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Characterizing Ligand-Microtubule Binding by Competition Methods

José Fernando Díaz and Rubén Martínez Buey

Summary

The knowledge of the thermodynamics and kinetics of drug-microtubule interaction is essential to understand the structure/affinity relationship of a given ligand family. When a ligand does not show an appropriate signal change (absorbance or fluorescence) upon binding, the extensive direct characterization of its binding affinities and kinetic rate constants of association and dissociation becomes a complex task. In those cases it is possible to obtain these parameters by competition of the ligand with a reference one of the same binding site that shows such change. Nevertheless, although the experimental setup of the competition measurements is easier, the treatment of the data is complex because simultaneous equilibrium/kinetic equations have to be solved. In this chapter, the taxoid-binding site of the microtubules will be used as an example to describe experimental competition and data analysis methods to determine the binding constants and kinetic rates of association and dissociation of ligands for microtubules.

Key Words: Microtubules; paclitaxel; epothilones; stopped flow; fluorescence; anisotropy.

1. Introduction

The pharmacopoeia of tubulin has been increased in the last decade with the discovery of a relatively wide range of the so called microtubule stabilizing agents (MSA); those compounds are paclitaxel mimetics in the sense that they mimic the paclitaxel mechanism of action, stabilizing the assembled form of tubulin. Although up to now two MSA binding sites are known (the taxoid-binding site and the laulimalide-binding site), most of the MSA discovered and developed target the taxoid-binding site (epothilones, eleutherobin, discodermolide, sarcodictyins, some steroid analogs, ciclostreptin, dictyostatin, and paclitaxel itself *[1–6]*).

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The binding affinity of the epothilones has been shown to correlate well with their cytotoxicity *(7)*, which makes determination of the binding affinity a very powerful tool for quick evaluation of a newly discovered compound or a large series of related synthetic compounds *(7)*.

The study of the interaction between paclitaxel and its mimetics with their binding site is greatly hampered by their mechanism of binding *(8,9)*. Because they preferentially bind to the assembled form of tubulin, inducing microtubule assembly, the assembly and binding are linked reactions *(8)*. Although empty sites can be assembled in the absence of ligand at high tubulin concentrations over the critical concentration (microtubule assembly is a noncovalent nucleated condensation polymerization, characterized by a cooperative behavior and the presence of a critical concentration Cr, below which no significant formation of large polymers takes place *(10)*, the high affinity of most of the MSAs makes it impossible to find conditions in which the reaction is not completely displaced towards the end state, thus making it difficult to obtain an exact measurement of the binding affinity of these compounds.

A procedure to stabilize assembled microtubules has made it possible to obtain taxoid-binding sites, which can be diluted to concentrations low enough to directly measure the binding affinity of two fluorescent derivatives of paclitaxel, Flutax-1 and Flutax-2 *(11)*, which could be used as reference ligands for a competition method that has been developed to measure the binding affinity of compounds that bind to the paclitaxel site *(7,12–15)*; and to distinguish them from compounds with similar effects, but which do not bind to the paclitaxel binding site, such as laulimalide *(13)* and peloruside A *(16)*.

The binding site of paclitaxel has been mapped in the β-tubulin subunit using photolabeling *(17–19)*. The labeled aminoacid residues are in agreement with the 3.5-Å resolution electron crystallographic structure of tubulin in paclitaxel-stabilized zinc-induced two-dimensional crystals *(20,21)*. Tubulin zinc-sheets, whose assembly is not GTP dependent *(22)*, consist of protofilaments similar to those that form the microtubules but organized in an antiparallel array. The docking of these protofilaments into electron microscopy density maps of paclitaxel-containing 14 and 15 protofilament microtubules *(23,24)* results in an atomic model of microtubules in which the binding site of paclitaxel is located on the microtubule inner surface *(24,25)*. Such luminal location will in principle make the binding site difficult to access for paclitaxel site ligands in assembled microtubules. However, it had previously been shown that paclitaxel modifies the flexibility of microtubules in a few seconds *(26)* and that the reversible binding of paclitaxel and its side chain analog docetaxel to an accessible site of microtubules changes the number of their protofilaments within a time range of 1 min *(27)*; so in principle, the binding site of paclitaxel seems to be accessible in preformed microtubules. The fluorescent derivatives of paclitaxel Flutax-1, Flutax-2, 7-Hexaflutax, and the stabilized binding sites

have been employed to study the problem of the accessibility of the taxoid-binding site in preformed microtubules. The kinetics constants of binding and dissociation of both Flutax-1 and Flutax-2 for the paclitaxel site have been measured using standard kinetic methods and the determined constants have been employed to determine those of paclitaxel itself to its site using a competition method *(11,15,28)*.

