

Effects of anthelmintics with different modes of action on the behavior and development of *Caenorhabditis elegans*

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Summary - The anthelmintic effects of four substances known to have different modes of action – two well-established nematicides (ivermectin, mebendazole) and two compounds presently in development (annonin, PF 1022) – were evaluated from the results of *in vitro* and *in vivo* tests with four different parasitic nematodes. To determine to what extent the test results also apply to free-living nematodes, the effects of the same drugs on locomotion, reproduction, development, and cellular structures of the well-studied free-living species *Caenorhabditis elegans* were analysed in detail. The role of culture conditions, exposure time necessary for induction of defects, and reversibility of drug effects were also studied. Each of the tested substances induces a specific defect pattern. Our data indicate that it is necessary to combine results from different tests in order to obtain a comprehensive picture of the anthelmintic effects of a substance and hence of its potential suitability as a nematicide. © Orstom/Elsevier, Paris

Résumé - Effets de produits antihelminthiques à modes d'action différents sur le comportement et le développement de *Caenorhabditis elegans* - En utilisant des tests *in vitro* et *in vivo*, les effets antihelminthiques de quatre substances connues pour leurs modes d'action différents ont été évalués : deux antihelminthiques bien réputés (ivermectine, mebendazole) et deux composés actuellement en expérimentation (annonine, PF 1022). Pour déterminer dans quelle mesure de tels résultats peuvent s'appliquer aussi à des nématodes libres, les effets de ces produits ont été analysés sur le nématode libre très étudié *Caenorhabditis elegans* en examinant leur influence sur la locomotion, la reproduction, le développement et les structures cellulaires. De plus, le rôle des conditions d'élevage, le temps d'exposition nécessaire pour l'induction des altérations et la réversibilité des effets des produits ont été étudiés. Chacun des produits testés induit un type d'altération spécifique. Nos données indiquent que seule la combinaison des données issues de tests différents peut fournir un tableau d'ensemble des effets antihelminthiques d'un composé et par là une idée sur son utilisation potentielle comme nematicide. © Orstom/Elsevier, Paris

Keywords: annonin, anthelmintic, *Caenorhabditis elegans*, *in vivo* screening, *in vitro* screening, ivermectin, mebendazole, nematicide, nematodes.

Parasitic nematodes are a serious threat to the health and life of many plants and animals, including man. Various compounds are available for the treatment of nematode infections and the different groups of anthelmintics have very different modes of action. Compounds in one group, including Levamisol, act via the nicotinic acetylcholine receptor of the parasites (Harrow & Gration, 1985). A second group comprising the benzimidazoles seems to impair microtubular integrity (Friedman & Platzer, 1980; Lacey, 1988, 1990). A third group of anthelmintics includes the 'modern' avermectinoids such as ivermectin (Chaballa *et al.*, 1980; Kass *et al.*, 1980; Champbell *et al.*, 1983; Cully & Pares, 1991). Accumulating evidence indicates that avermectinoids act via the stimulation of chloride channels (probably glutamate-gated), which induces muscle cell hyperpolarization (Martin, 1995).

A serious problem is the emerging resistance of parasites against antihelminthic compounds in a wide variety of animals, which jeopardizes the protective benefits. Therefore, the search for new chemical compounds with nematicidal action plays an important role in veterinary medicine. Various screening strategies using parasitic nematodes have been described for the evaluation of potential nematicidal effects of new substances (Jenkins & Carrington, 1981; Moltman, 1985; Rapson *et al.*, 1987; Coles, 1990). However, such studies are restricted by the often complex life cycle of the parasites and by difficulties encountered in monitoring all stages of development.

As an alternative, initial tests can be performed on free-living nematodes, which are much more accessible to analysis under controlled conditions. Best suited for such purposes appears to be the hermaph-

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rodentic soil nematode *Caenorhabditis elegans*, for which a vast amount of background data are available (Riddle *et al.*, 1997). This species has been cultured in the laboratory for decades at low cost and under simple conditions. Its anatomy and behavior are well known and its life cycle and development have been described in more detail than for any other metazoan organism. Also, thanks to its suitability for genetic analysis (Brenner, 1974) and micromanipulation, it has become a well established model for developmental biologists (Wood, 1988; Epstein & Shakes, 1995; McGhee, 1995; Riddle *et al.*, 1997).

The life cycle of *C. elegans* is the shortest of all studied nematodes (2-3 days at 25 °C). Its reproductive capacity is high and, because it is an internally self-fertilizing species, can typically each individual produce 200-300 offspring. Embryos and hatched animals are transparent, which means that major morphological defects can easily be detected under the light microscope in living specimens. These characteristics make it possible to perform mass screenings with many thousand individuals evaluated at the same time, and also detailed studies on individual specimens. Because of its hermaphroditic mode of reproduction leading to genetic uniformity and its essentially invariant development, even small test samples should lead to representative results.

This article first describes initial tests that demonstrate the general effectiveness of the investigated drugs on parasitic nematodes, then the results of more detailed studies on *C. elegans*. Starting from the effect of drugs on basic features such as mobility and reproductive capacity as addressed in earlier reports (Platzer *et al.*, 1977; Simpkin & Coles, 1981; Spence *et al.*, 1982; Novak & Vanek, 1992), we have studied additional aspects including role of culture conditions, exposure times needed, reversibility of drug-induced effects, and mode of drug uptake. These studies should give a better insight into the action spectrum of the substances tested and the sensitivity of the organism exposed to them. Another aim of this study is to determine whether it is possible to use simple test assays for differentiating different compound classes based on the defects they induce.

Materials and methods

DRUGS TESTED

The four drugs tested were dissolved in dimethylsulfoxide (DMSO) and stored in stock solutions in the refrigerator. Mebendazole (MBZ; Sigma), stock solution: 1mg/ml. Ivermectin (IVM; MSD) was used after HPLC purification of the cattle injection product; stock solution: 0.2 mg/ml. Annonin (ANN; Bayer), stock solution: 1 mg/ml. PF 1022 (Fukashe *et al.*, 1990; Tagaki *et al.*, 1991) was obtained from Dr. K.

Inuma, Meiji Seika Kaisha, Japan; stock solution: 10 mg/ml.

• *In vitro experiments using Trichinella spiralis larvae and adult Nippostrongylus brasiliensis*

In vitro experiments with *Trichinella spiralis* larvae (Jenkins & Carrington, 1981) and adult *Nippostrongylus brasiliensis* were performed as recently described (Martin *et al.*, 1996). For quantification of effects against *N. brasiliensis*, the activity of excreted acetylcholinesterase was determined according to Rapson *et al.* (1987).

