Biochemical Pharmacology, Vol. 39, No. 5, pp. 851–856, 1990.
Printed in Great Britain.

0006-2952/90 $3.00 + 0.00

DIETHYLCARBAMAZINE, ANTIFILARIAL DRUG, INHIBITS MICROTUBULE POLYMERIZATION AND DISRUPTS PREFORMED MICROTUBULES

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(*Received* 7 *June* 1989; *accepted* 10 *October* 1989)

Abstract—The effect of diethylcarbamazine (DEC) on microtubules was studied by using microtubule protein prepared from porcine brain. DEC inhibited assembly of microtubules and disassembled preformed microtubules *in vitro*. When the reassembled or disassembled products were examined in the presence of DEC by electron microscopy, ribbon-microtubules were frequently observed. Subsequently, the effect of DEC on the cytoplasmic microtubules complex was studied. The cells used in our study were LLC-MK_2. DEC inhibited proliferation of these cells, and cells grown in the presence of DEC were likely to separate from each other and became round in shape. Immunofluorescence microscopy revealed that the cells exposed to DEC were devoid of the delicate pattern of the cytoplasmic microtubule complex.

In our previous study [1], we reported that diethylcarbamazine (DEC) inhibited the development of *Brugia pahangi* larvae cultured *in vitro* in the presence of feeder cells (LLC-MK_2 cells). One of the possible explanations for this inhibition is that DEC induces some damages in the function of feeder cells.

In our preliminary experiments, we observed that DEC inhibited the proliferation of LLC-MK_2 cells and the cells exposed to DEC were likely to become round in shape. These results suggested that DEC interferes with microtubules, because microtubules are involved in a variety of cell functions, including mitosis, secretion, regulation of cell form, etc.

In the present communication, we report that DEC inhibits proliferation of LLC-MK_2 cells, disrupts the cytoplasmic microtubules complex, inhibits the assembly of microtubules *in vitro* and induces the disassembly of the preformed microtubules *in vitro*.

MATERIALS AND METHODS

Reagents. Diethylcarbamazine citrate (Supatonin) was purchased from Tanabe Ltd. The structure of the drug is shown in Fig. 1; guanosine triphosphate (GTP) from Seikagaku Co.; 2-(morpholino) etanesulfonic acid (MES) and ethyleneglycol-bis(2-aminoethylether)-*N,N,N',N'*-tetraacetic acid (EGTA) from Nakarai Chemical Ltd.

Cells and culture medium. LLC-MK_2 cells, an established cell line of rhesus monkey kidney, was obtained from Flow Laboratories, Inc. Culture medium used was RPMI 1640 buffered with Hepes (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid) and sodium bicarbonate (pH 7.4) and supplemented with 10% heat inactivated fetal bovine serum (KC Biological). Cells were grown at 37° in CO_2 incubator.

Cell proliferation in the presence or absence of DEC. Cells (10^5) were seeded in each tissue culture dishes (Falcon, 35 mm) with 2 mL of culture medium and grown for 96 hr. At this time, when cells were in exponential growth phase, the medium was replaced with the medium containing or lacking DEC (0.1 mg/mL and 1.0 mg/mL). Proliferation was continued for further 96 hr. The medium with or without DEC was changed every other day. In some culture dishes, cells exposed to DEC (1.0 mg/mL) for 48 hr were washed thoroughly with DEC-free medium and then grown for 48 hr in the absence of DEC. Cell counts were done every other day in a Türk-haematocytometer after detaching the cells with trypsin and EGTA and staining with Trypan blue.

Morphological observations of cells grown in the presence or absence of DEC. The cells seeded on glass coverslips were grown for 24 hr in the presence or absence of DEC (1.0 mg/mL). They were washed thoroughly with 0.1 M MES buffer (pH 6.8) containing 0.5 mM $MgCl_2$ and 1 mM EGTA, referred to as MES buffer thereafter. Then the specimens were fixed with 2% glutaraldehyde in MES buffer. They were stained with Haematoxylin–Eosin.

