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A COMPARISON OF THE INTERACTION OF ANTHELMINTIC BENZIMIDAZOLES WITH TUBULIN ISOLATED FROM MAMMALIAN TISSUE AND THE PARASITIC NEMATODE *ASCARIDIA GALLI*

PETER J. DAWSON, WINSTON E. GUTTERIDGE* and KEITH GULL†
Biological Laboratory, University of Kent, Canterbury, Kent CT2 7NJ, U.K.

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Abstract—Colchicine and a range of anthelmintic benzimidazoles inhibited the *in vitro* polymerization of tubulin purified from the parasitic nematode *Ascaridia galli*. In most cases, this inhibition was more pronounced than that detected when these drugs were incubated with tubulin purified from mammalian tissue. In particular, oxfendazole and thiabendazole had virtually no effect on mammalian tubulin assembly whereas they were both good inhibitors of nematode tubulin polymerization. Electron microscopic examinations revealed no morphological differences between microtubules from either nematode or mammalian tissues polymerized in the presence or absence of drug, though the length and number of microtubules was reduced in the drug-incubated samples. These results show that the benzimidazole group of anthelmintics interacts specifically with nematode tubulin and that their selectivity, at least in part, is a direct consequence of such interaction.

The anthelmintic benzimidazole carbamate group of drugs has a broad spectrum of activity against pathogenic nematodes, cestodes and trematodes. Experiments using mammalian brain microtubule protein have demonstrated that benzimidazole carbamates can inhibit the *in vitro* assembly of microtubules [1, 2]. Furthermore, these drugs have been found to bind specifically to tubulin and can be shown to compete with the classical antimicrotubule agent colchicine for its binding site on the tubulin molecule [1–4]. Cytological studies have shown that the benzimidazole carbamates can cause the disappearance of cytoplasmic microtubules from the cells of treated nematodes [5, 6] whilst cytoplasmic and spindle microtubules of the host were unaffected [5]. These observations raise the question of the apparent selective toxicity of these drugs.

Friedman and Platzer [7] found that colchicine binding to embryonic nematode cytosolic extracts could be inhibited by benzimidazole carbamates. Mebendazole and fenbendazole appeared to be competitive inhibitors of colchicine binding to bovine brain tubulin, and the inhibition constants were 250–400 times greater than those found for the interaction with the receptor identified in cell extracts of the embryonic nematode. These authors therefore suggested that the selective toxicity of the benzimidazole carbamates may be related to differential binding affinities between nematode and mammalian tubulin. However, Köhler and Bachmann [8], using cell extracts of adult nematodes in a similar series of experiments, showed little difference in binding affinities between the receptor in these extracts and brain tubulin. These workers have suggested therefore that differences in drug toxicity may be more dependent upon differential pharmacokinetics between parasite and host.

There are inherent problems in the use of crude cell extracts for competitive binding studies, and so we have attempted to make a more direct comparison of the action of the benzimidazole carbamates on tubulin from nematodes and mammals. We have recently developed a protocol for the purification and *in vitro* assembly of nematode tubulin [9]. We now report the effect of colchicine and a range of benzimidazoles on the *in vitro* polymerization of tubulin from the parasitic nematode *Ascaridia galli* and mammalian tissue.

MATERIALS AND METHODS

Materials. All chemicals were Analar or the purest grade available. Biochemicals were purchased from the Sigma Chemical Co. (Poole, Dorset, U.K.). Parbendazole, fenbendazole, mebendazole and thiabendazole were obtained through Pfizer Ltd. (Sandwich, U.K.) and oxfendazole was a gift from Wellcome Research Laboratories (Beckenham, U.K.).