• *In vivo experiments in mice using the nematodes Heligmosomoides polygurus, Heterakis spumosa, and Trichinella spiralis*

In vivo experiments were performed as recently described (Martin *et al.*, 1996). The level of anthelmintic activity was graded by determining the percentage of surviving nematodes in mice.

CULTURE OF *C. ELEGANS* ON AGAR PLATES

Nematodes were grown on agar plates seeded with a uracil-deficient strain of *E. coli* (OP50) as a food source, essentially as described by Brenner (1974). Before food was depleted, individual nematodes were transferred to a fresh plate with a sterile sharpened toothpick or a thin platinum wire fixed to a Pasteur pipette. The cultures were kept at room temperature.

• *Preparation of agar plates with drugs and exposure of test specimens to drugs*

Appropriate amounts of the stock solution of the drugs were added to melted agar (> 40°C) and stirred. Then, the solution was poured into Petri dishes and allowed to solidify. The final concentrations of the drugs tested were as follows. Mebendazole: 5, 10, 100, and 1000 mg/ml (the drug is only partially soluble at the highest concentration); ivermectin and annonin: 0.01, 0.1, and 1 µg/ml; PF 1022: 1, 10, and 100 µg/ml. The final concentrations of DMSO never exceeded 1%. At this concentration, we found that DMSO does not harm the nematodes in any detectable way. Test specimens were transferred to plates containing the drugs tested and left for as little as 1 min or as long as several weeks, depending on the drug and its concentration. The behavior of individual specimens was monitored under the dissecting microscope with illumination from below.

• *Liquid culture of C. elegans*

Nematodes were washed from agar plates with phosphate-buffered saline (PBS, Brenner, 1974) and separated from bacteria by brief centrifugation (300 g for 3 min). Animals were transferred to 3.5 cm plastic Petri dishes containing the test drugs dissolved in PBS.

If nematodes had to be tested for more than 1 h, a suspension of *E. coli* in PBS was used as a food supply. If bacteria were preincubated with the test drugs for 24 h the results were the same as without preincubation, which indicates that the drugs were not metabolized by the bacteria. Specimens were tested in liquid cultures with the same drug concentrations as used on agar plates. Unless otherwise stated, at least 100 and often more than 1000 adult animals were tested at each concentration with both agar plates and liquid culture assays.

DETERMINATION OF MOBILITY, REPRODUCTION AND LIFE SPAN

Definitions for the different categories of mobility are given in the legends of Figs 1-3. The velocity of specimens on the surface of agar plates was determined under the dissecting microscope by measuring the length of visible tracks in the bacterial lawn made per minute. To determine fecundity and life span, two-cell embryos were cut out of gravid adults and transferred with a pipette to a bacteria-seeded agar plate kept at room temperature (19-21°C). Starting when all the embryos had hatched (usually 15 ± 1 h), plates were monitored at 12 h intervals. As soon and as long as animals laid eggs, they were transferred to new plates daily and the number of eggs counted. Specimens were defined as dead when they did not feed (no pharynx-pumping) and failed to move even

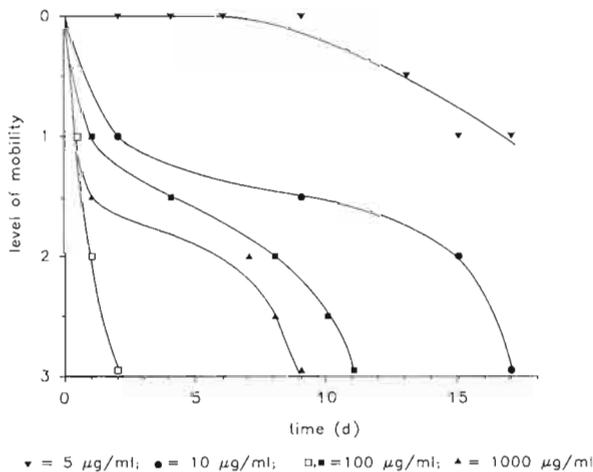


Fig. 1. Mobility of *Caenorhabditis elegans* on agar plates and in liquid culture at different concentrations of mebendazole. 0 = normal behavior; 1 = reduction of velocity; 2 = paralysis, but short movement when prodded; 3 = no reaction to prodding. A symbol placed at the level of a number (1-3) indicates that essentially all (> 95 %) specimens have reached the corresponding level of mobility. A symbol placed between numbers indicates that a certain fraction of specimens has reached the higher level, e.g. 50 % if the symbol is positioned at equal distance of two numbers. Closed symbols = agar plates; open symbols = liquid medium.

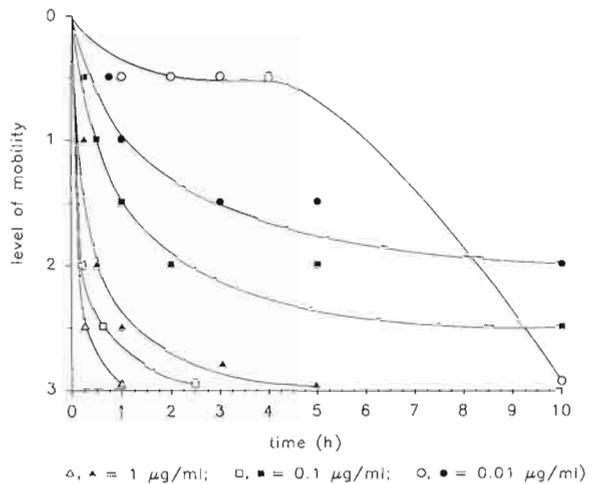


Fig. 2. Mobility on agar plates and in liquid culture at different concentrations of ivermectin: 0 = normal behavior; 1 = pharynx pumping stops; 2 = movement slows down and stops, but animals still show response to prodding; 3 = no reaction to prodding. For positioning of symbols, see legend to Fig. 1. Closed symbols = agar plates; open symbols = liquid medium.

when probed. This condition was then verified at the next inspection.

