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DIFFERENCES IN THE BINDING PROPERTIES OF VINCA ALKALOIDS AND COLCHICINE TO TUBULIN BY VARYING PROTEIN SOURCES AND METHODOLOGY*

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Abstract—We have evaluated the effects that methodology and species derivation have on the measurement of the interaction of tubulin with the alkaloids colchicine (CLC) and vinblastine (VLB). The results show that two different methods analyzed simultaneously differed significantly in their binding data. Further, variations in the measured association constants were observed from species to species as well as within a specie. On the basis of these results we conclude that differences in the processes for measuring binding of alkaloids to tubulin can influence the values determined and that differences in binding observed with the same methodology may result from differences in the source of tubulin.

The alkaloids vinblastine (VLB) and colchicine (CLC) are known to bind specifically to tubulin, the soluble subunit protein of microtubules. This tubulin–alkaloid interaction has been widely employed to quantitate tubulin content in tissues and cells, to afford insight into some of the biochemical characteristics of the tubulin molecule, and to provide information about drug–protein binding sites [1–4].

The interaction of these alkaloids with tubulin has been assayed by measuring the amount of tritium-labeled alkaloid drug (CLC or VLB) bound to the tubulin preparation [5–12]. Both the amount of tubulin present and the association constant of binding (K_a) have been determined by means of Scatchard plots [5, 6, 9, 11–13]. The association constants of binding are measured as the slope of this line, and the intercept of the abscissa provides an estimate of the amount of tubulin present, when corrected for the extent of binding (60 per cent of maximum is achieved with CLC after 3 hr and 83 per cent is achieved with VLB in 15 min) [9].

Separation of the radioactive drug–protein complex from unbound drug has been accomplished by a variety of methods; for example: the labeled drug–tubulin complex may be adsorbed on DEAE-cellulose filter discs and rinsed to remove the unbound material [2, 10, 13–15]; or separation may be accomplished by gel filtration [2, 10, 13, 14].

Numerous reports have appeared in the literature quantitating the affinity of both CLC and VLB to tubulin. The range of values reported for the association constants of binding for these drugs has differed greatly among various investigators. In particular, marked differences have been reported in data obtained using the technique of adsorbing the labeled drug–protein complex onto DEAE-cellulose filter discs. Specifically, several papers reported the K_a for VLB binding to porcine brain tubulin [9, 13] to be $5.2 \times 6.0 \times 10^6$ l/mole, while others reported K_a values of 2×10^5 l/mole when embryo chick brain tubulin was analyzed [3]. The CLC binding data also varied, depending upon the source of tubulin and method of assay, with K_a values ranging from 4×10^4 to 2.5×10^7 l/mole [6, 16, 17].

One of the problems in evaluating these data revolves around the variations encountered in the methodology employed, making it difficult to determine whether differences in binding constants reflected significant differences among tubulins or differences due to the methods themselves.

In this paper, we have explored the binding of CLC and VLB to tubulin derived from several mammalian sources and determined that minor changes in methodology affect the data obtained, and that there are measurable differences in the binding properties of tubulin dependent upon its source.

MATERIALS AND METHODS

Chick embryo brain crude tubulin extraction procedures

Chick embryos, 16- to 18-days-old, were sacrificed and their brains removed by dissection and placed in cold (0°) 20 mM sodium phosphate (NaH_2PO_4: Na_2HPO_4) buffer containing 100 mM sodium glutamate (pH 6.8). The brains were rinsed once with cold buffer, weighed (less 10 per cent for adherent buffer) and an equal amount by weight of the phosphate–glutamate buffer plus 2.5 mM GTP (Sigma Chemical Co.) was added. The mixture was then homogenized with a motor driven Teflon–glass mortar and pestle for ten strokes. The homogenate was centrifuged for 45 min at 40,000 rev/min and 4°. The supernatant was decanted and used immediately, simultaneously performing the radiolabeled VLB and CLC binding experiments.

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Porcine brain crude tubulin extraction procedures

Fresh porcine brain was obtained from a local distributor (Esskay, Inc., Baltimore, MD) within 3 hr of slaughter. The meninges were removed and the brain was washed once in cold (0°) 0.1 M piperazine-*N*,*N*'-bis-(2-ethane sulfonic acid): 1.0 mM ethyleneglyol-bis-(*β* aminoethyl ether)-*N*, *N*'-tetra-acetic acid buffer (EGTA) (pH 6.94). One and a half parts by weight of brain cortex was homogenized with one part by weight of the above buffer containing 2.5 mM GTP in a motor driven Teflon–glass homogenizer using ten strokes. The homogenate was centrifuged at 20,000 *g* for 30 min, and the supernatant was decanted and used immediately.