Preparation of microtubule proteins. Microtubule

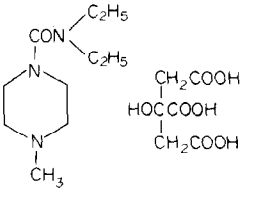

Fig. 1. The structure of diethylcarbamazine citrate (DEC).

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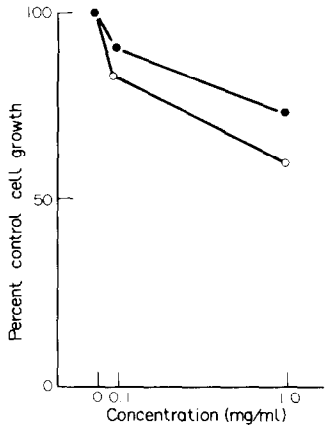

Fig. 2. Effect of DEC on LLC-MK_2 cells proliferation. Cells were seeded into Petri dishes (Falcon, 35 mm) at a density of 10^5 cells per dish and cultured at 37° in a 5% CO_2 incubator. On day 4 when they reached the exponential growth phase, the medium was replaced with the medium containing or lacking DEC. The cells were allowed to proliferate for a further 48 hours (●—●) or 96 hours (○—○) in the presence or absence of DEC. Numbers of cells cultured in the presence of DEC were plotted as percentage of control values. Number of control cells cultured for 48 hr or 96 hr was $12.6 \pm 0.91 \times 10^5$ or $22.6 \pm 1.14 \times 10^5$, respectively.

protein from porcine brain was purified by two cycles of assembly and disassembly essentially as described by Shelanski *et al.* [2] and stored at −80° in MES buffer without glycerol. All experiments were carried out on protein stored for less than 1 week. The microtubule protein was cycled once more before use. Concentration of microtubule protein was determined by Bio-Rad protein assay.

Preparation of antitubulin. The microtubular antibody preparation was obtained in rabbits using homogenous tubulin prepared from the microtubular protein by SDS–gel electrophoresis [3].

The preparation of IgG fraction was obtained by DEAE-Sephadex A 50 column chromatography. Antitubulin thus prepared was tested by the double immunodiffusion test and used for immunofluorescent visualization of cytoplasmic microtubules in LLC-MK_2 cells.

Visualization of cytoplasmic microtubules in LLC-MK_2 cells. Cells grown on glass coverslips in the presence or absence of DEC (1.0 mg/mL) were processed for immunofluorescent staining with tubulin antibody by the method of Brinkley *et al.* [4]. Briefly, cells were fixed in 3% formaldehyde in phosphate buffered saline (PBS), washed in PBS, immersed in absolute acetone at −20° for 7 min, and further washed in PBS. The cells were then incubated with antitubulin for 60 min, washed extensively in PBS and incubated with fluorescein-conjugated goat anti-rabbit IgG (COOPER Biomedical) for 60 min. After extensive washing in PBS, and draining the excess buffer, the coverslips were mounted in medium, containing 10% carbonate buffered glycerine. Usually slides were observed not later than 12 hr after mounting.

Assembly and disassembly of microtubules in vitro. Microtubule formation *in vitro* was determined by turbidity measurement [5] at 350 nm using Shimazu UV-240 spectrophotometer equipped with a thermostatically controlled cell holder. The path length of cuvette was 1 cm. Briefly, polymerization was initiated by adding 1 mM GTP to MES buffer and triggered by a temperature of 37°. For the determination of the inhibitory effect of DEC, the drug was dissolved in MES buffer. The microtubule protein concentration used in our experiments was about 3 mg/mL.

To examine the effect of DEC on the preformed microtubules, MES buffer containing DEC was added to the preformed microtubule and the reduction in turbidity was monitored.

Electron microscopy of microtubules. Microtubules assembled in the presence or absence of DEC and DEC-induced disassembled microtubules were examined by electron microscopy. A drop of the sample solution was applied to a carbon-coated collodion grid. After the specimens were washed with distilled water and the excess water was drained with a filter paper, they were stained with 1% uranyl acetate and examined by a JEM 100 CX operated at 80 kV.

