Biochemical Pharmacology, Vol. 43, No. 3, pp. 539–543, 1992.
Printed in Great Britain.

0006-2952/92 $5.00 + 0.00
© 1992. Pergamon Press plc

INTERACTION OF DOLASTATIN 10 WITH BOVINE BRAIN TUBULIN

RICHARD F. LUDUEÑA,*† MARY CARMEN ROACH,* VEENA PRASAD* and GEORGE R. PETTIT‡

*Department of Biochemistry, University of Texas Health Science Center at San Antonio, San Antonio, TX 78284-7760; and ‡Cancer Research Institute and Department of Chemistry, Arizona State University, Tempe, AZ 85287, U.S.A.

(*Received* 18 *June* 1991; *accepted* 25 *September* 1991)

Abstract—Dolastatin 10 is an unusual peptide of marine origin which binds to tubulin in the vinblastine/maytansine/phomopsin-binding region and potently inhibits mitosis. Using *N,N'*-ethylenebis(iodoacetamide) (EBI) and iodo[^{14}C]acetamide as probes for the effects of ligands on the thiol groups of tubulin, we found that dolastatin 10 has effects on the sulfhydryls indistinguishable from those of phomopsin A but quite different from those of vinblastine and maytansine. Using the binding of bis-5,5'-[8-(*N*-phenyl)aminonaphthalene-1-sulfonic acid] (BisANS) as a measure of tubulin decay, we found that dolastatin 10 resembled phomopsin A in that decay was not detectable by this assay in its presence. Interestingly, both otherwise very different peptides are among the most effective inhibitors of tubulin decay yet discovered.

Microtubules are cylindrical organelles, playing critical roles in a variety of cellular processes, such as mitosis [1]. They are composed of the 100 kDa protein tubulin, which is a dimer of two 50 kDa polypeptide chains designated α and β [2]. Tubulin is a target for a large number of anti-mitotic drugs which have served as excellent probes to obtain information on the structure and function of tubulin [3]. A rapid and discriminating assay for the interaction of a drug with the tubulin molecule is the effect of the drug on the relative reactivities of the tubulin thiol groups. We have found that when tubulin is allowed to react with the bifunctional alkylating agent *N,N'*-ethylenebis(iodoacetamide) (EBI§), this compound forms two intra-chain cross-links in the β subunit of tubulin [4, 5]. One cross-link, designated β^*, is between Cys^{239} and Cys^{354} [6]. The other cross-link, designated β^s, is between Cys^{12} and either Cys^{201} or Cys^{211} [7]. Formation of an intra-chain cross-link produces a species of β-tubulin with a distinct mobility when subjected to polyacrylamide gel electrophoresis; this makes the effect of the drug very easy to measure. Colchicine, together with podophyllotoxin and other drugs which inhibit colchicine binding, blocks formation of the β^* cross-link, while enhancing formation of the β^s cross-link [6, 8]. In contrast, vinblastine, maytansine, phomopsin A and rhizoxin inhibit formation of the β^s cross-link and enhance that of the β^* cross-link [6, 8–11]. Interestingly, vinblastine and other *Vinca* alkaloids only partially inhibit β^s formation, whereas maytansine, phomopsin A and rhizoxin completely inhibit this process [6, 10, 11]. Rhizoxin, maytansine and phomopsin A are inhibitors of the binding of vinblastine to tubulin [12–14]. Another way in which the specific effects of the drugs on tubulin thiol groups can be measured is by their effects on the alkylation of tubulin by iodo[^{14}C]acetamide. Vinblastine and phomopsin A are potent inhibitors of alkylation, while colchicine is somewhat weaker, podophyllotoxin still weaker, and maytansine has little or no effect [6, 8, 10]. The effect of a drug on the alkylation of tubulin by iodo[^{14}C]acetamide appears to be correlated with its effect on the decay of tubulin as measured by the effect of the drug on the interaction of tubulin with bis-5,5'-[8-(*N*-phenyl)aminonaphthalene-1-sulfonic acid] (BisANS), a fluorescent probe which binds to hydrophobic areas on the surfaces of proteins [15]. As the tubulin molecule decays, its binding to BisANS increases [15]. We have found previously that decay, as measured by BisANS binding, is blocked completely by phomopsin A, and then in decreasing order by vinblastine, colchicine, and podophyllotoxin, while maytansine and rhizoxin have no effect [11, 15, 16].