In this chapter, we will describe the methodology for the preparation of stabilized crosslinked microtubules and its use for the determination of the equilibrium binding constants and kinetic rate constants of paclitaxel binding site ligands. In principle, the methods are of general application to any ligand with a good signal change (absorbance, fluorescence intensity, fluorescence anisotropy) upon binding to its site. Nevertheless, it has to be pointed out that competition methods require the constants to be measured to be lower or not much higher than those of the reference ligand. Because the analysis is based on the stoichiometric displacement or perturbation of the binding of the reference ligand, any ligand over a certain affinity (1000 times larger than the reference) or association rate (100 times larger) will behave essentially identically because competition tests are based on the amount of reference ligand free and bound to the site. If we mix 100 n*M* of a problem ligand with 100 n*M* of a reference ligand with $10^7/M$ binding constant and 100 n*M* sites, the percentage of free reference ligand will depend on the binding constant of the problem ligand. If the constant is $10^7/M$, the percentage of free ligand will be 76.6%, if it is $10^8/M$, the free ligand will rise up to 81.9%, for $10^9/M$, the figure will be 92.8%, 97.5% for $10^{10}/M$, and 99.2 for $10^{11}/M$. So, with this reference ligand, it would be easy to determine with precision the binding constants of ligands in the interval between $10^7/M$ and $10^9/M$ but it would be very difficult to distinguish between two ligands of constants in the order of $10^{10}/M$. Because it is always possible to displace a ligand of high affinity with a large excess of one with lower affinity, reference ligands with the highest affinity possible are desired.

2. Materials

2.1. Buffers and Proteins

1. GAB, glycerol assembly buffer: 3.4 *M* glycerol, 10 m*M* sodium phosphate, 1 m*M* EGTA, 6 m*M* $MgCl_2$, 0.1 m*M* GTP, pH 6.5. Prepare the same day of use and keep in the cold.
2. Tubulin, purify it by the modified Weisenberg procedure (*[29–31] also see* Chapter 2) and store it in liquid nitrogen.

2.2. Measuring the Binding Affinities and Kinetic Binding Constants Using a Competition Assay

1. Crosslinked stabilized microtubules, prepare as described next and keep them frozen in liquid nitrogen. Once defrozen, the concentration of sites slowly decay at 4°C with a half-life of approx 50 d (average of four batches).

2. Flutax-1 (Calbiochem).
3. Docetaxel (Sigma).

2.3. Software for Data Analysis

1. Equigra v5.0 (Díaz et al. unpublished) for binding competition data analysis. Available from the authors upon request.
2. Software for kinetic data simulation and fitting. One of the most powerful ones is FITSIM *(32)*, available at Prof. Carl Frieden's web page: http://biochem.wustl.edu/cflab/.

3. Methods

3.1. Preparation of Stabilized Taxoid-Binding Sites

1. Load 20 mg of tubulin in a 25 × 0.9-cm column of Sephadex G-25 (GE Healthcare Bioscience, Upsala, Sweden) equilibrated with two volumes of buffer 10 m*M* phosphate, 1 m*M* EGTA, 0.1 m*M* GTP, 3.4 *M* glycerol, pH 6.8 (prepare 100 mL of the buffer and keep at 4°C).
2. Measure the absorbance at 295 nm of the fractions containing protein, pool those whose absorbance is higher than 1.0.
3. Clarify the solution by centrifugation at 50,000*g*, 4°C, for 10 min using TL100.2 or TL100.4 rotors in a Beckman Optima TLX centrifuge or similar.
4. Make a 1:20 dilution of the solution to measure the concentration of tubulin spectrophotometrically (extinction coefficient 107,000/*M*/cm at 275 nm) *(31)* in 10 m*M* sodium phosphate buffer containing 1% SDS pH 7.0.
5. Adjust the tubulin concentration to 50 μ*M* and add 6 m*M* $MgCl_2$ and up to 1 m*M* GTP to the solution, final pH 6.5.
6. Assembly the tubulin by incubating it 37°C for 30 min. The solution should become turbid and viscous.
7. Add 4 μL of 50% glutaraldehyde (EMscope, microscopy grade) per milliliter of tubulin (final concentration 20 m*M*) to the solution and mix it with a 1-mL pipet, incubate the solution at 37°C for another 10 min.
8. Place in a 250-mL beaker 60 μL of a 1 *M* $NaBH_4$ solution (Fluka) per milliliter of tubulin solution. Pour the assembled tubulin solution over it. Let the foam repose for 10 min degas it and recover the solution.
9. Dialyze the solution overnight using Slide-A-Lyzer 10K cassettes (Pierce) against GAB and drop freeze it in liquid nitrogen.

