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BINDING OF [^{3}H]BENZIMIDAZOLE CARBAMATES TO MAMMALIAN BRAIN TUBULIN AND THE MECHANISM OF SELECTIVE TOXICITY OF THE BENZIMIDAZOLE ANTHELMINTICS

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Abstract—The binding of tritiated benzimidazole carbamates ([^{3}H]BZCs) to mammalian brain tubulin was examined to investigate the kinetics of the BZC–tubulin interaction and to establish the mechanism of the selective toxicity of the BZC based anthelmintics. [^{3}H]BZC binding to tubulin was markedly greater at 4° than at 37° for all ligands. The association constant (K_a) and maximum amount of [^{3}H]BZC bound (B_{max}) were temperature dependent for [^{3}H]mebendazole ([^{3}H]MBZ), [^{3}H]oxibendazole ([^{3}H]-OBZ) and [^{3}H]oxfendazole ([^{3}H]OFZ). The K_a and B_{max} values obtained for [^{3}H]MBZ, [^{3}H]OBZ and [^{3}H]OFZ, and the comparatively weak binding of [^{3}H]carbendazim, reflected the known *in vitro* potency of these compounds as microtubule inhibitors. Dissociation of the [^{3}H]MBZ–tubulin complex was also temperature dependent, the first order dissociation rate constant being reduced by two orders of magnitude at 4° compared with that observed for 37°. These results indicate that the binding of BZCs to mammalian brain tubulin is temperature dependent and suggest that temperature induced conformational changes in the tubulin dimer influence the ability of the BZCs to form a stable BZC–tubulin complex. The temperature dependence of BZC binding and the affinity of the BZCs for mammalian tubulin are therefore unlike the BZC–tubulin interaction observed for parasitic nematodes, where optimum BZC binding occurs at 37° and results in the formation of a pseudo-irreversible complex.

Benzimidazole carbamates (BZCs§) are a class of anthelmintic agents that display activity against nematodes, cestodes and trematodes [1]. Within this class of compounds many analogues also have pronounced antifungal and antineoplastic activities [2]. This broad pharmacological profile reflects the potent action of BZCs as inhibitors of the tubulin–microtubule equilibrium in many species. Although BZCs inhibit the polymerization of mammalian brain tubulin at concentrations of less than 10 μM *in vitro*, these compounds are distinct from other microtubule inhibitors in that they display little or no acute toxicity towards the host when used as anthelmintics [1].

The BZCs have been shown to bind to the colchicine binding site of the tubulin dimer [3–5]. Although the precise location of the colchicine binding site within the tertiary structure of tubulin is yet to be identified, the biochemical interactions of a variety of microtubule inhibitors within this domain have been characterized extensively. Microtubule inhibitors known to bind to the colchicine binding site include: colchicine, podophyllotoxin, combretastatin, *cis*-tubulozole, the 1-deaza-7,8-dihydropteridines and the 6-benzyl-1,3-benzodioxoles [6-10]. Despite the structural heterogeneity of these compounds, all are competitive inhibitors of [^{3}H]colchicine binding to tubulin [6, 8–11].

The kinetics of the colchicine–tubulin interaction are well documented. The association constant (K_a) for the binding of [^{3}H]colchicine to tubulin derived from a number of species has been observed to range from $1 \times 10^4\ M^{-1}$ to $1.8 \times 10^6\ M^{-1}$ and is dependent upon the source of tubulin and the assay technique [12, 13]. The K_a for the binding of [^{3}H]colchicine to mammalian brain tubulin is typically $1–2 \times 10^6\ M^{-1}$. Optimum binding of [^{3}H]colchicine occurs at 37° and involves a slow, non-covalent, biphasic interaction, which induces conformational changes in both colchicine and tubulin resulting in the formation of an essentially irreversible colchicine–tubulin complex [14]. Equilibrium binding studies usually indicate a stoichiometry of 0.2 to 0.7 molar equivalents of [^{3}H]-colchicine per mole of tubulin, whereas equimolar stoichiometry has been observed in kinetic studies based upon colchicine-induced fluorescence [12, 14].

