Lethal and sublethal effects of ivermectin in a freshwater oligochaete, *Lumbriculus variegatus*

by

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This is to certify that the Master’s thesis of

Jing Ding

has met the thesis requirements of Iowa State University

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REVIEW OF LITERATURE

Ivermectin

Ivermectin (22,23-dihydroavermectin B₁) is a member of the avermectin family. It is the drug of choice for the treatment and prevention of many nematode and arthropod parasite infections (Chabala et al. 1980; Campbell et al. 1983, 1984, 1991, 1993; Ottesen et al. 1994). The anthelmintic activity of the avermectins was first described in 1979 (Burg et al. 1979; Egerton et al. 1979; Miller et al. 1979). Ivermectin was introduced commercially in 1981.

Structure, physical and chemical characteristics of ivermectin

Fermentation of the actinomycete Streptomyces avermitilis produces four homologous pairs of closely related, complex, 16-membered, macrocyclic lactones: avermectin A₁, A₂, B₁ and B₂. Based on the sidechain composition at carbon position 25 (Fig. 1), the four pairs are further divided into the major components “a” (a secondary butyl substituent at C-25, more than 80%) and minor components “b” (an isopropyl group, less than 20%). Avermectin B₁a has the highest potency against many of endo- and ectoparasites of animals, as well as many agricultural mite and insect pests. It is also the starting material for synthesizing ivermectin.

Because it is very difficult and impractical to separate the major components “a” from the minor components “b”, and because the biological activities of the two components are almost indistinguishable, the avermectins are often used as a mixture of the two components (more than 80% of component “a”, less than 20% of “b”). In such cases they are just named as A₁, A₂, B₁ and B₂. Ivermectin (22,23-dihydroavermectin B₁) is used as such a mixture.

The avermectins are highly lipophilic, and dissolve in most organic solvents. Their water solubility is correspondingly low – only 6 to 9 ppb (Fisher and Mrozik 1989).

Ivermectin contains two double bonds conjugated as an 8,9,10,11-diene function, which results in a strong UV absorption at 245 nm (Fisher and Mrozik 1989). This
property is useful in analytical detection. Another consequence is that exposure under UV light below 280 nm decomposes ivermectin rapidly (Mrozik et al. 1988).

Ivermectin is unstable under acidic and basic conditions. The disaccharide motif is subject to acidic hydrolysis. Weak acids cleave off the first sugar. Stronger acids are necessary to hydrolyze off the second sugar to give the aglycone (Fisher and Mrozik 1989). The proton at the asymmetric C-2 next to the lactone carbonyl group is acidic. A strong base aromatizes the 6-membered ring and opens the lactone ring (Pivnichny et al.)
1983, 1988). Consequently, strong bases can be used to destroy ivermectin residues for safe disposal.

**Application of ivermectin**

Synthetic compounds dominated the anthelmintic market until the late 1970s. The discovery of the avermectins changed that dramatically. Ivermectin has been used most widely in domestic animals in the control of parasitic diseases that cause losses of hundreds of millions of dollars annually. Its use in human medicine has also received considerable attention because of its control of onchocerciasis (river blindness), one of the most insidious and intractable of tropical diseases.

Ivermectin is used against a wide range of nematode and arthropod parasites in domestic animals (Chabala et al. 1980; Campbell et al. 1983, 1984, 1993), as well as in laboratory and exotic mammals and in birds, fish, and reptiles (Soll 1989). Essentially all important nematode species are susceptible, both immature and mature stages. In addition to its activity against nematode parasites, ivermectin is also active against several important ectoparasites, such as grubs, screwworms, lice, mites and ticks. Because of its activity against both endoparasitic and ectoparasitic organisms, it has become known as the first “endectocide”. Ivermectin is active when given orally, intramuscularly, subcutaneously, or even topically.