Rat brain crude tubulin extraction procedures

Adult or newborn (1- to 2-day-old) rats were sacrificed and their brains removed by dissection and placed in cold (0°) 0.24 M sucrose: 0.0005 M $MgCl_2$:0.001 M EGTA:0.01 M NaH_2PO_4:Na_2HPO_4 (pH 6.5) buffer. The brains were rinsed once and then homogenized in three times their weight of the above buffer plus 1.0 mM GTP. The homogenate was centrifuged at 100,000 *g* at 4° for 1 hr. The supernatant was decanted and used immediately, simultaneously performing the radiolabeled VLB and CLC binding experiments.

Sea urchin egg vinblastine–crystal tubulin

Vinblastine-tubulin paracrystals were induced in unfertilized *S. purparatus* eggs by incubation of an egg culture in sea water with 2×10^{-4} M vinblastine sulfate for 36 hr at 15°. Crystals composed of pure tubulin complexed to vinblastine in a molar ratio of 1 mole vinblastine/mole of tubulin were isolated as described by Bryan [6]. Crystals were solubilized by dilution into low ionic strength buffer (1.0 mM sodium phosphate, pH 6.8).

General procedures

VLB[^{3}H] was prepared (by the method of Beer and Richards [18]) with a specific activity of 80.7 mCi/m-mole and a purity of 96 per cent. CLC[^{3}H] was purchased from New England Nuclear (Boston, MA) and diluted with unlabeled CLC to a specific activity of 45.4 mCi/m-mole.

Association constants were determined by incubating varying concentrations of CLC[^{3}H] or VLB[^{3}H] at 37° (15 min for VLB, 3 hr for CLC) with 100 *μ*l of tubulin protein solution (1.2 mg/ml of tubulin) diluted with the appropriate buffer to a final volume of 1.00 ml.

Method 1. A 100-*μ*l aliquot of the above 1.0-ml incubation mixture was removed and placed on two premoistened DEAE-cellulose (Whatman DE 81) filter discs. After 10 min, the filter discs were washed by immersion in three separate changes of buffer (the same buffer used to prepare the protein solution less GTP and sucrose). The paper discs with adherent protein drug complex were placed directly in scintillation vials with 10 ml Bray's solution, cooled for 1 hr, and counted in a scintillation spectrophotometer (Packard model 3320). The external standard technique was used to convert to total dis/min.

Method 2. The remaining 900 *μ*l of original incubation mixture was placed on two premoistened DEAE paper discs. These discs were contained in a special filtering assembly (described in Ref. 13). The pads were moistened prior to use with 1 ml of the same buffer used to prepare the crude tubulin, less GTP and sucrose. The test tube containing the incubation mixture was rinsed with approximately 2 ml of this same buffer. The incubation mixture and rinse were allowed to filter by gravity. Each paper and assembly were rinsed under gentle suction with three 5-ml aliquots of the appropriate buffer, and the paper disc was counted as described previously.

Protein concentrations were determined by the method of Lowry *et al.* [19], standardized against crystalline bovine serum albumin. The K_a of binding of tritium-labeled drug to the protein preparations, and the amount of tubulin present were determined from Scatchard plots [20] from the slope of the line and the abscissa intercepts respectively.

RESULTS

Two important observations were made concerning the data presented in Tables 1 and 2. First, the binding data, both association constants and intercepts, for the alkaloids CLC and VLB differed between Method 1 and Method 2. Second, the binding of the alkaloids to tubulin, as measured by their association constants, varied from species to species.

