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Short communication

Contractile activity and motility responses of the dog heartworm *Dirofilaria immitis* to classical anthelmintics and other compounds

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Abstract

A variety of compounds including classical anthelmintics and avermectin analogs were screened for their effects on movements of adult heartworms (HW) (*Dirofilaria immitis*). Contractile activity was measured by tension recording of spontaneous movements of intact HW coil preparations (6 min compound exposure) and motility was evaluated by observation of spontaneous, free movements in culture (3 and 7 days compound exposure). Results for female HW indicated that some compounds caused spastic paralysis of contractile activity and inhibition of motility in culture (bephenium, DL-tetramisole, and pyrantel); some caused only spastic paralysis of contractile activity (methyridine and disophenol); and some caused only inhibition of motility in culture (chlorpromazine, dithiazanine, 1-ethoxycarbonylmethyl-1-methylpyrrolidinium, and 4-methyltropolone). Effects on motility in culture appeared to be lethal. The following compounds lacked effects: amprolium, 2-amino-2-thiazoline, bithionol, bitoscanate, bitriben, hexachlorophene, ivermectin, and 10 *H*-phenothiazine. A group of avermectin analogs was screened for effects only on motility in culture of both adult female and male HW. Several of the analogs affected motility, but the effects appeared to be non-lethal. Microfilaria release into the culture media was suppressed by two of the analogs (an aglycone and avermectin B2). The HW maintenance system used in the present study facilitated screening of compounds for effects on this parasite.

Keywords: Contractile activity; *Dirofilaria immitis*; Heartworms; Motility; Nematode; Pharmacology

1. Introduction

Movement ability of nematodes is a good indicator of their viability and can serve as a valuable parameter for evaluation of the spectrum of sensitivity of a specific nematode species to a variety of chemical

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agents. This information can be of great value in the identification of chemical structures that might prove effective in the development of macrofilaricides. In the present study, effects of some classical anthelmintics and other compounds were evaluated on contractile activity and motility in culture of dog heartworms (HW) (*Dirofilaria immits* Leidy, 1856). Techniques reported by Bowen and Vitayavirasuk (2004) for in vitro maintenance of adult HW facilitated screening for effects of compounds on both contractile activity and motility in culture. The term motility has been used in other reports to mean contractile activity (Terada et al., 1985), but a clear distinction was made in the application of these terms in the present study based on differences in evaluation procedures. Contractile activity measurements involved tension recording of spontaneous somatic muscle activity, whereas assessment of motility involved observation for presence of free, spontaneous movements in culture.

2. Materials and methods

2.1. Collection and maintenance of heartworms

Adult heartworms were harvested using sterile technique from dogs that had been experimentally infected by subcutaneous injection of 400 third stage larvae of *D. immitis* (McCall, 1981). This use of dogs was approved by the University of Georgia's Animal Care and Use Committee. The media for maintenance consisted of Eagle's minimum essential medium (MEM) (pH 7.4) supplemented with 10% horse serum, MEM vitamins, and a mixture of penicillin-streptomycin-amphotericin B (Gibco Life Technologies, Grand Island, NY). Culture dishes containing 20 ml of media and two female HWs or containing 50 ml of media and two male and two female HWs were stored in an incubator (37 °C) in a humidified atmosphere (5% CO_2:95% room air).

Table 1
Effect of classical anthelmintics and other compounds on contractile activity and motility of adult female heartworms

Compound	Concentration (μmol/l)[a]	Contractile activity[b]		Motility inhibition in culture[c]	
		Basal tension (% change)	Contraction index (% change)	Three days	Seven days
Pyrantel tartrate	48.5	SP[d]	–[e]	Yes[f]	Yes[f]
Bephenium embonate	22.5	SP	–	No	Yes[g]
DL-Tetramisole	49.0	SP	–	No	Yes[f]
Methyridine	72.9	SP	–	No	No
Disophenol	25.6	SP	–	No	No
Chlorpromazine	31.4	49.1	117	Yes[g]	Yes[f]
Dithiazanine iodide	19.3	3.1	−8.4	Yes[f]	Yes[f]
1-Ethoxycarbonylmethyl-1-methylpyrrolidinium iodide	33.4	−23.8	34.2	Yes[g]	Yes[g]
4-Methyltropolone	73.4	5.0	20.2	No	Yes[f]
Amprolium hydrobromide	24.7	−5.3	30.6	No	No
2-Amino-2-thiazoline	96.0	24.9	−17.0	No	No
Bithionol	28.1	−7.1	9.8	No	No
Bitoscanate	52.0	13.4	16.2	No	No
Bitriben	32.0	16.8	53.0	No	No
Hexachlorophene	24.6	−3.8	3.9	No	No
Ivermectin	11.5	0.0	−27.2	No	No
10 *H*-Phenothiazine	50.2	26.2	48.6	No	No

[a] Calculation based on a concentration of 10 μg/ml.
[b] Mean of two experiments; 10 μg/ml.
[c] Two female heartworms for each period and concentration and two control heartworms.
[d] SP: spastic paralysis.
[e] Not measurable in the presence of spastic paralysis.
[f] Effect noted for 1 and 10 μg/ml.
[g] Effect noted for 10 μg/ml only.