RESULTS

Effects of DEC on cell proliferation in vitro

Continuous presence of DEC at a concentration between 0.1 mg/mL and 1.0 mg/mL, incompletely but significantly inhibited LLC-MK_2 cell proliferation. In the medium with high concentration of drug (1.0 mg/mL), per cent inhibition of cell growth reached 26.5% for 48 hr and 41.0% for 96 hr. In the medium with low concentration of drug (0.1 mg/mL), the corresponding values were 8.4% for 48 hr and 16.7% for 96 hr (Fig. 2). When DEC was removed from the medium, cells proliferated again as the controls (data not shown).

Cell form observation of LLC-MK_2 exposed to DEC

Figure 3A shows the LLC-MK_2 cells which were grown in the medium, fixed in 2% glutaraldehyde in MES buffer and stained with Haematoxylin–Eosin. All cells were multiform and finely united to each other. When the cells were cultured in the medium containing DEC (1.0 mg/mL) for 24 hr, the tight junction between cells became loose and the cells were likely to separate from each other and became round in shape (Fig. 3B).

Cytoplasmic microtubule complex in LLC-MK_2 cells grown in the presence or absence of DEC

Cells grown on coverslips were processed for indirect immunofluorescence as described in Materials and Methods. Figure 4A shows a typical fluorescent pattern. The cells display a multitude of very thin, separate fluorescent fibers within the cytoplasm. When the cells were exposed to DEC, the microtubules pattern was affected. Figure 4B shows the fluorescent pattern of cells which were cultured in a high concentration of DEC (1.0 mg/mL) for 24 hr. The cells were devoid of the delicate

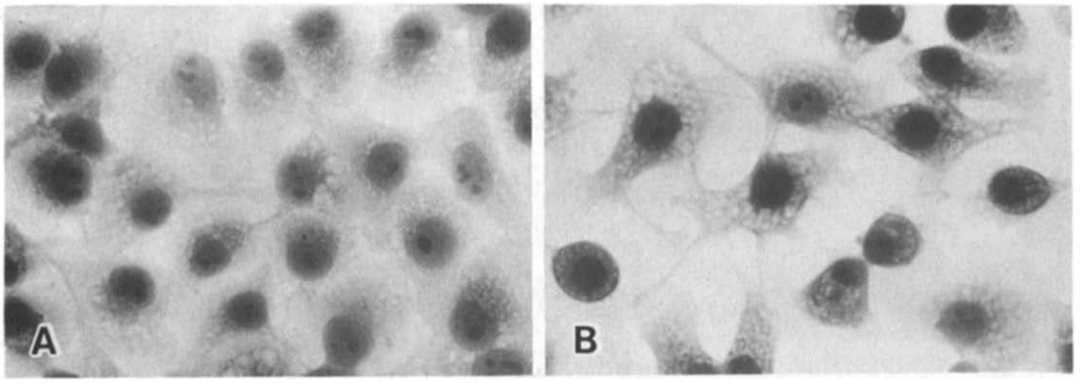

Fig. 3. Haematoxylin–Eosin staining of LLC-MK_2 cells which were grown on coverslip in the absence of DEC (A) and (B) in the presence of DEC (1.0 mg/mL).

