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Article

Quantification of Engagement of Microtubules by Small Molecules in Living Cells by Flow Cytometry

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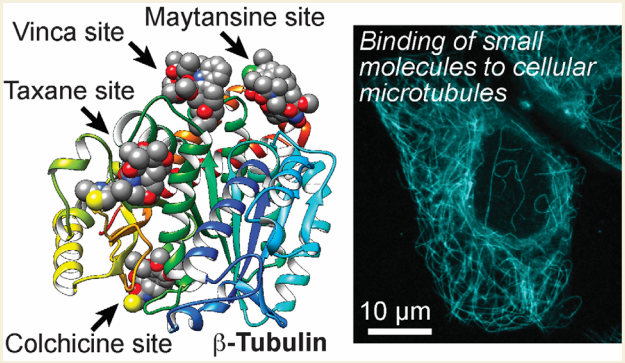
ABSTRACT: Drugs such as paclitaxel (Taxol) that bind microtubules are widely used for the treatment of cancer. Measurements of the affinity and selectivity of these compounds for their targets are largely based on studies of purified proteins, and only a few quantitative methods for the analysis of interactions of small molecules with microtubules in living cells have been reported. We describe here a novel method for rapidly quantifying the affinities of compounds that bind polymerized tubulin in living HeLa cells. This method uses the fluorescent molecular probe Pacific Blue-GABA-Taxol in conjunction with verapamil to block cellular efflux. Under physiologically relevant conditions of 37 °C, this combination allowed quantification of equilibrium saturation binding of this probe to cellular microtubules (K_d = 1.7 μM) using flow cytometry. Competitive binding of the microtubule stabilizers paclitaxel (cellular K_i = 22 nM), docetaxel (cellular K_i = 16 nM), cabazitaxel (cellular K_i = 6 nM), and ixabepilone (cellular K_i = 10 nM) revealed intracellular affinities for microtubules that closely matched previously reported biochemical affinities. By including a cooperativity factor (α) for curve fitting of allosteric modulators, this probe also allowed quantification of binding (K_b) of the microtubule destabilizers colchicine (K_b = 80 nM, α = 0.08), vinblastine (K_b = 7 nM, α = 0.18), and maytansine (K_b = 3 nM, α = 0.21). Screening of this assay against 1008 NCI diversity compounds identified NSC 93427 as a novel microtubule destabilizer (K_b = 485 nM, α = 0.02), illustrating the potential of this approach for drug discovery.



KEYWORDS: *drug discovery, cancer, fluorescence, taxoids, tubulin, cellular uptake, cellular efflux, equilibrium binding*

INTRODUCTION

Small molecules that bind microtubules can be effective anticancer therapeutics.[1] This class of compounds includes FDA-approved microtubule stabilizers such as taxanes and epothilones and microtubule destabilizers such as colchicine, vinblastine, and maytansinoids, which are delivered as antibody–drug conjugates (Figure 1).[2] The mechanism of action of taxane drugs involves the engagement of a 3.5 Å hydrophobic cleft of the protein β-tubulin when it heterodimerizes with α-tubulin to form tubular protein assemblies.[3,4] Binding of taxanes to polymerized microtubules is favored, and this binding induces conformational changes to microtubules that lower the critical concentration required for their assembly of these structures.[5−7] In contrast, destabilizers such as vinblastine weaken microtubule lattices, whereas colchicine inhibits the growth of microtubules by preventing conformational changes of dimers of α- and β-tubulin required for their polymerization.[2] Although microtubule-targeting drugs are effective first- and second-line therapies for numerous cancers, novel agents that bind microtubules are of substantial interest due to the emergence of drug resistance, the lack of efficacy for some cancers, and the complexity associated with the syntheses of some of these compounds.[8−10] Furthermore, dose-limiting side effects such as peripheral neuropathy associated with taxanes, epothilones, and their delivery vehicles continues to drive the discovery of novel agents with greater bioavailability and improved therapeutic windows.[11−13] Resistance to these drugs can be mediated by several mechanisms, including the overexpression of drug efflux transporters such as *p*-glycoprotein (MDR1), mutations in β-tubulin, and the expression of antiapoptotic proteins such as survivin.[13−15]

Small molecules that bind tubulin have been traditionally identified by their effects on the polymerization of purified microtubules[16] or by the displacement of radioactive[17] or fluorescent[18,19] derivatives. Subsequent cytotoxicity studies are used to confirm biological activity in cells,[20−23] which is generally highly correlated[16,22] with biochemical affinities for

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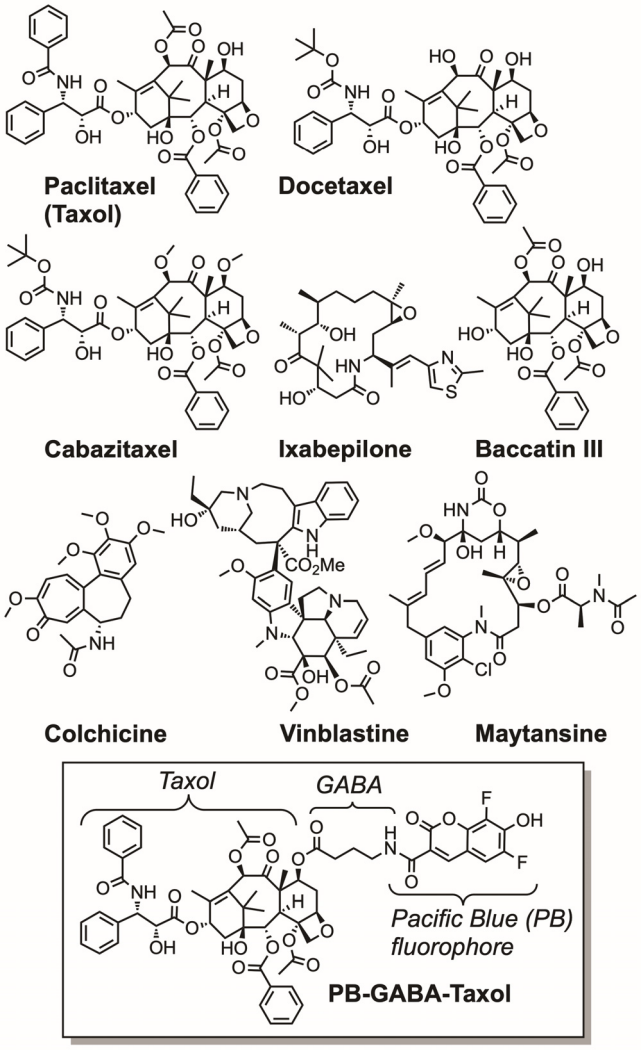

