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Hypothesis

Analysis of the colchicine-binding site of β-tubulin

Roy G. Burns

Biophysics Section, Blackett Laboratory, Imperial College of Science, Technology and Medicine, Prince Consort Road, London SW7 2BZ, UK

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Comparison of the β-tubulin sequences with the equilibrium colchicine K_a and the K_i for inhibition by podophyllotoxin suggests that residue β:316 is directly involved in binding the common trimethoxyphenyl- (or A-) ring. By contrast, the analysis indicates that the local hydrophobicity affects the rate of one of the two conformational changes associated with colchicine binding but does not determine the affinity of the colchicine-binding site.

β-Tubulin; Colchicine binding; Sequence analysis

1. INTRODUCTION

Colchicine binds to the tubulin dimer and inhibits microtubule assembly but does not bind to the assembled microtubule. The site is located on the β-subunit as colchicine- or colchemid-resistent *Chlamydomonas* or CHO cells express a modified β-tubulin [1,2], colchicine uncouples the assembly-dependent hydrolysis of GTP bound to the β-subunit [3], colchicine-binding is protected by this GTP [4] and is inhibited by alkylation of the β-subunit [5], while the binding kinetics are markedly affected by depleting bovine brain tubulin of the neuronal-specific β-III isotope [6]. Much higher colchicine concentrations are required in lower eucaryotes to inhibit mitosis and other microtubule-assembly dependent processes. The current analysis seeks to define part of the colchicine-binding site by correlating the experimentally defined association constants with the available primary sequences.

2. COLCHICINE BINDING: β-TUBULIN SEQUENCE ANALYSIS

The available values for the K_a of colchicine binding to various specific tubulins are remarkably consistent, yet there are marked differences between the different organisms (Table I). The mean vertebrate neuronal K_a is 1.8×10^6 M^{-1}, compared with 3.9×10^5 M^{-1} for sea urchin tubulin, and 1.65×10^5 M^{-1} for *Caenorhabditis* tubulin. Although there is some variation between the independent K_a determinations, the direct comparisons (chick brain vs. sea urchin axonemal tubulins [7,8] and ovine brain vs. *Caenorhabditis* and other nematode tubulins [9,10]) clearly confirm the higher values of the neuronal protein.

Primary β-tubulin sequences are only available for certain of these organisms, and the sequence comparison has been restricted to those β-tubulin isoforms which are expressed in those tissues used to purify the tubulin for the colchicine-binding studies. In particular, only certain β-tubulin isotypes are expressed in brain [11,12]: bovine brain consisting of 3% class I, 13% IV, 58% and II and 25% class III [13], while chick brain has a slightly different composition (50:0.1:25:25, Cleveland, personal communication). While the isoform composition of sea urchin egg or sperm axonemes is unknown, the available β-tubulin sequences are virtually identical (Table II). Three *Caenorhabditis* β-tubulins have been sequenced, of which one (mec-7) is only expressed in touch cells and so has been ignored from the current analysis. Finally, *Chlamydomonas* has been included in the analysis, despite the lack of a direct measure of the K_a, as an example of a non-metazoan tubulin with minimal colchicine binding [14,15] but with high homology to vertebrate tubulins (Table II).

Analysis of the β-tubulin sequences (Table II) strongly suggests that the relative affinities of different tubulins depends upon residues in the immediate vicinity of β:316. Bovine neuronal tubulin consists of a mixture of 16% Val^{316}, 58% Iso^{316} and 25% Thr^{315}/Val^{316}, while chick brain tubulin consists of 50% Val^{316}, 25% Iso^{316} and 25% Thr^{315}/Val^{316}. The available sequences show that the tubulins have a Iso^{316} in sea urchins, a Met^{316} in nematodes, a Ala^{313}/Ser^{314}/Leu^{316} in

Correspondence address: R.G. Burns, Biophysics Section, Blackett Laboratory, Imperial College of Science, Technology and Medicine, Prince Consort Road, London SW7 2BZ, UK. Fax: (44) (71) 589 9463.

Table I

Summary of the published equilibrium association constants for colchicine binding. No additional adjustments have been made to compensate for the differing assay conditions, such as the buffer, temperature or pH or for the intrinsic lability of the protein. The analysis has been restricted to the equilibrium values as the kinetic measurements are significantly different. The value for *S. cerevisiae* [34] is estimated as being 10% of that for colchemid-binding.