Preparation of tubulin. Details of the purification of tubulin from the pathogenic nematode *Ascaridia galli* have been described previously [9]. In order to provide a comparable protein, tubulin was purified from sheep brain in an identical manner. The nematodes or brain tissue were suspended in 0.1 M PIPES buffer [piperazine-*N*,*N*′-bis(2-ethone sulphuric acid)], pH 6.9, 1 mM EGTA [ethyleneglycol-bis-(2-aminoethyl ether)-*N*,*N*′-tetraacetic acid], 1 mM $MgCl_2$, 1 mM GTP and 20 μg/ml ribonuclease (Sigma Type 1-A) and homogenized using a Polytron. The

* Present address: The Wellcome Research Laboratories, Langley Court, Beckenham, Kent BR3 3BS, U.K.
† To whom correspondence should be addressed.

supernatant obtained after high speed centrifugation of the homogenate was applied to a DEAE-Sephadex A50 column equilibrated with 0.2 M KCl. The column was washed to remove unbound protein, and the salt concentration then raised to 0.5 M KCl to elute the tubulin. After subsequent concentration, centrifugation and glycerol dialysis, the protein in the presence of 1 mM GTP and 20% (v/v) DMSO (dimethyl sulphoxide) was warmed to 30° to assemble microtubules.

Microtubule assembly assays. Immediately before use, twice-cycled (HP_2) tubulin was resuspended in cold assembly buffer, 0.1 M PIPES, pH 6.9, 1 mM $MgCl_2$, 0.1 mM GTP and depolymerized at 0° for 45 min followed by centrifugation at 130,000 *g* for 30 min at 4°. The *A. galli* tubulin was polymerized into microtubules by the addition of GTP and DMSO [final concentrations 1 mM and 20% (v/v), respectively] to 30–50 μl aliquots (protein concentration, 2 mg/ml), and the mixture warmed to 30°. Mammalian tubulin at the same protein concentration was polymerized in the presence of 1 mM GTP at 30°. DMSO was included in some experiments to provide a strict comparison with the *A. galli* tubulin, which requires DMSO for polymerization (see Results). Water-insoluble drugs were first dissolved in DMF (dimethyl formamide) and then diluted into assembly buffer containing the tubulin at 0° to the desired final concentration and a final solvent concentration of 2% (v/v). This concentration of DMF does not inhibit microtubule assembly *in vitro*. After 40 min incubation, the resulting microtubules were pelletted by centrifugation at 100,000 *g* at 26° in a Beckman Airfuge air-driven ultracentrifuge for 5 min. Aliquots of the supernatant were diluted to determine the protein concentration using a sensitive dye-binding assay [10].

The presence of microtubules and the extent of microtubule assembly were also monitored by transmission electron microscopy. Aliquots (3–5 μl) of the sample were removed and placed on carbon-coated Formvar grids, left for a few sec, then washed with 3 drops of aqueous saturated uranyl acetate and examined in an AEI 801A electron microscope.

RESULTS

The antimitotic drug colchicine and the anthelmintic benzimidazoles oxfendazole, thiabendazole, fenbendazole, parbendazole and mebendazole (Fig. 1) were evaluated for their ability to inhibit the *in vitro* polymerization of tubulin purified from the nematode *Ascaridia galli* and a mammalian tissue.

Purification of mammalian tubulin is relatively straightforward and the protein can be obtained in large amounts. However, as with most lower organisms, tubulin is not an abundant protein in nematodes. It is difficult to purify and is only obtainable in very small amounts. Therefore, the yield of purified nematode tubulin makes turbidimetric analysis of polymerization studies impossible. Consequently, we developed a sensitive assay involving the rapid high speed centrifugation of very small amounts (30–50 μl) of sample, followed by the determination of the protein concentration of the supernatant. Microtubules are pelletted by this protocol: consequently, drug inhibition of tubulin polymerization will be detected by an increase in the soluble protein after centrifugation. We also used electron microscopy to confirm the identity of the polymerized material as microtubules.

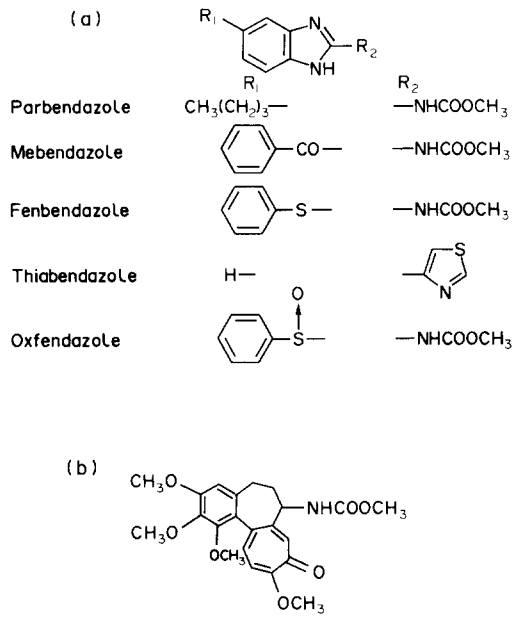

Fig. 1. Chemical structures of the antimicrotubule inhibitors used. (a) Benzimidazole group, (b) colchicine.