Figure 1. Structures of microtubule stabilizers (paclitaxel, docetaxel, cabazitaxel, ixabepilone, and baccatin III) and destabilizers (colchicine, vinblastine, and maytansine) and the fluorescent molecular probe PB-GABA-Taxol.

microtubules. Given the structural complexity of many microtubule-targeting compounds, a wide variety of simpler analogues have been designed and screened using these assays. However, simpler analogues that engage the taxane site of tubulin in vitro and exhibit potent on-target cytotoxicity in cancer cells have been difficult to identify.[16] Taxanes are actively taken up by cells via organic anion transporter polypeptides (OATP)[24,25] and can be actively effluxed by ATP-binding cassette transporters such as MDR1, transporters of the MRP family (ABCC), and BCRP (ABCG2).[26,27] Limited cellular uptake, enhanced active efflux, and the involvement of other cellular factors likely contribute to challenges associated with the discovery of synthetic mimics of taxanes.

The very low success rates[28] of anticancer drug candidates in clinical trials suggest that improved methods of evaluating the selectivity of interactions in living systems are needed. Quantitative studies of interactions between destabilizers such as colchicine and microtubules can be challenging,[29] and measurements of the affinities of these compounds for microtubules have been primarily limited to binding assays with costly purified proteins,[19] ultracentrifugation methods,[30] or measuring the cellular microtubule content via antibody labeling of fixed cells.[31,32] Existing methods that use derivatives of paclitaxel can be effective but are low throughput, involving radioactive [^{3}H]-Taxol,[33] the competitive displacement of fluorescent paclitaxel probes[34,35] such as Flutax-2[18,36] and SirTub by microscopy,[37,38] or transfection with genes that encode tubulin fusion proteins.[39] The commercially available Flutax-2, comprising paclitaxel linked at the 7-position to the fluorophore Oregon Green via a β-Ala (or L-Ala) linker, exhibits high affinity for tubulin, and Flutax-2 (L-Ala) binds cross-linked microtubules with biochemical K_d = 14 nM as measured by fluorescence anisotropy.[36] Competition experiments with this probe have been used to measure biochemical binding affinities of paclitaxel (K_d = 27 nM) and docetaxel (K_d = 17 nM) for glutaraldehyde-cross-linked microtubules.[40] Other fluorescent taxoids that link fluorophores to the primary amine of the side chain of docetaxel, such as BODIPY 564/570 Taxol (Botax, biochemical K_d = 2.2 μM)[41] and silicon rhodamine (SiR)-tubulin (SirTub),[37,38] exhibit lower affinities for microtubules, but SirTub has been used for both super-resolution imaging of these structures[37] and to measure cellular K_i values of small molecules for the taxane binding site in living cells by confocal microscopy.[38]

To provide an alternative higher-throughput flow-cytometry approach in living cells, we describe here a novel method that allows the quantification of apparent cellular affinities of small molecules that bind microtubules. This approach uses the fluorescent probe Pacific Blue-GABA-Taxol (PB-GABA-Taxol,[42,43] Figure 1). This molecular probe exhibits sufficiently high cellular permeability and affinity for microtubules to allow saturation binding assays under equilibrium conditions. This enabled the measurement of its cellular K_d for microtubules by flow cytometry in living cells on 96-well plates. In conjunction with adaptations of the Cheng–Prusoff equation[44] and the allosteric equation[45] implemented in GraphPad Prism, the cellular K_d of PB-GABA-Taxol can be used to measure cellular competitive K_i and allosteric K_b values of unlabeled compounds that engage microtubules at the orthosteric site or distinct sites that are cooperatively coupled. We further used this approach to screen a library of 1008 NCI diversity compounds and identified a novel microtubule destabilizer, illustrating the potential of this approach for drug discovery.

RESULTS AND DISCUSSION

PB-GABA-Taxol was investigated because of its relatively high affinity (biochemical K_d = 265 nM) for microtubules, its high cellular permeability, its low cytotoxicity, and the unique cellular and photophysical properties of its linked Pacific Blue (PB) fluorophore.[42] This PB derivative of paclitaxel is monoanionic under physiological conditions (pH 7.4), making it substantially more hydrophobic compared with dianionic Flutax-2 and related compounds.[46] PB is also fairly bright when bound to proteins in living cells and can be efficiently excited at 405 nm with the violet lasers commonly found on confocal microscopes and flow cytometers. Additionally, in the presence of verapamil, which inhibits MDR1- and MRP-family transporters,[47] PB-GABA-Taxol binds with a high specificity to microtubules of living HeLa cells, as imaged by super-resolution confocal laser scanning microscopy, and can be readily detected in cells by flow cytometry (Figure 2). In cells treated with PB-GABA-Taxol, the addition of excess paclitaxel as a specific competitor substantially reduced cellular