3.2. Measuring the Binding Sites Concentrations

1. Make a 1:20 dilution of the solution of crosslinked microtubules to measure the concentration of tubulin spectrophotometrically (extinction coefficient 107,000/*M*/cm at 275 nm, *(31)* in 10 m*M* sodium phosphate buffer containing 1% SDS pH 7.0.
2. Make the calibration curves. Employing the previously determined concentration, prepare 0.2, 0.5, 1, 2, and 5 μ*M* solutions (tubulin concentration) of crosslinked microtubules in 10 m*M* sodium phosphate buffer containing 1% SDS

pH 7.0 and in a mixture 4:1 of 10 m*M* sodium phosphate buffer containing 1% SDS pH 7.0 and GAB and measure their fluorescence intensity (λ_{exc} 280 nm λ_{ems} 323 nm) to get a fluorometric tubulin concentration calibration curve.

3. Prepare 0.2, 0.5, 1, 2, and 5 μ*M* solutions of Flutax-1 in 10 m*M* sodium phosphate buffer containing 1% SDS pH 7.0 and in a mixture 4:1 of 10 m*M* sodium phosphate buffer containing 1% SDS pH 7.0 and GAB and measure their fluorescence intensity (λ_{exc} 495 nm λ_{ems} 520 nm) to get a fluorometric Flutax-1 concentration calibration curve.
4. Centrifuge two-hundred microliters of the following samples in GAB in a Beckman TL100 rotor in Beckman TLX Optima ultracentrifuge at 50,000 rpm for 20 min at 25°C:
   a. 20, 10, 5, and 2 μ*M* crosslinked microtubules.
   b. 2 μ*M* Crosslinked microtubules plus 5 μ*M* Flutax-1.
   c. 2 μ*M* Crosslinked microtubules plus 5 μ*M* Flutax-1 plus 100 μ*M* docetaxel.
5. Collect supernatants and dilute them 1:4 in 10 m*M* sodium phosphate buffer containing 1% SDS pH 7.0. Add 200 μL of 10 m*M* sodium phosphate buffer containing 1% SDS pH 7.0 to the pellets to resuspend them and dilute them 1:4 in 10 m*M* sodium phosphate buffer containing 1% SDS pH 7.0.
6. Take the dilutions of supernatants and pellets of the tubes containing only crosslinked microtubules (**step 4a**) and measure the tubulin concentrations fluorometrically employing the appropriate calibration curves to know the percentage of tubulin polymerized, which should be more than 80%.
7. Take the dilutions of supernatants and pellets of the tubes containing crosslinked microtubules and Flutax-1 (**step 4b,4c**) and measure the Flutax-1 concentrations fluorometrically employing the appropriate calibration curves. The difference between the Flutax-1 concentration in tube b and c is the concentration of binding sites.

3.3. Measuring of the Binding Constant of the Problem Ligand

1. Take a 96-well plate suitable for florescence measurements (**Scheme 1**). Don't use the border wells: columns 1 and 12 and rows A and H. In the first five rows (B to F) dispense 200 μL of the mixture of 50 n*M* Flutax-1 and 50 n*M* binding sites. In row G dispense 200 μL of 50 n*M* binding sites in GAB as blank in wells 2 and 3 and 200 μL of 50 n*M* Flutax-1 in GAB in wells 4 and 5 as anisotropy standard.
2. In wells 2–11 of rows B–F, add the following problem ligand concentrations: 0 n*M*, 5 n*M*, 10 n*M*, 20 n*M*, 50 n*M*, 100 n*M*, 500 n*M*, 1 μ*M*, 2 μ*M*, and 5 μ*M*.
3. Set the plate at the desired temperature (note that the binding constant of the reference ligand should be known at this temperature **[Table 1]**) and measure F_{VV} (fluorescence intensity with excitation 490 nm vertically polarized, emission 520 nm vertically polarized) and F_{VH} (fluorescence intensity with excitation at 490 nm vertically polarized, emission at 520 nm horizontally polarized) of the wells, subtract the blank and calculate the G factor (the correction for the effi-

Scheme 1
Distribution of the 96-Well Plate for the Binding Constant Determination Assay

Ligand A n*M*	0	5	10	20	50	100	500	1000	2000	5000
Ligand B n*M*	0	5	10	20	50	100	500	1000	2000	5000
Ligand C n*M*	0	5	10	20	50	100	500	1000	2000	5000
Ligand D n*M*	0	5	10	20	50	100	500	1000	2000	5000
Ligand E n*M*	0	5	10	20	50	100	500	1000	2000	5000
	Blank	Blank	Standard	Standard						

1. All the border wells are empty and all the wells containing problem ligands contain 200 µL of 50 n*M* Flutax-1 and 50 n*M* sites in GAB.

