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Ultrastructural evaluation of mebendazole action in *Pseudodactylogyrus bini* (Monogenea), gill parasites from European eel *Anguilla anguilla*

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ABSTRACT: The broad-spectrum anthelmintic mebendazole (MBZ) has demonstrated its efficacy against a range of fish-parasitizing monogeneans. The action of this drug in the gill parasitic monogenean *Pseudodactylogyrus bini* has been elucidated by the use of transmission electron microscopy (TEM). Parasites were exposed *in vitro* to MBZ (1 and 10 mg l^{-1}) for up to 42 h whereafter they were fixed and prepared for TEM studies. It was demonstrated that the tegument and subtegumental layers in parasites exposed to 10 mg MBZ l^{-1} for 36 h were disorganized and partly destroyed, and that the drug interfered with the organization of the flame-cell microtubule system. This is in accordance with the suggestion that tubulin and thereby the cytoskeleton is the main MBZ target, although other drug action mechanisms cannot be excluded.

KEY WORDS: Benzimidazoles · Mebendazole · Monogenea · *Pseudodactylogyrus* · Mode of action · Ultrastructure · Microtubules

INTRODUCTION

Benzimidazoles have been extensively used as anthelmintics in human medicine (Cook 1990) and in traditional livestock industries (Campbell 1990). Recently this drug group has also been used against helminth infections in aquaculture systems (Goven & Amend 1982, Székely & Molnár 1987, Buchmann & Bjerregaard 1990a, b, Mellergaard 1990, Tojo et al. 1992). However, although the mode of action of benzimidazoles in parasites from homoiothermic animals has been the subject of numerous investigations (Lacey 1988) similar studies for fish parasites have not been published. The study elucidates the action of mebendazole (MBZ) in the gill monogeneans *Pseudodactylogyrus bini* parasitizing the European eel *Anguilla anguilla*.

MATERIALS AND METHODS

A total of 32 *Pseudodactylogyrus bini* were removed from the gills of 1 large eel (100 g body weight), rinsed in freshwater to remove mucus and cell debris and transferred to a 96 well cell incubation plate (Nunc, Denmark) kept at 24 °C.

The parasites were incubated in groups of 8 in 1 or 10 mg MBZ l^{-1}, in freshwater alone or in freshwater containing the solvent formic acid (10 µl l^{-1}) (Table 1). The drug used was pure MBZ. Parasites were incubated for 36 h (10 mg MBZ l^{-1}) or 42 h (1 mg MBZ l^{-1}). Half of the control worms were kept for 36 h and the rest for 42 h. After the experiment all parasites were fixed in 2.5 % glutaraldehyde in 0.1 M cacodylate buffer (pH 7.4) for 2 h. They were then postfixed in 1 % osmium tetroxide for 1 h at 4 °C and block stained for 2 h at room temperature in 0.5 % aqueous uranyl acetate. Epon was used as embedding medium. The sections were cut with a diamond knife on a Reichert Jung ultracut ultramicrotome, stained with uranyl acetate and lead citrate and examined with a Jeol 1200 Ex electron microscope at 60 kV. Controls (non-treated and formic-acid-exposed parasites) were handled likewise. In order to reduce artefacts and secondary postmortem changes of parasite structure, only parasites showing some motility were used for further studies.

The exposure of parasites to MBZ for up to 42 h is

Table 1. *Pseudodactylogyrus bini*. Number of parasites exposed to various media for 36 or 42 h at 24 °C. The number of dead parasites after exposure in brackets. MBZ: mebendazole

	MBZ		Formic acid	Water
	1 mg l^{-1}	10 mg l^{-1}	10 µl l^{-1}	
Exposure time:	42 h	36 h	36/42 h	36/42 h
P. bini	8 (3)	8 (6)	8 (0)	8 (0)

relevant for study as this drug is slow-acting and at least 3 d (Buchmann & Bjerregaard 1990) or up to 4 to 6 d (Székely & Molnár 1987) are needed for parasite eradication. In addition both concentrations used have shown high efficacy (Székely & Molnár 1987).