(A) Method 1 vs Method 2

The alkaloid tubulin interaction was simultaneously assayed by two methods, which differed in the amount of reaction material placed on the DEAE-

Table 1. Binding of CLC and VLB to crude tubulin preparations

Source of protein	Method 1 K_a (l/mole, 10°)	Method 1 Intercept (%)	Method 2 K_a (l mole, 10°)	Method 2 Intercept (%)	P*
CLC					
Pig	1.8 ± 0.2†	17 ± 4	3.0 ± 0.7	13 ± 2	0.025
Chick	1.2 ± 0.3	41 ± 16	2.3 ± 0.9	24 ± 6	0.05
	P = 0.01‡		P = 0.20		
VLB					
Pig	0.23 ± 0.04	18 ± 4	2.9 ± 0.2	4.2 ± 0.2	
Chick	0.094 ± 0.035	48 ± 2	1.1 ± 0.6	8.1 ± 0.3	
	P = 0.01		P = 0.005		

* Comparison of Method 1 and Method 2, using Student's *t*-test.

† All numbers are the average of three runs.

‡ Comparison of pig and chick, using Student's *t*-test.

cellulose filter discs and in the method of rinse. The two methods are comparable for there is no evidence that in the larger amount of protein placed on the DEAE-cellulose filter discs in Method 2 saturated the ability of the paper to bind the drug–protein complex. Pilot experiments were conducted in which the number of discs in a given determination were varied from 1 to 4. Only a slight rise in associated dis/min was found, identical to the expected increase in background counts for each individual disc. In Method 1, further rinsing caused no change in the counts obtained.

Table 1 compares these two methods using two protein solution sources (porcine and chick embryo brain). Association constants and intercept data for CLC and VLB binding were the criteria used to compare Methods 1 and 2.

Association constants. Using crude porcine brain tubulin, the K_a for CLC binding differed between Method 1 and Method 2 by a ratio of 3:5. This difference between the two methods was significant at the 0.025 level. When chick embryo brain was substituted for porcine brain the resulting association constants for CLC with Method 1 were half those with Method 2, again significant at the 0.05 level.

VLB association constants were determined for each of the protein sources at the same time as those for CLC. The association constants for VLB using Method 1 vs Method 2 differed by a factor of ten for both the porcine brain and chick embryo brain crude tubulin extracts.

Intercepts. The intercept data (see Table 1) provided another parameter where significant differences were demonstrated between Method 1 and Method 2. The intercept on the Scatchard plot reflects the percentage of the soluble protein that is tubulin, bound to the DEAE-cellulose filter discs. Assuming 120,000 daltons for the molecular weight of tubulin, a relationship between the amount of drug bound to the tubulin molecule can be ascertained by comparing the intercepts obtained with CLC and those obtained with VLB, since it is known that CLC binds 1 mole for each 120,000 daltons of tubulin [2].

Method 1 intercept data consistently had higher values than the data of Method 2, and in addition indicated that both CLC and VLB bind to tubulin to the same extent. The relationship between intercepts was 1:1, meaning that there was 1 mole VLB bound/mole of tubulin. When the intercepts obtained under the conditions of Method 2 were compared, the relationship between intercepts was a little less than 1:2, indicating that about half the amount of VLB was bound to tubulin when compared to CLC. These latter data are consistent with our prior experience [9, 13].

The alteration in K_a values, indicating a selective elution of a less tightly bound component, shows that the differences in intercepts may not result from simple elution of more drug–protein complex. Whether this difference is due to the affinity of the drug–protein complex for DEAE-cellulose discs, or more likely a dissociation of a weaker drug–protein complex into dissociated elements, we cannot determine at present. Significant was the fact that all Scatchard plots were linear.

(B) *Tubulin binding differences*

Inter- and intraspecies. In Section A, we demonstrated that methodology can influence the measurement of alkaloid–tubulin binding. Table 2 indicates that the source of the protein solution, i.e. the species, can also affect alkaloid–tubulin binding.

In Method 1, three species—porcine, chick embryo, and outer doublet tubulin and solubilized VLB-induced crystals from sea urchin sperm and eggs—were used as sources for tubulin. CLC and VLB binding to the first two was significantly different at the 0.01 level. Specifically, the K_a for CLC binding in the porcine extract was roughly one and a half times that found for chick. With sea urchin egg tubulin, the K_a is quite different from both pig and chick. Moreover, the K_a for the sea urchin sperm tail outer doublet tubulin is quite different from that of the sea urchin egg solubilized vinblastine-crystal tubulin, demonstrating a significant intraspecies difference. The K_a of VLB binding for the porcine extract was better than twice that found for chick.