The kinetics of the binding of other classes of microtubule inhibitors to the colchicine binding site of tubulin are less well characterized. In contrast to colchicine, the binding of podophyllotoxin to mammalian brain tubulin is rapid and reversible, and less subject to temperature constraints [6]. The binding of BZCs to mammalian brain tubulin has

§ Abbreviations: BSA, bovine serum albumin; BZC, benzimidazole carbamate; DMSO, dimethyl sulfoxide; EGTA, ethyleneglycol-bis-(β-aminoethylether)*N,N,N',N'*-tetraacetic acid; MBC, carbendazim (methyl 2-benzimidazole carbamate); MBZ, mebendazole (methyl 5-benzoyl-2-benzimidazole carbamate); MES, 4-morpholineethanesulfonic acid; OBZ, oxibendazole (methyl 5-propoxy-2-benzimidazole carbamate); and OFZ, oxfendazole (methyl 5-(phenylsulfinyl)-2-benzimidazole carbamate).

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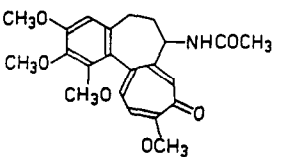
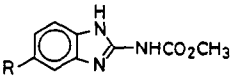


Colchicine



Benzimidazole carbamates

		R
Mebendazole	(MBZ)	-C(=O)-C$_6$H$_5$
Oxibendazole	(OBZ)	-OCH$_2$CH$_2$CH$_3$
Oxfendazole	(OFZ)	-S(=O)-C$_6$H$_5$
Carbendazim	(MBC)	H

Fig. 1. Structures of colchicine and the benzimidazole carbamates: mebendazole, oxibendazole, oxfendazole and carbendazim.

been investigated by a number of indirect methods such as the inhibition of microtubule polymerization [4, 5, 15–17] or the inhibition of [^{3}H]colchicine binding [3, 4, 18]. Previous studies of the binding of [^{3}H]BZCs to mammalian tubulin suggest that the BZC–tubulin interaction is reversible [3, 5, 17]; however, no study has directly addressed the possible relationship between the temperature dependence, rate and stability of formation of the BZC–tubulin complex and the selective toxicity of BZC anthelmintics.

It has been postulated that species differences exist in the binding of BZCs to tubulin and that these differences are responsible for the selective toxicity of the BZC anthelmintics [18]. In this study we report the development of a rapid and sensitive gel filtration technique which has been used to examine the interactions between the commonly used BZCs (Fig. 1) and mammalian tubulin. Results from this study support the hypothesis that the kinetics of the BZC–tubulin interaction differs between species and that these differences are responsible for the efficacy and selective toxicity of the BZCs as anthelmintics.

MATERIALS AND METHODS

Chemicals. Pure samples of mebendazole (MBZ), oxibendazole (OBZ) (SmithKline Beecham Animal Health, Sydney, Australia), and oxfendazole (OFZ) (Syntex Agribusiness, Sydney, Australia) were tritiated and purified as described by Lacey *et al.* [19]. Synthesis of [^{3}H]carbendazim ([^{3}H]MBC) has been described previously [19]. Bovine serum albumin (BSA, Fraction V), 4-morpholine-ethanesulfonic acid (MES), Norit A activated charcoal and ethylene glycol-bis-(β-aminoethyl ether)*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA) were purchased from the Sigma Chemical Co., St. Louis, MO, U.S.A. GTP (dilithium salt) was obtained from Boehringer Mannheim, Sydney, Australia. Bio-gel P-6 was from Bio-Rad Laboratories, Sydney, Australia. Bovine brain calmodulin was purchased from Calbiochem-Behring, Sydney, Australia. All other solvents and reagents were of analytical grade.

