Research Report

Rapid Colchicine Competition-Binding Scintillation Proximity Assay Using Biotin-Labeled Tubulin

BioTechniques 29:156-160 (July 2000)

Stephen K. Tahir, Peter Kovar, Saul H. Rosenberg and Shi-Chung Ng
Abbott Laboratories, Abbott Park, IL, USA

ABSTRACT

We have developed a rapid [^{3}H]colchicine competition-binding scintillation proximity assay (SPA) to evaluate antimitotic compounds that bind to the colchicine-binding site on tubulin. The premise of our assay is that compounds will compete with radiolabeled colchicine for the tubulin-binding domain. Biotin-labeled tubulin is incubated first with unlabeled compound and radiolabeled ligand. Streptavidin-labeled SPA beads are added, and the radiolabel associated with tubulin is directly counted with no separation steps. Under our experimental conditions, the dissociation constant of binding (K_d) for colchicine to tubulin was determined to be 1.4 mM, which was consistent with previously reported values. Assay validation was performed by competitively inhibiting [^{3}H]colchicine binding to tubulin with known microtubule inhibitors and comparing their inhibition constants (K_i). Our SPA bead method is a powerful tool since it overcomes the disadvantage of traditional filtration techniques, as there are no separation steps. It is extremely easy to set up, multiple samples can be assayed and supply and labor costs are reduced because of the minimal volume and test reagents used.

INTRODUCTION

Currently, there are several natural products and their analogs with antimitotic activity under development for the treatment of neoplastic diseases (11, 13–15). Colchicine, derived from the plant *Colchicum autumnale*, was the first antimitotic agent to be characterized. Isolated in the 19th century, it was instrumental in the isolation and characterization of microtubules (11). Microtubules are dynamic, labile, polar polymers composed primarily of tubulin heterodimers consisting of one α- and one β-tubulin subunit noncovalently linked (3,9,21). Microtubules are a major intracellular component of cells involved in cell shape, cellular movement and division (3,9,12). During cell division, microtubules form the mitotic spindle that lines up and properly separates the chromosomes to each new "daughter" cell (26). Compounds such as colchicine interfere with the ability of cells to properly form the mitotic spindle causing the cells to arrest at metaphase. Cells arrested at metaphase with colchicine eventually die by the process of apoptosis (2,4).

The exact mechanism of colchicine on microtubule dynamics is unclear; however, it is known to first bind to a specific site on the β-tubulin subunit of the tubulin heterodimer. The tubulin-colchicine complexes then become incorporated into the microtubule ends, leading to changes in the rate and extent of growing and shortening of microtubules (14,15,21,27). Tubulin-binding assays have been a useful screening tool to identify several new classes of structurally unrelated antimitotic compounds that can interact with tubulin at the colchicine-binding domain. Antimitotic compounds, such as combretastatin A4 and E7010, which bind to tubulin at the colchicine-binding domain, are either under development or in clinical trials as antineoplastic agents (7,28).

Several colchicine-binding assays, such as the DEAE-cellulose filter paper, gel filtration or activated charcoal assays, require the separation of bound colchicine from unbound colchicine (5,8,18,24). However, there are disadvantages to using separation techniques, for example, the introduction of extra steps for separating and washing, plus the potential decay of the complex. A previously developed fluorometric assay does not require the separation of the tubulin-colchicine complex with free colchicine. However, it is not as sensitive as [^{3}H]colchicine assays (1). To overcome the disadvantages inherent with separation assays, we developed a novel [^{3}H]colchicine competition-binding scintillation proximity assay (SPA) using yttrium beads to evaluate antimitotic compounds in vitro. SPA technology overcomes the disadvantage of traditional filtration techniques because there are no separation steps and the reaction and counting are done in the same well.