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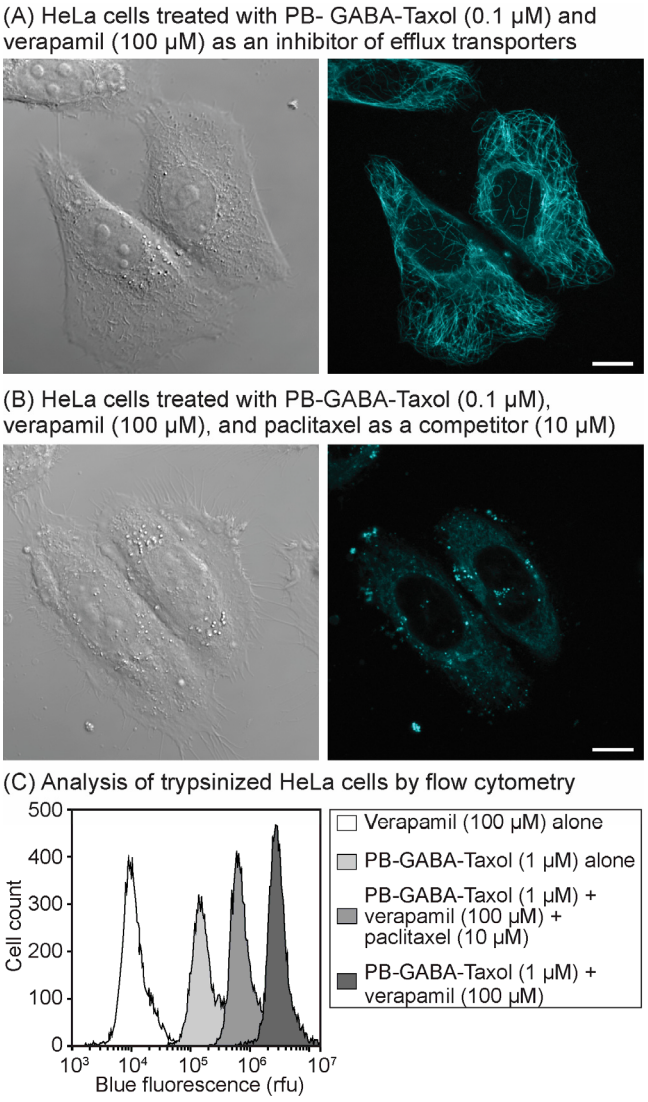

Figure 2. Analysis of living HeLa cells treated with PB-GABA-Taxol and other small molecules (3 h, 37 °C). (A and B) Differential interference contrast micrographs (DIC, left) and Leica lightning super-resolution confocal laser scanning micrographs (right, 140 nm resolution, Ex. 405 nm, Em. 425−500 nm). Scale bars represent 10 μm. (C) Flow cytometry for quantification of interactions with microtubules. Enhanced cellular uptake of the fluorescent probe was observed in the presence of verapamil, and the addition of excess paclitaxel as a specific competitor blocked the uptake of the probe.

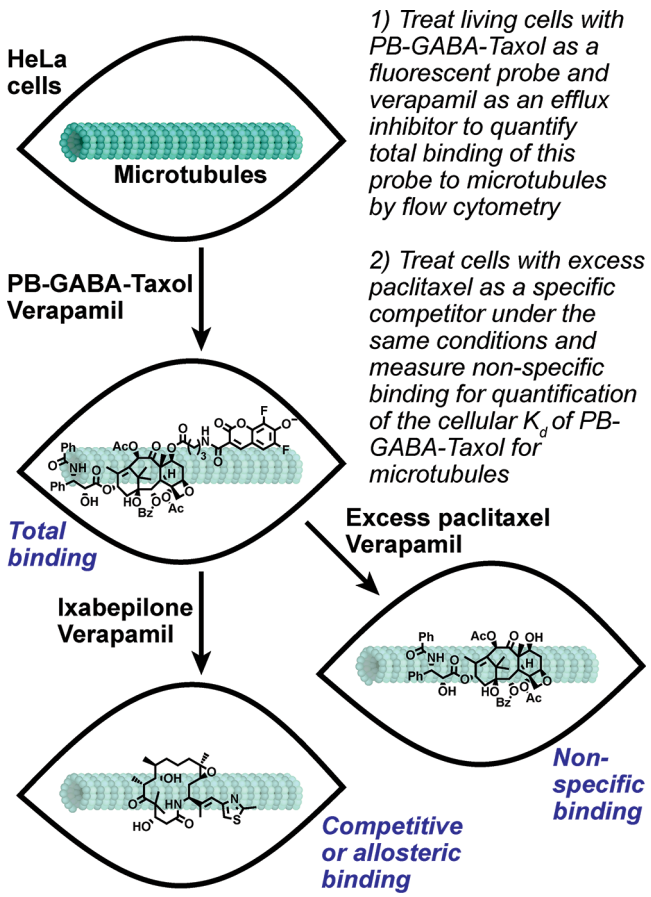

Figure 3. PB-Taxoid method for the quantification of cellular affinities of small molecules that bind microtubules by flow cytometry. Trypsinized HeLa cells were treated in a suspension at 37 °C for equilibrium binding measurements.

fluorescence without appreciable short-term (≤3 h) effects on cellular viability, illustrating the low nonspecific binding of this probe (Figures 2 and S1).