In short, the region of the tubulin molecule to which vinblastine binds undergoes rather complex interactions with drugs. Three groups of drugs bind here. First, there are the *Vinca* alkaloids, which partially inhibit β^s cross-link formation and strongly inhibit decay; second, there are maytansine and rhizoxin, competitive inhibitors of the binding of vinblastine to tubulin, which completely block β^s formation and have little effect on decay; and third, there is phomopsin A, a non-competitive inhibitor of the binding of vinblastine, which strongly inhibits β^s formation and is an even stronger inhibitor of decay than is vinblastine.

Dolastatin 10 (Fig. 1) is a modified peptide with strong anti-mitotic activity isolated from the marine mollusk *Dolabella auricularia* [17]. Extracts of these

† Corresponding author: Dr. Richard F. Ludueña, Department of Biochemistry, University of Texas Health Science Center, 7703 Floyd Curl Drive, San Antonio, TX 78284-7760. Tel. (512) 567-3732; FAX (512) 567-3719.

§ Abbreviations: EBI, *N,N'*-ethylenebis(iodoacetamide); and BisANS, bis-5,5'-[8-(*N*-phenyl)aminonaphthalene-1-sulfonic acid].

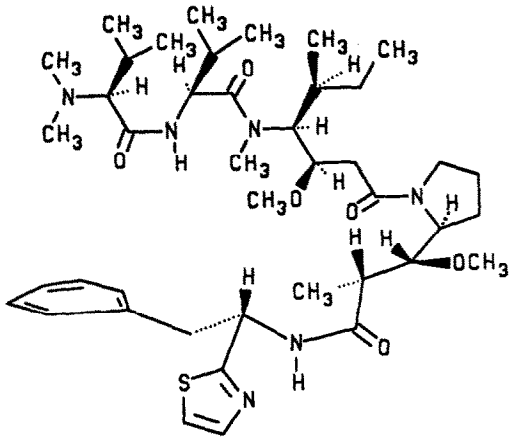

Fig. 1. Structure of dolastatin 10.

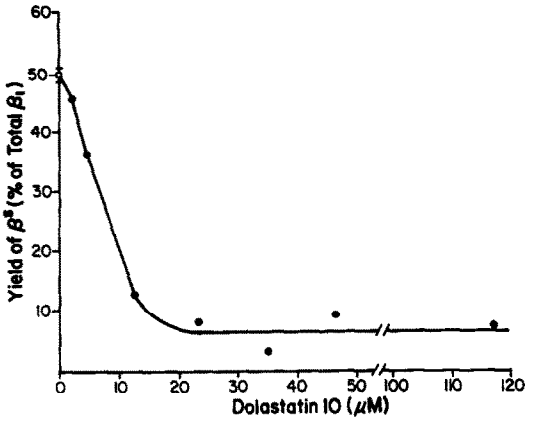

Fig. 3. Effect of dolastatin 10 on the formation of the β^s cross-link by EBI. Aliquots (250 μL) of tubulin (0.66 mg/mL) containing reduced and carboxymethylated conalbumin (0.2 mg/mL) were incubated for 1 hr at 30° in the absence (○) or presence (●) of the indicated concentrations of dolastatin 10. The samples were then reduced and carboxymethylated and subjected to polyacrylamide gel electrophoresis using the system of Laemmli [28] as modified by Banerjee *et al.* [29]. The yield of the β^s cross-link was calculated as previously described [6]. The sample lacking dolastatin 10 was incubated in duplicate; the range of values is shown for that sample.