Organism	Association constant	
Chick brain, embryonic	2×10^6	[7]
Chick brain, embryonic	2×10^6	[16]
Chick brain, embryonic	1.1×10^6	[8]
Rat brain	1×10^6	[27]
Rat brain	1.8×10^6	[19]
Rat brain	3.3×10^6	[28]
Bovine brain	9×10^5	[29]
Porcine brain	1.8×10^6	[18]
Porcine brain	2×10^6	[30]
Neuronal mean: $1.76 \pm 0.73 \times 10^6$ M^{-1}		
Rat thyroid	6×10^5	[27]
KB tissue culture cells	1.1×10^6	[31]
Axolotl egg	$2–9 \times 10^5$	[17]
Sea urchin axonemes	4.8×10^5	[8]
Sea urchin axonemes	6.3×10^5	[7]
Sea urchin axonemes	(3.2×10^6)	[31]
Sea urchin egg	2.9×10^5	[8]
Sea urchin egg	2.9×10^5	[31]
Sea urchin egg	(2.3×10^6)	[4]
Sea urchin egg, paracrystals	2.7×10^5	[22]
Echinoderm mean: $3.92 \pm 1.58 \times 10^5$ M^{-1}*		
Caenorhabditis	$1.6–1.7 \times 10^5$	[9]
Ascaris, early embryos	$3.1–5.5 \times 10^5$	[32]
Ascaris, late embryos	$1.5–2.2 \times 10^5$	[32]
Ascaris, intestinal cells	5.9×10^4	[33]
Hymenolepis	7.6×10^4	[16]
Saccharomyces cerevisiae	$\approx 5 \times 10^2$	[34]

*The anomolously high Echinoderm values shown in parentheses have been omitted in calculating the mean and standard deviation.

Chlamydomonas, and a Phe^{316} in *S. cerevisiae*. Comparison of these residues with the experimental association constants indicates that tubulins with Iso^{316} must bind colchicine significantly more weakly than those with Val^{316} and/or Thr^{315}/Val^{316}, but more strongly than the Met^{316}, $Ala^{313}/Ser^{314}/Leu^{316}$, or Phe^{316} substitutions. Significantly, the bovine brain K_a (58% Iso^{316}, 9×10^5 M^{-1}) is much lower than that for chick brain tubulin (25% Iso^{316}, 1.7×10^6 M^{-1}), consistent with the Val^{316} isoform binding colchicine more tightly (calculated as $1.6–2.2 \times 10^6$ M^{-1} by assuming that the Val^{316} and Thr^{315}/Val^{316} isoforms have identical binding constants). Finally, the tubulin(s) of axolotyl eggs bind colchicine with a similar affinity as the echinoderm protein (Table I). Although no axolotyl β-tubulins have been sequenced, the only available amphibian β-tubulin sequence (*Xenopus*) resembles the echinoderm protein by having a Iso^{316}. The identity of β:316 therefore appears to have a

Table II

Comparison of the sequences of those β:tubulin classes (Classes I–IV; [11,12]) expressed in vertebrate brain with those of echinoderms (*Strongylocentrotus*, *Lytechinus* β1, β2 and β3, and *Paracentrotus*), *Caenorhabditis* (ben-1 and tub-7), *Chlamydomonas*, and *Saccharomyces cerevisiae*. Only the residues flanking β:316 are shown as analysis of the full tubulin sequences shows that only this peptide correlates with the colchicine-binding activity. The top line shows the single letter code of the vertebrate Class I sequence. The letters on the lower lines indicate those residues which are altered in all members of that Class or organism. Also shown (A) are the number of residues within the conserved sequences up to β:430 which are invariably different from the Class I sequence, and (B) the number of additional residues within this sequence which vary in one or more of the individual β:tubulins.

	316	A	B
Class I	GRYLTVAAVFRGRMSMK	0	4
Class IV	.....	4	12
Class II	.....I.....	10	7
Class III	.....T.....	21	6
Echinoderms	.....I.....	9	1
Caenorhabditis	.....M.....R	24	17
Chlamydomonas	.....AS.L.....T.	38	0
S. cerevisiae	.....F...KV.V.	102	–

profound effect on the K_a for the colchicine binding observed under quasi-equilibrium conditions.