Ivermectin was first reported in human trials against onchocerciasis in 1982 (Aziz et al. 1982a, 1982b). Its clinical trials and usage have been well reviewed (Campbell 1991, 1993). Its potent microfilaricidal activity against the other major filarial parasites of humans has also been recognized (Campbell 1991; Ottesen et al. 1994). Preliminary studies have shown that ivermectin is effective against some human intestinal nematodes, *Ascaris lumbricoides, Strongyloides stercoralis, Trichuris trichiura* and *Enterobius vermicularis*, but weak against other species, *Ancylostoma duodenale* and *Necator americanus* (Campbell 1993). The efficacy of ivermectin against ectoparasitic infestations (mites, lice) of humans has also been reported (Ottesen et al. 1994).
Antiparasitic effect of ivermectin and mechanism of action

Ivermectin reduces motor activity of the parasites so that they are excreted from the host (Martin 1993). Early electrophysiological studies using muscle or nerve preparations from lobsters, insects, large nematodes or mammalian brains (Fritz et al. 1979; Kass et al. 1980, 1984; Duce and Scott 1985; Mellin et al. 1983; Abalis et al. 1986; Albert et al. 1986; Krusek and Zemkova 1994; Huang and Casida 1997) suggested that ivermectin interferes with synaptic transmission, and that this is the result of increased cell membrane permeability to chloride ions, which is explained by the opening of GABA-gated Cl⁻ channels.

It has recently been shown, however, that ivermectin also activates Cl⁻ channels that are not regulated by GABA. Specific ivermectin binding sites have been identified in the free-living nematode *Caenorhabditis elegans* and in mammalian brain tissue. The affinity of ivermectin for the nematode site (apparent dissociation constant, $K_d = 0.26$ nM), is about 100 times greater than that for the mammalian brain site (Schaeffer and Haines 1989). The failure of GABA to compete with ivermectin for binding to the nematode (Cully and Paress 1991) suggests that the GABA and ivermectin receptors are significantly different. The mode of action of ivermectin has also been studied in specially prepared oocytes of the frog *Xenopus laevis*. After mRNA of *C. elegans* is injected into an oocyte, a Cl⁻ current can be activated in the membrane of the oocyte by ivermectin and L-glutamate, but not GABA (Arena et al. 1991, 1992). These studies together suggest that GABA plays little, if any, part in the antiparasitic action of ivermectin. They also lead to the discovery of a novel type of Cl⁻ channels, namely a glutamate-gated ivermectin-sensitive Cl⁻ channel.

Glutamate-gated ivermectin-sensitive Cl⁻ channels have been described in many invertebrates, such as nematodes, insects, crustaceans, and mollusks (Cleland 1996). Although their physiological role has not been fully determined, they are reported to be involved in generating rhythmic firing of the neurons within the crustacean stomatogastric ganglion (Cleland and Selverston, 1995, 1998). Two subunits (GluClα and GluClβ) have been cloned from *C. elegans* (Cully et al. 1994). Gene structure comparisons and phylogenetic analyses reveal that these channels are related to
vertebrate glycine channels/receptors. Since these channels only exist in invertebrates, they may mediate the powerful anthelmintic and insecticidal action of ivermectin, while accounting for the low toxicity to mammals.

Other actions of ivermectin

It was reported that ivermectin specifically binds to retinol-binding protein isolated from parasitic worms of the family *Filarioidea*, but not the host organism (Sani and Vaid 1988). Ivermectin was also reported to be a specific inhibitor of protein kinase C isolated from rat brain (Ellis *et al.* 1987). The high concentration of ivermectin required for this action (IC$_{50}$: 1 µM) suggests that this may not be of physiological significance. In sarcoplasmic reticulum (SR) of rat and rabbit skeletal muscle, ivermectin directly activates ryanodine receptor Ca$^{2+}$ channels, and reduces Ca$^{2+}$ uptake by the SR Ca$^{2+}$-Mg$^{2+}$-ATPase (Ahern *et al.* 1999). Once again, the high dose of ivermectin (10 µM) may not be pharmacologically significant.

Safety of ivermectin in mammals

Clinical signs of the acute toxicity of ivermectin in mammals may be related to its effect on GABA-gated Cl$^{-}$ channels in the mammalian brain and spinal cord. Common signs include ataxia, tremors, and in severe cases, coma and death (Lankas and Gordon 1989; Pulliam and Preston 1989). Rodents are more sensitive to ivermectin toxicity than are other species. Severe reactions and death occur in mice at dosages as low as 200 µg/kg—the dosage that is routinely used in treating parasitic nematodes in animals and onchocerciasis in human (LD$_{50}$ = 25 mg/kg) (Campbell 1993). However, doses of 2.0 mg/kg or higher are needed for acute toxicity in other species, such as rats, rabbits, rhesus monkeys, horses, cattle, sheep, goats, swine and dogs (Lankas and Gordon 1989; Pulliam and Preston 1989).