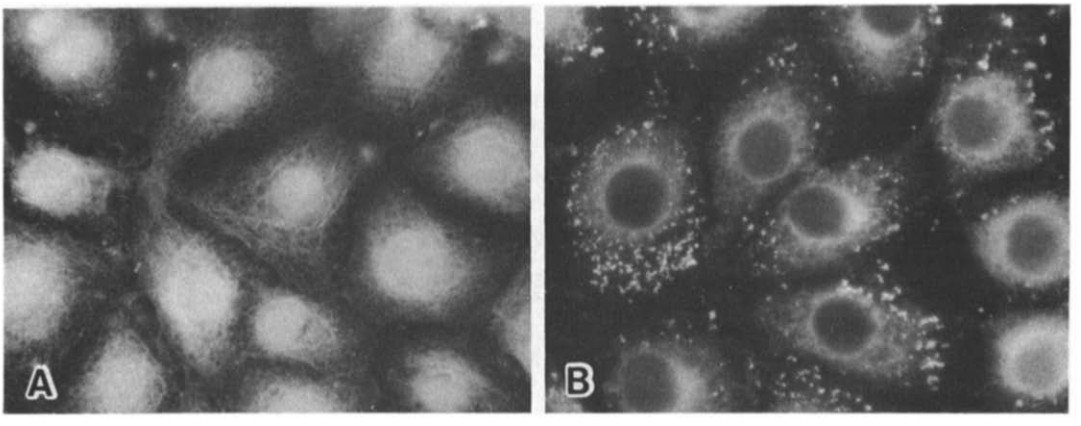

Fig. 4. Cytoplasmic microtubules visualized by indirect immunofluorescence. (A) Intact LLC-MK_2 cells; (B) LLC-MK_2 cells exposed to a high concentration of DEC (1.0 mg/mL) for 24 hr.

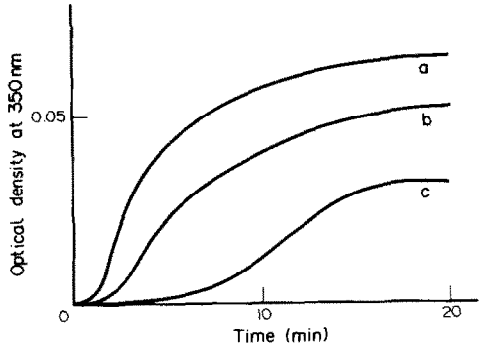

Fig. 5. Effects of DEC on microtubules polymerization *in vitro* in the absence of DEC (a), in the presence of a low concentration (0.1 mg/mL) of DEC (b) and a high concentration (1.0 mg/mL) of DEC (c). Microtubule protein was mixed with 0.1 M MES buffer (pH 6.8) containing 0.5 mM $MgCl_2$, 1 mM EGTA on ice. Polymerization was initiated by adding 1 mM GTP and raising temperature to 37°.

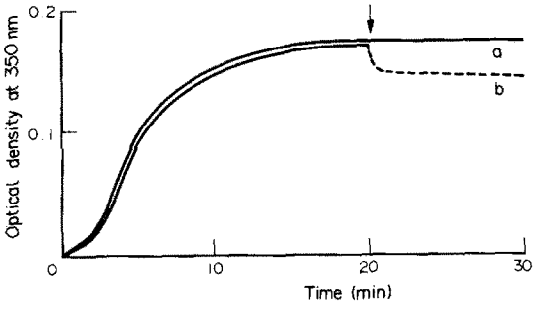

Fig. 6. Effects of DEC on preformed microtubules. Microtubules protein was polymerized in 0.99 mL of 0.1 M MES buffer (pH 6.8) containing 0.5 mM $MgCl_2$, 1 mM EGTA and 1 mM GTP at 37°. Twenty minutes later, 10 μL of the MES buffer lacking (a) or containing DEC (100 mg/mL) (b) was added (arrow) and the turbidity change was monitored.