Quantification of the Binding of PB-GABA-Taxol to Microtubules in Living HeLa cells

To quantify the affinity of PB-GABA-Taxol for microtubules in living cells (cellular K_d), we developed the saturation binding method shown in Figure 3. In this assay, the cellular K_d was measured by varying the concentration of the fluorescent probe added to cells at equilibrium, as established by kinetic assays of probe uptake. The total binding of this probe to specific and nonspecific sites in cells in the presence of the efflux inhibitor verapamil was determined by flow cytometry. Nonspecific binding was quantified separately by adding excess paclitaxel with the probe under the same conditions. The linear nonspecific binding contribution was subtracted from the total binding curve to analyze specific binding of the probe to tubulin of microtubules. Measuring this cellular dissociation constant under equilibrium conditions further allowed the half-maximal inhibitory concentration (IC_{50}) of unlabeled compounds to be converted to cellular inhibitory constants using an adaptation of the Cheng−Prusoff equation[44] for competitive modulators (K_i) or the allosteric equation[45] for noncompetitive modulators (K_b and the cooperativity factor α), as implemented by GraphPad Prism.

In general, accurate measurements of the affinities of small molecules for proteins require that systems be at equilibrium.[48−50] Most biochemical microtubule binding assays are conducted at room temperature and reach equilibrium in less than 1 h.[18,51] However, in cells, the presence of the plasma membrane and transporters such as *p*-glycoprotein can play a major role in reducing microtubule binding by limiting the intracellular concentrations of compounds such as paclitaxel.[52] To determine the time required for PB-GABA-Taxol to reach equilibrium in cells, we treated HeLa cells in suspension with this probe at 37 °C and fit fluorescence data obtained by flow cytometry to an exponential growth model to measure half-times under different experimental conditions (Figure 4, panels

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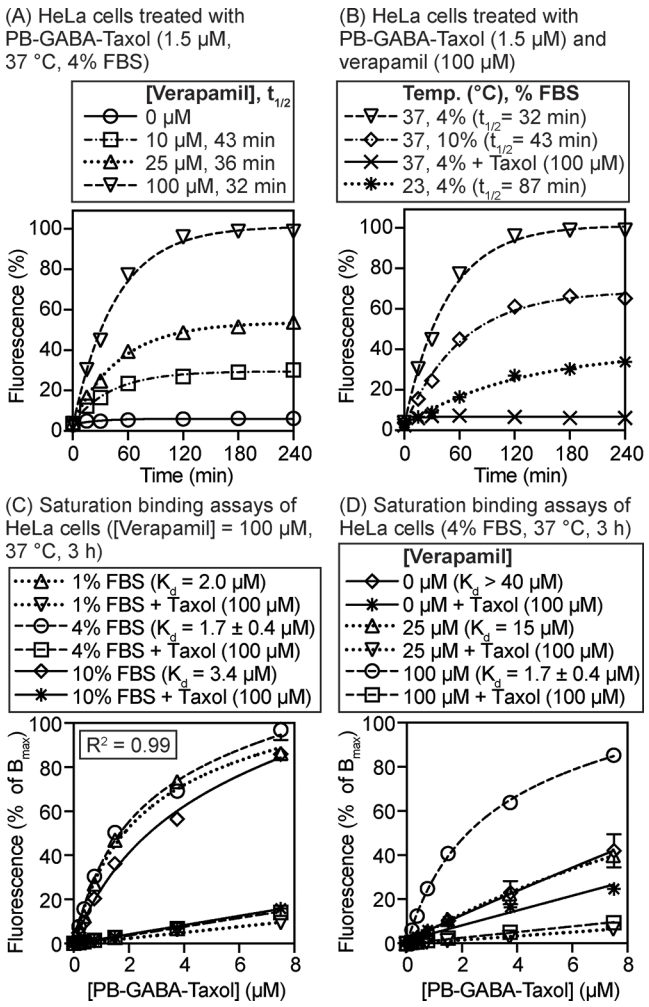

Figure 4. Quantification of PB-GABA-Taxol in living HeLa cells by flow cytometry. (A and B) Time required for PB-GABA-Taxol (1.5 μM) to reach equilibrium with varying [verapamil], % FBS in media, temperature, and competition by Taxol (100 μM). (C and D) Saturation binding assays of PB-GABA-Taxol to intracellular Taxol-binding sites with varying % FBS and [verapamil]. The K_d of PB-GABA-Taxol under optimized conditions (1.7 ± 0.4 μM, 4% FBS, 37 °C, [verapamil] = 100 μM) is shown as the mean ± SD (N = 8, independent replicates).

A and B). The addition of the efflux inhibitor verapamil at 100 μM was found to enhance cellular fluorescence 32-fold compared to that in the absence of verapamil, where essentially only nonspecific binding was observed. Lower concentrations of verapamil enhanced fluorescence 17-fold at 25 μM and 9-fold at 10 μM. The most rapid equilibration was observed at 37 °C with 100 μM verapamil ($t_{1/2}$ = 32 min), whereas incubation at room temperature (23 °C) substantially slowed the time to equilibration ($t_{1/2}$ = 87 min).

For saturation binding assays, HeLa cells were incubated with PB-GABA-Taxol at 37 °C for 180 min in the presence of verapamil (100 μM). These conditions achieved more than 96.6% equilibration (five half-times).[48] Analysis of the cellular cytotoxicity by flow cytometry with propidium iodide (3 μM) revealed that cells remained >90% viable under these conditions (Figure S1). As shown in Figure 4 (panels C and D), this was used to examine the influence of % fetal bovine serum (FBS) in media and the impact of verapamil on cellular K_d values of PB-GABA-Taxol. The total binding of PB-GABA-Taxol was measured by treating HeLa cells with 0−7.5 μM of this probe, and nonspecific binding was measured by an additional cotreatment with excess Taxol (100 μM) as a competitor.[51] Cellular K_d values were measured by nonlinear regression with a one-site total and nonspecific binding model (GraphPad Prism 9).