Only a small number of the known non-metazoan β-tubulin sequences contain Iso^{316}. All are plants or fungi (*Colletotrichum graminicola* β2, *Aspergillus* benA, *Neurospora*, *Histoplasma*, *Arabdopsis* tub5, *Erysipha* tub2, *Epichloe*), and in each case the immediate sequence is Ala^{313}/Cys^{313}·Ser·Ala·Iso^{316} in contrast to the usual metazoan Val^{313}·Ala·Ala·Iso^{316}. There is only a single known non-metazoan β-tubulin sequence with

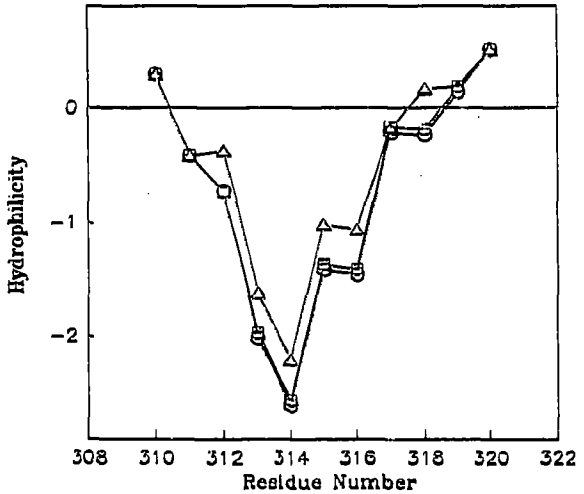

Fig. 1. The calculated hydrophilicity (Kyte-Doolittle, averaged over a window of seven residues) of β-tubulin sequences between residues 310 and 320. The Val^{316} isoform with a high colchicine K_a (□) is virtually identical to the more weakly binding Iso^{316} isoform (○), and both differ from the slow binding [6] neuronal-specific β:III $Thr^{315}.Val^{316}$ isoform (△).

Val^{316} (*Arabdopsis* tub5), with a surrounding peptide of Arg^{313}·Ser·Ala·Val^{316}. The restriction of Iso^{316} and Val^{316} to β-tubulins from organisms which lack flanking amino acid substitutions and which are highly colchicine-sensitive strongly supports the proposal that this residue is intimately involved in specifying the colchicine-binding site.

One apparent anomoly of this correlation is that while the vertebrate β-tubulins are heterogeneous at β:316 the available Scatchard plots are approximately linear [16–19], indicating that the protein contains a single class of binding site. This may be artifactual, because the different isoforms bind colchicine at differing rates and may have differing labilities [6].

3. HYDROPHILICITIES OF THE DIFFERENT ISOFORMS

The calculated hydrophilicity plot shows that the lowest value within the entire sequence is centered on β:314 (Fig. 1), i.e. immediately adjacent to a residue implicated in colchicine binding. Sequences containing Ala^{315}·Val^{316} or Ala^{315}·Iso^{316} have virtually identical predicted hydrophilicities, while the Thr^{315}·Val^{316} substitution of the neuronal-specific βIII is significantly less hydrophobic (and closely resembles that for the Ala^{315}·Met^{316} *Caenorhabditis* and Ala^{315}·Phe^{316} *S. cerevisiae* sequences, not shown).

4. PODOPHYLLOTOXIN AND COLCHICINE BINDING

Podophyllotoxin binds to rat brain tubulin (K_a: 1.8–3.6 × 10^6 M^{-1}; [20,21]) and competitively inhibits colchicine binding to a variety of tubulins [7–9, 16,20,22].