RESULTS

All parasites exposed to pure freshwater or freshwater containing solvent (formic acid) for both 36 and 42 h survived the exposure time. Some of the treated parasites survived the MBZ exposure (Table 1).

TEM examination findings of non-treated worms

The non-treated parasites showed a well-defined structure (Figs. 1 & 2). The outer tegument with a thickness of up to 2.2 µm (2200 nm) is constructed of an outer membrane and an inner membrane connected with a basal lamina with finger-like invaginations. Under the basal lamina, circular and longitudinal muscle layers are found, under which a syncytium with nuclei, numerous larger mitochondria with well-developed cristae and Golgi bodies are located. Smaller mitochondria are found in the outer tegument where membrane stacks, a large numbers of vesicles with a granular content and a few electron lucent vesicles are also located. A few vacuolar sacs bounded by a double membrane were detected in the tegument. Numerous bacteria associated with the outer membrane were frequently observed.

The intact flame bulb of an untreated *Pseudodactylogyrus bini* is composed of a terminal cell containing a large nucleus and surrounded by large mitochondria with well-developed cristae. Internal ribs extend apically and connect with the capillary at their external ribs. The flame consists of more than 20 cilia extending into the collecting duct.

The control sections comprised both highly contracted worms with extended invaginations of the basal lamina and the outer tegument in addition to relaxed specimens with smooth basal lamina and outer tegumental membrane.

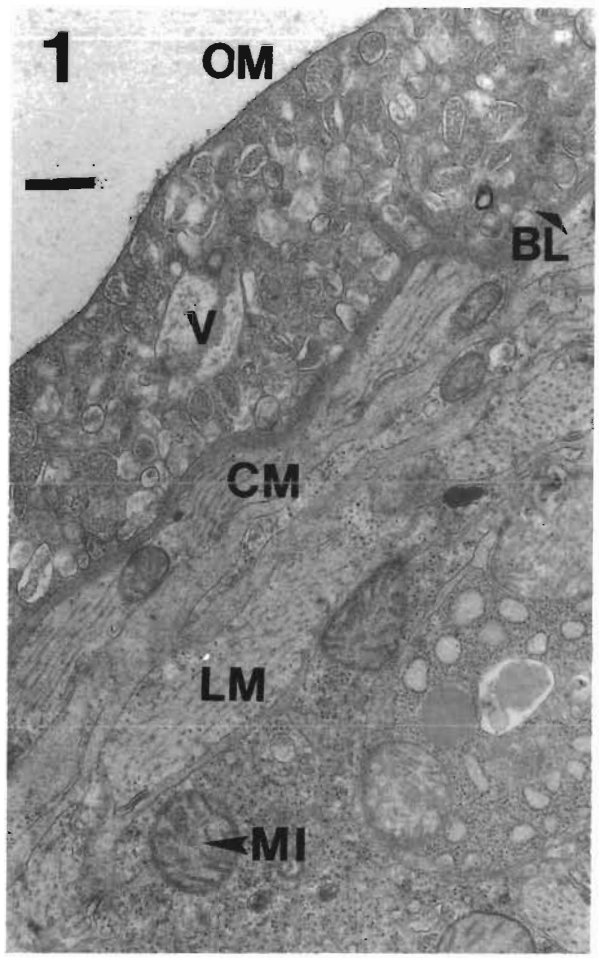


Fig. 1. *Pseudodactylogyrus bini*. Tegument of an untreated specimen. OM: outer membrane; V: vacuole; BL: basal lamina with inner membrane; CM: circular muscle; LM: longitudinal muscle; MI: mitochondria. Scale bar = 0.5 µm (500 nm)

The formic acid treated parasites did not show aberrations from the untreated control group.