Paclitaxel is known to bind albumin (K_d = 120 nM),[53] which can comprise up to 60% of proteins in fetal bovine serum (FBS).[54] We hypothesized that albumin in FBS might lead to ligand depletion[48−50] by reducing the concentration of free PB-GABA-Taxol available to bind to microtubules.[55] As shown in Figure 4 (panel C), analyzing the effects of different concentrations of serum revealed that the cellular K_d of PB-GABA-Taxol in 4% FBS was 1.7 ± 0.4 μM (mean ± SD, N = 8 independent replicates in triplicate). A lower concentration of 1% FBS gave a similar K_d of 2.0 μM, but greater cellular aggregation was observed under these conditions. In contrast, in the presence of 10% FBS, ligand depletion caused this apparent affinity to be reduced by approximately twofold. These reduced serum conditions additionally decreased nonspecific binding of PB-GABA-Taxol to cells (6% nonspecific binding at 1.5 μM in 4% FBS versus 8% nonspecific binding at 1.5 μM in 10% FBS, Figure S2 and Supporting Information). To maximize cellular viability and minimize cellular aggregation observed in the absence of serum, 4% serum in media was used for further cellular binding assays. As expected, co-treatment with excess paclitaxel abolished specific binding without affecting the cellular viability by more than 10% after 3 h (Figure S1). Substantial reductions in the apparent cellular affinity of PB-GABA-Taxol were seen with verapamil concentrations below 100 μM (Figure 4, panel D) because this probe was such an efficient substrate of efflux transporters.[42,43] Optimal conditions were found to be incubation for 3 h at 37 °C in media containing 4% serum and 100 μM verapamil. Although the cellular K_d of PB-GABA-Taxol (1.7 μM) is sixfold higher than its biochemical K_d (265 nM) for chemically cross-linked microtubules in solution at room temperature,[42] this apparent cellular affinity includes contributions from the complex and dynamic environment of living cells.

Quantitation of the Number of Specific Binding Sites for PB-GABA-Taxol in HeLa Cells

In HeLa cells, tubulin is highly abundant, representing ca. 4% of total cellular protein,[56,57] with an estimated concentration of 20 μM.[38] By promoting the polymerization of tubulin, paclitaxel associates specifically with microtubules, and less than 5% of this hydrophobic drug is observed in cellular membranes.[53] Because these high concentrations of Taxol-binding sites have the potential to lead to ligand depletion at low probe concentrations, we used PB-GABA-Taxol to quantify the number of specific binding sites per cell by flow cytometry. This was achieved using a standard curve constructed with calibration particles bearing a standardized number of blue coumarin 30 fluorophores per bead (Figure S3A). We confirmed that the emission of PB-GABA-Taxol is similar to that of the coumarin 30 dye immobilized on these beads, which is blue-shifted compared to coumarin 30 dye alone (Figure S3B). These studies revealed 55 × 10^6 PB-GABA-Taxol molecules/cell at saturation. Assuming that one molecule of PB-GABA-Taxol binds each tubulin heterodimer, similar to paclitaxel,[58] we converted these binding sites to mole units and divided by the volume of a HeLa cell (4.5 pL, as

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measured by confocal microscopy).[59] Using this method, the average concentration of saturable binding sites occupied by PB-GABA-Taxol in a HeLa cell was determined to be 22 ± 4 μM.

Optimization of Cellular Assays for the Quantitative Profiling of Microtubule Modulators by Flow Cytometry

Based on its cellular K_d of 1.7 ± 0.4 μM, PB-GABA-Taxol added to HeLa cells at a concentration of 1.5 μM will occupy approximately 50% of its binding sites in HeLa cells at equilibrium. To optimize conditions for competition binding assays, we explored the use of concentrations of PB-GABA-Taxol below its K_d, which is typically used for equilibrium competition binding assays.[48] However, the stabilization of microtubules by low concentrations of paclitaxel derivatives can complicate binding studies for some probes, and previously reported assays with Sir-Tub by confocal microscopy[38] required the incorporation of an exponential relaxation equation that simulated the change in microtubule mass across different concentrations of the microtubule-bound probe to derive apparent equilibrium binding constants.[33,37,38] We found that the ability of PB-GABA-Taxol to achieve equilibrium within 3 h allowed studies at concentrations where microtubule mass did not change appreciably.[60−62] When PB-GABA-Taxol was used at a concentration of 1.5 μM, near its measured cellular K_d value, changes in microtubule concentrations during competitive equilibrium binding assays were minimal, and no upward trends in fluorescence were observed when paclitaxel was added as a competitor.[38] These conditions also provided an outstanding assay signal window (SW)[63] of 79, offering the greatest sensitivity for detection of differences in affinities of competitors. In contrast, increasing the probe concentration above the cellular K_d to 4.5 μM resulted in a 12-fold underestimation of the cellular K_i of paclitaxel (SW = 20), whereas lower probe concentrations of 450 nM (SW = 25) or 150 nM (SW = 3) substantially reduced the SW (Figure S4, Supporting Information).

Ligand depletion can affect high-throughput screening when assays are miniaturized on multiwell plates. When high concentrations of the receptor are needed to increase sensitivity or low concentrations of the probe are needed to conserve resources, the concentration of the free ligand can be reduced via ligand depletion.[49] Under these conditions, the free ligand will not be equivalent to the concentration added to the well, causing errors in the determination of K_d or K_i values. To determine whether ligand depletion might affect competitive binding assays with PB-GABA-Taxol, we calculated the concentration of binding sites for PB-GABA-Taxol in each well of a 96-well plate to be 30 nM for the assays shown in Figure 4 (60 000 cells/200 μL/well). This was accomplished using the concentration of binding sites for PB-GABA-Taxol per cell measured by flow cytometry. As shown in Figure 5A, to evaluate ligand depletion (σ), we treated cells with PB-GABA-Taxol (1.5 μM) and measured the competitive cellular IC_{50} and K_i values of paclitaxel using different numbers of cells per well. This varied the estimated concentration of β-tubulin from 30 to 300 nM per well. At 30 nM total binding sites, ~3% ligand depletion was observed using 1.5 μM of the PB-GABA-Taxol probe, a value well within the 10% limit considered acceptable[49] for accurate competitive binding assays.