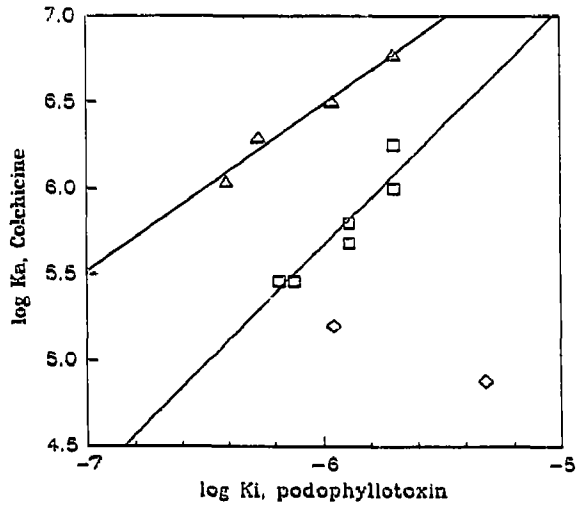

Fig. 2. The published K_a values for colchicine binding to tubulin as a function of the published K_i values for the inhibition of colchicine-binding by podophyllotoxin. The data has been arbitarily divided into two groups (△ and □). The two additional points relate to the binding to *Ascaris* late embryos and to *Hymenolepis*.

The observed podophyllotoxin inhibition constants have been compared with the parallel determinations of the K_a for colchicine binding (Fig. 2). Leaving aside the values for *Ascaris* and *Hymenolepis*, the K_i and K_a values can be fitted to two regression lines. The lower line includes the echinoderm axoneme and egg data and that from rat brain. The upper line includes data from embryonic chick brain, rat thyroid, and the single echinoderm axonemal determination with an anomolous colchicine K_a (see Table I). Unfortunately, the current analysis cannot be extended to the podophyllotoxin-binding as the isoform expression in rat thyroid is unknown, and as an insufficient number of sea urchin and rat β-tubulin sequences are available.

5. DISCUSSION

The competitive binding of colchicine and podophyllotoxin has indicated that both drugs bind to tubulin by the shared trimethoxy-substituted phenyl ring, and the observed correlations (Table I and Fig. 2) strongly suggest that β:316 is intimately involved in specifying the binding site for this ring. Indeed, the Iso^{316} and Val^{316} isoforms only differ in the presence or absence of a methyl group, which suggests that the 3-fold difference in the colchicine K_a values may be due to a steric effect involving the methoxy/methyl groups. Such a steric effect would be greater with the Met^{316} and Phe^{316} isoforms, consistent with their lower colchicine-binding K_as. Significantly, there is no difference in the colchicine dissociation rates from sea urchin (Iso^{316}) and porcine brain (Val^{316} and Iso^{316}) tubulins (2.9 vs. 3.2 ± 0.6 × 10^{-4} min^{-1} [23]), indicating that the difference in the K_a values specifically involves the association rate constants. This is the first identification of a residue involved with the binding of a specific part of colchicine, since point mutations which confer resistance in *Chlamydomonas* (β:Lys^{350} to Glu^{350} or Met^{350}) have additional pleotrophic effects [24].

The high hydrophobicity of residues immediately adjacent to β:316 (Fig. 1) is consistent with the evidence that colchicine binding involves hydrophobic interactions [8,19,22] and, from studies of *N*-acetyl mescaline binding [25], that much of the binding energy is derived from the interaction of the A-ring. Such studies have suggested that the colchicine binding site is bifocal, and that the interaction of a ligand with one (probably the tropolone or C-ring) induces a conformational change which facilitates the binding of the A-ring. There is also evidence that an additional conformational change is required to accomodate the B-ring since the alkylation of tubulin by iodoacetamide is inhibited by a variety of ligands including colchicine and podophyllotoxin but not by structural analogues of the A- and the C-rings [5]. Comparison of the binding kinetics of B-ring analogues [26] shows that the slow colchicine-binding kinetics

are due to the conformational change required to accommodate the B-ring.

The observed biphasic kinetics of colchicine-binding is primarily due to differences in the rate of binding by the different tubulin isoforms, with the neuronal-specific βIII binding much more slowly [6]. Comparison of the primary sequence of this with the other neuronally-expressed isoforms shows that there are only seventeen residues which are invariably different, one of which (the Ala^{315}:Thr^{315} substitution) results in the decreased predicted hydrophobicity at β:314. The lack of any correlation between the calculated hydrophobicities at β:316 (Fig. 1) with the determined K_as for colchicine binding (Table I) establishes that it is the amino acid substitution rather than the consequential change in the hydrophobic environment which specifies the association constant. It is therefore probable that β:316 contributes to the colchicine/podophyllotoxin binding site and that the local hydrophobicity specifies the rate of the conformational change required to accomodate the B-ring.