2.2. Recording and evaluation of contractile activity

Quantitative measurement of contractile activities of HW was made by myographic recording of tension changes of a HW coil (1-cm diameter) (Bowen and Vitayavirasuk, 2004). The coil was placed in a jacketed organ bath filled with a solution (45 ml) that contained the following (mmol/l): NaCl (115), KCl (4.6), $CaCl_2$ (1.5), $MgCl_2$ (1.0), NaH_2PO_4 (1.2), $NaHCO_3$ (22), and glucose (22). The bath solution was pH 7.4 when gassed with a mixture of 5% CO_2:95% O_2 and was maintained at 37 °C. The coil was connected to a Grass FT.03C force displacement, isometric transducer (Grass Instruments Co., Quincy, MA, USA) and changes in tension were recorded continuously with a polygraph (Grass Instrument Co.).

The numerical values for basal tension (g) and contraction index (mm/min) were determined before and during exposure to a compound. Results are expressed in Table 1 as percent change from control values. The contraction index was measured using an opisometer (Model 2962, Brookstone, Petersborough, NH) to measure the length of tracing (line integral or straight-line equivalent) associated with the record evaluated. If spastic paralysis occurred, spontaneous activity ceased and the contraction index was not measurable. Additional details on recording and evaluation of HW contractile activity are available in a previous report (Bowen and Vitayavirasuk, 2004).

2.3. Evaluation of motility in culture

Motility was evaluated after 3 and 7 days continuous exposure to a compound. Media with or without test compound was changed daily (Table 1) or on alternate days (Table 2). Presence of motility was based on observation of HW in the culture dishes immediately after their removal from the incubator and prior to exchange of culture media. An immotile HW in culture was considered dead if, when lifted on a glass rod (2-mm diameter) at the worm's midpoint, the worm conformed closely to the curvature of the rod. Turgor of the body of living worms produced an arc of the body, which limited its ability to conform to the curvature of the rod. For study of the avermectin analogs (Table 2), release of microfilaria into the media was noted based on cloudiness of the media, which was confirmed by microscopic examination. The heartworms used in the evaluation of the avermectin (AVM) analogs were all obtained from one dog.

2.4. Compounds evaluated

Two groups of compounds were provided for evaluation using a coded format by Merck & Company. One group of compounds (Table 1) was evaluated for their effect on contractile activity (10 μg/ml) and on motility in culture (1 and 10 μg/ml). The second group of compounds (Table 2) was evaluated for effect only on motility (1 and 10 μg/ml). The identity of the compounds in both groups was not revealed until after completion of the experiments. Compounds that were

Table 2
Effect of avermectin analogs on motility in culture of adult female and male heartworms

Analog	Inhibition of motility in culture[a]			
	Three days		Seven days	
	1 μg/ml	10 μg/ml	1 μg/ml	10 μg/ml
Avermectin B1 (abamectin)	No	Yes (male only)	Yes	Yes
13-Deoxy-13-methoximino ivermectin aglycone	No	No	No	No
4″-Deoxy-4″-epi-acetylamino-avermectin B1 (eprinomectin)	No	No	No	No
4″-Deoxy-4″-epimethylamino avermectin B1 (emamectin)	No	No	Yes	Yes
Avermectin B1 8,9-oxide	No	No	Yes	No
4″-Deoxy-4″ epi-1,1-dimethyl hydrazino avermectin B1	No	No	No	Yes (male only)
4″-Deoxy avermectin B1-4″-dimethyl semicarbazone	No	No	Yes	No
Avermectin B2	No	Yes	Yes	Yes
Ivermectin monosaccharide	No	No	Yes	Yes

[a] Two female and two male heartworms for each period and concentration.

not soluble in the bath solution or culture media were first dissolved in DMSO. This mixture was diluted with bath solution or culture medium and then vigorously shaken. An amount of this stock solution was added to the bath or culture medium containing HW to provide the desired final concentration. The final concentration of DMSO, when added alone, had no visible effects on contractile activity or motility in culture. After 6 min exposure to a compound in the contractile activity experiments, the bath was flushed with at least three volumes of fresh solution.

3. Results

Results for evaluation of the effects of the classical anthelmintics and other compounds on contractile activity and motility in culture are presented in Table 1. Pyrantel, bephenium, DL-tetramisole, methyridine, and disophenol produced spastic paralysis. Among these compounds, pyrantel, bephenium, and DL-tetramisole also inhibited motility in culture. Methyridine and disophenol did not affect motility in culture. Chlorpromazine, dithiazanine, 1-ethoxycarbonylmethyl-1-methylpyrrolidinium, and 4-methyltropolone inhibited motility in culture, but lacked effect on contractile activity although chlorpromazine appeared to have some stimulatory effect on contractile activity when compared with all other compounds that did not produce spastic paralysis. Effects on motility in culture appeared to be lethal based on loss of hydrostatic turgor.