2. The blank wells contain 200 µL 50 n*M* sites in GAB and the standard wells contain 200 µL 50 n*M* Flutax-1 in GAB.

Table 1
Equilibrium Constants of Flutax-1 Binding to Microtubules

	27°C	32°C	37°C	40°C	42°C
$Ka \times 10^7/M$	6.0 ± 0.2	4.3 ± 0.4	2.9 ± 0.3	2.1 ± 0.2	1.5 ± 0.1
v_o	0.57	0.51	0.45	0.39	0.33

Data from **ref. *11***.

v_o (Flutax-1)$_{bound}$/(Flutax-1)$_{total}$ is calculated from the concentration of Flutax-1, binding sites and the binding constant of Flutax-1 at the different temperatures for 50 n*M* binding sites and 50 n*M* Flutax-1.

ciency of each optical channel) ***(33)*** directly if your plate reader can measure F_{HV} (fluorescence intensity with excitation at 490 nm horizontally polarized, emission at 520 nm vertically polarized) and F_{HH} (fluorescence intensity with excitation at 490 nm horizontally polarized, emission at 520 nm horizontally polarized); otherwise calculate it from the standard (Flutax-1 free in GAB $r = 0.05$) and use it to calculate the anisotropy of each well using **Eq. 1**:

$$r_x = \frac{\frac{Fvv}{Fvh * G} - 1}{\frac{Fvv}{Fvh * G} + 2} \quad (1)$$

4. Check whether the anisotropy value has reached the minimum value (that of the free Flutax-1) at the maximum problem ligand concentration; otherwise repeat the measurement with increased problem ligand concentrations in **step 2**, avoiding going over the solubility limit of the problem ligand, because precipitated ligand may cause scattering, which is polarized and will interfere with the measurement.
5. Transform the anisotropy values into fractional saturation values. The fractional saturation of Flutax-1 (reference ligand) (v_x=|Flutax-1|$_{bound}$/|Sites|) in the presence of the competitor ligand is calculated from the fluorescence anisotropy measurements as follows:
   Because the anisotropy is an additive property, the anisotropy of a given mixture is the sum of the anisotropy of their components. The anisotropies of Flutax-1 in the two reference states (the bound $r_o = 0.24 \pm 0.02$ and the fully displaced $r_{min} = 0.05 \pm 0.02$ ***(11)***, and the fractional binding of the reference ligand Flutax-1 in the absence of competitor (v_o, **Table 1**) are known, so the measured anisotropy values r_x can transformed into fractional saturation values v_x employing **Eq. 2**.

$$v_x = \frac{vo * (rx - r\min)}{(ro - r\min)} \quad (2)$$

The binding constant of the problem ligand K(l) can be determined from the known values of the binding constant of the reference ligand K(r) and the total concentrations of binding sites, Flutax-1 and problem ligand by solving the simultaneous mass action equations **(Eqs. 3–7)**.

$$K(l)=[Ligand]_{bound}/[Sites]_{free}*[Ligand]_{free} \quad (3)$$

$$K(r)=[Flutax\text{-}1]_{bound}/[Sites]*[Flutax\text{-}1]_{free} \quad (4)$$

$$[Flutax\text{-}1]_{free}=[Flutax\text{-}1]_{total}-[Flutax\text{-}1]_{bound} \quad (5)$$

$$[Ligand]_{free}=[Ligand]_{total}-[Ligand]_{bound} \quad (6)$$

$$[Sites]_{free}=[Sites]_{free}-[Ligand]_{bound}-[Flutax\text{-}1]_{bound} \quad (7)$$

In **Fig. 1** the fractional saturation values of Flutax-1 at different competitor concentrations are best fitted (assuming unitary stoichiometry) to the equilibrium binding constant of different competitors (Epothilone B, paclitaxel, and an steroid analog of paclitaxel) and the binding constant of the reference ligand (Flutax-1) with a program developed *ad hoc* (Equigra v5) (*see* **Note 1**).