A key criterion for achieving equilibrium is the stability of measured inhibition constants over time. To further confirm that equilibrium was achieved after 3 h, we measured cellular

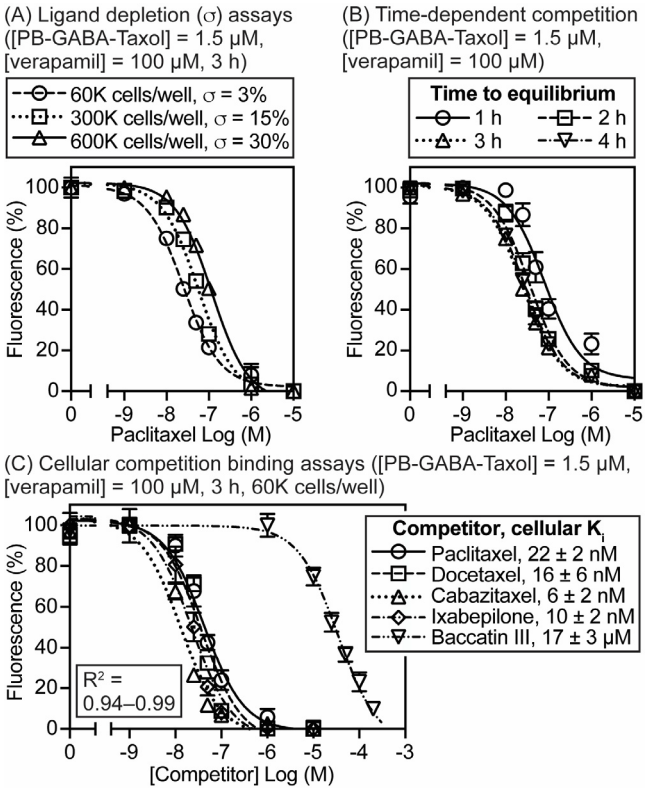

Figure 5. Competitive binding assays in living HeLa cells treated with PB-GABA-Taxol (1.5 μM) and verapamil (100 μM, 37 °C, 4% FBS). (A) Competition by paclitaxel with a variable number of cells per well (60K−600K) to calculate ligand depletion (σ). [β-tubulin/well] = 30 nM (σ = 3% at 60K cells/well), 150 nM (σ = 15%), and 300 nM (σ = 30%). The apparent affinity (cellular K_i) of paclitaxel decreased 2.3-fold at σ = 15% and 4.7-fold at σ = 30%. 60K cells/well (200 μL) was used in other experiments to minimize ligand depletion. (B) Effect of the incubation time on cellular K_i values. Compared with an incubation time of 3 or 4 h, an incubation time of 1 h decreased the apparent affinity threefold (twofold decrease at 2 h). (C) Competitive binding of microtubule-stabilizing drugs and the low-affinity analogue baccatin III. Cellular K_i values (N = 3, independent replicates) were calculated with a competitive binding site Fit K_i model (GraphPad Prism) using cellular K_d (PB-GABA-Taxol) = 1.7 μM.

K_i values of paclitaxel at different time points (Figure 5). Measurements of these values taken after only 1 h led to a threefold decrease in the apparent affinity for microtubules compared to measurements taken at 3 or 4 h, where these values stabilized.

Quantitative Profiling of Microtubule Stabilizers That Engage the Taxane Binding Site

This approach was used to measure cellular K_i values of four approved microtubule-stabilizing drugs and a low affinity precursor to paclitaxel. As shown in Figure 5C, cabazitaxel exhibited the highest affinity for microtubules with cellular K_i = 6 ± 2 nM, essentially identical to its previously determined[17] biochemical K_i = 7.4 ± 0.9 nM. The more recently developed microtubule-stabilizing drug ixabepilone exhibited a cellular K_i of 10 ± 2 nM, similar to a measurement previously reported by confocal microscopy (K_i = 7.6 ± 1.6 nM).[38] The cellular K_i of docetaxel was 16 ± 6 nM, similar to many previous reports of its biochemical affinity (biochemical K_d = 6.8 ± 0.2 nM,[17] K_i = 17 ± 6 nM,[40] and K_d = 25 ± 0.4 nM[64]). Additionally, the value

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measured for paclitaxel (cellular K_i = 22 ± 2 nM) was similar to several previously reported biochemical K_i values for cross-linked microtubules (biochemical K_d = 15 nM,[65] K_i = 19 nM,[66] 27 ± 11 nM,[40] K_i = 31 nM,[21] K_d = 50 nM,[67] and K_d = 70 ± 0.6 nM[64]). Analysis of lower-affinity baccatin III, a precursor of paclitaxel missing the C-13 side chain that engages the taxol-binding site, (cellular K_i = 17 ± 3 μM) provided a value within threefold that of a reported biochemical K_d (6.7 ± 2 μM).[21] These values obtained in living cells are remarkably consistent with previously reported biochemical affinities of these competitors.[21,38,68,69]

Quantitative Profiling of Destabilizers of Microtubules

Whereas microtubule stabilizers such as paclitaxel and ixabepilone bind the taxane site of β-tubulin,[70] colchicine, vinblastine, and maytansine destabilize microtubules by binding distinct sites (Figure 6A).[71,72] To evaluate whether PB-GABA-Taxol could be used as a quantitative probe of these allosteric modulators, we used the allosteric modulator equation[45] implemented by GraphPad Prism to measure the affinity of these compounds. In this model, two compounds that engage tubulin at different binding sites influence each other's binding through cooperativity. Compounds that disrupt the binding of PB-GABA-Taxol to the Taxol-binding site will have negative cooperativity (α < 1), whereas agents that stabilize the binding of PB-GABA-Taxol will have positive cooperativity (α > 1). Smaller cooperativity factors (α) represent stronger effects on the binding of the orthosteric probe. For these allosteric modulators, the apparent cellular affinity was defined as K_b, where the mathematical relationship between K_b and α is provided in the Supporting Information (eq 3).