Using this methodology, our first priority was to determine the kinetics of tubulin polymerization such

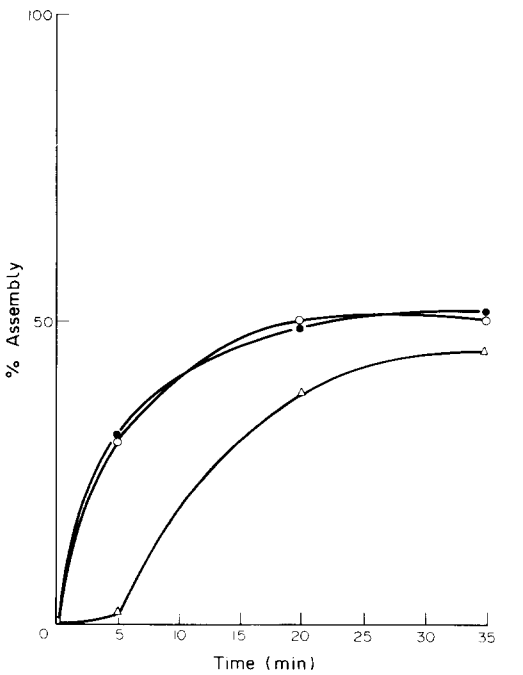

Fig. 2. Rate of polymerization of tubulin (2 mg/ml) as a function of time at 30°. (△) Mammalian brain tubulin, (○) mammalian brain tubulin in the presence of 20% DMSO, (●) *Ascaridia galli* tubulin in the presence of 20% DMSO.

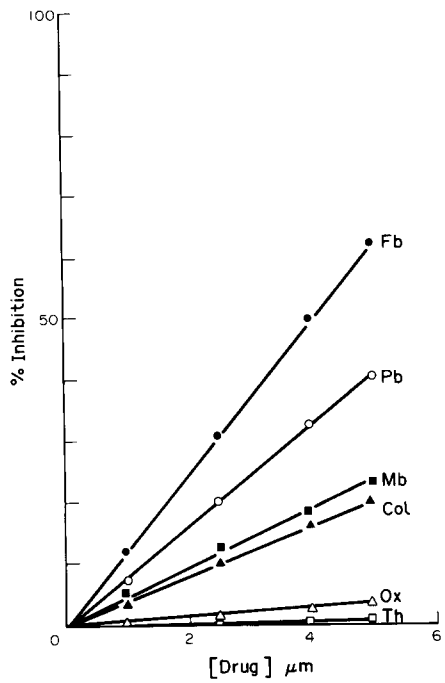

Fig. 3. Inhibition of benzimidazole carbamates and colchicine on the polymerization of tubulin from mammalian brain tissue. Fb, Fenbendazole; Pb, parbendazole; Mb, mebendazole, Ox, oxfendazole; Th, thiabendazole; Col, colchicine.

that all determinations involving drug inhibition could be made when tubulin polymerization had reached equilibrium. Furthermore, as previously described [9], *A. galli* tubulin requires DMSO for *in vitro* polymerization. Therefore it was necessary to determine the effects of DMSO on the kinetics of tubulin polymerization in order to make comparisons of drug inhibition of nematode and mammalian tubulin. Samples of *A. galli* tubulin were incubated at 30° for 5, 20 and 35 min followed by centrifugation and subsequent determinations of the protein concentration of the resulting supernatants. Microtubules were already present at 5 min, and polymerization had reached equilibrium by 35 min (Fig. 2). When this experiment was repeated using mammalian tubulin minus DMSO, it was found that microtubules were slow to form, there being few at 5 min. However, by 35 min they were numerous and again polymerization appeared to have reached a plateau (Fig. 2). Mammalian tubulin was also polymerized in the presence of DMSO. In this case polymerization kinetics were essentially identical to *A. galli* tubulin. Microtubules observed by electron microscopy revealed no morphological differences between the nematode and mammalian systems.