TEM examination of MBZ-treated worms

Specimens of *Pseudodactylogyrus bini* which were exposed to MBZ (1 mg l^{-1}) for 42 h showed slight aberrations from the normal structure of the tegument and the described flame cell composition. Only few blebs were observed in the treated tegument and in the flame cell the connection between the capillary and the internal ribs of the terminal cell became loosened (Fig. 3). Some of the moribund specimens of *P. bini* exposed to 10 mg MBZ l^{-1} for 36 h showed a total disintegration and sloughing of the tegument in some places, leaving only the basal lamina enclosing cytoplasmic residues, interstitial fibers and deteriorating muscle

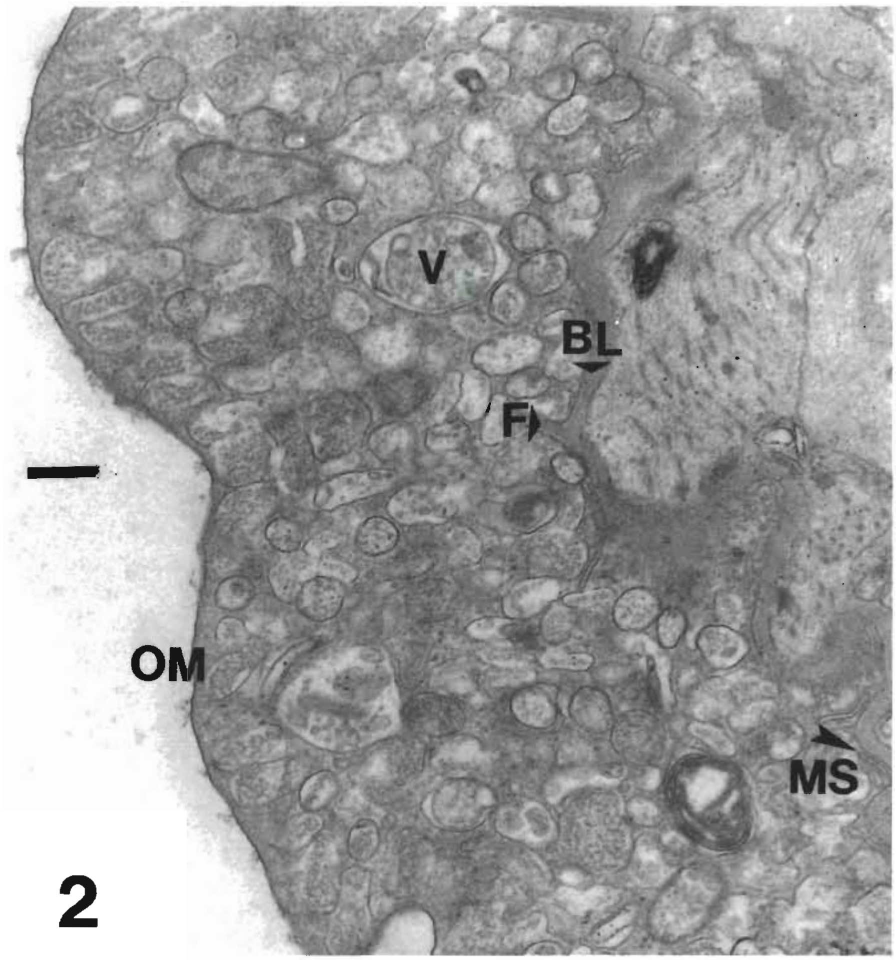


Fig. 2. *Pseudodactylogyrus bini*. Tegument of an untreated specimen. MS: Membrane stack; F: finger-like invaginations of basal lamina and inner membrane. Other symbols as in Fig. 1. Scale bar = 0.25 µm (250 nm)

layers (Fig. 4). One specimen exhibited partial destruction of the microtubule arrangement in the flame cell and disintegration of the terminal cell (Fig. 5).

DISCUSSION

As was previously demonstrated in the genera *Pseudodactylogyrus* (Schmahl & Mehlhorn 1988) and *Dactylogyrus* (El-Naggar & Kearn 1983), we often found the intact tegument of *Pseudodactylogyrus* to have a monogenean structure consisting of the outer anucleate layer under which the basal lamina and muscle layers are located (see Fried & Haseeb 1991).