This paper describes our novel 96-well [^{3}H]colchicine SPA using biotin-labeled tubulin and streptavidin-yttrium beads. The assay can be used to identi-

Table 1. Effect of Various Microtubule Inhibitors on [^{3}H]Colchicine Binding to Tubulin

Compound	Observations	K_i (μM)	Reported K_i (μM)	Reference No.
Colchicine	competitive inhibition	0.53 ± 0.9	0.34–2.0	20
combretastatin A4	competitive inhibition	0.18 ± 0.3	0.12	19
E7010	competitive inhibition	2.6 ± 0.2	3.3	29
colcemid	competitive inhibition	4.56 ± 0.6	14	22
mebendazole	competitive inhibition	2.1	3.9	23
podophyllotoxin	competitive inhibition	0.2	0.51	16
vinblastine	non competitive	-	-	-
taxol	non competitive	-	-	-

fy novel compounds with potential antimitotic properties that compete with colchicine for the colchicine-binding site on tubulin.

Biotin-labeled tubulin is incubated first with unlabeled compound and radiolabeled ligand (Figure 1). Streptavidin-labeled SPA beads are added, and the radiolabel associated with tubulin is directly counted with no separation step. Our SPA bead method is a powerful tool—it overcomes the disadvantage of traditional filtration techniques because there are no separation steps. It is extremely easy to set up, multiple samples can be assayed and supply and labor costs are reduced as a result of the minimal volume and test reagents used.

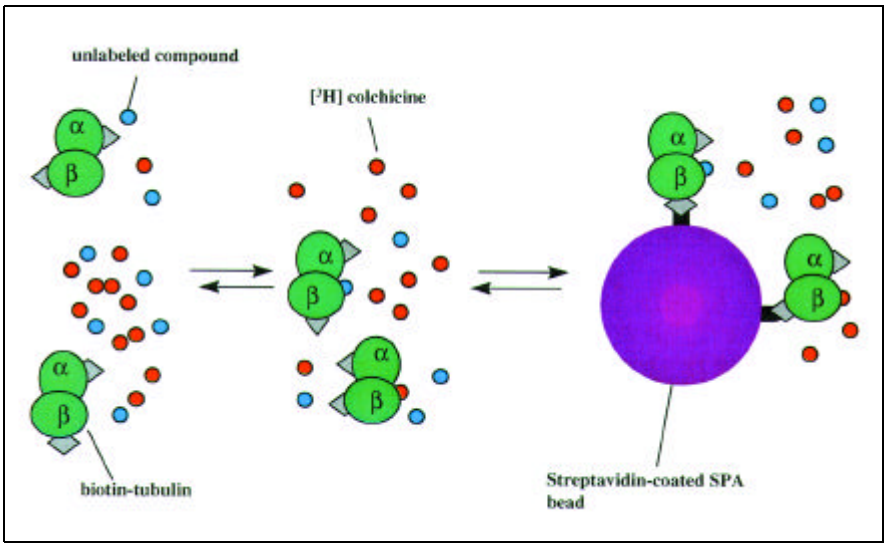

Figure 1. Diagrammatic representation of colchicine-tubulin SPA system. Biotin-labeled tubulin is incubated first with unlabeled compound and radiolabeled ligand. Streptavidin-labeled SPA beads are then added, and the radiolabel associated with tubulin is directly counted without the need to separate bound from free ligand. Any [^{3}H]colchicine-tubulin complexes bound to SPA beads will be close enough to stimulate the bead and emit light. Any unbound radioligand is too distant from the bead to transfer energy and therefore is not detected.

MATERIALS AND METHODS

Chemicals

Special long-chain, biotin-labeled tubulin was purchased from Cytoskeleton (Denver, CO, USA). [^{3}H]colchicine in ethanol [Specific Activity = 2.3 TBq/mmol (61.4 Ci/mmol); 1 μCi/μL] was purchased from New England Nuclear (Boston, MA, USA). Unlabeled colchicine, podophyllotoxin, mebendazole, colcemid, vinblastine and paclitaxel were purchased from Sigma (St. Louis, MO, USA). Combretastatin A4 and E7010 were synthesized at Abbott Laboratories. Unlabeled colchicine, podophyllotoxin, mebendazole, paclitaxel, vinblastine, combretastatin A4 and E7010 were used for the competition-binding assays. Stock solutions of all unlabeled compounds (1 mM) were prepared in ethanol before use.