mollusks have an ancient and dramatic history [18, 19]; they are speculated to have been employed in 54 A.D. by the Roman empress Agrippina the Younger and her son Nero to poison her husband Claudius and step-son Britannicus in order to facilitate the accession of Nero to the throne [20]. Dolastatin 10 is a non-competitive inhibitor of the binding of vinblastine to tubulin ($K_i = 1.4\ \mu M$) [21]. Phomopsin A (Fig. 2), which is also a modified peptide, though otherwise quite different from dolastatin, is also a non-competitive inhibitor of the binding of vinblastine to tubulin ($K_i = 2.8\ \mu M$) [21] To determine if dolastatin 10 has effects similar to those of phomopsin A, we examined its effects on the thiol groups of tubulin and on tubulin decay. We found that dolastatin 10 is like phomopsin A, in that it completely blocks β^s formation, and strongly inhibits alkylation and decay.

METHODS

Materials. Dolastatin 10 was synthesized as recorded earlier [22]. EBI was synthesized from ethylenediamine and iodoacetic anhydride as previously described [23]. Vinblastine was the gift of the Eli Lilly Corp., Indianapolis, IN. BisANS was obtained from Molecular Probes, Inc. (Junction City, OR). Microtubules were prepared from bovine cerebra by cycling, and tubulin was purified from the microtubules by phosphocellulose chromatography according to the method of Fellous *et al.* [24]. For the experiment with EBI, tubulin was purified from microtubules by chromatography on phosphocellulose and Sephadex G-25 as described by Roach and Ludueña [5]. Unless otherwise indicated, all experiments were done in 100 mM 2-(*N*-morpholino)ethanesulfonic acid, pH 6.4, 1 mM ethylene glycol bis(β-aminoethyl ether)-*N,N'*-tetraacetic acid, 0.1 mM ethylenediaminetetraacetic acid, 0.5 mM $MgCl_2$, 1 mM GTP and 1 mM β-mercaptoethanol. In experiments involving EBI and iodo[^{14}C]acetamide, the buffer lacked β-mercaptoethanol. All other materials were obtained as previously described [23].

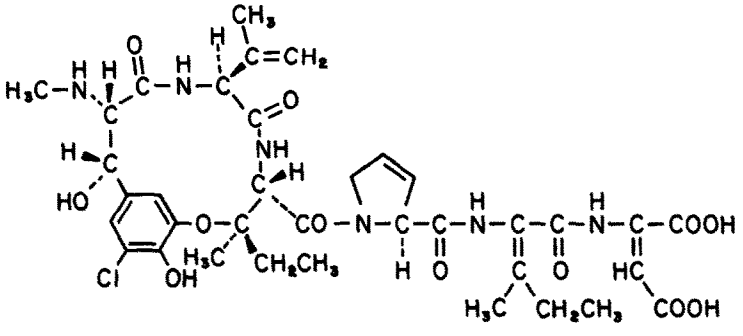

Fig. 2. Structure of phomopsin A.

Table 1. Effects of dolastatin 10 and vinblastine on the alkylation of tubulin by iodo[^{14}C]acetamide*

Addition	moles ^{14}C/100 kDa	% of Control
None	3.39 ± 0.14 (4)†	100 ± 4 (4)
Dolastatin 10, 50 μM	1.38 ± 0.06 (3)	41 ± 2 (3)
Vinblastine, 50 μM	1.76 ± 0.06 (4)	52 ± 3 (4)

* Aliquots (250 μL) of tubulin (0.66 mg/mL) were incubated with 1.36 mM iodo[^{14}C]acetamide (0.54 Ci/mol) for 1 hr at 37°07. They were then precipitated and filtered, and the radioactivity of the filters was determined as previously described [9]. Values are means ± SD.