Purification of mammalian brain tubulin. Ovine brain tubulin was purified by arginine-Sepharose 4B affinity chromatography as described by Lacey and Snowdon [20]. Freshly isolated ovine brain (30 g) was homogenized in 0.4 M MES, 1.0 mM EGTA, 0.5 mM $MgSO_4$ at pH 6.9 (buffer A), centrifuged at 100,000 *g* for 45 min at 4° in a Kontron TGA-75 Ultracentrifuge (Zurich, Switzerland) and the crude supernatant (300–400 mg protein) applied to a 5 mL arginine-Sepharose 4B (Pharmacia-LKB, Sydney, Australia) column equilibrated with buffer A. The column was eluted sequentially with 50 mL of buffer A and 4 × 5 mL of 0.1 M MES, 1.0 mM EGTA, 0.5 mM $MgSO_4$ at pH 6.9 (buffer B) containing 0.19 M $(NH_4)_2SO_4$. GTP (1 mM) was added to the relevant tubulin fractions which were kept on ice for less than 3 hr prior to use. Purified tubulin fractions (> 95% purity) were diluted with cold buffer B containing 1 mM GTP to a final concentration of 0.5 to 0.78 mg/mL prior to use in binding studies. Protein concentration was determined using the Bio-Rad Dye Assay (Bio-Rad) based upon the method of Bradford [21] using BSA as a standard.

Binding assay. A 2.5 mM [^{3}H]BZC stock solution, in dimethyl sulfoxide (DMSO), was diluted 1:10 with a 2.5 mM solution of each unlabelled BZC in DMSO to give a final specific activity of 24, 3.1, 1.1 and 4.5 GBq/mmol for [^{3}H]MBC, [^{3}H]MBZ, [^{3}H]-OBZ and [^{3}H]OFZ, respectively. To examine the stoichiometry of [^{3}H]BZC binding, 98 μL of tubulin or buffer B (blank) was routinely incubated in duplicate with 2 μL of the respective [^{3}H]BZC (final concentration range 0.625 to 30 μM) for 30 min at either 4° or 37°. The association of [^{3}H]MBZ with tubulin was examined at various times at either 4° or 37° using a final concentration of 1 μM [^{3}H]MBZ or 20 μM [^{3}H]MBZ at 20 min to determine the number of binding sites present. The dissociation of [^{3}H]MBZ from tubulin was examined by an initial incubation with 1 μM [^{3}H]MBZ for 30 min at either 4° or 37° followed by the addition of 50 μM unlabelled MBZ and incubation for various time periods at the required temperature.

The charcoal extraction assay is based on the method described by Sherline *et al.* [22]. Binding was terminated by a 5-min incubation with 0.5 mL of a 2 mg/mL suspension of charcoal in 1% BSA. Charcoal was sedimented by centrifugation (10,000 rpm for 10 min) in a Mikro-Rapid centrifuge (Hettich, Tuttlingen, F.R.G.) and 0.4-mL aliquots of the supernatant were counted in 5 mL Biofluor (New England Nuclear, Boston, MA, U.S.A.) as described below.

The gel filtration assay was a modification of the micro-column method described by Penefsky [23]. Gel filtration tubes consisted of 0.5 mL of 10% (w/v) hydrated Bio-gel P-6 in 0.7 mL microtubes

Table 1. Effect of gel volume on [3H]MBZ background levels and [3H]MBZ binding to tubulin

Gel volume (mL)	Background [3H]MBZ* (pmol/assay)	[3H]MBZ–tubulin (pmol/assay)
1.0	0.14 (0.7)	18.9
0.9	0.22 (1.0)	22.4
0.8	0.45 (1.9)	24.2
0.7	0.59 (2.4)	24.6
0.6	0.51 (1.8)	28.0
0.5	1.29 (4.4)	29.2
0.4	2.91 (9.0)	32.4
0.3	6.07 (16.9)	36.0

* Background [3H]MBZ as a percentage of tubulin specific binding is indicated in parentheses.

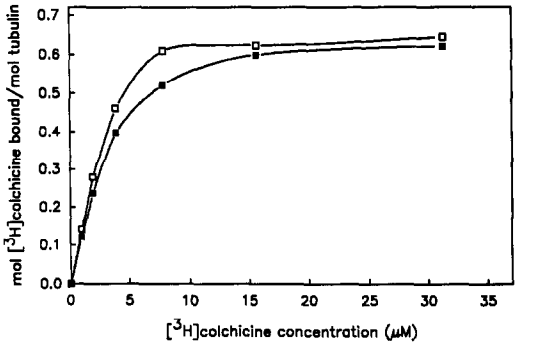


Fig. 2. Stoichiometry of colchicine binding to tubulin as determined by charcoal extraction or gel filtration assays. Mixtures were incubated for 1 hr at 37°. Key: (■——■) charcoal extraction and (□——□) gel filtration.