Results of some subchronic, developmental and reproductive toxicity studies are summarized in Table 1 (Lankas and Gordon 1989). It is concluded that ivermectin is well tolerated in most mammals.
Table 1. No-observed-effect level (mg/kg/day) of toxicity studies with ivermectin

<table>
<thead>
<tr>
<th>Species</th>
<th>Subchronic studies</th>
<th>Developmental and reproductive studies</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mouse</td>
<td>-</td>
<td>0.1 (maternotoxicity)</td>
</tr>
<tr>
<td>Rat</td>
<td>0.4 (splenic enlargement)</td>
<td>5.0 (maternotoxicity and developmental toxicity)</td>
</tr>
<tr>
<td>Rabbit</td>
<td>-</td>
<td>1.5 (developmental toxicity)</td>
</tr>
<tr>
<td>Dog</td>
<td>0.5 (mydriasis)</td>
<td>-</td>
</tr>
<tr>
<td>Rhesus monkey</td>
<td>&gt;1.2 (treatment-related effects)</td>
<td>-</td>
</tr>
</tbody>
</table>

Environmental fate and concern of ivermectin

When ivermectin is administered (orally, topically, or subcutaneously) to food-producing animals, the drug enters the environment as a result of excretion. Most of it (at least 90%) is excreted as intact drug in the feces of the treated animal. The remainder is excreted in urine (Campbell 1993).

The distribution and movement of a chemical in the environment depends on the compound's physical and chemical properties. Ivermectin's low water solubility predicts tight binding to soils, as evidenced by the high partition coefficient ($K_D = \text{concentration in soil}/\text{concentration in water} = 227$ to $333$) and high organic-carbon binding constant ($K_{OC} = 12,600$ to $15,700$) (Halley et al. 1989). The strong soil binding results in the minimal leaching into ground water. But run-off in surface water with eroding sediments should be expected.

Ivermectin is degraded by sunlight and soil microbes. Depending on the environmental conditions, the degradation half-life of ivermectin varies from 7 - 14 d in summer to 91 - 217 d in winter (Halley et al. 1989).

Because of its potent antiparasitic effect mediated through glutamate-gated $\text{Cl}^-$ channels, which are present in many invertebrates (Cleland 1996), ivermectin raises concerns about its impact on non-target organisms, especially invertebrates, and associated environmental consequences. It has been shown that dung from ivermectin-treated animals failed to degrade in the normal way and this failure was associated with
the absence of dung-degrading invertebrate community, which includes many species of insects and oligochaetes (Wall and Strong 1987; Strong 1993). This may exert a threat to pastureland ecology.

Although lethal levels of ivermectin have been determined in some non-target organisms – daphnia, fish, earthworms, birds and insects (Halley et al. 1993), the effects of sublethal doses have hardly been recognized at the present time. In the present study, we examined the lethal and sublethal behavioral and electrophysiological effects of ivermectin in a freshwater oligochaete, *Lumbriculus variegatus*.

**Freshwater oligochaete, *Lumbriculus variegatus***

Blackworms, *Lumbriculus variegatus* (Phylum Annelida, Class Oligochaeta), are common in ponds, lakes, and marshes of North America and Europe. They freely crawl within and feed on submerged and decaying vegetation, such as rotting leaves and logs (Drewes and Fourtner 1989).

**Locomotor behaviors in *L. variegatus***

Generally, *L. variegatus* moves toward food sources and away from strong light and open spaces. When suddenly touched or threatened, it uses a variety of responses to move to safety. The first response, seen when worms are covered or in contact with substrate, is an escape reflex involving rapid body shortening and withdrawal of the worm’s head or tail end. However, other reflex responses may occur depending on the worm’s environmental context. On wet surfaces or in confined spaces under water, worms crawl forward or backward when lightly touched at tail or head end, respectively. However, when worms are submerged in “open water” condition, tail stimulation evokes rhythmic waves of helical swimming, while head stimulation evokes a body reversal response (Drewes, 1999a). Each of these reflex behaviors is apparently controlled by a different neural pathway (Fig. 2).
Giant-interneuron pathways and escape reflex