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REFERENCES

[1] Lee, V.D. and Huang, B. (1990) Plant Cell 2, 1051–1057.
[2] Cabral, F., Sobel, M.E. and Gottesman, M.M. (1980) Cell 20, 29–36.
[3] David-Pfeuty, T., Simon, C. and Pantaloni, D. (1979) J. Biol. Chem. 254, 11696–11702.
[4] Borisy, G.G. and Taylor, E.W. (1967) J. Cell Biol. 34, 535–548.
[5] Roach, M.C., Bane, S. and Luduena, R.F. (1985) J. Biol. Chem. 260, 3015–3023.
[6] Banerjee, A. and Luduena, R.F. (1991) J. Biol. Chem. 266, 1689–1691.
[7] Wilson, L. and Meza, I. (1973) J. Cell Biol. 58, 709–719.
[8] Pfeffer, T.A., Asnes, C.F. and Wilson, L. (1967) J. Cell Biol. 69, 599–607.
[9] Russell, G.J. and Lacey, E. (1989) Biochim. Biophys. Acta 993, 233–239.
[10] Dawson, P.J., Gutteridge, W.E. and Gull, K. (1984) Biochem. Pharmacol. 33, 1069–1073.
[11] Joshi, H.C., Yen, T.J. and Cleveland, D.W. (1987) J. Cell Biol. 105, 2179–2190.
[12] Burns, R.G. and Surridge, C. (1990) FEBS Lett. 271, 1–8.
[13] Banerjee, A., Roach, M.C., Wall, K.A., Lopata, M.A., Cleveland, D.W. and Luduena, R.F. (1988) J. Biol. Chem. 263, 3029–3034.
[14] Burns, R.G. (1973) Exptl. Cell Res. 81, 285–292.
[15] Flanagan, D. and Warr, J.R. (1977) FEBS Lett. 80, 14–18.
[16] Wilson, L., Bamburg, J.R., Mizel, S.B., Grisham, L.M. and Creswell, K.M. (1974) Fed. Proc. 33, 158–166.
[17] Raff, E.C. (1977) Dev. Biol. 58, 56–75.
[18] Owellen, R.J., Owens, A.H. and Donigian, D.W. (1972) Biochem. Biophys. Res. Commun. 47, 685–691.
[19] Bhattacharrya, B. and Wolff, J. (1974) Proc. Natl. Acad. Sci. USA 71, 2627–2631.
[20] McClure, W.O. and Paulson, J.C. (1977) Mol. Pharmacol. 13, 560–575.
[21] Cortese, F., Bhattacharrya, B. and Wolff, J. (1977) J. Biol. Chem. 252, 1134–1140.
[22] Bryan, J. (1972) Biochemistry 11, 2611–2616.
[23] Garland, D. and Teller, D.C. (1975) Ann. N.Y. Acad. Sci. 253, 232–238.
[24] Schibler, M.J. and Huang, B. (1991) J. Cell Biol. 113, 605–614.
[25] Andreu, J.M. and Timasheff, S.N. (1982) Biochemistry 21, 534–543.
[26] Ray, K., Bhattacharyya, B. and Biswas, B. (1981) J. Biol. Chem. 256, 6241–6244.
[27] Bhattacharrya, B. and Wolff, J. (1975) J. Biol. Chem. 250, 7639–7646.
[28] Watts, S.D.M. (1981) Biochim. Biophys. Acta 667, 59–69.
[29] Detrich, H.W., Williams, R.C., Macdonald, T.L., Wilson, L. and Puett, D. (1981) Biochemistry 20, 5999–6005.
[30] Garland, D.L. (1978) Biochemistry 17, 4266–4272.
[31] Borisy, G.G. and Taylor, E.W. (1967) J. Cell Biol. 34, 525–533.
[32] Friedman, P.A., Platzer, E.G. and Carroll, E.J. (1980) Dev. Biol. 76, 47–57.
[33] Kohler, P. and Bachmann, R. (1981) Mol. Biochem. Parasitol. 4, 325–336.
[34] Haber, J.E., Peloquin, J.G., Halvorsen, H.O. and Borisy, G.G. (1972) J. Cell Biol. 55, 355–367.