[^{3}H]Colchicine-Tubulin Binding

Except where noted, radiolabeled colchicine (0.5 μL; 1 μCi/μL) was first diluted with 19.5 μL ethanol for a total transfer volume of 20 μL/well (the final concentration of [^{3}H]colchicine/well was approximately 0.08 μM). For K_d experiments, the appropriate volume of radiolabeled colchicine (0.03–16 μCi/reaction) was added to a final transfer volume of 20 μL/well. Serial dilutions of unlabeled compounds (1×10^{-3}–1×10^{-11} M) were prepared in ethanol for a total transfer volume of 100 μL/well. The radiolabeled colchicine and unlabeled compounds were transferred to a 96-well plate, and the ethanol was evaporated using a SpeedVac® centrifuge for 15 min. The 90-μL binding buffer [80 mM PIPES (pH 6.8), 1 mM EGTA, 1 mM $MgCl_2$ and 1 mM GTP] was added and followed by 0.5 μg special long-chain, biotin-labeled tubulin prepared in 10 μL binding buffer. The plates were incubated for 2 h at 37°C with gentle shaking. After 2 h, 0.08 mg streptavidin-labeled yttrium SPA beads (Amersham Pharmacia Biotech, Piscat-

away, NJ, USA) in 20 µL binding buffer were added, and the bound radioactivity was determined using a Packard TopCount™ Microplate Scintillation Counter (Packard Instrument, Meriden, CT, USA). The cost of the beads on a per-well basis was approximately $0.16 (or about $15/96-well plate). Nonspecific binding was determined in the presence of excess unlabeled colchicine (>100× K_d). Nonlinear regression was used to analyze the data using GraphPad Prism™, Version 2.0.

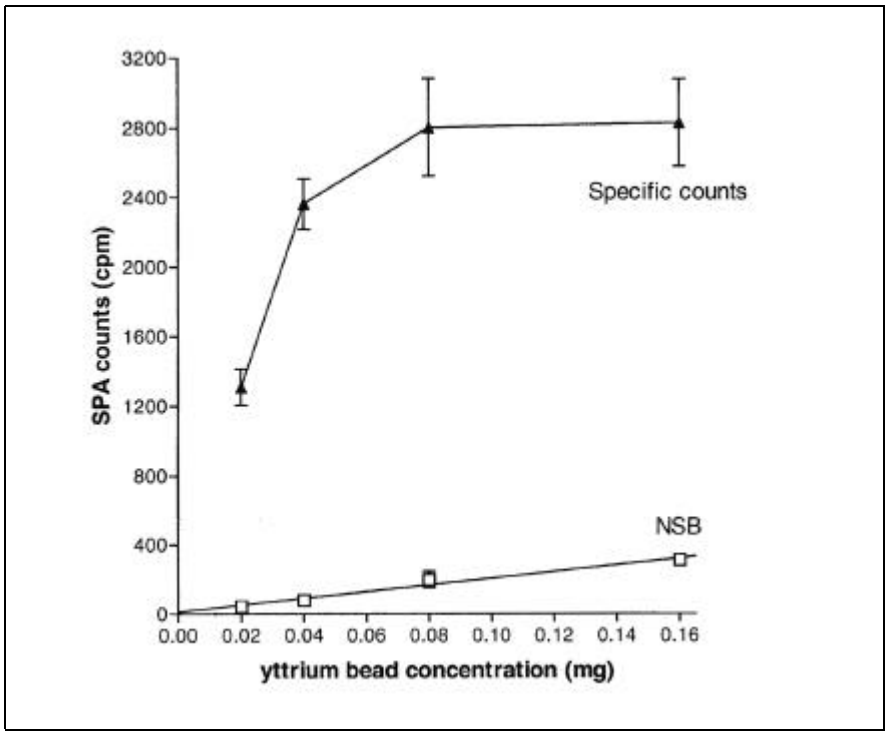

Figure 2. Optimization of bead concentration. Optimal bead concentration was determined by first incubating tubulin with [^{3}H]colchicine for 2 h. Various amounts of yttrium beads were added and the specific counts and NSB were determined after 15 min of gentle shaking. Each point represents mean ± SD of $n = 9$.