In some moribund parasites exposed to 10 mg MBZ l^{-1}, part of the tegument was sloughed off. However, a low concentration (1 mg l^{-1}) for 42 h did not elicit severe changes in the tegument as only few blebs were observed in the outer anucleate layer. The impact of the drug on the tegument could play an important part in the parasiticidal effect of MBZ. However, the present study did not establish a direct causality between MBZ treatment and tegumental damage as these injuries at least theoretically could be explained as secondary. Thus, Bresciani (1973) demonstrated that the tegument of *Polystoma integerrimum* in old specimens was empty and that the teguments often were damaged during fixation. Rohde (1975) even found that some areas of the body wall of *Polystomoides* sp. were without tegument.

The protonephridial system of monogeneans has been extensively studied by a number of authors (Rohde 1973, 1993, Rohde et al. 1989a, b, 1992) and the flame bulbs of *Pseudodactylogyrus bini* conform with descriptions given for other monogeneans. The flame cells and the protonephridial capillary showed a well-defined structure in the intact parasite. In this study adverse effects on the flame cells were seen associated with mebendazole exposure, which interfered with the organization of microtubule associated structures. The slightest effect was seen on the connection between the terminal cell and the capillary, which was found

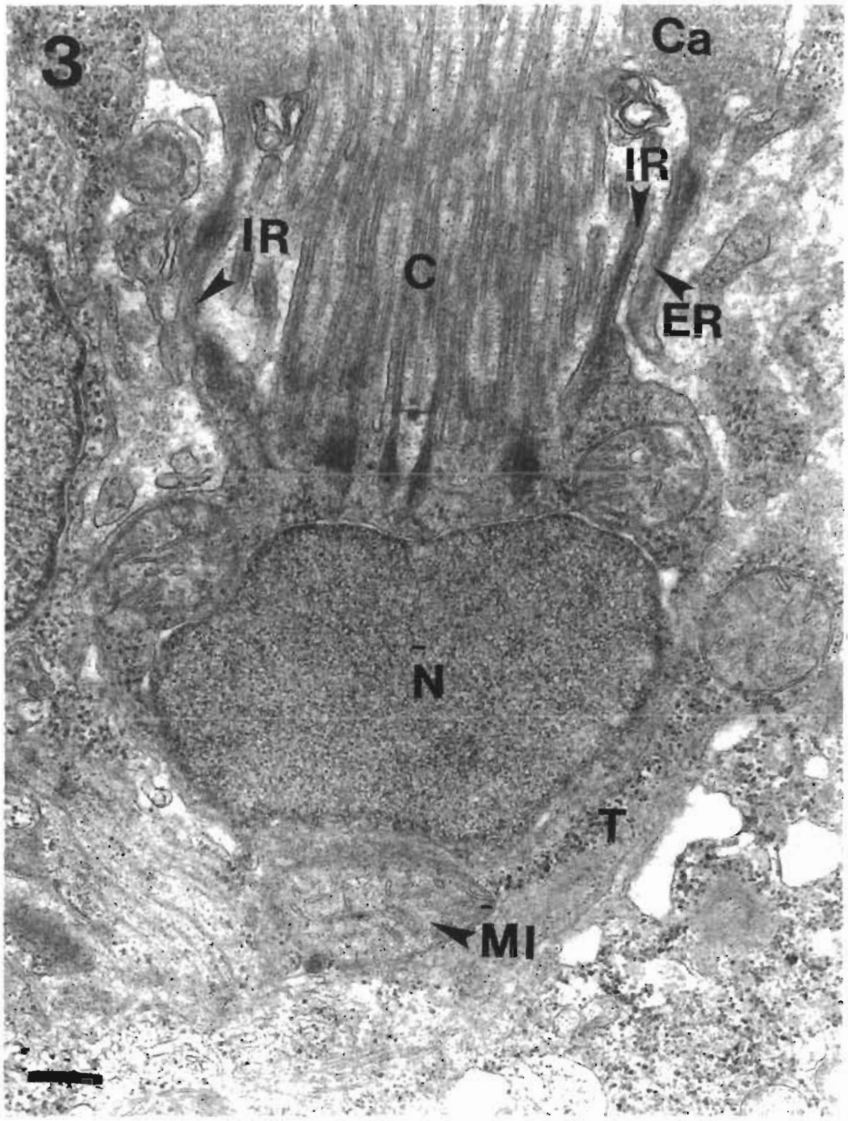
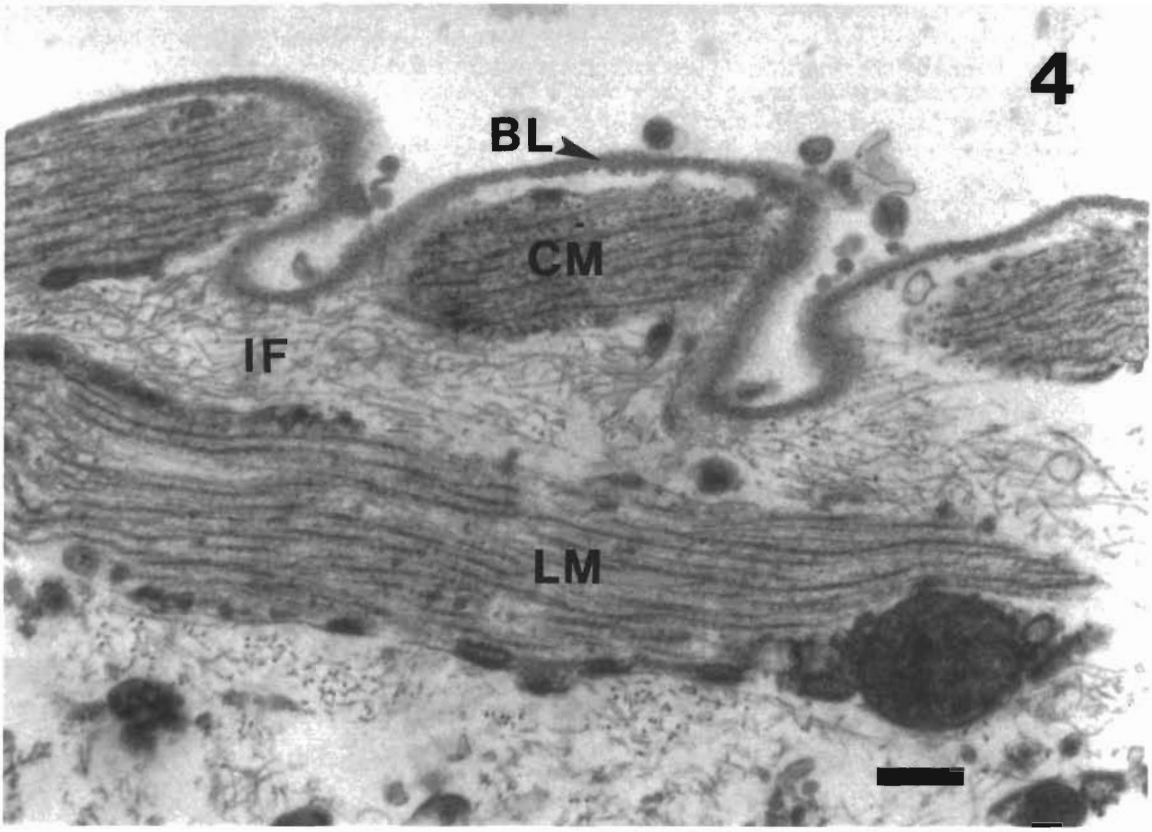