INHIBITION OF DRUG UPTAKE THROUGH THE INTESTINAL TRACT

We tested whether a drug needs to be ingested to induce the effects observed. For this, a regular bac-

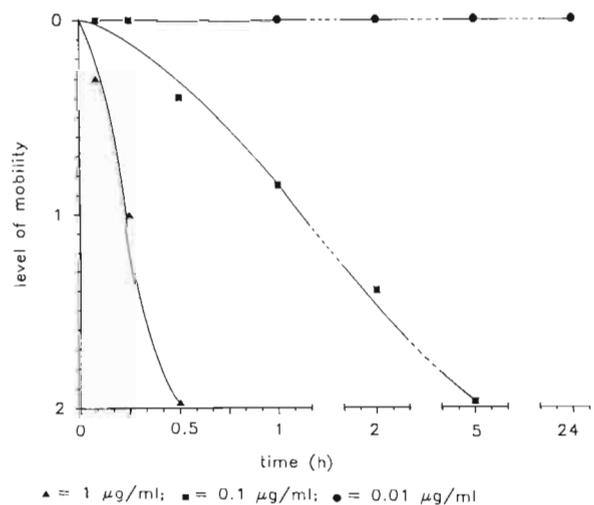


Fig. 3. Mobility on agar plates at different concentrations of annonin: 0 = normal behavior; 1 = no pharynx pumping and retarded movement; 2 = animals paralyzed, no response to prodding. For positioning of symbols, see legend to Fig. 1.

teria-seeded agar plate with a mixed population of nematodes was placed in the refrigerator (4°C) for 30 min. The chilled animals stop feeding and become reversibly paralysed. Chunks of agar containing at least 100 animals each were cut off the chilled plate and dipped into cold PBS containing 1mg/ml of either ivermectin or annonin. Specimens floated away into the test solution and were put back into the refrigerator. To remove the drug, the solution with worms was centrifuged (5000 g/20 s) at low temperature, the supernatant was removed, and cold PBS added to wash the worm pellet. This procedure was repeated twice and the specimens were transferred back to regular plates. The number of mobile individuals was counted after 10 min, 1 h, and 12-15 h at room temperature. To verify that no oral uptake of drugs occurred under these conditions, a few drops of acridine orange (AO; 10⁻² % in PBS; Sigma) were added to the drug solution (final concentration approx. 10⁻⁴ %). After the washing procedure, 10-20 specimens/experiment were immediately examined with epifluorescence (see below) for absence of feeding-related AO-induced fluorescence in the gut lumen. Prior to the experiment, we had determined that *i*) worms kept in PBS at cold temperature for 7 days (and longer) quickly recover after transfer to room temperature, *ii*) even brief feeding (pharynx pumping) on agar plates containing the above-mentioned concentration of AO causes bright green fluorescence in the lumen of the intestinal tract, which is absent in non-feeding specimens (pharyngeal lumen may fluoresce) and animals reared on regular plates, and *iii*) even long-term exposure to AO at the concentrations applied does not interfere with normal mobility of nematodes.

EFFECT OF DRUGS ON EMBRYOGENESIS

To test whether embryos are sensitive to drugs, early embryonic stages were placed on agar plates that contained the drug. Progression of cell divisions and hatching were monitored under the dissecting microscope after 30 min, 2 h, and 24 h. When embryonic arrest was induced, pulse experiments with exposure times as short as 1 min were performed.

To penetrate the eggshell and the underlying protective vitelline membrane, early embryonic stages were transferred into a drop of cell culture medium on a microscope slide. A few pulses of a N2-pumped dye laser coupled to the microscope created a temporary hole in the eggshell and allowed the drug to reach the embryo (Schierenberg & Junkersdorf, 1992).

MICROSCOPE ANALYSIS

To assess drug effects on test specimens, individual worms or embryos cut out of gravid adults were inspected under a dissecting microscope with illumina-

tion from below. For morphological analysis, they were transferred to a microscope slide coated with a thin protective layer of 5 % agar (Sulston & Horvitz, 1977), covered with a coverslip, and sealed with Vaseline for examination with Nomarski optics (magnifications: animals, × 10-40; eggs, × 100). Fluorescence of acridine orange was studied at an excitation wavelength of 450-490 nm. Nuclei were stained with the DNA-specific dye diamidinophenylindole hydrochloride (DAPI; 1mg/ml; Boehringer, Mannheim) for 10 min and analysed at an excitation wavelength of 340-380 nm.

VIDEORECORDING AND PHOTO DOCUMENTATION

Animals and embryos were recorded on a time-lapse video recorder (Panasonic AG-6720E) with a CCD video camera (Panasonic WV-BC700). Selected images of the recorded specimens were printed directly with a video copy processor (Mitsubishi P66E).

Results

ANTHELMINTHIC EFFECTS AGAINST ANIMAL PARASITIC NEMATODES

To compare the *in vivo* and *in vitro* activity on parasitic helminths of anthelmintic compounds with different modes of action, representative compounds from the major nematocidal classes such as the benzimidazole - mebendazole, and the avermectin, ivermectin were used. In addition, two new compounds with previously unknown modes of action were investigated. Only a brief summary of our results is given to serve as a reference for the studies with *C. elegans* described below. More extensive descriptions have been published (Martin *et al.*, 1996) or will be published elsewhere.

Ivermectin proved to be very effective against *Heligmosomoides polygyrus* in mice at oral dosages as low as 1 mg/kg (Table 1) and against *Heterakis spumosa* at 0.5 mg/kg. Mebendazole is active against *H. spumosa* at dosages as low as 10 mg/kg and also against *Trichinella spiralis* at 100 mg/kg. PF 1022 is active against *H. polygyrus* and *H. spumosa* at 50 mg/kg (Table 1).

T. spiralis and *Nippostrongylus brasiliensis* were examined *in vitro* (Table 2). While ivermectin showed no activity against *T. spiralis*, mebendazole and PF 1022 displayed activities at concentrations of 0.01-0.001 µg/ml. Annonin had minor effects at 100 µg/ml. With *N. brasiliensis*, ivermectin had by far the best anthelmintic activity, followed by mebendazole and PF 1022. Annonin had only low activity at 100 µg/ml.

Thus, each of the four tested drugs induced at least some visible effects, but the tested parasites displayed significant differences in sensitivity.

Table 1. In vivo anthelmintic activity of ivermectin, mebendazole and PF1022 against different helminths in experimentally infected mice after oral application.

Compound	Dosage (mg/kg)	<i>Heligmosomoides polygyrus</i>	<i>Heterakis spumosa</i>	<i>Trichinella spiralis</i>
Ivermectin	2.5	3 ^a	3	0
	1.0	3	3	0
	0.5	2	3	0
	0.25	0	2	0
	0.1	0	0	0
Mebendazole	100	0	3	3
	50	0	3	1
	25	0	3	0
	10	0	3	0
	5	0	0	0
PF1022	100	3	3	0
	50	3	3	0
	25	2	0	0
	10	1	0	0
	5	0	0	0

^a 3 = cure (no parasites detectable); 2 = effective (< 20 % of parasites remaining); 1 = trace effect (< 50 % but > 20 % of parasites remaining); 0 = ineffective (> 50 % of parasites remaining).