(Eppendorf, Hamburg, F.R.G.) which had been perforated by an 18-gauge needle and plugged with glass wool. Gels were centrifuged at 10,000 rpm for 5 min in a Mikro-Rapid Centrifuge, placed inside 1.5 mL microtubes (Eppendorf) and stored on ice prior to use. Binding was terminated by applying 50 μL of the assay mixture to the gel and eluting the [3H]BZC–tubulin complex by centrifugation (10,000 rpm for 5 min). The eluent was diluted with 0.4 mL of glass distilled and deionized water, transferred to scintillation vials, and counted in 5 mL Biofluor in a Tricarb 2650 Liquid Scintillation Spectrophotometer (Packard, Downers Grove, IL, U.S.A.). The association constant (K_a) and the maximum amount of [3H]BZC bound at infinite free ligand concentration (B_{max}) were calculated using the EBDA/LIGAND computer analysis programs for radioactive ligand binding studies [24, 25].

RESULTS

Investigation of gel filtration variables. The gel filtration assay was standardized using a final concentration of 1 μM [3H]MBZ and 290 pmol tubulin per assay to determine the optimum conditions for detecting the binding of [3H]BZCs to tubulin. Variation in background [3H]MBZ and elution of the [3H]MBZ–tubulin complex with differing gel volumes is indicated in Table 1. Larger gel volumes reduced protein recovery and decreased the detection of the [3H]MBZ–tubulin complex with little reduction in the background [3H]MBZ. Gel volumes below 0.5 mL produced unacceptably high levels of background [3H]MBZ. A gel volume of 0.5 mL produced the best compromise between protein recovery and background [3H]MBZ and was selected for routine use. Maximum stoichiometry of 1 μM [3H]MBZ binding was approximately 10% for a gel volume of 0.5 mL. At this gel volume, background [3H]MBZ levels were routinely less than 5% of the total [3H]MBZ detected, while 77 ± 6% of the applied protein was recovered over a protein range of 12 to 80 μg. Compensation for the reduced recovery of protein was achieved using a correction factor of 1.33, assuming a routine elution of 75% of the applied protein.

An investigation of the effect of solution variables on [3H]MBZ binding to tubulin demonstrated that variations in ionic strength (5 mM to 0.5 M MES), $(NH_4)_2SO_4$ concentration (0.05 to 1.0 M) and pH (5.5 to 7.5 pH units) had no effect on total [3H]-MBZ binding. Standard solution conditions of 0.1 M MES, 1.0 mM EGTA, 0.5 mM $MgSO_4$ at pH 6.9 containing ≤ 0.19 M $(NH_4)_2SO_4$ and 1 mM GTP were used for the analysis of the binding of [3H]-BZCs to mammalian brain tubulin.

Comparison of colchicine binding using gel filtration and charcoal extraction assays. To establish the validity of this technique for the analysis of binding interactions, the binding of [3H]colchicine to tubulin was examined using both the gel filtration assay and the charcoal extraction assay as described by Sherline *et al.* [22]. Profiles for the binding of [3H]colchicine to tubulin as measured by both assay techniques are compared in Fig. 2. Analysis of the [3H]colchicine binding data indicated a stoichiometry of 0.65 and 0.67 mol [3H]colchicine bound per mol of tubulin (assuming a molecular weight of 100,000 for dimeric tubulin) for the charcoal extraction and gel filtration techniques, respectively. These results are consistent with the "molar" stoichiometry of [3H]colchicine binding to tubulin observed in other studies under equivalent conditions [12, 26]. The association constant (K_a) was calculated as $0.9 \times 10^6\ M^{-1}$ and $1.8 \times 10^6\ M^{-1}$ for the charcoal extraction and gel filtration assays, respectively. These values are in good agreement with published data for K_a determinations using these techniques [12].