Rapid escape responses in oligochaetes are mediated by two independent giant interneuron pathways, the medial giant fiber (MGF) system and the lateral giant fiber (LGF) system (Drewes 1984; Zoran and Drewes 1987; Drewes and Fourtner 1989; Drewes and Brinkhurst 1990). Both MGF and LGF pathways consist of tandemly aligned large caliber axons derived from interneurons in each segment. The tandem axonal units are connected at intersegmental boundaries by electrical synapses (gap junctions). These connections cause the fibers to act as a functional syncytium, allowing uninterrupted conduction of action potentials along the whole length of the ventral nerve cord (Drewes 1999b). The MGF is excited by tactile stimulation to the anterior 30% of the worm’s body, while the sensory field for the LGF extends over the posterior 70% of the body (Drewes and Fourtner 1990). The LGF is also excited by photosensory inputs (Drewes and Fourtner 1989). In each segment the giant fibers activate motor neurons, which innervate body wall longitudinal muscles and bring about body shortening (Drewes 1999b).
Non-giant interneuron pathways and other locomotor behaviors

Tactile stimulation anywhere within the posterior one-half of the worm consistently evokes swimming responses when the worm is in “open water” conditions. Swimming movements consist of a series of alternating left-handed and right-handed, rapid and rhythmic waves of helical body bending. Each wave rapidly progresses from head to tail, thus propelling the worm forward (Drewes 1999a).

Touching to anterior segments while in “open water” evokes body reversal, which consists of a stereotyped sequence of bending movements that effectively reverses head and tail positions. Although there is little net movement of the body center, reversal may reposition the head away from the predatory threat and prepare the worm for swimming away from the threatening stimulus (Drewes 1999a).

Crawling movements are seen when the worm is in substantial contact with substrate or sediment, thus restricting other movements, such as swimming. Crawling consists of a series of rhythmic peristaltic waves of circular and longitudinal muscle contraction. Each wave begins at the anterior or posterior end and rapidly progresses posteriorly or anteriorly along the body (forward or backward crawling, respectively).

The specific network of neurons in an animal’s nervous system that controls rhythmic movements, such as the worm’s swimming and crawling, is called a central pattern generator (Young 1989; Drewes 1999b). Central pattern generators for locomotion in invertebrates, such as annelids and arthropods, are usually in the ventral nerve cord but do not involve giant nerve fibers.
LETHAL AND SUBLETHAL EFFECTS OF
IVERMECTIN IN A FRESHWATER OLIGOCHAETE,
*LUMBRICULUS VARIEGATUS*

**Introduction**

Ivermectin, (22, 23-dihydroavermectin B$_{1a}$), a semisynthetic avermectin analog, is a potent anthelmintic and insecticide against nematode and arthropod parasites. It is widely used to improve the health care of livestock and companion animals (Chabala et al. 1980; Campbell et al. 1983, 1984, 1993). Its use also extends to human medicine (Campbell et al. 1991; Ottesen et al. 1994). Although ivermectin’s mode of action is not fully understood, it is generally believed that ivermectin reduces excitability of muscle or nerve through the opening of Cl$^-$ channels (Arena et al. 1995; Brownlee et al. 1997; Duce & Scott 1985; Kass et al. 1980; Martin et al. 1997).

The environmental effects and fates of ivermectin have also been studied (Halley et al. 1989, 1993; Roncalli 1989; Strong 1993; Wall & Strong 1987). In the vast majority of these studies, mortality has been the endpoint. *Daphnia magna* was particularly sensitive to ivermectin with 48 h LC$_{50}$ of 0.025 ppb (~ 0.03 nM, Halley et al. 1993). Fish were less sensitive [48 h LC$_{50}$ for bluegill (*Lepomis macrochirus*) and rainbow trout (*Oncorhynchus mykiss*) were 4.8 ppb and 3.0 ppb, respectively], and earthworms (*Eisenia fetida*, 28 d LC$_{50}$ 315 ppm in soil) were relatively insensitive to ivermectin (Halley et al. 1993). Without analysis of sublethal end-points (e.g., effects on growth, development, fecundity, morphology, behavior, or physiology), we can not fully understand the complex biological actions or predict ecological impacts of environmental toxicants. This is especially true for a chemical like ivermectin, which exerts antiparasitic effects not by immediately killing the target organisms but by reducing their motor activities so that the parasites are excluded from the host (Martin 1993).