EFFECTS OF ANTHELMINTIC DRUGS ON *C. ELEGANS*

For easy reference purposes, a general account of the results from various experiments is given in Table 3. Details and quantitative data are found in the text and corresponding figures.

Table 2. In vitro anthelmintic activity of ivermectin, mebendazole, PF1022 and annonin against *Trichinella spiralis* larvae and *Nippostrongylus brasiliensis* adults.

Concentration (µg/ml)	<i>Trichinella spiralis</i> larvae				<i>Nippostrongylus brasiliensis</i> adults			
	Ivermectin	Mebendazole	PF1022	Annonin	Ivermectin	Mebendazole	PF1022	Annonin
100	0*	3	3	2	3**	3	3	1
10		2	3	0	3	3	3	0
1		2	3		3	3	2	
0.1		2	2-3		3	2	0	
0.01		2	2		2	1		
0.001		1	2		2	1		
0.0001		0	0-1		1	0		

* For *T. spiralis*: 3 = full activity (100 % larvae killed); 2 = good activity (> 50 % larvae killed); 1 = weak activity (< 50 % larvae killed but > controls); 0 = no activity (number of living larvae = controls).

** For *N. brasiliensis*: 3 = full activity (95 - 100 % inhibition of excreted acetylcholinesterase); 2 = good activity (inhibition 75-95 %); 1 = weak activity (50 - 75 % inhibition); 0 = negligible activity (< 50 % inhibition).

EFFECTS ON MOBILITY: AGAR PLATES VS LIQUID CULTURE

Mebendazole: Under normal conditions, animals move on the surface of agar plates in sinusoidal waves at a velocity of 12-17 mm/min. This behavior changes after addition of MBZ. We defined three different phases of MBZ-induced effects which are easy to observe under the dissecting microscope: *i*) reduction of velocity to 2-4 mm/min; *ii*) animals stop moving but pharynx pumping continues; they react with body contraction after being probed with a toothpick; *iii*) animals are completely paralyzed and defined as dead as they cannot be reactivated by probing or transfer to normal plates. Our findings are summarized in Fig. 1. Depending on the concentration, it takes 7-15 days to paralyze the animals and up to 17 days to kill them.

In liquid culture, animals show twitching contractions instead of sinusoidal movement. The retarding effect of MBZ is similar to that on agar plates but the onset of drug activity becomes visible much sooner. At 1000 mg/ml it takes less than 12 h to kill the animals (data not shown) compared to 9 days on agar, and at 100 µg/ml it takes only 2 days compared to 11 days on agar (Fig. 1). First juvenile stages were found to be considerably less sensitive than adults, but these differences have not been studied in detail.

Ivermectin: IVM causes in *C. elegans* a sequence of characteristic behavioral changes that are similar but not identical to those caused by MBZ. During phase 1, ingestion of food with frequent pharynx pumping stops. Then (phase 2), animals slow down their movement and come to a stop. However, they still respond

Table 3. Effects and features of anthelmintic drugs on *Caenorhabditis elegans*.

Drug	Effect of drug on				Feature of drug		
	Mobility ^a	Life span	Reproduction	Development oocytes/embryos ^b	Necessary exposure ^c	Reversibility ^d	Ingestion required ^e
Mebendazole	+	++	++	yes/no	long	yes	n.d.
Ivermectine	+++	+++	+++	no/no	short	partial	no
Annonin	+++	+++	+++	yes/yes	short	yes	yes
PF 1022	-	-	+	no/no	/	/	/

Effects: -, no; +, some; ++, considerable; +++, strong. a: after 24 h at intermediate (drug-dependent) concentration; b: effect on intact embryos; c: time required to induce significant effects on adult behavior; d: reversibility of retarded movement and/or pharynx pumping; e: for paralysis, according to low temperature test; n.d.: not determined; /: does not apply. For details, see text.

to probing with a needle or toothpick by moving a short distance before stopping again. Finally (phase 3), they exhibit complete paralysis with body relaxed and no movement after probing. Fig. 2 shows that the effect of IVM depends on concentration: phase 2 is reached in more than 5 h at 0.01 µg/ml, but in less than 1 h at 1 µg/ml. As observed with MBZ, IVM acts several times faster in liquid culture than on agar plates: at 1 mg/ml, phase 3 is reached in more than 3 h on plates, but in less than 1 h in liquid medium (Fig. 2). First juvenile stages are less sensitive to IVM, i.e. higher concentrations and/or longer times were needed to inactivate them, as was observed with MBZ.

Annonin: We tested the mobility of animals on agar plates with three different concentrations of ANN. In contrast to MBZ and IVM, only two categories of impairment were defined. The first sign was interruption of pharynx pumping and nearly concomitant slowdown of movement (phase 1). Eventually, animals stopped moving completely (phase 2). However, once immobilized they no longer responded to prodding, contrary to what was observed with IVM. Our results are summarized in Fig. 3. While 0.01 µg/ml of ANN did not produce any obvious effect even after 24 h, at 0.1 µg/ml interruption of pharynx pumping began after 15-30 min and slowdown of movement began after 30-60 min. After 2 h about 40 % of the animals had stopped, and after 5 h all had become immobilized. At 1 µg/ml, pumping stopped and movement slowed down within the first 15 min and after 30 min all animals were immobilized. Unlike MBZ and IVM, with ANN there was no significant difference observable between juveniles and adults.

Also in contrast to MBZ and IVM, the effect of ANN in liquid culture was found to be significantly less dramatic than on agar plates (data not shown). While on agar plates (at 1 µg ANN/ml) all animals

were paralysed after 30 min (see above), in liquid culture with the same concentration of ANN, a minority of adults and the majority of juveniles still moved vigorously after 5 h.

PF 1022: We tested three different concentrations (1, 10, 100 µg/ml) of PF 1022, a new anthelmintic drug. For *C. elegans*, we could not detect any significant effects on co-ordination and velocity of movement, neither on agar plates nor in liquid culture.

EFFECTS ON REPRODUCTION, LIFE SPAN, AND DEVELOPMENT

Mebendazole: It has been reported (Spence *et al.*, 1982; Enos & Coles, 1990) that MBZ negatively influences reproduction in *C. elegans*. Our findings

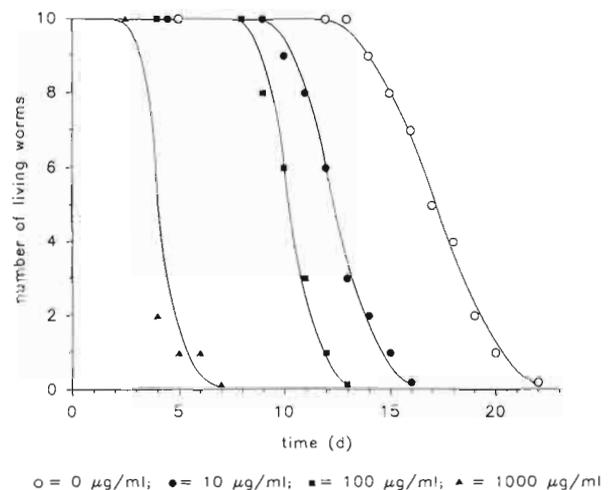


Fig. 4. Life span on agar plates at different concentrations of mebendazole. For each concentration ten specimens were tested.