Temperature dependence of [3H]BZC binding to tubulin. Incubation temperature had a pronounced effect on the extent of [3H]MBZ binding to tubulin. At 37° the binding of 1 μM [3H]MBZ reached a maximum of 178 pmol/mg at 1 min, decreasing to an equilibrium level of 155 pmol/mg. [3H]MBZ binding increased 3-fold to an equilibrium level of 510 pmol/mg by 10 min upon incubation at 4° (Fig. 3). The stoichiometry of [3H]MBZ binding reflected the temperature dependence of the formation of the [3H]MBZ–tubulin complex when assayed by rapid gel filtration (Table 2, Fig. 4). The stability of the

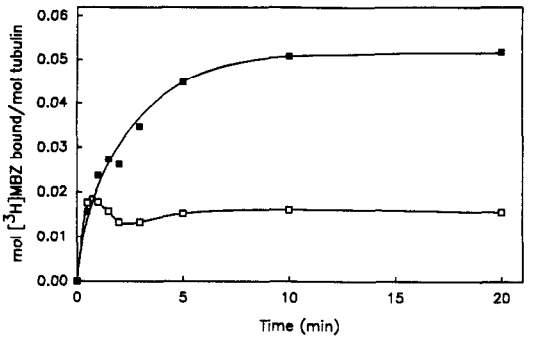


Fig. 3. Time dependence of mebendazole binding to tubulin. Assays were performed at 4° (■——■) or 37° (□——□) using a final concentration of 1 μM [^{3}H]MBZ.

[^{3}H]MBZ–tubulin complex was also reflected in the ability of each assay to detect [^{3}H]MBZ binding. Binding of [^{3}H]MBZ to mammalian tubulin was detected at 37° and 4° using the gel filtration assay; however, no [^{3}H]MBZ binding was observed using the charcoal extraction assay (Fig. 4).

Dissociation of the [^{3}H]MBZ–tubulin complex was also temperature dependent. More than 50% of the [^{3}H]MBZ bound at 4° or 37° dissociated during a 30-sec incubation at 37° (Fig. 5). In contrast, only 10% of the initially bound [^{3}H]MBZ had dissociated after a 5-min incubation at 4°. First-order dissociation rate constants were calculated as being 2.7 min^{-1} at 37° and 0.02 min^{-1} at 4° following the addition of excess unlabelled MBZ.

To investigate the possibility that the reduced binding of [^{3}H]MBZ to tubulin at 37° resulted from the formation of oligomeric tubulin, several experiments were designed to assess the effects of 1 μM calmodulin

Table 2. Comparison of K_a and B_{max} for [^{3}H]BZC binding to tubulin at 4° and 37° with the inhibition of microtubule polymerization *in vitro*

	4°		37°		
Compound	K_a (M^{-1})	B_{max} (mol [^{3}H]BZC bound/mol tubulin)	K_a (M^{-1})	B_{max} (mol [^{3}H]BZC bound/mol tubulin)	IC_{50}* (μM)
Mebendazole (MBZ)	3.8×10^5	0.48	2.5×10^5	0.24	
	3.8×10^5	0.36	1.8×10^5	0.33	
	3.8×10^5	0.42	2.2×10^5	0.29	6.1
Oxibendazole (OBZ)	1.1×10^5	0.44	9.7×10^4	0.25	
	1.3×10^5	0.31	7.0×10^4	0.3	
	1.2×10^5	0.38	8.4×10^4	0.28	2.4
Oxfendazole (OFZ)	3.3×10^4	0.29	2.3×10^4	0.19	
	2.8×10^4	0.28	2.3×10^4	0.19	
	3.1×10^4	0.29	2.3×10^4	0.19	NI†100

* Data from Lacey and Watson [16].
† No inhibition at the maximum concentration tested.

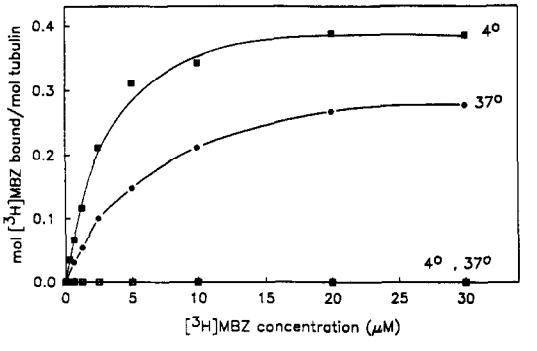


Fig. 4. Stoichiometry of mebendazole binding to tubulin. Mixtures were incubated for 30 min at 4° or 37° using charcoal extraction (open symbols) or gel filtration (filled symbols) assays.