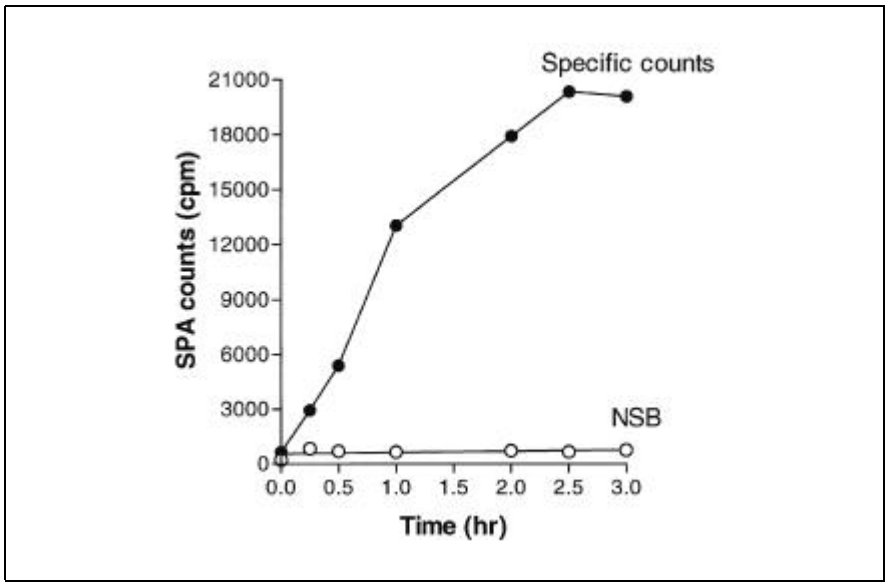

Figure 3. Time course for colchicine binding to tubulin. [^{3}H]colchicine (0.163 µM) was incubated with biotin-tubulin (1 µg) for varying times at 37°C. The specific and nonspecific counts were determined 15 min after the addition of beads.

RESULTS AND DISCUSSION

Bead Concentration

The optimal bead concentration to obtain the maximum specific signal with low nonspecific background (NSB) was determined by adding varying amounts of yttrium beads to a fixed amount of [^{3}H]colchicine and tubulin. As Figure 2 shows, the specific signal increased linearly and became saturated above 0.08 mg/well. The NSB increased linearly from 0.01 to 0.16 mg/well. We chose 0.08 mg/well as our optimal bead concentration because it gave us a high specific signal while still having a low NSB (5%). Given the low NSB, our assay is suitable for high-throughput screening. Assays having a specific binding component of greater than 80% are suitable for high-volume screening because they are reproducible and reliable as drug discovery tools (25).

Incubation Time

We examined the binding characteristics of colchicine to tubulin over time at 37°C. Colchicine was shown previously to reversibly bind to tubulin in a two-step manner involving a fast initial binding phase of relatively low affinity followed by a slow conformational change of the complex (10,17,20). As previously described, we observed that colchicine binding to tubulin was biphasic using our SPA system. Our data corroborates the results of Luyckx et al. (20) in that there was a relatively quick phase in which approximately 60% of the binding occurred, followed by a slower phase (Figure 3).

Effect of Solvents

Two solvents commonly used to dissolve drugs (DMSO and ethanol) were tested for their effect on the SPA counts. It was determined that the specific signal decreased as the concentration of ethanol or DMSO concentration was increased above 1% (Figure 4).

Determination of Colchicine Dissociation Constant of Binding (K_d) to Tubulin

The K_d for colchicine binding to tubulin was determined by increasing the concentration of radioligand (0.005 –2.6 μM) with a constant amount of tubulin (0.5 μg). GraphPad Prism was used to determine the B_{max} (total receptor number) and K_d using nonlinear regression to fit the data to a one-site binding curve using the formula

$$\text{specific binding} = \frac{B_{max} \times [L]}{K_d + [L]}$$

where [L] is the concentration of free radioligand, B_{max} is the total number of