(unless otherwise stated, the data in this and all following paragraphs refer to studies on agar plates) are in line with these results. More precisely, we found that egg production was reduced by 10-90 % in the range of 1-100 $\mu\text{g/ml}$. But, even at the highest concentrations tested, most hermaphrodites produced at least a few eggs, indicating that MBZ might not be able to completely inhibit reproduction.

Life span is also significantly affected by MBZ. We tested three different concentrations and found that the life span was reduced by 30-70 % (Fig. 4). Animals appear to age faster under the influence of MBZ as they show relatively early markers typical for old animals such as slow movement, dark pigmented intestine and inability to lay eggs rapidly.

To better understand the detrimental effect of MBZ on reproduction, we analysed the structure of the gonad in living specimens which had been grown in MBZ (100 $\mu\text{g/ml}$) from hatching onward. We found gross defects in the structure of oocytes and embryos.

Mature oocytes adjacent to the spermatheca frequently showed an abnormal cytoplasmic texture (Fig. 5C) with nuclei in peripheral instead of central positions (Fig. 5B). In addition, the oocytes were often of variable size and contained multiple large nuclei and/or various numbers of small vesicle-like structures which looked like nuclei of oogonia (Fig. 5D). DAPI staining confirmed that these structures contain DNA plus a non-staining central region (nucleolus) as found in nuclei of regular oogonia. Thus, at the concentrations tested, MBZ severely affects oogenesis by interfering with proper formation of oocytes. Most prominently, the separation process of syncytially-connected oogonia into individual cells appears to be hampered. In addition, MBZ also seems to interfere with meiosis as indicated by the absence of one or both polar bodies typically present prior to first cleavage.

These abnormalities became visible after 1-2 h exposure to 100 $\mu\text{g/ml}$ MBZ, in contrast to the slow

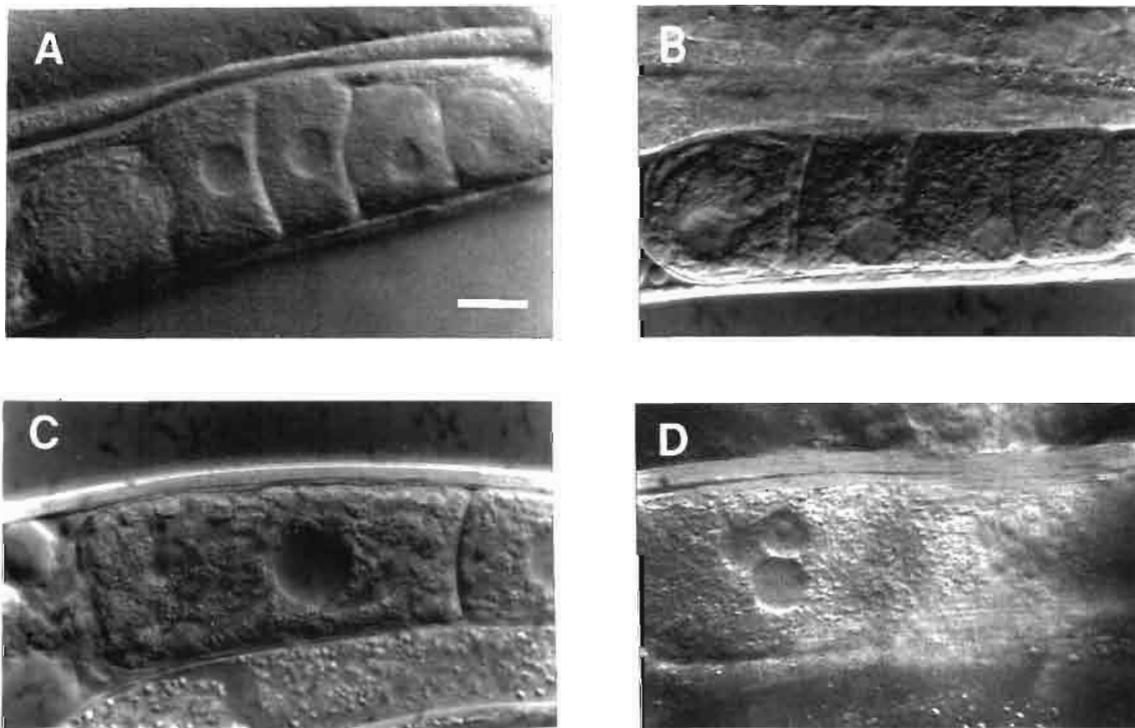


Fig. 5. Defective oocytes in *Caenorhabditis elegans* hermaphrodites raised on agar plates containing 100 $\mu\text{g/ml}$ mebendazole (MBZ). A: Animals grown in the absence of mebendazole produce normal oocytes with centrally located nuclei (control). B-D: Oocytes exposed to MBZ; B: oocytes with abnormal texture of the cytoplasm and peripheral positioning of nuclei. Intestine is visible in the upper part of the picture; C: Mature oocyte prior to fertilization with abnormal cytoplasmic texture. Intestine visible in the lower part and spermatheca at the left margin of the picture; D: Two mature oocytes, with three large nuclei (left) and many small nuclei (right).