Among a few studies concerning sublethal effects of ivermectin on non-target organisms, most have examined development and reproduction, especially in dung-dwelling insect populations, that may be threatened by drug residues in the manure from

Locomotor capabilities are logical focal points for studies of sublethal effects of ivermectin, because the most predominant effects shown in target organisms are reduced motor activities (Martin 1993). However, effects of ivermectin on motor activity on non-target organisms are poorly understood. In this study, we examined sublethal effects of ivermectin on locomotor behaviors of a non-target invertebrate, *Lumbriculus variegatus* (Family Lumbriculidae, common name: mud worm, blackworm).

Features that make *L. variegatus* especially suitable for this study include: (i) their ubiquitous inhabitation in North America and Europe, and introduction into Africa, Australia, and New Zealand (Brinkhurst and Jamieson 1971); (ii) their freshwater benthic habitat, in which they are vulnerable to the possible runoff of ivermectin within eroding sediments; (iii) their ease in laboratory rearing, maintenance and handling; (iv) their defined patterns of locomotor behaviors, namely helical swimming, body reversal (Drewes 1999a), and crawling (Drewes and Cain 1999); (v) the presence of giant nerve fibers (interneurons) that mediate rapid escape responses (Drewes 1984; Drewes and Fourtner 1989; Drewes and Brinkhurst 1990); (vi) and the capability of non-invasive electrophysiological testing of escape reflex function (Drewes 1984; Zoran and Drewes 1987; Rogge and Drewes 1993).

Our specific objectives were to: (i) determine lethal concentrations of ivermectin in *L. variegatus*; (ii) examine sublethal effects of ivermectin on locomotor behaviors: swimming, reversal and crawling; (iii) verify the involvement of Cl⁻ channels using picrotoxin, a Cl⁻ channel blocker, and (iv) examine electrophysiologically the effects of ivermectin on giant nerve fiber pathways.
Methods and Materials

Materials

The following chemicals were used: ivermectin stock solution (10 mg/ml in 40% glycerol formal and 60% propylene glycol, Merck AgVet, Rahway, NJ) and picrotoxin (Sigma Chemical Co., St. Louis, MO).

Animal maintenance and selection

*L. variegatus* were reared in the laboratory from asexually reproducing colonies. The worms were kept in aerated aquaria containing pieces of brown paper towel (21 - 23°C) and fed three to five times per week with sinking fish food.

Medium-sized worms (≥ 4 - 5 cm long) were removed from rearing tanks 12 - 24 h prior to testing and placed in Petri dishes containing distilled water to allow clearance of gut contents. Worms were visually screened for uniformity in segmentation pattern. Worms showing recent segment regeneration or any obvious morphological defects were not used.

Treatment

Ivermectin solutions were prepared by diluting ivermectin stock solution in distilled water. Additional propylene glycol was added to all the solutions of lower ivermectin concentrations so that the volume of vehicle in all solutions was the same. Control solutions also had the same volume of vehicle as the ivermectin solutions. In ivermectin and picrotoxin antagonism experiments, picrotoxin was dissolved in distilled water. After worms were exposed in the picrotoxin solutions for 60 min, ivermectin and/or propylene glycol were added to the solutions. Concentration levels of ivermectin and/or picrotoxin were determined according to preliminary range-finding experiments (Table 2). All concentrations reported are nominal; no analytical procedures were performed to verify the actual concentrations of the chemicals. However, all aqueous solutions were freshly prepared immediately before the experiments.
Table 2. Treatments used in the present study

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Treatments</th>
</tr>
</thead>
<tbody>
<tr>
<td>LC$_{50}$</td>
<td>Ivermectin: 180, 320, 560, 1000, 1800 nM</td>
</tr>
<tr>
<td>Swimming and reversal</td>
<td>Ivermectin: vehicle, 0.3, 3, 30, 300 nM</td>
</tr>
<tr>
<td>Swimming frequency</td>
<td>Ivermectin: vehicle, 0.03, 0.3, 3, 30 nM</td>
</tr>
<tr>
<td>Antagonism by picrotoxin of ivermectin on</td>
<td>All combinations of two levels of ivermectin (vehicle, 30 nM) and six</td>
</tr>
<tr>
<td>swimming frequency</td>
<td>levels of picrotoxin (0, 1, 10, 100, 1000, 10000 nM)</td>
</tr>
<tr>
<td>Crawling frequency and speed</td>
<td>Ivermectin: 0, 10, 30, 100, 300 nM</td>
</tr>
<tr>
<td>Antagonism by picrotoxin of ivermectin on</td>
<td>All combinations of two levels of ivermectin (vehicle, 300 nM) and six</td>
</tr>
<tr>
<td>crawling frequency and speed</td>
<td>levels of picrotoxin (0, 1, 3, 10, 30, 100 µM)</td>
</tr>
</tbody>
</table>