6. If the binding constant of the ligand at a different temperature is required, change the temperature setting of the plate reader and incubate the plate until it reaches equilibrium (usually 20 min is enough) and go back to **step 3**, (check Flutax-1 photobleaching after three to four measurements). If the binding constant of a ligand is measured at several temperatures, apparent thermodynamic parameters can be calculated using the van't Hoff and Gibbs' equations.

3.4. Measuring the Association Rate Constant of the Problem Ligand

1. Place in one of the syringes of the stop-flow device a 1.2-μ*M* solution of taxoid-binding sites in GAB.
2. Prepare 1 μ*M* solutions of Flutax-1 in GAB containing 0 and 1 μ*M* of the problem ligand.
3. In the second syringe, load the solution containing Flutax-1 and no competitor and measure the kinetic association curve (final concentrations 600 n*M* sites and 500 n*M* Flutax-1). Be sure that the reaction has reached equilibrium. Repeat the measurement with the solution containing 1 μ*M* of the problem ligand.
4. Compare the kinetic association curves and check if there is a significant decrease of the amplitude and velocity in the presence of the problem drug; otherwise increase the competitor concentration until the amplitude of the curve decreases by around 75%.
5. Prepare another four 1 μ*M* solutions of Flutax-1 in GAB containing 2, 1/2, 1/4, 1/8, and 1/16 of the problem ligand concentration determined in **step 4**. Measure the kinetic association curves of Flutax-1 to the taxoid sites in the presence of increasing concentrations of the problem ligand.
6. Scale the first curve to 1 at the equilibrium and to 0 in the origin (at the concentrations of Flutax-1 and sites employed, the half-life of the reaction is 2–3 s, so

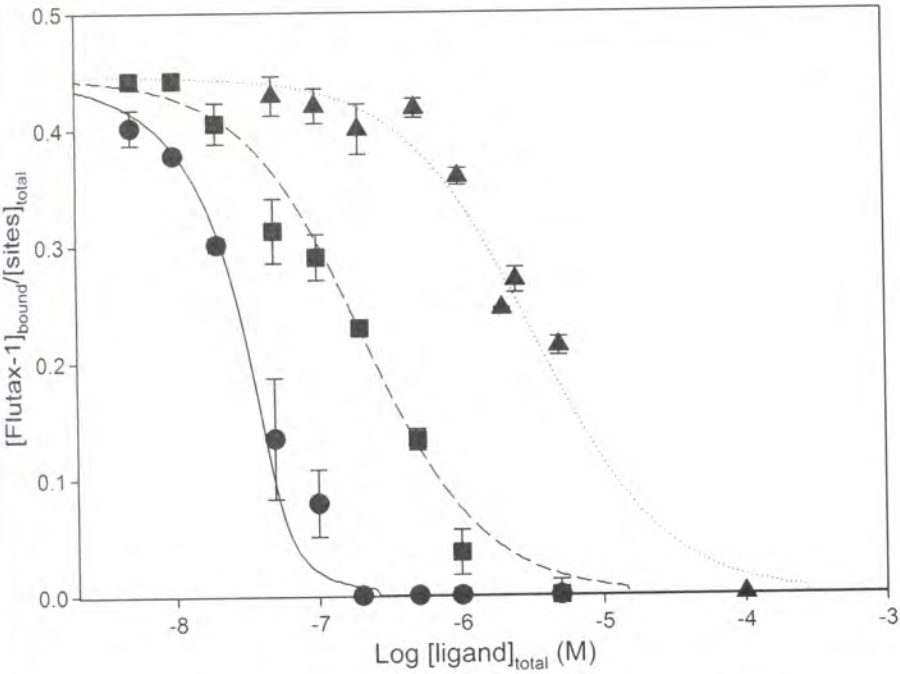

Fig. 1. Displacement of the fluorescent taxoid Flutax-1 (50 n*M*) from microtubule binding sites (50 n*M*) by taxoid-binding site ligands at 37°C. The points are data and the lines were generated with the best fit value of the binding equilibrium constant of each competitor, assuming a one to one binding to the same site. Ligands: Epothilone B (circles, solid line), Paclitaxel (squares, dashed line), 2-ethoxyestradiol analog (3,17βd-diacetoxy-2-ethoxy-6-oxo-B-homo-estra-1,3,5[10]-triene; [4]) (triangles, dotted line). The determined binding constants were $8 \times 10^8 M^{-1}$ for Epothilone B, $2 \times 10^7 M^{-1}$ for paclitaxel and $8.3 \times 10^5 M^{-1}$ for 2-ethoxyestradiol analog.

no significant part of the reaction should be observed within the typical dead time of the stopped flow instrument) and scale the other curves accordingly. Check that the proportion between the curves at equilibrium roughly corresponds with the equilibrium binding constant previously determined.