Figure 3 shows the effects of various anthelmintic drugs and colchicine on the *in vitro* assembly of mammalian tubulin without the addition of DMSO. Fenbendazole was the most active benzimidazole, causing 60% inhibition of microtubule assembly at a concentration of 5 μM. Parbendazole and mebendazole both gave significant inhibition of assembly at 5 μM (40 and 22%, respectively). However, oxfendazole and thiabendazole showed only very little inhibition of assembly. Colchicine produced a 20% inhibition of assembly at 5 μM and complete inhibition was seen at around 20 μM. In all experiments electron microscopy of the drug-treated microtubules revealed no morphological differences in their substructure compared with the controls, although the number and length of microtubules were reduced in the drug-treated samples (Fig. 4). These effects of colchicine and the benzimidazoles were essentially unaffected by polymerizing the mammalian tubulin in the presence of DMSO (Fig. 5). Fenbendazole was again the most potent inhibitor of assembly whilst parbendazole, mebendazole and colchicine all produced significant levels of inhibition at concentrations of 5 μM. However, again thiabendazole and oxfendazole showed little inhibition of mammalian tubulin assembly.

Inhibition of assembly of *A. galli* tubulin polymerization by these drugs is seen in Fig. 6. All drugs tested showed a significant inhibition of assembly. Colchicine was the most inhibitory compound. However, all the benzimidazoles gave inhibitions of assembly. Fenbendazole and mebendazole both produced around 50% inhibitions of microtubule assembly at 5 μM. Parbendazole and oxfendazole produced 45% inhibitions at a concentration of 5 μM, and at this concentration thiabendazole inhibited microtubule polymerization by 35% as compared with controls. Electron microscopy of the *A. galli* microtubules polymerized under these various drug treatments revealed no differences in substructure but there was, of course, a difference in the number of microtubules and their lengths (Fig. 4). Consequently, the major difference detected between the mammalian tubulin and that of *A. galli* was the latter's susceptibility to inhibition of polymerization by the benzimidazole anthelmintics.

Drug concentrations causing 50% inhibition in mammalian and nematode tubulin assemblies are summarized in Table 1.

DISCUSSION

Many mammalian tissues contain relatively abundant amounts of the microtubule subunit protein tubulin. Although helminths do not contain such

Table 1. Relative effectiveness of anthelmintic benzimidazoles and colchicine on assembly of mammalian and nematode microtubules *in vitro* (IC_{50})

Drug	Mammal	IC_{50}* Mammal + DMSO	*A. galli* + DMSO
Fenbendazole	4	3.5	4.5
Parbendazole	6	6.5	6
Mebendazole	8	7	5
Oxfendazole	200†	200†	6
Thiabendazole	950†	950†	8
Colchicine	10	9	4

* Concentration of drug ($\times 10^6$ M^{-1}) causing 50% inhibition of microtubule assembly.

† Limit of solubility.