The three species—rat, porcine and chick—were used as sources for the crude tubulin preparations in Method 2. CLC binding to the porcine vs chick crude tubulin preparations suggested a difference, but it was not statistically supported (P value 0.2). How-

Table 2. Species differences—binding constants at 37°

	CLC	VLB
	K_a (l/mole × 10^6)	K_a (l/mole × 10^6)
Method 1		
Pig	1.8 ± 0.2	
Chick	1.2 ± 0.3	
Sea urchin-outer doublet tubulin*	0.63	
Sea urchin eggs solubilized VLB-induced crystals	0.23	
Method 2		
Pig	3.0 ± 0.7	2.9 ± 0.2
Chick	2.3 ± 0.9	1.1 ± 0.6
Rat (newborn)	0.59 ± 0.16	1.7 ± 0.6
(adult)	0.84 ± 0.14	1.4 ± 0.4

* From Wilson and Meza [21].

ever, the porcine and chick CLC binding values (K_a values of 3.0 and 2.3 × 10^6 l/mole, respectively) were strikingly different than those found for the rat (0.84 × 10^6 l/mole for adult and 0.59 × 10^6 l/mole for newborn rats). VLB binding to the porcine extract was almost three times that found in the chick and this difference was significant at the 0.0005 level. The rat data fell between the porcine and chick extract data, with a K_a of 1.4 to 1.7 × 10^6 l/mole, vs 2.9 × 10^6 l/mole for porcine and 1.1 × 10^6 l/mole for chick.

DISCUSSION

The specific and non-competitive nature of CLC and VLB binding to tubulin has made these alkaloids important probes in the investigation of microtubules and their involvement in various cellular processes. The literature concerning the relationship of these alkaloids binding to tubulin is varied in the range of association constants reported, in the sources of tubulin, and in the methodology used to assay the association constants [2, 5, 6, 9, 13, 16, 21–27]. This variability in data raises questions concerning what effect each of these variables, i.e. methodology or tubulin source, has on ascertaining the binding relationship, as expressed by the association constant [28]. The extent to which differences in K_a values reflect differences in the tubulin dimers and/or differences in methods was until now uncertain. This study has for the first time compared methodologies and found significant differences, and it has identified specific species and intraspecies differences using identical methods. Earlier reports of conflicting data on K_a values for VLB are now resolved as related to differences in methodology [12].

The differences seen between Methods 1 and 2 arose either due to preferential adsorption on DEAE-cellulose filter discs, i.e. affinity of the DEAE for a particular drug–protein complex, or more likely, due to the dissociation of a weaker drug–protein complex into its dissociated elements (this latter, however, despite the fact that the Scatchard plots remained linear throughout). Earlier work using the method of gel filtration on chick embryo brain gave data comparable to that found in this study using Method 1 [14].

Experimental conditions, i.e. pH and the ionic strength of the buffers, have been reported to affect binding activity [14]. These variables were eliminated as a potential cause of altered binding in our experiments. We suggest the possibility that the force and extent of rinsing, which differed between the two methods, permitted only the high affinity bound drug–protein complex to remain in Method 2, while Method 1 included low affinity binding drug–protein complex or complexes. The existence of high and low affinity tubulin binding sites for CLC has been reported in the literatures [23]. The low affinity sites for CLC, however, were apparently seen only at very high CLC concentrations, and probably do not represent the same phenomenon seen in our present work. In addition, evidence for two functional classes of cytoplasmic microtubules has been described [29].

The heterogeneity of tubulin, identified as an equi-

Table 3. Reported binding constants for tubulins from various sources, determined by various methods

Source of tubulin	CLC K_a	Technique*	Reference
Pig brain—pure	1.8 × 10^6	2	9
Pig—pure	2.5 × 10^7	4	17
Chick embryo brain—purified	1.1–2.0 × 10^6	1	2
Chick embryo brain			
Pure w/VLB	2 × 10^6	1 and 3	3
Crude	1.2 × 10^6	1	
Crude	2.3 × 10^6	2	
Chick, 13-day-old	2.3 × 10^6	3	16
Chick, 13-day-old—purified	1–2 × 10^6	3	22
Human cells—KB strain	1.1 × 10^6	4	5
Human brain			
Gray matter	2.3 × 10^6	3	16
White matter	2.5 × 10^6	3	16
Toad bladder—epithelial cells	1 × 10^6	3	†
Sea urchin			
Tubulin VLB crystals	~4 × 10^4	6	6
Solubilized VLB crystals	2.9–2.3 × 10^5	1	21
Solubilized outer doublet	6.3 × 10^5	2	
Rat liver			
Normal	6 × 10^5	5	24
48-hr Regenerated	3.3 × 10^5	5	23
Rat brain membranes	1 × 10^6	2	25
Beef thyroid	0.6 × 10^6	2	25