7. Fit the kinetic curve in the absence of competitor, employing FITSIM *(32)* (*see* **Note 2**) and compare the kinetic rate of association obtained with the published one *(11)*.
8. Fit all kinetic curves simultaneously employing FITSIM *(32)* (*see* **Note 2**). **Figure 2A** shows the fitting of the association curves of Flutax-1 to the taxoid-binding site of the microtubules in the presence of different concentrations of Epothilone A.
9. If the association rate constant of the ligand at a different temperature is required, change the temperature setting of the stopped flow device and go back to **step 1**.

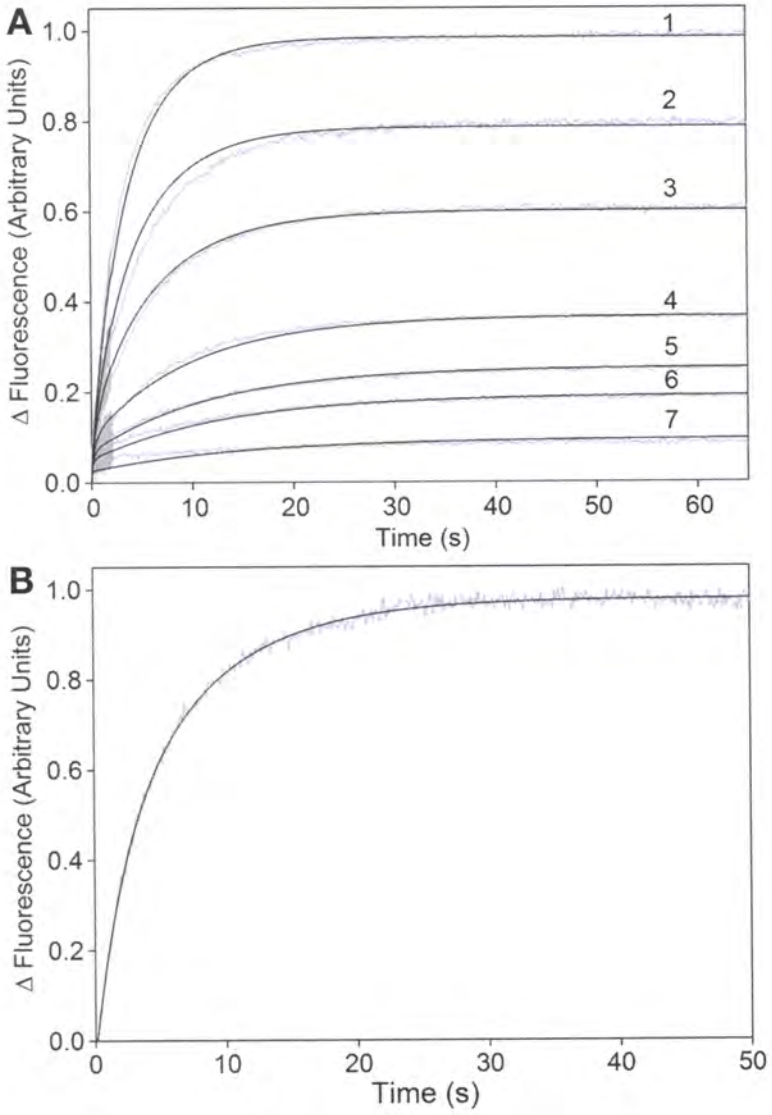

Fig. 2. (**A**) Kinetics of association of Epothilone A to microtubules at 37°C. In the stopped flow device, a solution of crosslinked microtubules containing 1.2 μ*M* sites was mixed 1:1 with a 1 μ*M* solution of Flutax-1, containing different concentrations of Epothilone A (curve 1) 0 μ*M*, (curve 2) 0.5 μ*M*, (curve 3) 1.0 μ*M*, (curve 4) 2.0 μ*M*, (curve 5) 3.0 μ*M*, (curve 6) 4.0 μ*M*, (curve 7) 8.0 μ*M*. The gray lines are the experimental data and the solid lines are the result of the simultaneous fitting of all curves to a kinetic model of a single step association of Epothilone A to the paclitaxel binding site. The kinetic association rate constant of Epothilone A to the taxoid-binding site of