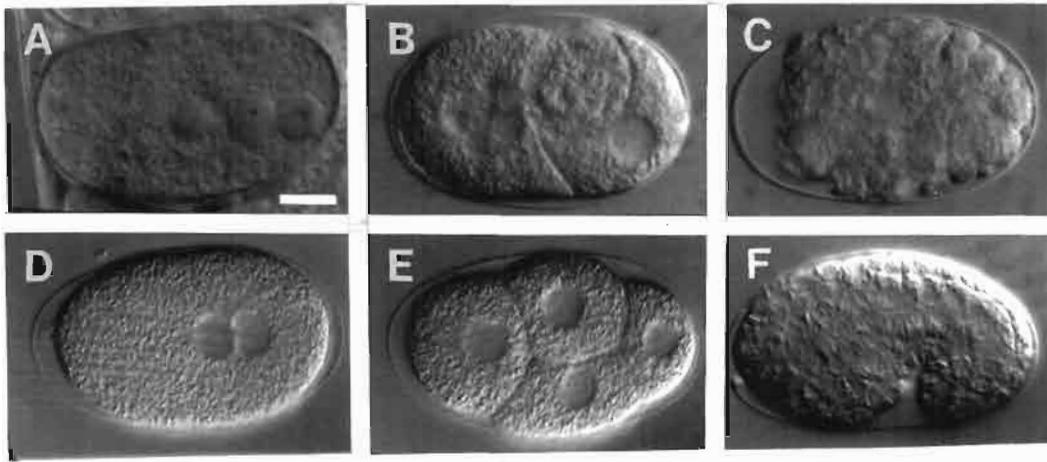


Fig. 6. Defective embryos from *Caenorhabditis elegans* hermaphrodites raised on agar plates containing 100 $\mu\text{g/ml}$ mebendazole. A-C: Defective embryos. A: One-cell embryo with three pronuclei; B: Four-cell embryo with supernumerary nuclei and aberrant positioning of blastomeres; C: Embryo arrested with several hundred cells. D-F: corresponding normal stages (control). D: One-cell stage, two pronuclei on their way to fuse; E: Four-cell stage; F: Stage with several hundred cells after the start of visible morphogenesis.

effect on mobility reported above. When animals were transferred back to normal agar plates, even after exposure to this MBZ concentration for several days, they were able to recover and soon started to produce exclusively healthy oocytes that, after fertilization, developed into reproducing adults.

In general, embryos produced under the influence of MBZ had above normal variation in size, *i.e.* extraordinarily large or small eggs were found (data not shown). Blastomeres often contained abnormal numbers of nuclei (Fig. 6A, B) with several hundred cells but without initiation of a proper morphogenesis (Fig. 6C). To determine whether these abnormalities are the result of defects initiated during oogenesis or are related to a sensitivity of embryos themselves to MBZ, one- to four-cell embryos were placed on agar plates with MBZ. No effect could be detected even at concentrations as high as 1 mg/ml, and reproducing adults developed from these embryos when transferred to normal plates after hatching. This indicates that the drug cannot penetrate the eggshell. It has been shown that the thin vitelline membrane underneath the eggshell proper functions as a chemical barrier in *C. elegans* (Schierenberg & Junkersdorf, 1992).

Ivermectin: We found that adult animals that had been immobilized with IVM were still able to produce some offspring. Microscopical analysis revealed that the typical contraction waves of the gonad, which push oocytes toward the spermatheca was blocked by IVM treatment so that no new fertilizations took place. However, development of eggs fertilized before treatment continued and juveniles hatched inside their mother.

To determine whether the cuticle of the mother protects the embryos or the embryos themselves are insensitive to IVM, we placed early embryos ($n=23$) into 1 $\mu\text{g/ml}$ IVM. In no case was embryogenesis affected and all embryos hatched. To test the assumption that this is due to the eggshell barrier (see above), we opened the eggshell with a laser microbeam in a medium containing 10 $\mu\text{g/ml}$ IVM. Development of early-stage embryos continued and they reached a final phenotype of at least several hundred cells (7/17) with various signs of differentiation (*e.g.*, muscle twitching) and most embryos even hatched (10/17). These results indicate that IVM does not significantly affect embryonic cell division and differentiation.

Annonin: In those animals which were paralyzed on agar plates containing 1 $\mu\text{g/ml}$ ANN, oogenesis was affected, too. Parallel to the onset of paralysis, transport of oocytes along the gonadal tube ceased and no further fertilization took place. However, there were no abnormalities of oocytes similar to those seen after MBZ treatment (Fig. 5).

To test whether fertilized eggs inside the eggshell are protected as we found for MBZ and IVM, early stage embryos (one- to four-cell embryos) were placed on plates with 1 $\mu\text{g/ml}$ ANN. Development of all embryos (16/16) was arrested early at 2-50 cells. The same result was obtained in liquid medium (20/20). With pulse experiments we determined that a 1 min exposure is sufficient to irreversibly induce an early embryonic arrest.

Our finding that ANN can penetrate the eggshell made it possible to address additional questions by exposing advanced-stage embryos to 1 and 10 $\mu\text{g/ml}$

ANN. It has been shown that, in *C. elegans*, the formation of gut-specific birefringent granules does not require mitosis or DNA replication after the first division of the gut precursor cell (Laufer *et al.*, 1980; Edgar & McGhee, 1988). Therefore, we tested whether such gut differentiation can take place under the influence of ANN. The result was that the birefringent granules never developed (0/73).

In another experiment, we investigated whether morphogenetic processes are sensitive to annonin. During the second half of the embryogenesis of *C. elegans*, a worm develops from a ball of cells essentially without additional cell divisions (Sulston *et al.*, 1983). We found that this process was inhibited by ANN as well (37/37), so that morphogenesis terminated at the stage when the drug was applied.

The last two experiments showed that ANN exhibits a quick-acting, general effect on various developmental processes consistent with its presumed action on the respiratory chain (Londershausen *et al.*, 1991).

PF 1022: To evaluate the effect on reproduction, we transferred L4 juveniles onto agar plates containing three different concentrations of PF 1022 and monitored the number of offspring on each plate. At 1 and 10 $\mu\text{g/ml}$, 150–200 offspring were produced per adult (a small but significant reduction compared to fecundity on normal agar plates), while at 100 $\mu\text{g/ml}$ the number of offspring was only about 50 % of the above number. In addition, egg-laying was retarded by about 1 day and hatched juveniles took 1–2 days longer than controls to start egg production, which indicates that high concentrations of PF 1022 generally affect the rate of development.

We also observed that developing embryos accumulated in the uterus of their mothers and that, in general, no early-stage embryos were laid. This is typical of old or starving adults in untreated cultures and is indicative of a dysfunctional vulva musculature.

We studied the effects of PF 1022 on the development of fertilized eggs. No abnormalities were observed on agar plates or in liquid culture (100 $\mu\text{g/ml}$ PF 1022) and all embryos hatched ($n=20$). To investigate any potential effect on oogenesis we grew freshly hatched juveniles on agar plates containing 100 $\mu\text{g/ml}$ PF 1022 and studied these animals after they had reached the adult stage. Oogonia and oocytes appeared to be normal at the light microscopic level. Thus, the reduced fecundity does not seem to be due to defective germ cell development as found for MBZ (Fig. 5).