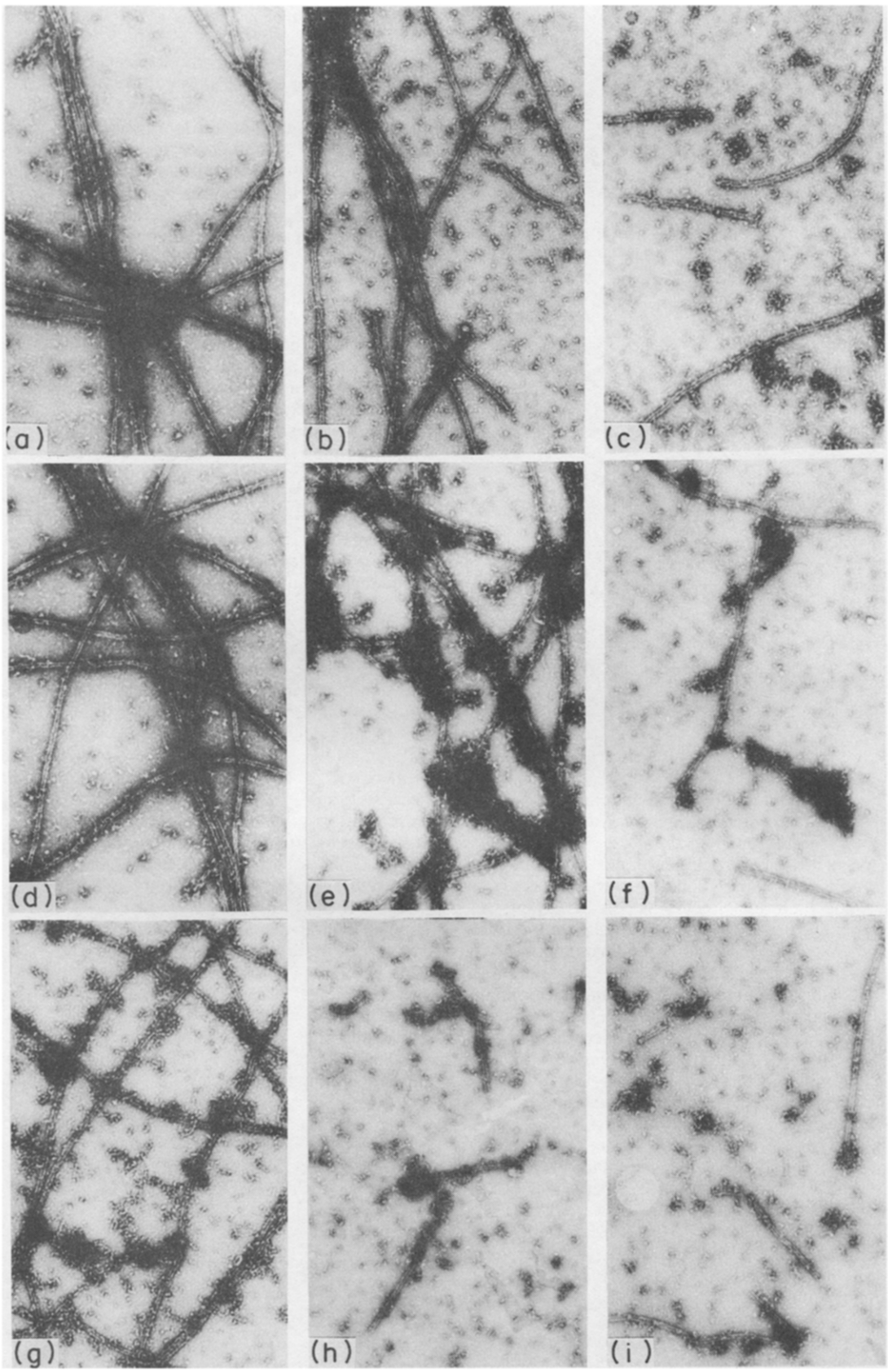

Fig. 4. Electron micrographs of control and drug-treated microtubules. (a) Control, mammalian brain microtubules; (b) mammalian brain microtubules assembled in the presence of 5 μM oxfendazole; (c) mammalian brain microtubules assembled in the presence of 5 μM colchicine; (d) control, mammalian brain microtubules assembled in the presence of 20% DMSO; (e) as for (d), with the addition of 5 μM oxfendazole before assembly; (f) as for (d), with the addition of 5 μM colchicine before assembly; (g) control, *Ascaridia galli* microtubules; (h) *Ascaridia galli* microtubules assembled in the presence of 5 μM oxfendazole; (i) *Ascaridia galli* microtubules assembled in the presence of 5 μM colchicine.