* Method: (1) DEAE-cellulose assay, immersion rinse (Method 1 of this paper); (2) DEAE-cellulose paper assay, filtration (Method 2 of this paper); (3) gel filtration (Biogel p 10); (4) gel filtration (Sephadex-kinetic analysis); (5) charcoal adsorption; and (6) centrifugation of intact crystals; loss of labeled colchicine from suspension.

† L. Wilson and A. Taylor, unpublished data.

Table 4. Reported binding constants for tubulins from various sources, determined by various methods

Source of tubulin	VLB K_a	Technique*	Reference
Pig—pure	$5–6 \times 10^6$	2	9
Rat brain—crude	3.3×10^6	2	9
Rat brain—pure reassembled	5.2×10^6	2	13
Chick embryo brain	$3–5 \times 10^6$	1 and 3	24
Chick embryo brain			
Pure fresh	4.5×10^5	4	
12-hr Stored	2.4×10^4	4	24
12-hr Stored	$1–3 \times 10^5$	1	
Sea urchin—soluble outer doublet	1×10^5	3	24, 26
Calf brain	2.3×10^4	4	27
Rat brain membrane	$\sim 8 \times 10^{-6}$	2	25

* Method: (1) DEAE-cellulose paper assay, immersion rinse (Method 1 of this paper); (2) DEAE-cellulose paper assay, filtration (Method 2 of this paper); (3) gel filtration (Biogel p 10); and (4) gel filtration (Sephadex-kinetic analysis).

molar mixture of α and β forms, has been well reported in the literature [30]. Although tubulin appears to be fairly well conserved phylogenetically in the amino acid sequencing of the two tubulin subunits [31], there are some differences, shown in at least four species—embryo chick, rat, mouse and sea urchin [32]. In addition, it has been reported that calf and rat brain tubulin have important biochemical and structural differences. Calf brain tubulin contains a disulfide bridge per subunit and rat brain tubulin does not [33, 34]. Tables 3 and 4 list some of the methods, tubulin sources, and association constants which have been reported. Note that calf tubulin differs from rat brain tubulin in its binding of VLB by at least two orders of magnitude. Although subtle differences in experimental technique cannot be ruled out as responsible for the relatively low K_a, it is consistent with the species-related differences that we observed with porcine, chick and rat tubulin, since the methodology was generally the same.

An interesting clinical observation has been with the difference in neuro-toxicity between adults and children seen with the drug VCR [35]. Young patients show a greater tolerance for this drug, where dose levels are two to four times higher than for adult patients. We suggest that these differences may be related to differences in the K_a of binding to adult vs young tubulin, and suggest that a different tubulin protein is present, or at least predominates in children. The data in Table 2 suggest such a difference in the rat but do not establish it beyond question. Age-related differences in tubulin have already been reported, where significant changes in the concentration of microtubules and in the decay rate of CLC binding activity of tubulin have been correlated with maturation in rat brain and chick embryo brain [22, 36].

Rapaport *et al.* [23] reported that, in regenerating liver, there was a 2.1 to 1 ratio of CLC binding to normal rat liver vs 48-hr regenerated liver. This, together with our finding of different K_a values within a species, i.e. outer doublet vs solubilized egg VLB crystals in sea urchins, suggests that there may be different forms of tubulin within the same species.

In summary, there are at least two variables in operation affecting the binding of alkaloids to tubulin. Methodology has been shown to influence the binding affinity, and depending on the technique will measure either the high or low affinity properties. The second concerns variability in the tubulin. At the interspecies level, we can demonstrate biochemical and structural differences between tubulins, but the specific effect these have on the binding of alkaloids is unknown. Within a species we have encountered differences in the binding of alkaloids dependent upon the tubulin source. Whether the differences in alkaloid binding tubulin reflect differences in affinity of the tubulin–drug complex for DEAE, or different classes of binding sites, affinities, mechanism or regulators that activate or inactivate tubulin and thereby affect binding is speculative. Further investigation is warranted to clarify these issues.

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