† Number of aliquots.

Cross-linking. Tubulin was incubated with EBI, reduced and carboxymethylated, and subjected to polyacrylamide gel electrophoresis, and the relative yields of the β^s and β^* bands were calculated as previously described [8]. The yields of β^s and β^* are expressed as a percentage of the total β_1. This is based on previous observations that both the β^s and β^* cross-links are formed from the β_1 band and not from the β_2 band [6, 23]. The β_1 band consists of the β_I, β_{II} and β_{IV} isotypes of tubulin and the β_2 band is formed from the β_{III} isotype [25].

Alkylation with iodo[^{14}C]acetamide. Tubulin was incubated with iodo[^{14}C]acetamide, precipitated, and filtered, and the filters were counted according to the procedure of Ludueña and Roach [9].

Fluorescence. Tubulin was incubated at 37°, and then at intervals aliquots were removed and mixed with BisANS and placed in a SLM/Aminco SPF-500C spectrofluorometer set in the ratio mode. Excitation was at 385 nm and emission was at 490 nm.

Other methods. Protein concentrations were determined, using bovine serum albumin as a standard, by the method of Lowry *et al.* [26] as modified by Schacterle and Pollack [27]. Unless otherwise indicated, gel electrophoresis was carried out on 5.5% polyacrylamide gels using the system of Laemmli [28]. When an accurate estimation of the yield of the β^s band was desired, samples were analyzed by the modified Laemmli system of Banerjee *et al.* [29] in which the buffer in the resolving gel is 0.74 M Tris–HCl, pH 9.1. In this system, the band containing the β^s cross-link is well-resolved from the β_1 band, which is not the case in the unmodified system [29]. However, in the modified system, the band containing the β^* cross-link co-migrates with the band containing the β^s cross-link; therefore, the reactions of these samples with EBI needed to be carried out in the presence of podophyllotoxin to prevent formation of the β^* cross-link.

RESULTS AND DISCUSSION

Preliminary results indicated that dolastatin 10 blocked β^s formation and enhanced β^* formation. In one experiment, the yield of β^s, measured in the

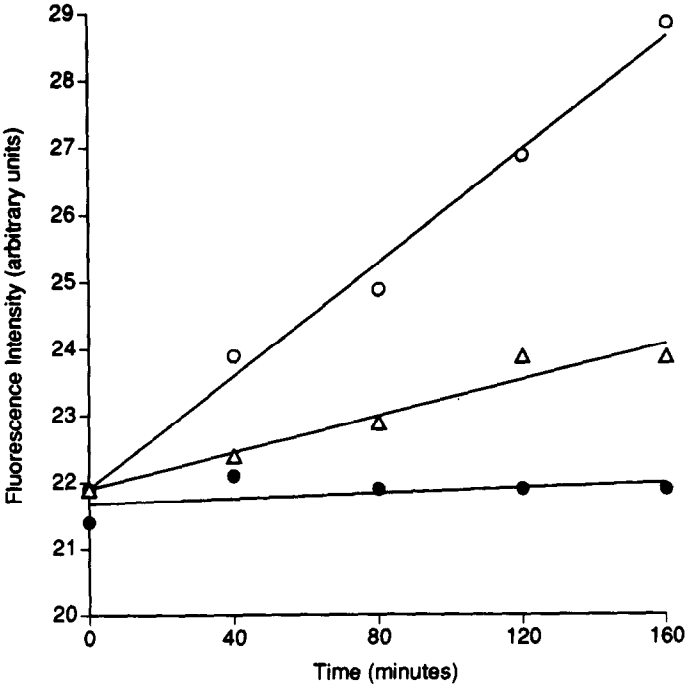

Fig. 4. Effects of dolastatin 10 and vinblastine on the binding of BisANS to tubulin. Three 5-mL samples of tubulin (0.2 mg/mL) were incubated at 37° in the absence (○) or presence of 20 μM concentrations of either vinblastine (△) or dolastatin 10 (●). At the times shown, 1-mL aliquots of each solution were removed, made 10 μM in BisANS and placed in fluorescence cuvettes; the fluorescence was measured as described in the text.