3.5. Measuring the Dissociation Rate Constant of the Problem Ligand

1. First, determine the binding constant of the problem ligand. If it is more than 20–30 times that of the reference ligand at the temperature of the measurement, it will not be possible to displace it with a 10 times excess making it not possible to measure the dissociation rate constant.
2. Place in one of the syringes of the stop-flow device a 1-μM solution of taxoid-binding sites in GAB plus 1.2 μM of the problem ligand. Using the association-binding constant, calculate that a significant percentage of the binding sites are filled with the problem ligand. Otherwise increase the concentration accordingly.
3. In the other syringe, place a 12 μM solution of Flutax-1 in GAB (final concentrations 500 nM taxoid-binding sites, 600 nM problem ligand, 6 μM solution of Flutax-1; if the concentration of binding sites and problem ligand have been increased and the Flutax-1/sites ratio is under 5:1 increase the Flutax-1 concentration accordingly). Measure the dissociation constant from the small change of fluorescence intensity upon Flutax-1 binding to the binding sites that are left empty by the problem ligand.
4. Analyze the kinetic curve as a sum of exponentials. If all the binding sites are filled with the problem ligand, only the kinetics of dissociation of the problem ligand should be observed. If a significant fraction of binding sites are empty, a fast initial phase corresponding to the filling of these sites by Flutax-1 will be observed. The dissociation of Epothilone A from the taxoid-binding site of the microtubules is shown in **Fig. 2B**.
5. If the dissociation rate constant of the ligand at a different temperature is required, change the temperature setting of the stop flow device and go back to **step 1**.

4. Notes

1. Equigra v5 may work both by introducing the data manually and by reading them from a file. If you want to introduce the data manually, the program will prompt you for:
   a. Ligand name.
   b. Total sites concentration (which should be equal for all data sets).
   c. Binding affinity of the reference ligand.

Fig. 2. *(continued)* microtubules at 37°C was found to be $3.30 \pm 0.03 \times 10^6$ $M^{-1}s^{-1}$. **(B)** Kinetics of dissociation of Epothilone A from microtubules at 37°C. At time 0 s, 10 μM Flutax-1 was added to 1-μM binding sites in crosslinked microtubules that contained 1.2 μM Epothilone A. The reaction was followed by the change in fluorescence intensity (average of nine curves). The data were fitted to a double exponential (solid line) and the dissociation of Epothilone A from its site in the microtubules was found to be biphasic indicating the existence of two different species with kinetic constants of dissociation of 0.138 ± 0.37 s^{-1} and 0.463 ± 0.32 s^{-1}, respectively.

d. The number of data sets (with different reference ligand concentrations).
e. The number of problem ligand concentrations for each data set (which should be equal for all data sets).
f. The lower and upper limits for the search of the binding constant.
g. The concentrations of the reference ligand for each data set.
h. The concentrations of the problem ligand for each data set.
i. The fractional saturation of reference ligard for each pair or reference ligand/problem ligand concentration.

If you wish to prepare an input data file (which is much more useful) you should create a file with a name less than eight characters long and with .dat extension. The file should contain the same data except the ligand name **(Note 1a)**, which will be taken from the name of the input file. The format of the data should be the following (delete the comments in parenthesis):

```
50e-9     (Concentration of binding sites)
3.0e7     (Binding constant of the reference ligand)
1         (One data set, multiple data sets with different reference ligand
          concentration can be employed)
10        (Ten problem ligand concentrations for each data set)
1e7       (Lower limit for the problem ligand binding affinity search)
1e10      (Upper limit for the problem ligand binding affinity search)
50e-9     (Reference ligand concentration)
          0.0000 5e-9 10e-9 20e-9 50e-9 100e-9 200e-9 500e-9 1000e-9
          5000e-9 (Problem ligand concentrations, M)
0.4510    (Fractional saturation for problem ligand concentration 0.0000)
0.3806    (Fractional saturation for problem ligand concentration 5e-9)
0.3140    (Fractional saturation for problem ligand concentration 10e-9)
0.2112    (Fractional saturation for problem ligand concentration 20e-9)
0.1731    (Fractional saturation for problem ligand concentration 50e-9)
0.1379    (Fractional saturation for problem ligand concentration 100e-9)
0.0352    (Fractional saturation for problem ligand concentration 200e-9)
0.0376    (Fractional saturation for problem ligand concentration 500e-9)
0.0417    (Fractional saturation for problem ligand concentration 1000e-9)
0.0347    (Fractional saturation for problem ligand concentration 5000e-9)
```

Run the program and input the filename (without the .dat extension). The program will iteratively calculate the binding constant of the problem ligand, stopping after 100 iterations showing the quality of the fitness D^2/N (the square of the deviations divided by the number of points) and the calculated binding constants and will prompt you to do another 100 iterations; type "y" until the quality of the fitness remains constant. When no more iterations are needed, type "n" and a plot of the fitting will appear. Press "e" to exit the program.