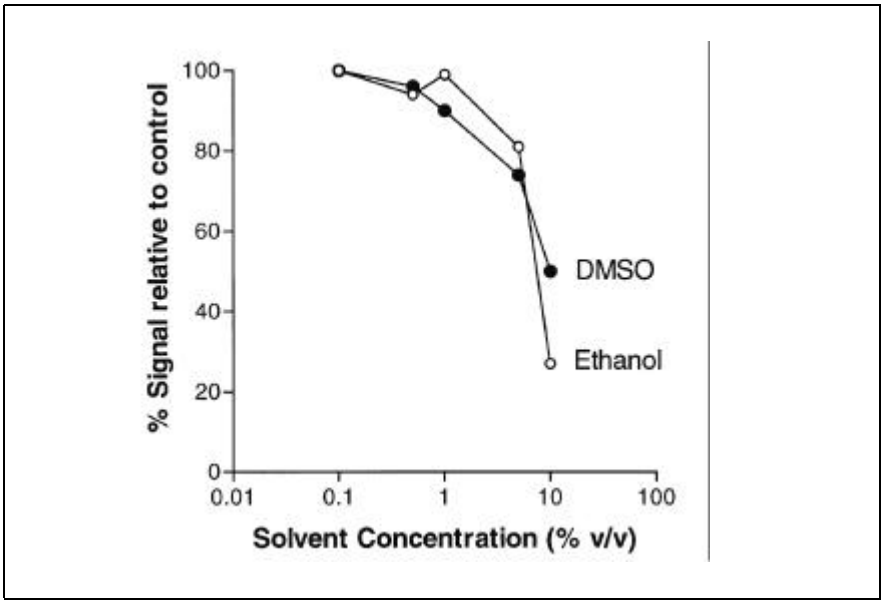

Figure 4. Effect of solvents. [^{3}H]colchicine was incubated with biotin-tubulin for 1 h with or without varying concentrations of solvent. The specific counts in the presence of solvent was determined and expressed relative to the specific counts in the controls. A DMSO or ethanol concentration at or below 1% did not adversely affect the assay.

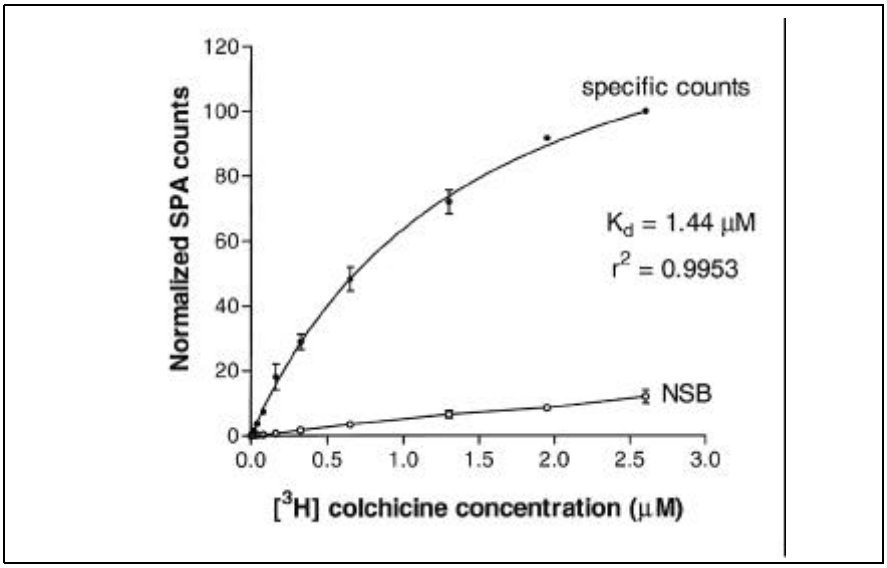

Figure 5. Determination of colchicine dissociation constant of binding (K_d) to tubulin. The K_d for colchicine binding to tubulin was determined by increasing the concentration of radioligand with a constant amount of tubulin (0.5 μg). Nonlinear regression was used to analyze the data using GraphPad Prism. Data represents mean ± SD.

receptors expressed in the same units as specific binding and K_d is the equilibrium dissociation constant (expressed in the same units as [L]).