The allosteric microtubule destabilizers[2,8] colchicine, vinblastine, and maytansine were investigated with PB-GABA-Taxol using previously optimized cellular binding conditions (Figure 6). Potent allosteric binding affinities were observed for colchicine (K_b = 80 ± 12 nM, α = 0.08), vinblastine (K_b = 7 ± 2 nM, α = 0.18), and maytansine (K_b = 3 ± 1 nM, α = 0.21). This apparent cellular affinity of colchicine was lower but within threefold of previously reported values measured with purified microtubules (K_d = 24 nM)[29] and fixed cells (IC_{50} = 22 nM).[32] In contrast, the cellular affinity of vinblastine was higher than reported biochemical affinities toward purified GDP-bound microtubules (K_d = 190−1000 nM).[73,74] The apparent cellular affinity of maytansine was slightly higher but within about twofold of the affinity of a fluorescent maytansine for purified tubulin measured by fluorescence anisotropy (K_d = 6.8 ± 0.8 nM).[19] Colchicine showed greater negative cooperativity compared with vinblastine and maytansine in these assays, but the basis for this difference is unknown.

Pilot Screening of Diversity Compounds with PB-GABA-Taxol Identified a Novel Microtubule Destabilizer

To explore the potential of PB-GABA-Taxol in HeLa cells as an assay for drug discovery, we performed a pilot small-molecule screen with a 1008-compound subset of the NCI Diversity Set VI library using flow cytometry. Analysis of the assay performance with paclitaxel (10 μM) as a positive control on each plate revealed Z' values of 0.60−0.85 across 12 96-well plates. Baccatin III (25 μM) was also included on each plate as a weakly binding control. Library compounds with activities greater than that of baccatin III (>28% inhibition) were

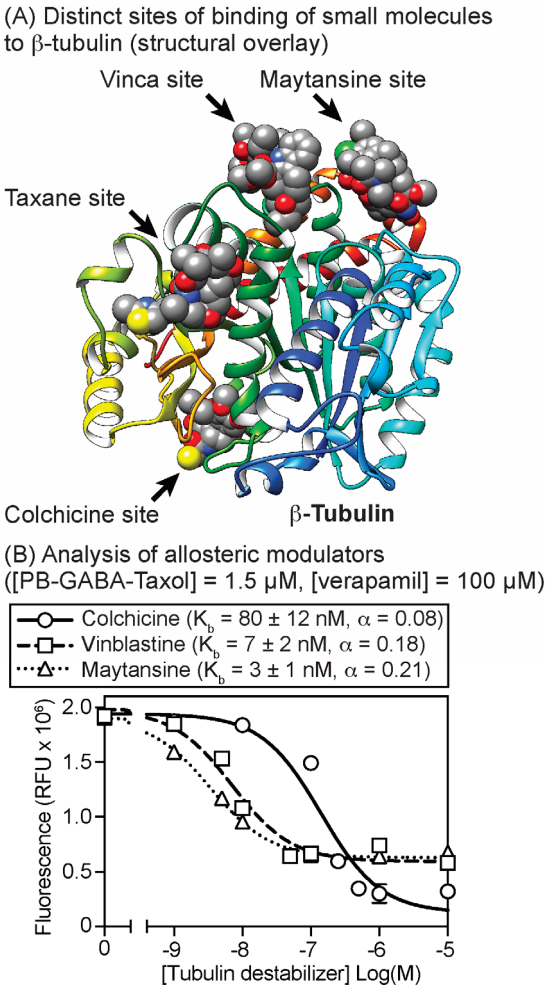

Figure 6. (A) Overlay of the structures of small molecules (spacefilling models) bound to β-tubulin (PDB IDs 7DAF, 4TV8, and 1Z2B) illustrating the distinct sites of binding of tubulin stabilizers and destabilizers. Ixabepilone is shown bound to the taxane site, colchicine is shown bound to the colchicine site, vinblastine is shown bound to the vinca site, and maytansine is shown bound to the maytansine site. (B) Analysis of microtubule destabilizers using an allosteric modulator model (GraphPad Prism). Living HeLa cells (60 000 cells/well) were treated with PB-GABA-Taxol (1.5 μM) and verapamil (100 μM) for 3 h (37 °C), and cellular K_b and α values were calculated (N = 3) using cellular K_d (PB-GABA-Taxol) = 1.7 μM.

considered hits. Of the 1008 compounds screened, seven hits were obtained (Figure S5). The hit that exhibited the greatest effect on fluorescence, termed NSC 93427, was further validated with the PB-GABA-Taxol assay as a dose-dependent microtubule modulator (K_b = 483 ± 50 nM, α = 0.02, Figure 7A). Consistent with this activity, as shown in Figure 7B, NSC 93427 was cytotoxic toward HeLa cells after 48 h (IC_{50} = 554 ± 87 nM). However, colchicine was more potent as a cytotoxic control (IC_{50} = 20 ± 8 nM). Verapamil (25 μM) enhanced the cytotoxicity of these compounds (IC_{50} (NSC 93427) = 237 ± 22 nM; IC_{50} (colchicine) = 13 ± 0.4 nM), suggesting that both are substrates of efflux transporters.