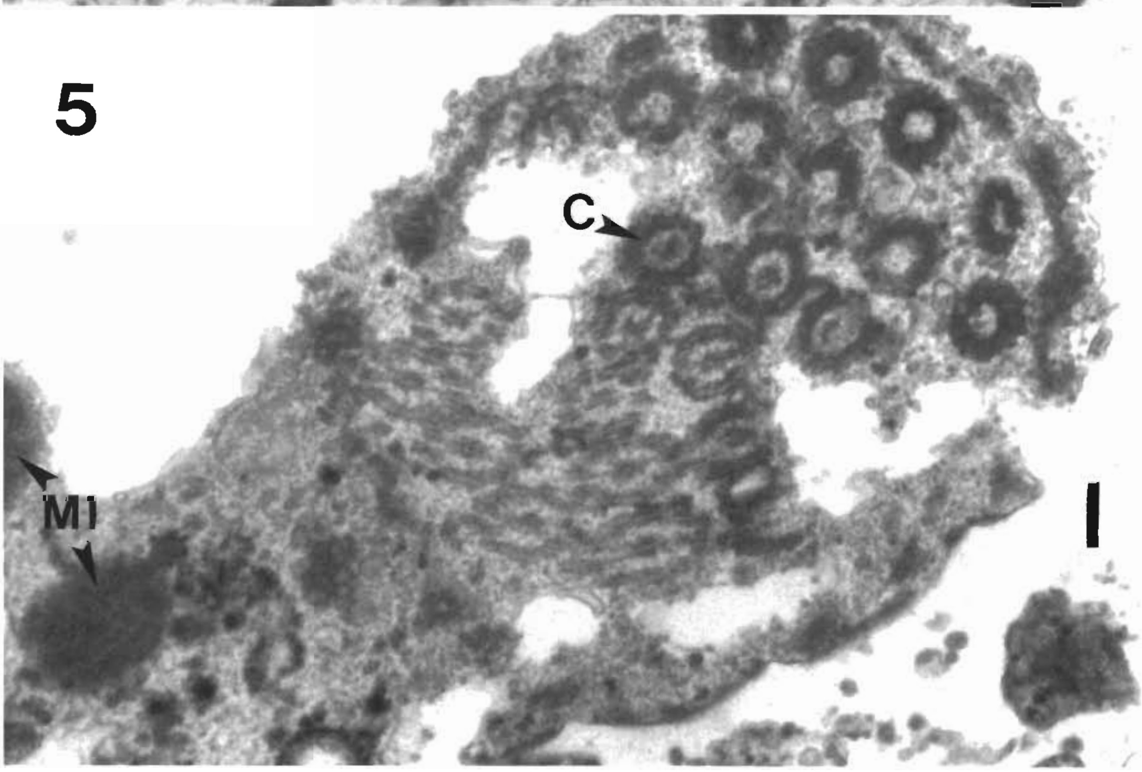
Fig 3 *Pseudodactylogyrus bini* Flame bulb after treatment with mebendazole (MBZ) (1 mg l^{-1}, 42 h). T terminal cell; N: nucleus; C cilia, Ca: capillary; IR internal rib. ER external rib; MI mitochondria Scale bar = 0.25 µm (250 nm)