One preliminary observation we made may give a clue to the cause of reduced egg production. Many adults appeared transparent to a variable degree in the posterior 10–25 % of their body. Analysis with Nomarski optics at high magnification revealed that gut cells in the posterior part of the animal contained

a reduced amount of cytoplasmic granules even under optimal food conditions, which suggests insufficient posterior uptake or leakage of nutritive components. In addition, the gut lumen in this region was wide open. This is a characteristic also found in starving animals. Thus, the reason for the reduced number of offspring and egg-laying defects under the influence of PF 1022 could be a starvation syndrome, the cause of which remains to be elucidated.

NECESSARY EXPOSURE TIME AND REVERSIBILITY OF INDUCED DEFECTS

Only IVM and ANN, which express quick acting effects on mobility, were tested.

Ivermectin: In order to determine the time of exposure to IVM that induces irreversible defects, specimens were temporarily placed on agar plates containing different concentrations of the drug. Our results demonstrate concentration-dependent effects (Fig. 7). At the highest concentration tested (1 $\mu\text{g/ml}$), 5 min exposure was already enough to cause later paralysis in 50 % of the animals. At 0.01 $\mu\text{g/ml}$, even 5 h exposure irreversibly affected only a minority of specimens: 24 h after transfer to normal plates most animals moved and ingested food. However, when animals were exposed for 12 h to this concentration, they were also completely paralysed. In no case have we been able to reactivate completely paralysed specimens by transferring them to normal agar plates. However, slow moving animals in which pharynx

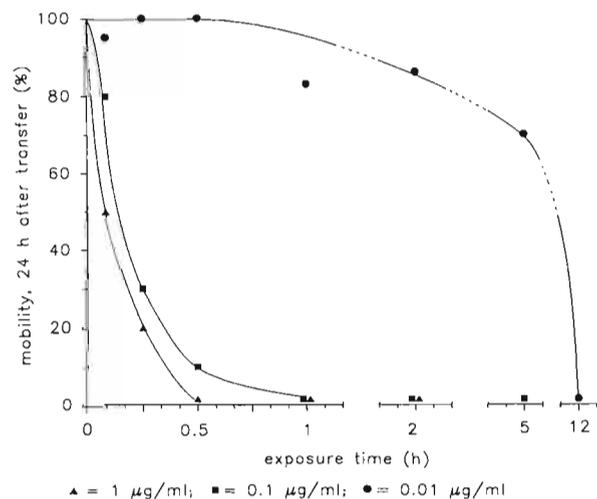


Fig. 7. Necessary time of exposure to ivermectin on agar plates to induce irreversible defects. Specimens were exposed to the drug for various times ('pulsed') and then transferred to normal plates. Percentage of mobile animals was tested on normal plates 24 h after transfer. Each symbol reflects the behavior of at least twenty adult animals.

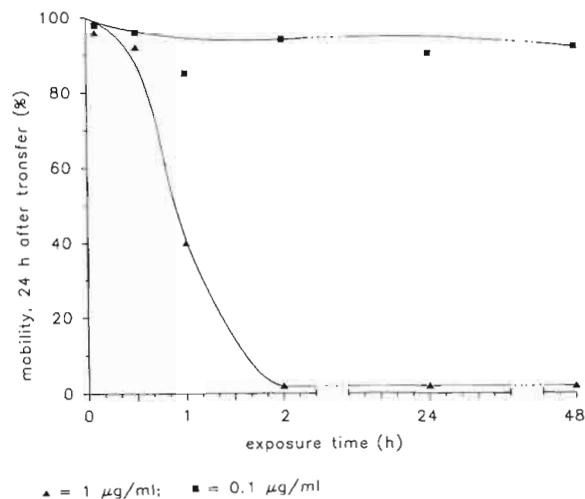


Fig. 8. Necessary time of exposure to annonin on agar plates to induce irreversible defects. Specimens were exposed to the drug for various times ('pulsed') and then transferred to normal plates. Percentage of mobile animals was tested on normal plates 24 h after transfer. Each symbol reflects the behavior of at least twenty adult animals.

pumping had ceased can at least partially recover and resume pharynx pumping if only at a lower than normal frequency.

Annonin: To determine the minimum time of exposure to ANN causing irreversible defects, we placed animals on plates with this drug for different times. The results are shown in Fig. 8. Surprisingly, nearly all specimens recovered after exposure to 0.1 µg/ml even for 48 h, despite the fact that they were paralysed after just a few hours (see Fig. 3). Thus, and in contrast to IVM, completely paralysed animals can recover. However, exposure to 1 µg/ml for 2 h or more resulted in the death of all animals.

MODE OF DRUG UPTAKE

To determine whether a drug needs to be taken with feeding to be effective or it can penetrate through the cuticle, we exposed animals to the drugs at low temperature (4°C) where they could neither move nor feed. Only IVM and ANN, which have quick acting effects on mobility, were tested.

Ivermectin: We found that all adults exposed to 1 µm/ml IVM at low temperature for 3 days were irreversibly inactivated without any oral uptake, while at least 50 % of young juveniles (L1 and L2) remained mobile, even after 1 week exposure. This reduced sensitivity of juveniles is in line with the results of the standard test assay described above. We also tested dauerlarvae, a special form of L3 larvae which does

not feed (Cassada & Russell, 1975), and we obtained results similar to the results with adults. Our findings indicate that IVM does not have to be ingested and can be taken through the cuticle. This conclusion is in accordance with permeability studies on isolated nematode cuticle (Thompson *et al.*, 1993).

Annonin: Experiments in cold medium with 1 mg/ml ANN gave different results. A mixed population exposed to the drug for 1 week recovered rapidly with at least 95 % of the test specimens starting to move immediately after transfer to a normal plate at room temperature and later reproducing. This indicates that ANN does not enter through the cuticle and that it must be ingested to be effective. Additional observations support our conclusions: when gravid animals were exposed to 1 µg/ml ANN for 24 h at room temperature, juveniles hatched inside their dead mothers and moved vigorously in the body cavity of the corpse for many hours. As we found that embryos quickly arrest after exposure to ANN (see above), we conclude that they must be protected inside the mother, most likely by an impenetrable cuticle. Those larvae by themselves are sensitive to ANN and their movement stops within 30 min when they are cut out of dead mothers and placed on plates with ANN.

Discussion

Our results illustrate the opportunities provided by *C. elegans* as an *in vivo* test system for anthelmintic drugs because many parameters can only be studied under controlled laboratory conditions with an organism for which the range of what is 'normal' is well known.

We found that each of the four tested drugs induces a characteristic pattern of defects (Table 3). Our studies show that a combination of various tests gives a more comprehensive picture of the effectiveness of a drug than any single test. Together, they seem to be a useful tool for initial screening of new compounds.