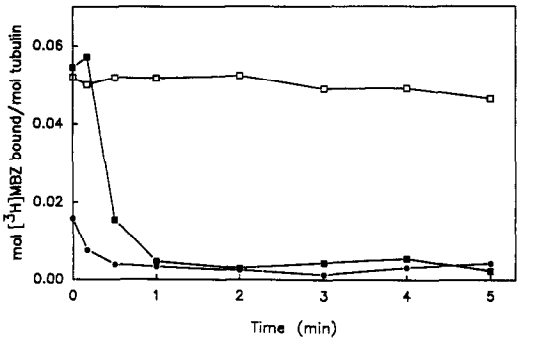


Fig. 5. Dissociation of the [^{3}H]MBZ–tubulin complex. Determined by a 30 min preincubation with 1 μM [^{3}H]-MBZ at either 4° (squares) or 37° (circles) and a 30-min incubation with 50 μM MBZ at 4° (□——□) or 37° (■——■ and ●——●).

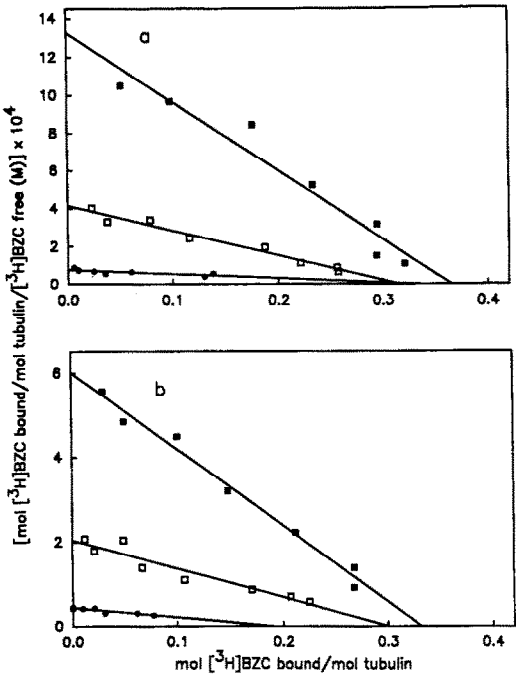


Fig. 6. Scatchard plots for the binding of [^{3}H]BZCs to tubulin. Mebendazole (■), oxibendazole (□) and oxfendazole (●) were incubated at 4° (a) and 37° (b). Data points from a typical experiment are shown.

and/or 1.5 mM Ca^{2+} on the extent and dissociation of [^{3}H]MBZ binding to tubulin. Neither 1 μM calmodulin and 1.5 mM Ca^{2+} nor 1.5 mM Ca^{2+} alone had any effect on the extent or dissociation of [^{3}H]MBZ binding to tubulin at 4° and 37°.

Binding of other anthelmintic BZCs to mammalian tubulin was also investigated to determine the role of the 5(6)-substituent in the formation and stability of the BZC–tubulin complex. The binding of [^{3}H]-MBZ, [^{3}H]OBZ and [^{3}H]OFZ to mammalian brain tubulin was best described as operating at a single site when analysed by the EBDA/LIGAND analysis program. Scatchard plots for the binding of [^{3}H]-MBZ, [^{3}H]OBZ and [^{3}H]OFZ to tubulin at 4° and 37° are shown in Fig. 6. Binding of [^{3}H]MBC to tubulin was not detected at drug concentrations $\leq$ 30 μM and was only within detection limits at 50 μM [^{3}H]MBC (the maximum concentration tested), where binding was observed to be 0.03 mol [^{3}H]-MBC per mol of tubulin. Calculation of K_a and B_{max} values for [^{3}H]MBZ, [^{3}H]OBZ and [^{3}H]OFZ from replicate experiments at 4° and 37° are shown in Table 2. All K_a and B_{max} values were reduced at 37° compared with those observed at 4°.