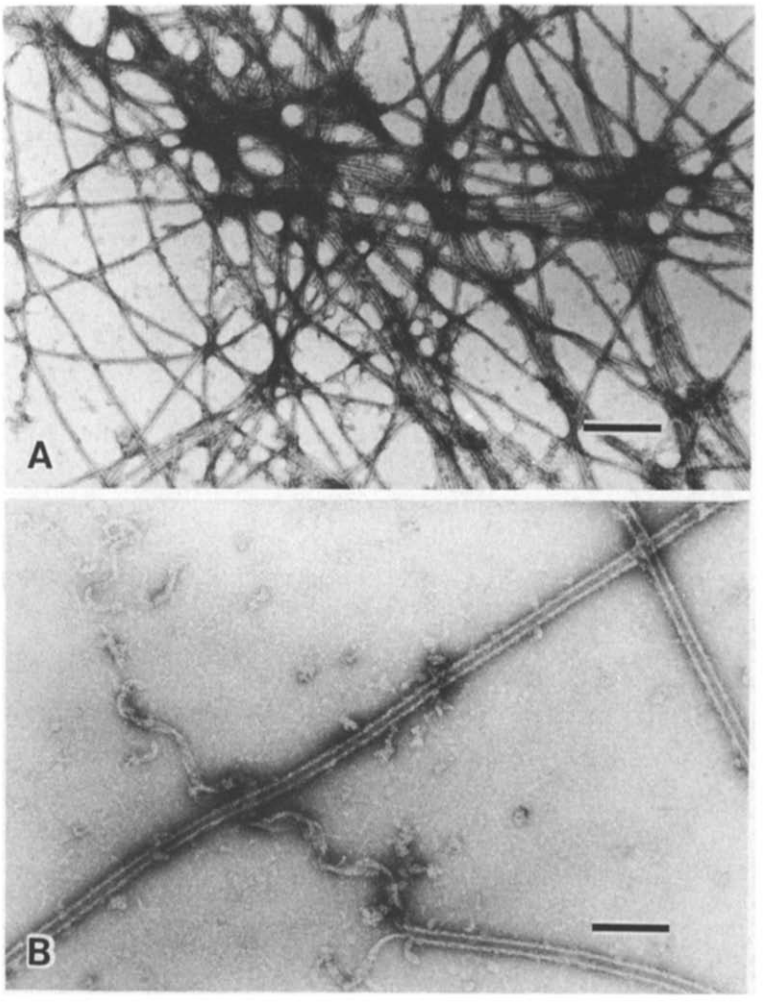

Fig. 7. Electron micrographs of microtubules protein incubated in the absence (A) bar = 6 μm or in the presence of DEC (1.0 mg/mL) (B) bar = 3 μm.

pattern of cytoplasm fibers and showed rod-like aggregates in the cytoplasm.

Effect of DEC on microtubule polymerization and preformed microtubules in vitro

All solutions tested in our experiment were adjusted to pH 6.8, because the polymerization of microtubular protein is very sensitive to pH [6]. Microtubular polymerization curves are shown in Fig. 5. It can be seen that DEC induces a strong inhibition of microtubule polymerization. Indeed, at a high concentration of drug (1.0 mg/mL), the polymerization was preceded by a pronounced lag period and subsequent inhibition of the order 50%.

To examine the interaction of DEC with preformed microtubules *in vitro*, an aliquot of concentrated DEC was added to MES buffer after the formation of microtubules. A small but reproducible decrease in the absorbance occurred (Fig. 6).

Electron microscopy of microtubule protein

The polymerization and depolymerization products in the presence of DEC were examined by electron microscopy. Figure 7A shows the microtubules assembled in MES buffer containing 1 mM GTP. When DEC was contained in the buffer, at a concentration of 1.0 mg/mL, in addition to the microtubules protein, ribbon microtubules were frequently formed (Fig. 7B). These ribbon-microtubules were formed again when preformed microtubules were mixed with a high concentration of DEC (data not shown).

DISCUSSION

DEC is a drug which has been used for treatment of filariasis for many years. Beside its powerful filaricidal action, DEC shows other pharmacological actions against a variety of mammalian cells. DEC inhibits the release of histamine or SRS-A/LTC4 from rat peritoneal cavity [7], human lung [8], monkey lung tissue [9], rat basophil leukemia cells [10], mouse bone marrow derived mast cells [11] and mastocytoma cells [12]. Stevens *et al.* [13] reported that DEC inhibited proteoglycan synthesis and exocytosis in the swarm rat chondrocyte.

The present report is the first to document that DEC inhibits microtubule polymerization and disrupts preformed microtubules. It is well known that a range of benzimidazoles not only inhibits the assembly of mammalian and nematode microtubules but also reversed microtubule assembly *in vitro* [14–16]. The IC_{50} values of fenbendazole, parbendazole and mebendazole are in the range of 3.5 to 8.6 μM for sheep and bovine brain tubulin and that of thiabendazole is 720 to 950 μM [16, 17]. While, the estimated IC_{50} of DEC is 2×10^{-3} M, so that DEC acts on mammalian microtubules assembly at concentrations 2–500 times higher than the concentration of a group of benzimidazoles. For the experimental treatment of the rodents infected with filarial worm by DEC, however, a large dose of DEC (300 mg/kg body wt) is usually used and plasma concentration of DEC reached up to a level of 61 μg/mL 30 min after the administration of the drug [18]. Therefore, a concentration of 0.1 mg/mL which inhibited partially polymerization of microtubules is near physiological concentration of DEC. Friedman and Platzer [17] reported the selective toxicity of benzimidazoles which is related to differential binding affinities between nematode tubulin and mammalian tubulin. Studies on the sensitivity of filarial worm microtubules to DEC are expected to be done. Recently the tubulin was estimated to account for about 3% of soluble protein in Brugian filarial worm [19].