Some of the tests presented here could also be performed with suitable parasitic nematodes to investigate whether results obtained with free-living nematodes apply to them as well. It is true that differences in the reaction to drug treatment have been found (Russell & Lacey, 1991), probably due to differences in life style arising from specific adaptations of the parasites. It remains that many similarities exist between parasitic nematodes and *C. elegans* (Howells & Johnstone, 1991; Politz & Philipp, 1992).

We found that different parasitic species can react very differently to the same treatment (Tables 1, 2). IVM is ineffective against *T. spiralis* (larvae) but it is very effective against *N. brasiliensis* (adults), *H. polygyrus*, and *H. spumosa*. MBZ is very active against the latter but shows no effect against *H. polygyrus*. In con-

trast, early development of the parasitic *Parascaris equorum* (Boveri, 1899) is quite similar to that of *C. elegans* (although slower) and embryogenesis in the alternating free-living and parasitic generations of *Rhabdias bufonis* differs only marginally (Spieler & Schierenberg, 1995), while significant variations were found among free-living nematodes (Skiba & Schierenberg, 1992; Malakhov, 1994). This comparison suggests that an *a priori* separation of nematodes into two groups, free-living and parasitic, is not very helpful and that the question of how much two species have in common, must be determined in each case.

While our tests show that mobility can be most rapidly examined in liquid medium, a precise description of animal behavior after or during drug exposure is difficult or impossible in this medium. Consequently, we prefer agar plates where various aspects can be evaluated, including general 'healthy' appearance, co-ordinated movement, velocity, egg production, and feeding (rate of pharynx pumping). Nevertheless, it is important to recognize that the effect of a drug may vary with culture conditions. We found that after application of MBZ and IVM in liquid medium a paralyzing effect occurs considerably sooner than on solid medium (Figs 1, 2), while the opposite is true for ANN. The stronger effect of MBZ and IVM in liquid medium may be attributed to a higher metabolic turnover rate and/or a faster transfer of the drug to the target site in rapidly moving specimens. As ANN acts on the respiratory chain that affects the availability of ATP (Londershausen *et al.*, 1991), the rapid paralysis of all stages on agar plates is not surprising. The limited effect of ANN (which must be ingested; see above) in liquid culture may indicate the disruption of pharynx pumping as a stress response in an unusual environment for this soil-dwelling nematode. Why, in contrast to agar plates, only some of the specimens are immobilized in a reasonable time in liquid medium may be explained with age-dependent but also individual differences in oral drug uptake.

We found that *C. elegans* juveniles express a reduced sensitivity to certain drugs (MBZ; IVM). This may be due to structural changes in the cuticle leading to a different penetrance in larvae and adults. Such stage-specific variations in the composition of the cuticle, *e.g.*, the pattern of collagen species, have been well documented (Cox *et al.*, 1981; Kramer *et al.*, 1985; Johnstone, 1994). It is also possible that a switch in the pathway of incorporation (from oral to cuticular or vice versa) takes place with increasing age as described for the uptake of pyrantel pamoate in *Toxocara canis* (Mackenstedt *et al.*, 1993).

Quite complex procedures have been developed to investigate the mode of drug uptake, including diffusion chambers with isolated nematode cuticle (Thompson *et al.*, 1993) and tracing with radioac-

tively marked test substance (Mackenstedt *et al.*, 1993). If the drug induces an easy to score effect, the simple *in vivo* test presented here (where low temperature blocks feeding and unwanted ingestion can be controlled with a marker dye), appears sufficient to show whether the drug can penetrate the cuticle or not.

In *Ascaris suum*, the first MBZ-induced lesions detected with the electron microscope 6 h or more after drug exposure occurred in the intestine (Borgers & De Nollin, 1975). With the light microscope we found abnormalities in unfertilized eggs already 1-2 h after application of the drug (Fig. 5), which indicates that it reaches its target within a reasonable time. Significant effects on mobility and growth, however, were seen only after several days under the same conditions (Fig. 1; see also Spence *et al.*, 1982). This and our microscopic observations indicate that in *C. elegans* the effect on the intestine is not as strong as found in *Ascaris*. The quick damage to germ cells can be explained by the mode of drug action. MBZ appears to interfere with microtubule synthesis or integrity (Friedman & Platzer, 1980; Woods *et al.*, 1988; McKellar & Scott, 1990; Savage & Chalfie, 1991). An intact cytoskeleton is indispensable for proper meiosis, which explains why oocytes are particularly affected while embryos are protected by their eggshell. Surprisingly, postembryonic cell divisions (Sulston & Horvitz, 1977; Kimble & Hirsh, 1979) do not seem to be seriously affected, as young juveniles develop into mature adults under the influence of MBZ, as also observed by Spence *et al.* (1982). It remains to be determined whether this is due to different microtubule populations or to differences in the bioavailability of the drug in germ cells on the one hand and somatic cells on the other. We conclude from our findings that the quickly proliferating germ cells are potentially sensitive targets for drugs. Consequently, these cells should be inspected during any test.

An important question is whether nematodes can still reproduce normally after drug treatment, particularly in the case of self-fertilizing hermaphrodites such as *C. elegans* that do not depend on mating. Our data indicate that, in all cases where body musculature became dysfunctional with subsequent paralysis of the animals, fertilization of eggs also stopped, probably because the contractile gonadal sheath cells necessary for transport of germ cells along the gonadal tube (Sulston & Horvitz, 1977) were inactivated as well.

At first glance we could not detect any effect of PF 1022 on *C. elegans*. However, under closer examination we found that several, though not very prominent effects may be induced, which point towards an impaired utilization of food. Tests of various parasitic helminths with PF 1022 yielded very different results. While the drug had a strong effect on mobility of

H. polygyrus and *H. spumosa*, no effects were observed with *T. spiralis*. *In vitro*, however, PF 1022 was strongly active against both *T. spiralis* and *N. brasiliensis* (Tables 1, 2). Whether this can be correlated to differences in the presumed target site of PF 1022 (GABA receptors; Terada, 1992; Terada *et al.*, 1995) or to other factors that affect bioavailability or the influence of pharmacokinetics in experimentally mixed-infected mice requires further studies.

The analysis of drug-resistant mutants in *C. elegans* is a very promising approach for the study of the molecular processes of drug action (Lewis *et al.*, 1980; Chalfie *et al.*, 1986; Woods *et al.*, 1988; Schaeffer & Haines, 1989; Enos & Coles, 1990; Russell & Lacey, 1991). Therefore, we have started a search for *C. elegans* mutants resistant to some of the compounds investigated here. The analysis of the genes involved and the products for which they encode will help us to better understand the underlying mechanisms and, hopefully, the events leading to natural drug resistance in parasitic nematodes.

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