DISCUSSION

The interaction of [^{3}H]BZCs with mammalian brain tubulin was examined by a rapid gel filtration technique. This assay is a reproducible and sensitive method for investigating the binding of both [^{3}H]-colchicine and [^{3}H]BZCs to mammalian brain tubulin. The association and dissociation of the [^{3}H]-MBZ–tubulin complex were temperature dependent, with significantly greater binding occurring at 4° than at 37°. Binding was rapidly reversible at 37°, however, maintenance of the [^{3}H]MBZ–tubulin complex at 4° markedly reduced the dissociation rate. The K_a and B_{max} values determined for [^{3}H]MBZ, [^{3}H]OBZ and [^{3}H]OFZ were dependent upon incubation temperature, with lower values being recorded at 37° than at 4° for all compounds. These values are consistent with the observed potency of the BZCs as determined by the inhibition of microtubule polymerization or displacement of [^{3}H]colchicine binding [3–5, 12, 15–18]. Studies of the structure–activity relationships of the BZCs as microtubule inhibitors, *in vitro*, have shown that the hydrophobicity and/or size of the 5(6)-substituent is a critical factor in determining activity [16]. The differences between the affinity and extent of binding of the highly active microtubule inhibitors [^{3}H]MBZ and [^{3}H]OBZ, and that of the comparatively weak inhibitors [^{3}H]OFZ and [^{3}H]MBC, determined in this study, reflect this relationship. Our inability to detect [^{3}H]MBZ binding to mammalian brain tubulin using the charcoal extraction assay is consistent with the results previously reported by Lacey [27] for both rat and ovine brain cytosolic supernatants.

Colchicine binding to mammalian brain tubulin is at an optimum level at 37° and undetectable at 4° [28]; conversely, BZC binding is increased at 4°. While the reasons for these differences are not understood at present, Ventilla *et al.* [29] have demonstrated that native mammalian brain tubulin contains approximately 22% α-helix, 30% β-pleated sheet and 48% random coil at 4°, and that gross conformational changes occur as temperature increases, leading to a loss in α-helix at 37°. Temperature-induced conformational changes in the tubulin dimer at regions influencing the colchicine binding site are presumably responsible for the different protein–drug interactions observed for colchicine, podophyllotoxin and the BZCs. Indeed, Head *et al.* [17] have observed that the initial binding of nocodazole to tubulin was favoured by a reduction in temperature and also identified two identical sites per tubulin dimer. Both sites were characterized by identical $K_{d(app)}$ such that equilibrium techniques indicated a single binding site and were unable to detect the apparent heterogeneity within the system. The existence of a second identical binding site which is involved in the rapid and reversible binding of BZCs to tubulin was not observed using the equilibrium techniques in the present study.

Both the temperature dependence of binding and the affinity of the [^{3}H]BZCs for mammalian tubulin observed in this study contrast with those observed for tubulin derived from parasitic nematodes. Optimum binding of [^{3}H]MBZ to tubulin in the parasitic nematodes *Haemonchus contortus* and *Trichostrongylus colubriformis* occurs at 37°, being of relatively high affinity ($K_a \geq 1 \times 10^6\,M^{-1}$) and forms a pseudo-irreversible complex that is stable to charcoal extraction [30]. Our results demonstrate that the binding of [^{3}H]MBZ to mammalian tubulin is not stable to charcoal extraction, is rapidly reversible and possesses a K_a at least one order of

magnitude lower than that observed for helminth tubulin. It is apparent that there are species differences in the rate of dissociation, and hence the stability, of the BZC–tubulin complex as the first-order dissociation rate constant observed for [^{3}H]-MBZ was 1.6×10^{-3} min^{-1} for helminth tubulin* as opposed to 2.7 min^{-1} for mammalian tubulin. Therefore the selective toxicity of the BZCs as anthelmintics is directly related to differences between the affinity of host and helminth tubulins for these compounds. It is with interest that we now await the resolution of the structural differences between tubulins which yields the inherent species specificity of drug action against this highly conserved protein.

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