The program will generate two output files .out and .tab. The .out file contains the result of the fitting for the program to represent the result of a previous calculation. The .tab file is an ASCII file with the calculated competition curve.

2. This note does not aim to be a user guide for FITSIM *(32)* but just a small walkthrough of how to process competition kinetic data using this powerful tool. We can not give support on the tool and any question to the authors concerning the use of FITSIM will be unattended.
FITSIM is a program based on iterative solving of the differential equations that govern the kinetics of chemical reactions. In this way no pseudofirst order conditions are required for the experiments and a wide range of experimental setups can be analyzed.

   To use FITSIM to analyze your data, the first thing you need is to set up a mechanism. This can be done with a text editor such as Windows notepad.

   For the case of the competition of a ligand with Flutax-1 for the taxoid-binding site your mechanism will be as follow:

   ```
   $Simple competition mechanism
   A + T == AT
   B + T == BT
   *OUTPUT
   F*AT
   ```

   where A is Flutax-1, B the problem ligand, AT and BT the complexes between the ligands and the taxoid site and F is a factor to convert concentration of AT to the observed fluorescence change. Although Flutax-1 binding to microtubules follows a two-step mechanism *(11)*, only the first step is visible by fluorescence intensity change and useful for our purposes. Save the file with the extension .mec. Open KINSIM40 and compile the mechanism (Option O), then you will get a .sim file that will be required for the simulation.

   Second, the data produced by the stopped flow instrument should be converted to the FITSIM format. To do this, the data should be in a two-column ASCII format: time and fluorescence change value (scaled from 0 to 1). Once the data are in ASCII format, they should be converted to FITSIM format using option A of KINSIM40, save the files with the extension .rdf.

   Third, the parameter file of each data file has to be created. Use the C, F, and T options of the KINSIM40 menu to input the concentration of sites and ligands (C), the output factors (F) and the time factors for the calculation (T), and the K option to input any rate constant that wants to be fixed during the simulation.

   The output factor is a conversion factor between the Flutax-1 concentration and the fluorescence signal. Because at the concentration of 500 n*M* employed, the reaction is more than 99% displaced towards the bound state, it can be considered that, at the equilibrium in the absence of competitor all Flutax-1 is in the bound state (AT). Because the value of the association binding curve in the absence of competitor has been scaled to 1 at the equilibrium, the F factor for AT should be approx 1/500e-9, i.e. 2e6; note that the curve has been scaled to 0 at origin, so only the change of fluorescence is considered and therefore the F factor for A should be 0. In any case the F factor is a parameter that will be fitted later.

The time factors: (delta time, integrations [default = 1], run time, Ymax, flux tolerance [default = 0.02] and integral tolerance [default = 1E-6]) are parameters for the simulations that are used to calculate the constants. The user should introduce the run time of the curve in run time, Y max is always 1 and you should let the other parameters in the default values. Delta time determines the number of points in the simulation, i.e., if run time is 1 s and delta time 0.01 s there will be 100 points, but you can set it to a relatively large value (like 1) and the program will search for the smallest delta time that falls within an integral tolerance range. Save the data set as a .sav file.

Once you have the .sim file for the mechanism, a .rdf file and a .sav file for each file you may start the fitting.

Run FITSIM40 and choose 1 to make a .fdt file, which will contain the parameters for the fitting. Input the mechanism filename.sim (be sure of typing it with the .sim extension) and the program will prompt for the constants to be fitted (those that were selected to be fixed should be specified in the .sav file of the parameter file previously saved). Then it will prompt for the data sets to be employed, if you are not familiar with FITSIM it is recommended to just fit one measurement to learn the structure of the .fdt files, which are used for fitting. Afterwards, the program will ask if all the default parameters will be employed; answer "n" and include the output factor as a fitted parameter, let the other parameters in the default values unless you are familiar with them. Once the .fdt file is ready the system will return to the main menu, type 2 to fit the data curves and 3 to see the results of the fitting.

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