The binding of colchicine to tubulin was saturable (Figure 5). The K_d for colchicine under our experimental conditions was 1.4 μM, which was consistent with previously reported values (16, 20). NSB increased in a linear manner as the concentration of radioligand was increased, and background accounted for only 3%–10% of the total counts. The portion of the curve to the right showing binding at higher radioligand concentrations did not reach complete saturation. We did not go higher because of the expense of the radioligand and the NSB might be too high a fraction of total binding.

Inhibition of Colchicine Binding to Tubulin

Validation of the assay was performed by competitively inhibiting [^{3}H]colchicine binding to tubulin with known microtubule inhibitors and comparing their K_i values when possible. The competition assays were performed by holding the protein and radioligand concentrations constant and varying the concentration of the unlabeled competitor. The K_i values for each compound were used to compare their effect on colchicine binding since IC_{50} values tend to vary between laboratories because of the different methodologies used and variation in radioligand concentration. K_i values were calculated using the Cheng-Prussof equation (6). Nonlinear regression analysis was used to fit the data to a one-site competition-binding curve using GraphPad Prism. The K_i values for combretastatin A4, E7010, podophyllotoxin, mebendazole, colcemid and colchicine, reported to bind to tubulin at the colchicine site range between 0.2–4.6 μM, are listed in Table 1. Taxol and vinblastine, which bind to different sites on tubulin, did not compete with [^{3}H]colchicine binding to tubulin at concentrations below 100 μM. The values of various microtubule inhibitors in our SPA assay were consistent with K_i values reported by others (Table 1).

In summary, our assay is useful for evaluating antimitotic compounds in vitro and can overcome the disadvantage of traditional filtration techniques since there are no separation steps. Our SPA bead method is extremely easy to set up, multiple samples can be assayed, supply and labor costs are minimal and it has utility as a high-throughput screening tool.