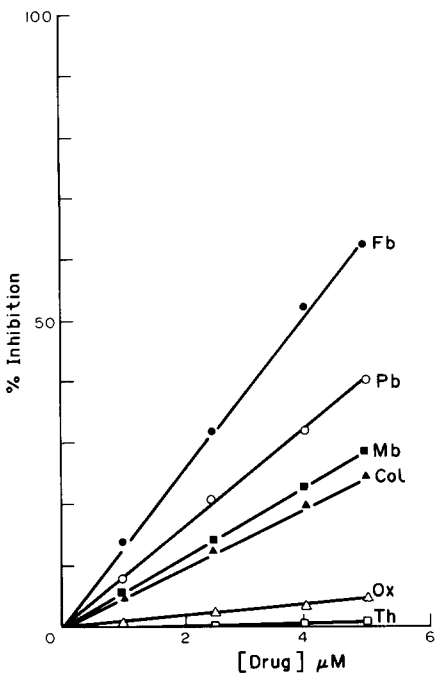

Fig. 5. Inhibition of benzimidazole carbamates and colchicine on the polymerization of tubulin from mammalian brain tissue incubated with 20% DMSO. Abbreviations as for Fig. 3.

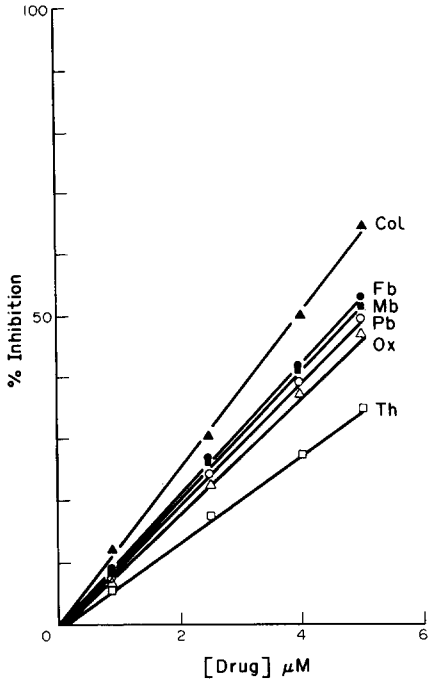

Fig. 6. Inhibition of benzimidazole carbamates and colchicine on the polymerization of tubulin from *Ascaridia galli*. Abbreviations as for Fig. 3.

large amounts of this protein, we have been able to purify enough for use in a direct assay of the relative potency of the benzimidazole group of drugs as inhibitors of microtubule polymerization. The small quantities of helminth tubulin made turbidimetric assays of microtubule polymerization undesirable. Consequently, we used an air-driven centrifuge to provide a fast ultracentrifugation of microtubules and coupled this to a direct assay of the amount of protein that remained unassembled in the supernatant. We tested this protocol with mammalian brain tubulin and found that it gave a reliable estimate of microtubule assembly. Furthermore, our results using this protocol on the effectiveness of various inhibitors of *in vitro* polymerization of mammalian tubulin were in very reasonable accord to previously published work using turbidimetric methods of assaying polymerization [1, 2].

Purified nematode tubulin appears to have slightly different requirements for *in vitro* polymerization than mammalian tubulin. We have previously attempted the assembly of the nematode tubulin under a very wide range of conditions and have so far only been able to induce assembly by the inclusion of DMSO in the assembly buffers. The method of production of the nematode tubulin makes it likely that microtubule-associated proteins will not be co-purified. Consequently, we were careful to compare the *in vitro* polymerization of nematode tubulin with mammalian tubulin purified by exactly the same protocol [9] and assembled under identical conditions.

Our results show that the nematode tubulin polymerization is generally more susceptible to inhibition by a range of inhibitors than is mammalian tubulin. Differences in susceptibility to colchicine have been shown previously for yeast [11] and *Physarum* [12] tubulins. However, both of these cases involve tubulins which are much less susceptible to the effects of colchicine than is mammalian tubulin. *Ascaridia galli* tubulin appears to be slightly more susceptible to colchicine inhibition of polymerization than mammalian tubulin. The previous reports of binding of benzimidazole drugs to nematode tubulins have relied on competition studies using radiolabelled colchicine and crude cell extracts [7, 8]. This approach has yielded two rather conflicting views. Friedman and Platzer [7] have indicated that there is increased binding of some benzimidazoles to nematode tubulin, whilst Köhler and Bachmann [8] have reported that nematode tubulin bound the benzimidazole with kinetics similar to mammalian tubulin. There are differences in the source of material used in these studies. However, such studies are difficult to assess in that the binding was being assessed indirectly, and the tubulin was contained in crude extracts. We have sought to look at the direct effects of the benzimidazoles on microtubule assembly *in vitro* using highly purified tubulin from mammalian tissue and the nematode *Ascaridia galli*. Our results clearly show that very low concentrations of the benzimidazole anthelmintic compounds are very effective inhibitors of nematode tubulin polymerization. In fact, all the inhibitors we used