The electron microscopy of the products assembled in the presence of DEC was expected to reflect the DEC-inhibited polymerization of microtubules. The only notable change induced by DEC was the formation of ribbon-like microtubules. The ribbon-microtubules are transit intermediates during normal assembly of microtubules [20]. However, they are usually detected in an abnormal process of polymerization [21]. The experiment should be attempted to disclose whether the ribbon-like microtubules formed in the presence of DEC is a normal or abnormal process of polymerization of microtubules. Benzimidazoles did not cause any morphological alteration in microtubules, though the length and number of microtubules were reduced in the drug-incubated samples [16]. The study should also be designed to examine actually the length and number of microtubules formed in the presence of DEC.

The microtubules are involved in a vareity of cell function including chromosome movement, secretion, regulation of cell form and anchorage of surface receptors into the plasma membrane [4]. The present study describes DEC-inhibited LLC-MK_2 cell proliferation and DEC-induced alterations of the tight junctions between cells and in cell form. These alterations probably are attributed to DEC-disrupted spindle microtubules and microtubules complex in cytoplasm.

Physical and chemical treatment, such as cold temperature, colchicine or vinblastine, disrupt the cytoplasmic microtubule complex [4]. In the cultured cells treated with colchicine, no organized fluorescent microtubules appeared in the cytoplasm [22]. Vinblastine induces paracrystalline formation in cytoplasm of cultured cells [23]. Benzimidazole carbamates caused the disappearance of cytoplasmic microtubules from the cells of treated nematodes [24, 25]. Our immunofluorescence microscopy revealed that DEC induces rod-like aggregates in cytoplasm of LLC-MK_2 cells. We used homogenous tubulin for immunization and purified IgG from serum for staining the cytoplasmic microtubules. Therefore DEC-induced aggregates in our cells are of tubulin and not non-specific staining.

In our previous report [1], we dealt with the DEC-inhibited development of filarial larvae cultured *in vitro* using feeder cells. DEC-inhibited development of larvae may now be explained by the possible assumption that DEC interacts with microtubules of feeder cells and finally these cells lose their supporting functions for filarial larvae.

Acknowledgements—This work was supported in part by Grant-in-Aid (No. 62570178) for Scientific Research from Ministry of Education, Science, and Culture of Japan.

REFERENCES

1. Fujimaki Y, Shimada S, Kimura E and Aoki Y, DEC-inhibited development of third-stage *Brugia pahangi* in vitro. *Parasitol Res* **74**: 299–300, 1988.
2. Shelanski ML, Gaskin F and Cantor CR, Microtubule assembly in the absence of added nucleotides. *Proc Natl Acad Sci USA* **70**: 765–768, 1973.
3. Aubin JE, Subrahmanyan L, Kalnins VI and Ling V, Antisera against electrophoretically purified tubulin stimulate colchicine-binding activity. *Proc Natl Acad Sci USA* **73**: 1246–1249, 1976.
4. Brinkley BR, Fistel SH, Marcum JM and Pardue RL, Microtubules in cultured cells; indirect immunofluorescent staining with tubulin antibody. *Int Rev Cytol* **63**: 59–95, 1980.
5. Gaskin F and Cantor CR, Turbidimetric studies of the *in vitro* assembly and disassembly of porcine neurotubules. *J Mol Biol* **89**: 737–758, 1974.
6. Matsumura F and Hayashi M, Polymorphism of tubulin assembly: *in vitro* formation of sheet, twisted ribbon and microtubule. *Biochim Biophys Acta* **453**: 162–175, 1976.
7. Orange RP, Valentine MD and Austen KF, Inhibition of the release of slow-reacting substance of anaphylaxis in the rat with diethylcarbamazine. *Proc Soc Exp Biol Med* **127**: 127–132, 1968.
8. Orange RP, Austen WG and Austen KF, Immunological release of histamine and slow-reacting substance of anaphylaxis from human lung. *J Exp Med* **134**: 136s–148s, 1971.
9. Ishizaka T, Ishizaka K, Orange RP and Austen KF, Pharmacologic inhibition of the antigen-induced release of histamine and slow reacting substance of anaphylaxis (SRS-A) from monkey lung tissues mediated by human IgE. *J Immunol* **106**: 1267–1273, 1971.
10. Bach MK and Brashler JR, Inhibition of the leukotriene synthetase of rat basophil leukemia cells by diethylcarbamazine, and synergism between diethylcarbamazine and piriprost, a 5-lipoxygenase inhibitor. *Biochem Pharmacol* **35**: 425–433, 1986.
11. Razin E, Romeo LC, Krilis S, Liu F-T, Lewis RA, Corey EJ and Austen KF, An analysis of the relationship between 5-lipoxygenase product generation and the secretion of preformed mediators from mouse bone marrow-derived mast cells. *J Immunol* **133**: 938–945, 1984.
12. Mathews WR and Murphy RC, Inhibition of leukotriene biosynthesis in mastocytoma cells by diethylcarbamazine. *Biochem Pharmacol* **31**: 2129–2132, 1982.

