technique in everyday oncology practice. Furthermore, we believe that the described bioassay can also be used as a tool to monitor the next generation of antimitotic drugs that are paclitaxel analogs and show antitumor activity.

Acknowledgments

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