unmodified Laemmli system in the absence of drug, was 18.3 ± 1.4%; in the presence of 50 μM dolastatin 10, no β^s cross-link was generated. Where the yield of the β^* cross-link in the control was 23 ± 1%, the yield of β^* in the presence of dolastatin 10 was 57%, a significant increase. In this respect, dolastatin 10 differs strikingly from colchicine and podophyllotoxin which inhibit β^* formation and enhance β^s formation. In the same experiment, in which β^s cross-link formation was measured in the modified Laemmli system, the yield of β^s in the absence of added drug was 29.6 ± 0.1% and, in the presence of 50 μM concentrations of vinblastine, maytansine and dolastatin 10, the yields of β^s were, respectively, 16, 6 and 6%. Therefore, dolastatin 10 appears to cause as complete an inhibition of β^s formation as do maytansine and phomopsin A, in contrast to vinblastine, which causes only partial blockage of β^s formation. Based on these effects, dolastatin 10 should be classified with maytansine, rhizoxin, and phomopsin A in terms of the nature of its interaction with the tubulin molecule. The concentration-dependence of the inhibition of dolastatin of β^s formation is shown in Fig. 3. The results suggest that half-maximal inhibition of β^s formation in 6.6 μM tubulin is caused by 7 μM dolastatin.

When the effect of dolastatin 10 on the reaction of tubulin with iodo[^{14}C]acetamide was measured, the results shown in Table 1 were obtained. Dolastatin 10 caused a 59% decrease in alkylation as opposed to the 48% decrease caused by vinblastine. This result distinguishes dolastatin 10 from maytansine and rhizoxin which have little or no effect on the reaction of tubulin with iodo[^{14}C]-acetamide [9, 30] and places dolastatin 10 in the same category as phomopsin A whose effect on tubulin alkylation is comparable to that of vinblastine.

The effect of dolastatin 10 on tubulin decay as measured by BisANS binding is shown in Fig. 4. The results suggest that dolastatin 10 is a potent inhibitor of decay. The almost flat line seen in Fig. 4 is virtually the same as that obtained with phomopsin A. Both drugs seem to inhibit decay very effectively, whereas vinblastine is a weaker inhibitor. Vinblastine and dolastatin 10 decreased the rate of BisANS binding to tubulin by 67 and 95%, respectively. Previous work suggested that phomopsin A reduced decay to a level that could not be detected by BisANS binding; however, some decay of [^{3}H]colchicine binding was apparent, although this was much slower than that observed in the presence of vinblastine [10, 16]. Hamel and colleagues [21] have found that dolastatin 10 stabilizes [^{3}H]colchicine binding about as much as does phomopsin A and significantly more than does vinblastine.