REFERENCES

1. **Arai, T. and T. Okuyama.** 1975. Fluorometric assay of tubulin-colchicine complex. Anal. Biochem. *69*:443-450.
2. **Basu, A. and S. Haldar.** 1998. Microtubule-damaging drugs triggered bcl2 phosphorylation-requirement of phosphorylation on both serine-70 and serine-87 residues of bcl2 protein. Int. J. Oncol. *13*:659-664.
3. **Bershadsky, A.D. and J.M. Vasiliev.** 1989. Cytoskeleton. Plenum Press, New York.
4. **Blagosklonny, M.V., P. Giannakakou, W.S. El-Deiry, D.G.I. Kingston, P.I Higgis, L. Neckers and T. Fojo.** 1997. Raf-1/bcl2 phosphorylation: a step from microtubule damage to cell death. Cancer Res. *57*:130-135.
5. **Borisy, G.G.** 1972. A rapid method for quantitative determination of microtubule protein using DEAE-cellulose filters. Anal. Biochem. *50*:373-385.
6. **Cheng, Y. and W.H. Prusoff.** 1973. Relationship between the inhibition constant (K_i) and the concentration of an inhibitor which causes 50 per cent inhibition (I_{50}) of an enzymatic reaction. Biochem. Pharmacol. *22*:3099-3108.
7. **Chaplin, D.J., G.R. Pettit and S.A. Hill.** 1999. Anti-vascular approaches to solid tumour therapy: evaluation of combretastatin A4 phosphate. Anticancer Res. *19*:189-195.
8. **Detrich, H.W., III, S.A. Berkowitz, H. Kim and R.C. Williams, Jr.** 1976. Binding of glycerol by microtubule protein. Biochem. Biophys. Res. Commun. *68*:961-968.
9. **Dustin, P**. 1984. Microtubules. Springer-Verlag, Berlin.
10. **Garland, D.** 1978. Kinetics and mechanism of colchicine binding to tubulin. Evidence for ligand-induced conformational change. Biochemistry *17*:4266-4271.
11. **Hamel, E.** 1996. Antimitotic natural products and their interactions with tubulin. Biochemistry *16*:207-231.
12. **Hyams, J.S. and C.W. Lloyd.** 1994. Microtubules. Wiley-Liss, New York.
13. **Jordan, M.A., J.A. Hadfield, N.J. Lawrence and A.T. McGown.** 1998. Tubulin as a target for anticancer drugs: agents which interact with the mitotic spindle. Med. Res. Rev. *18*:259-296.
14. **Jordan, M.A. and L. Wilson.** 1998. Microtubules and actin filaments: dynamic targets for cancer chemotherapy. Curr. Opin. Cell Biol. *10*:123-130.
15. **Jordan, M.A. and L. Wilson.** 1998. Use of drugs to study role of microtubule assembly dynamics in living cells. Methods Enzymol. *298*:252-276.
16. **Kelleher, J.K.** 1977. Tubulin binding affinities of podophyllotoxin and colchicine analogues. Mol. Pharmacol. *13*:232-241.
17. **Lambier, A. and Y. Engelborghs.** A quantitative analysis of tubulin-colchicine binding to microtubules. 1980. Eur. J. Biochem. *109*:619-624.
18. **Liliom, K., A. Lehotzky, A. Molnar and J. Ovadi.** 1995. Characterization of tubulin-alkaloid interactions by enzyme-linked immunosorbent assay. Anal. Biochem. *228*:18-26.
19. **Lin, C.M., H.H. Ho, G.R. Pettit and E. Hamel.** 1989. Antimitotic natural products combretastatin A-4 and Combretastatin A-2: studies on the mechanism of their inhibition of the binding of colchicine to tubulin. Biochemistry *28*:6984-6991.
20. **Luyckx, M., C. Brunet and M. Cazin.** 1984. Binding of colchicine to beef brain tubulin: influence of methodology on binding constants. Methods Find. Exp. Clin. Pharmacol. *6*:679-684.
21. **Nogales, E., S.G. Wolf and K.H. Dowing.** 1998. Structure of the αβ tubulin dimer by electron crystallography. Nature *391*:199-202.
22. **Ray, K., B. Bhattacharyya and B.B. Biswas.** 1981. Role of B-ring of colchicine in its binding to tubulin. J. Bio. Chem. *256*:6241-6244.
23. **Russell, G., J.H. Gill and E. Lacey.** 1992. Binding of [^{3}H]benzimidazole carbamates to mammalian brain tubulin and the mechanism of selective toxicity of the benzimidazole anthelmintics. Biochem. Pharmacol. *43*:1095-1100.
24. **Sherline, P., C.K. Bodwin and D.M. Kipnis.** 1974. A new colchicine binding assay for tubulin. Anal. Biochem. *62*:400-407.
25. **Sweetnam, P.M., L. Caldwell, J. Lancaster, C. Bauer, Jr., B. McMillan, W.J. Kinnier and C.H. Price.** 1993. The role of receptor binding in drug discovery. J. Natural Products *56*:441-455.
26. **Valdivia, M. and B.R. Brinkley.** 1984. Biochemical studies of the kinetochore/centromere of mammalian chromosomes, p. 79-86. *In* G.G. Borisy, D.W. Cleveland and D.B. Murphy (Eds.), Molecular Biology of the Cytoskeleton. CSH Laboratory Press, Cold Spring Harbor, New York.
27. **Wilson, L. and K.W. Farrell.** 1986. Kinetics and steady state dynamics of tubulin addition and loss at opposite microtubule ends:the mechanism of action of colchicine. Ann. NY Acad. Sci. *466*:690-708.
28. **Yamamoto, K., K. Noda, A. Yoshimura, M. Fukuoka, K. Furuse and H. Niitani.** 1998. Phase I study of E7010. Can. Chemother. Pharmacol. *42*:127-134.
29. **Yoshimatsu, K., A. Yamaguchi, H. Yoshino, N. Koyanagi and K. Kitoh.** 1997. Mechanism of action of E7010, an orally active sulfonamide antitumor agent: inhibition of mitosis by binding to the colchicine site of tubulin. Cancer Res. *57*:3208-3213.

Received 18 October 1999; accepted 10 February 2000.

Address correspondence to:
Dr. Shi-Chung Ng
Abbott Laboratories
Dept. 4N6, AP9-2
100 Abbott Park Rd
Abbott Park, IL 60064, USA
e-mail: shi-chung.c.ng@abbott.com