13. Stevens RL, Parsons WG, Austen KF, Hein A and Caulfield JP, Novel inhibition of proteoglycan synthesis and exocytosis by diethylcarbamazine in the swarm rat chondrocyte. *J Biol Chem* **260**: 5777–5786, 1985.
14. Friedman PA and Platzer EG, Interaction of anthelmintic benzimidazoles and benzimidazole derivatives with bovine brain tubulin. *Biochim Biophys Acta* **544**: 605–614, 1978.
15. Laclette JP, Guerra G and Zetina C, Inhibition of tubulin polymerization by menendazole. *Biochem Biophys Res Commun* **92**: 417–423, 1980.
16. Dawson PJ, Gutteridge WE and Gull K, A comparison of the interaction anthelmintic benzimidazoles with tubulin isolated from mammalian tissue and the parasitic nematode. *Ascaridia galli*. *Biochem Pharmacol* **33**: 1069–1074, 1984.
17. Friedman PA and Platzer EG, Interaction of anthelmintic benzimidazoles with *Ascaris suum* embryonic tubulin. *Biochim Biophys Acta* **630**: 271–278, 1980.
18. Kimura E, Aoki Y, Nakajima Y and Niwa M, Plasma level of diethylcarbamazine in jirds and hamsters. *Southeast Asian J Trop Med Pub Hlth* **15**: 74–79, 1984.
19. Tang L and Prichard RK, Characterization of tubulin from *Brugia malayi* and *Brugia pahangi*. *Mol Biochem Parasitol* **32**: 145–152, 1989.
20. Kirschner MW, Honig LS and Williams RC, Quantitative electron microscopy of microtubule assembly *in vitro*. *J Mol Biol* **99**: 263–276, 1975.
21. Sato Y, Murai T, Oda T, Saito H, Kodama M and Hirata A, Inhibition of microtubule polymerization by synthetic estrogens: formation of a ribbon structure. *J Biochem* **101**: 1247–1252, 1987.
22. Weber K, Pollack R and Bibring T, Antibody against tubulin: the specific visualization of cytoplasmic microtubules in tissue culture cells. *Proc Natl Acad Sci USA* **72**: 459–463, 1975.
23. Manfredi JJ and Horwitz SB, Vinblastine paracrystals from cultured cells are calcium-stable. *Exp Cell Res* **150**: 205–212, 1984.
24. Borgers M, De Nollin S, Verheyen A, Vanparijs O and Thienpont D, Morphological changes in cysticerci of *Taenia taeniaeformis* after mebendazole treatment. *J Parastiol* **61**: 830–843, 1975.
25. Borgers M, De Nollin S, De Brabander M and Thienpont D, Influence of the anthelmintic mebendazole on microtubules and intracellular organelle movement in nematode intestinal cells. *Am J Vet Res* **36**: 1153–1166, 1975.