inhibited *A. galli* tubulin polymerization whilst mammalian tubulin was relatively unaffected by oxfendazole and thiabendazole. It has previously been reported that thiabendazole and methyl benzimidazole carbamate were bound more effectively by the tubulin of mycelial fungi than by mammalian tubulin [13, 14]. Two other benzimidazoles, parbendazole and mebendazole, again showed a general tendency to inhibit nematode tubulin assembly more effectively than the polymerization of mammalian tubulin.

Data published previously [2] showed no absolute correlation between anthelmintic efficacy and antimicrotubule activity using mammalian tubulin. This has also been ascertained from our experiments with nematode tubulin. Comparisons with data for *in vivo* drug effects show good correlation between antimicrotubule activity against nematode tubulin and anthelmintic efficacy for parbendazole, fenbendazole and oxfendazole but not for mebendazole and thiabendazole which may be due in part to pharmacodynamic behaviour or alternatively metabolic effects imposed on the drug. The slightly reduced *in vitro* sensitivity of nematode tubulin towards fenbendazole compared to mammalian tubulin may suggest that the *in vivo* situation greatly influences drug–tubulin interactions and the ability to inhibit nematode and microtubule assembly. However, at present there is insufficient data to explain the result or support this view.

Our results provide evidence that the benzimidazole group of anthelmintic drugs do interact, at low concentrations, with tubulin purified from a nematode and cause an inhibition of its polymerization. Furthermore, there is a general tendency for the nematode tubulin to be more susceptible to inhibition of polymerization than mammalian tubulin. These results should provide a basis for understanding the nature of the resistance to benzimidazoles recently developed by some nematodes in terms of possible alterations in microtubule protein. Furthermore, the interaction of these drugs with nematode tubulin *in vivo* may be expected to produce a variety of cytopathic effects. We are presently studying the consequences of treatment with benzimidazoles on the microtubule-based functions of nematodes.

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REFERENCES

1. P. A. Friedman and E. G. Platzer, *Biochim. biophys. Acta* **544**, 605 (1978).
2. C. M. Ireland, K. Gull, W. E. Gutteridge and C. I. Pogson, *Biochem. Pharmac.* **28**, 2680 (1979).
3. J. Hoebeke, G. Van Nijen and M. De Brabander, *Biochem. biophys. Res. Commun.* **69**, 319 (1976).
4. J. C. Havercroft, R. A. Quinlan and K. Gull, *J. Cell Sci.* **49**, 195 (1981).
5. M. Borgers, S. De Nollin, A. Verheyen, M. De Brabander and D. Thienpont, in *Microtubules and Microtubule Inhibitors* (Eds. M. Borgers and M. De Brabander), p. 497. North Holland/Elsevier, Amsterdam (1975).
6. C. Atkinson, R. J. Newsam and K. Gull, *Protoplasma* **105**, 69 (1980).
7. P. A. Friedman and E. G. Platzer, *Biochim. biophys. Acta* **630**, 271 (1980).
8. P. Köhler and R. Bachmann, *Molec. biochem. Parasit.* **4**, 325 (1981).
9. P. J. Dawson, W. E. Gutteridge and K. Gull, *Molec. biochem. Parasit.* **7**, 267 (1983).
10. M. Bradford, *Analyt. Biochem.* **72**, 248 (1976).
11. J. V. Kilmartin, *Biochemistry* **20**, 3629 (1981).
12. R. A. Quinlan, A. Roobol, C. I. Pogson and K. Gull, *J. gen. Micro.* **122**, 1 (1981).
13. L. C. Davidse and W. Flach, *J. Cell Biol.* **72**, 174 (1977).
14. L. C. Davidse and W. Flach, *Biochim. biophys. Acta* **543**, 82 (1978).