Apparently, in its interaction with tubulin, dolastatin 10 is very similar to phomopsin A as Hamel and his colleagues [21] proposed. Both phomopsin A and dolastatin 10 are modified peptides; nevertheless, their sequences are not the same. The sequence of phomopsin A can be described as a modification of YVVPVD. That of dolastatin 10 can be described as a modification of VVIPF. The results presented here and those of Bai *et al.* [21] suggest that the binding sites on the tubulin molecule of both dolastatin 10 and phomopsin A are the same. There is also likely to be some relationship among this site and the binding sites for maytansine and the *Vinca* alkaloids. Based on our results with dolastatin 10 and phomopsin A [10, 16], one can postulate that there is a region(s) on the tubulin molecule which controls the decay of tubulin. Whether one or all of these regions contain clefts which open, exposing hydrophobic areas, as tubulin decays, or whether they allosterically control such a cleft-containing area, is not clear. Possibly one of these regions interacts differently with different portions of the phomopsin/dolastatin–maytansine–vinblastine binding area. Presumably, this decay-controlling region would not impinge on the maytansine portion of the binding area, thereby explaining why maytansine does not slow down decay. However, this decay-controlling region would have a small overlap with the vinblastine-binding site to account for the ability of vinblastine to significantly slow down the decay of tubulin. Finally, this decay-controlling region would overlap greatly with the phomopsin/dolastatin binding site, thereby accounting for the ability of these drugs to almost completely block tubulin decay. Prasad *et al.* [15] have proposed a hypothesis that microtubule assembly is accompanied by an orderly conformational change in the tubulin molecule, exposing hydrophobic tubulin–tubulin binding sites, and that when normal microtubule assembly cannot take place, a similar but less ordered conformational change occurs, and that this change results in decay. If this hypothesis is correct, then the binding site for dolastatin 10 and phomopsin A may play an important role in regulating microtubule assembly. This supposition is consistent with the observation of Bai *et al.* [21] that dolastatin 10 and phomopsin A are more effective than maytansine at inhibiting the binding to tubulin of GTP, which is required for, and whose hydrolysis accompanies, normal microtubule assembly.

Acknowledgements—We are grateful to Mohua Banerjee for her assistance. This research was supported by Grants GM23476 from the National Institutes of Health and AQ-0726 from the Robert A. Welch Foundation to R.F.L. The ASU CRI research was supported by Outstanding Investigator Grant CA44344-01A1-02, the Arizona Disease Control Research Commission and the Fannie E. Rippel Foundation. For other assistance we are also pleased to thank Dr. Cherry L. Herald.

REFERENCES

1. Dustin P, *Microtubules*. Springer, Berlin, 1988.
2. Ludueña RF, Shooter EM and Wilson L. Structure of the tubulin dimer. *J Biol Chem* **252**: 7006–7014, 1977.
3. Ludueña RF, Biochemistry of tubulin. In: *Microtubules* (Eds. Roberts K and Hyams J), pp. 65–116. Academic Press, London, 1979.
4. Ludueña RF and Roach MC, Interaction of tubulin with drugs and alkylating agents. 1. Alkylation of tubulin by iodo[^{14}C]acetamide and *N,N'*-ethylene-bis(iodoacetamide). *Biochemistry* **20**: 4437–4444, 1981.
5. Roach MC and Ludueña RF, Different effects of tubulin ligands on the intrachain cross-linking of β_1-tubulin. *J Biol Chem* **259**: 12063–12071, 1984.
6. Little M and Ludueña RF, Structural differences

between brain β1-and β2-tubulins: Implications for microtubule assembly and colchicine binding. *EMBO J* **4**: 51–56, 1985.