To examine the mechanism of microtubule modulation mediated by NSC 93427, we imaged HeLa cells transiently transfected to express the fluorescent protein mScarlet-α-Tubulin (Figure 7C).[75] When these cells were treated with

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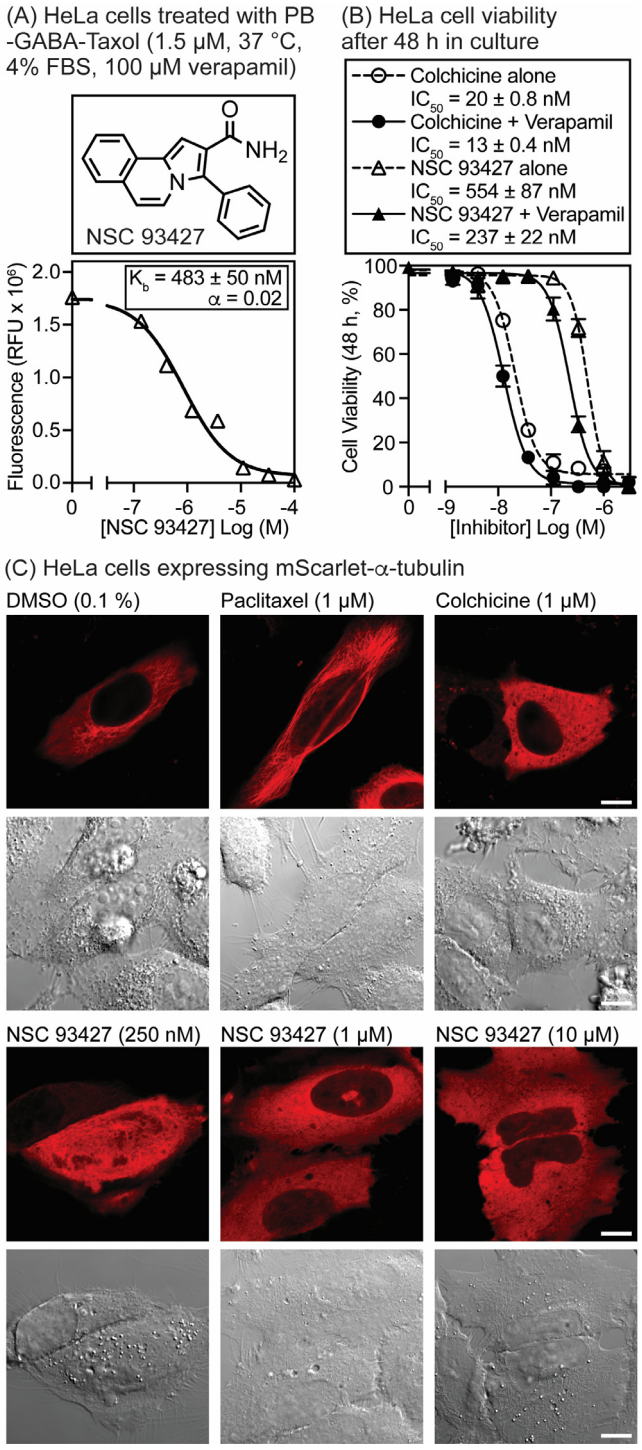

Figure 7. Characterization of NSC 93427 in HeLa cells by flow cytometry (A and B) and confocal (top) and DIC (bottom) microscopy (C). (A) Cellular K_b and α values calculated with the allosteric modulator model using cellular K_d (PB-GABA-Taxol) = 1.7 μM (N = 3). Error bars (SD) for three technical replicates are smaller than the symbols shown. (B) Cytotoxicity after 48 h in the presence and absence of verapamil (25 μM) by flow cytometry. (C) Fluorescent microtubule phenotypes in HeLa cells transiently transfected to express mScarlet-α-Tubulin and treated with the microtubule modulators (1 h at 37 °C). Paclitaxel enhanced the formation of long microtubule fibers, but NSC 93427 disrupted the microtubule network, similar to colchicine. Scale bars represent 10 μm.

paclitaxel as a tubulin-stabilizing control, an increased number of microtubule fibers could be observed by confocal microscopy. In contrast, treatment with colchicine disrupted the microtubule network. Treatment with NSC 93427 afforded a cellular phenotype similar to colchicine, where a dose-dependent disruption of microtubules of live cells was observed.

CONCLUSIONS

The molecular probe PB-GABA-Taxol can be used to quantitatively measure interactions of small-molecule stabilizers and destabilizers with microtubules in the physiologically relevant environment of living cells. Given that variations in the expression of β-tubulin isoforms[17] and influx or efflux transporters[11,13] play key roles in the action of many of these agents, profiling small molecules in living cells may better predict differences in activities in vivo. This probe also has potential for drug discovery applications, where small molecules can be screened by flow cytometry or confocal microscopy.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsbiomedchemau.2c00031.

Additional figures, methods, and data (PDF)

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CRediT: **Angelo E. Andres** conceptualization (supporting), data curation (equal), formal analysis (equal), investigation (lead), methodology (supporting), validation (equal), writing-original draft (equal), writing-review & editing (supporting); **Andres Mariano** investigation (supporting); **Digamber Rane** investigation (supporting), writing-review & editing (supporting); **Blake R. Peterson** conceptualization (lead), data curation (equal), formal analysis (equal), funding acquisition (lead), investigation (supporting), methodology (lead), project administration (lead), resources (lead), supervision (lead), validation (equal), visualization (lead), writing-original draft (equal), writing-review & editing (lead).

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Notes

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