Results for effects of the AVM analogs on motility in culture are presented in Table 2. The aglycone and eprinomectin lacked effects, while the other AVM analogs appeared to inhibit motility. Abamectin and the hydrazino AVM B1 had effects only on males at certain concentrations and exposures. A lower density of microfilaria based on cloudiness of the culture media was readily observed for the aglycone and AVM B2 at 7 days. When compared with effects of compounds on motility that were noted in Table 1, the effects of the AVM analogs on adult HW did not appear to be lethal.

4. Discussion

Several of the classical anthelmintics and related compounds demonstrated important differences in effects on contractile activity and motility. Some of the compounds affected both contractile activity and motility, while some others affected only one of these response systems. These differences in responses emphasize the value of screening compounds for anthelmintic potential on both contractile activity and motility in culture and also the importance of applying clear definitions of the terms contractile activity and motility. Analysis of effects on contractile activity reflects effects having relatively rapid onset and, for many compounds, this probably is the result of the neuromuscular system being the target site. Motility effects represent slower onset and for many compounds, a compromise of energy generating mechanisms is probably involved.

Although the experimental repetitions were limited to two and dose-response relationships were not investigated, the results of the present study raise interesting questions regarding mechanisms of action. Compounds that caused both spastic paralysis and inhibition of motility in culture (bephenium, DL-tetramisole, and pyrantel) may have produced the motility effect through loss of energy reserves because of the muscle contraction associated with the spastic paralysis. Bacon et al. (1998) reported that energy depletion could lead to relaxation of nematode somatic muscles. Compounds that caused spastic paralysis, but lacked an effect on motility may have had a less intense degree of spastic paralysis or their paralytic effect may not have been sustained with continued exposure to the compound. Progression of the energy loss may explain why the inhibitory effect of bephenium and DL-tetramisole were noted at 7 days and not at 3 days. The 3 and 7 days inhibitory effects differed markedly from viability for control HW in the present study and a previous study demonstrated that the maintenance procedures used enabled HW motility in culture to continue for prolonged periods (Bowen and Vitayavirasuk, 2004). The cyanine dye dithiazanine has been reported to have effects on some tissue dwelling filarial parasites (Saz and Bueding, 1966). Its inhibitory effect on HW motility in culture may be associated with its ability to reduce energy reserves by inhibition of glucose and oxygen uptake.

Bephenium and methyridine have structural similarities to acetylcholine, which is probably the excitatory neurotransmitter in HW. This may explain their excitatory effect. The competitive nitric oxide

synthase inhibitor 2-amino-2-thiazoline had no effect on contractile activity or motility in culture although nitric oxide is produced by HW (Kaiser et al., 1998). The catechol-*o*-methyl-transferase inhibitor 4-methyl-tropolone inhibited motility in culture, but not contractile activity. This suggests aminergic involvement in motility. The phenolic anthelmintics bithionol and hexachlorophene, which are both capable of uncoupling oxidative phosphorylation, lacked effect on contractile activity and motility in culture. Disophenol, also an uncoupler of oxidative phosphorylation and a phenolic anthelmintic, produced spastic paralysis, perhaps by a mechanism unrelated to uncoupling of oxidative phosphorylation in view of the lack of effect of the other uncouplers of oxidative phosphorylation. Rew (1978) reported that uncouplers of electron transport-associated phosphorylation were effective in vivo against flukes and cestodes, but not nematodes. Bithionol affects canine hookworms (*Ancylostoma caninum*) (Sano et al., 1981), but it did not affect HW in the present study. In contrast, disophenol is active against hookworms as well as HW. Neither ivermectin nor 10 *H*-phenothiazine affected HW contractile activity or motility in culture although both have anti-nematodal activity against certain stages and species of nematodes. The phenothiazine tranquilizer chlorpromazine inhibited motility and appeared to stimulate contractile activity. Zinser et al. (2002) reported that chlorpromazine had a weak antagonistic effect on somatic ACh receptors in *Ancylostoma suum*.

The AVM analogs were not tested for effect on contractile activity based on the general belief that they lack such an effect on adult HW and that any effect might require a longer time of onset. The fact that ivermectin did not affect motility in contrast to several of the AVM analogs may have been the result of different culture conditions and different ages of the adult HW used in these experiments. A major difficulty in the evaluation of compounds on in vitro systems can be the poor solubility of the compound in biological fluids. Solubilization procedures such as use of DMSO may not fully solubilize the compound and may have subtle influences on compound effects. Lack of complete solubility of some of the AVM analogs might explain the variation in concentration effects noted for some of the analogs in Table 2. In addition, variations in binding of some analogs to plasma proteins could have influenced the results (Fisher and Mrozik, 1992).

HW males appeared to be more sensitive to certain AVM analogs than HW females (Table 2). Court et al. (1986) noted that *Brugia pahangi* males were more resistant to ivermectin than *B. phangi* females, whereas gender sensitivity for *Dipetalonema viteae* was similar. Two of the AVM analogs suppressed release of microfilaria into the culture medium. A similar effect was reported for ivermectin on *B. pahangi* and *D. viteae* (Court et al., 1986).

Evaluation of effects of compounds on both contractile activity and motility in culture is especially helpful in screening anthelmintics for species susceptibility, will help prevent overlooking new potential anthelmintics, and can lead to better understanding of mechanisms of action.

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