7. Little M and Ludueña RF, Location of two cysteines in brain β_1-tubulin that can be cross-linked after removal of exchangeable GTP. *Biochim Biophys Acta* **912**: 28–33, 1987.
8. Ludueña RF and Roach MC, Interaction of tubulin with drugs and alkylating agents. 2. Effects of colchicine, podophyllotoxin, and vinblastine on the alkylation of tubulin. *Biochemistry* **20**: 4444–4450, 1981.
9. Ludueña RF and Roach MC, Contrasting effects of maytansine and vinblastine on the alkylation of tubulin sulfhydryls. *Arch Biochem Biophys* **210**: 498–504, 1981.
10. Ludueña RF, Roach MC, Prasad V and Lacey E, Effect of phomopsin A on the alkylation of tubulin. *Biochem Pharmacol* **39**: 1603–1608, 1990.
11. Sullivan AS, Prasad V, Roach MC, Takahashi M, Iwasaki S and Ludueña RF, Interaction of rhizoxin with bovine brain tubulin. *Cancer Res* **50**: 4277–4280, 1990.
12. Bhattacharyya B and Wolff J, Maytansine binding to the vinblastine sites of tubulin. *FEBS Lett* **75**: 159–162, 1977.
13. Lacey E, Edgar JA and Culvenor CCJ, Interaction of phomopsin A and related compounds with purified sheep brain tubulin. *Biochem Pharmacol* **36**: 2133–2138, 1987.
14. Takahashi M, Iwasaki S, Kobayashi H, Okuda S, Murai T and Sato Y, Rhizoxin binding to tubulin at the maytansine-binding site. *Biochim Biophys Acta* **926**: 215–223, 1987.
15. Prasad ARS, Luduena RF and Horowitz PM, Bis(8-anilinonaphthalene-1-sulfonate) as a probe for tubulin decay. *Biochemistry* **25**: 739–742, 1986.
16. Ludueña RF, Prasad V, Roach MC and Lacey E, The interaction of phomopsin A with bovine brain tubulin. *Arch Biochem Biophys* **272**: 32–38, 1989.
17. Bai R, Pettit GR and Hamel E, Dolastatin 10, a powerful cytostatic peptide derived from a marine animal. Inhibition of tubulin polymerization mediated through the vinca alkaloid binding domain. *Biochem Pharmacol* **39**: 1941–1949, 1990.
18. Pliny the Elder. *The Historie of the World: commonly called, the Naturall Historie of C. Plinius Secundus* (Trans. Holland P). Impensis G.B., London, 1601.
19. Aetius of Amida, *Contractae ex Veteribus Medicinae Sermones XVI*. Ioannis Gryphius, Venice, 1553.
20. Donati G and Porfirio B, Marine pharmacology and toxicology. The dolastatins. *Conchiglia* **16**: May 1984.
21. Bai R, Pettit GR and Hamel E, Binding of dolastatin 10 to tubulin at a distinct site for peptide antimitotic agents near the exchangeable nucleotide and vinca alkaloid sites. *J Biol Chem* **265**: 17141–17149, 1990.
22. Pettit GR, Singh SB, Hogan F, Lloyd-Williams P, Herald DL, Burkett DD and Clewlow PJ, The absolute configuration and synthesis of natural (−)-dolastatin 10. *J Am Chem Soc* **111**: 5463–5465, 1989.
23. Ludueña RF, Roach MC, Trcka PP, Little M, Palanivelu P, Binkley P and Prasad V, β_2-Tubulin, a form of chordate brain tubulin with lesser reactivity toward an assembly-inhibiting sulfhydryl-directed cross-linking reagent. *Biochemistry* **21**: 4787–4794, 1982.
24. Fellous A, Francon J, Lennon AM and Nunez J, Microtubule assembly *in vitro*. *Eur J Biochem* **78**: 167–174, 1977.
25. Banerjee A, Roach MC, Wall KA, Lopata MA, Cleveland DW and Ludueña RF, A monoclonal antibody against the type II isotype of β-tubulin. Preparation of isotypically altered tubulin. *J Biol Chem* **263**: 3029–3034, 1988.
26. Lowry OH, Rosebrough NJ, Farr AL and Randall RJ, Protein measurement with the Folin phenol reagent. *J Biol Chem* **193**: 265–275, 1951.
27. Schacterle GR and Pollack RL, A simplified method for the quantitative assay of small amounts of protein in biologic material. *Anal Biochem* **51**: 654–655, 1973.
28. Laemmli UK, Cleavage of structural proteins during the assembly of the head of bacteriophage T_4. *Nature* **227**: 680–685, 1970.
29. Banerjee A, Jordan MA, Little M and Luduena RF, The interaction of cystamine with bovine brain tubulin. *Eur J Biochem* **165**: 443–448, 1987.
30. Sullivan AS, Prasad V, Roach MC, Takahashi M, Iwasaki S and Luduena RF, The interaction of rhizoxin with tubulin. *J Cell Biol* **107**: 671a, 1988.