partly separated after 42 h in MBZ (1 mg l^{-1}). The moribund parasites (36 h in 10 mg l^{-1}) showed a disintegration of flame bulbs and microtubules in the flame.

It could be suggested that the *Pseudodactylogyrus bini* protonephridial system, containing essential microtubule structures, is affected by mebendazole, whereby the osmoregulatory ability of the parasite deteriorates and the parasite eventually is killed. A possible direct effect of MBZ on cytoplasmic microtubules in the tegument which would elicit blebbing and sloughing of the tegument in addition to disorganization of muscle layers could assist this killing. Schmahl (1993) indicated that MBZ is able to interfere with the tegument, muscle and nerve structures of *Gyrodactylus bullatarudis*. This is partly in accordance with our results.

Studies on the effects of mebendazole on the ultrastructure of *Ascaris suum* (Borgers & De Nollin 1975) and of other microtubule inhibitors on *Fasciola hepatica* (Stitt & Fairweather 1992, 1993) also disclosed that organelles became disorganized due to treatment with these substances. However, although the microtubule system is considered as the main target for benzimidazoles (Lacey 1988) this drug group has been shown to affect a range of enzymatic reactions in helminths (Kohler & Bachmann 1978, Sarwal et al. 1989) as well, and it cannot be excluded that a number of other action mechanisms are involved in the described effect on the monogenean.





Figs. 4 & 5. *Pseudodactylogyrus bini* after treatment with mebendazole (MBZ, 10 mg l^{-1}, 36 h). Fig. 4. Moribund specimen after sloughing of tegument due to treatment. BL: basal lamina, CM: circular muscle layer, LM: longitudinal muscle layer. IF: interstitial fibres. Scale bar = 0.2 µm (200 nm). Fig. 5. Flame bulb after MBZ. C: transverse section of the cilia forming the flame. MI: mitocondria. Scale bar = 100 nm

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