Treatment was carried out in covered glass Petri dishes (9 cm in diameter, 2 cm in depth) with one worm per container of 100 ml (for swimming and reversal tests) or 50 ml (for other tests) solution. Individual worms were randomly assigned to the treatments. Each treatment was replicated ten to 21 times. In swimming and reversal tests, the worms were examined directly in the treatment dishes. In other tests (swimming frequency, crawling and electrophysiology), the worms were quickly rinsed twice in distilled water and temporarily removed from the treatment dishes for behavioral or electrophysiological testing.

**Lethal effect**

Fifty worms were randomly assigned to five treatments (Table 2), ten worms/treatment. Observations were made after 24 and 72 h of exposure. Mortality was determined as decomposition of the worms.

**Behavioral testing**

**Swimming and Reversal**

Helical swimming and body reversal behaviors in *L. variegatus* were studied as previously described (Drewes 1999a). The worms’ ability to initiate swimming and/or reversal episodes was tested before and at various times after treatment (0, 1, 3 and 8 h).
In each test, a worm was touched ten times with a thin rubber probe alternately at its anterior or posterior end to evoke reversal and swimming, respectively. The interval between successive touches was three to five seconds. A response to a touch stimulus was scored as successful only when the worm showed stereotypical patterns of swimming or reversal movements.

**Swimming frequency and pattern**

To quantify possible effects of ivermectin on swimming frequency, a worm was placed in the middle of a plastic Petri dish (14 cm in diameter, 2.5 cm in depth) containing 200 ml of distilled water. Swimming responses were evoked twice by tactile stimulation to the posterior end of the worm using a rubber probe (Drewes 1999a). The worm was allowed to rest about 2 min after it was moved into the dish and between the two trials. The process was recorded on VHS videotape using a video cassette recorder (MITSUBISHI, model HS-U650) connected to a camcorder (LXI, model 934.53796290), and replayed frame-by-frame on a video monitor (NEC, model XM-2950) after testing to examine the swimming pattern and frequency (number of helical body waves produced per second). Each worm’s responses were measured before and at a selected time after treatment (0 and 3 h in ivermectin-alone experiment, 0 and 1.5 h in ivermectin-picrotoxin antagonism experiment). The ratio of the mean frequency after treatment to the mean frequency before treatment was defined as relative swimming frequency for each worm. When exposed to higher concentrations of ivermectin, some worms failed to swim in one trial or both trials. In such cases, only successful trials were used to calculate relative swimming frequency. If the worm failed twice, the failures were recorded as a separate category. These failures were not used for calculation of mean swimming frequency.

**Crawling**

In the crawling test, a worm was placed next to a smooth strip of Plexiglass (180 × 40 × 6 mm) which rested on a piece of thoroughly wetted filter paper (Whatman #1). Any excess water was removed, thus confining the worm within the surface tension of a narrow band of water between the Plexiglass and paper. A straight rubber band (5 mm long, 0.5 mm in diameter, attached to a wooden applicator stick) was used to brush the worm’s tail, so the worm would crawl forward in a straight line along the Plexiglass. The
frequency of brushing was 3.6 ± 0.1 strokes/sec (n = 20) as determined from videotape replay. This stimulation lasted 10 - 15 sec, or until the worm had crawled 4 - 6 cm. The same procedure was repeated once, and the worm was allowed to rest for about 1 minute between trials. Crawling behavior was recorded on videotape, and later replayed, frame-by-frame, after testing to measure the crawling speed (distance moved per second) and frequency (number of peristaltic waves of contraction produced per second). Each trial consisted of one to three episodes of continuous crawling movements. Episodes that had relatively constant crawling frequency were used to calculate speed and frequency. Only the episode with the highest crawling speed was used for analysis. Each parameter was measured twice, once before (0 min) and once after the treatment (15, 30, 60, 120 or 180 min in ivermectin-alone experiment, 90 min in ivermectin-picrotoxin antagonism experiment). Each of these values represents the highest value obtained from one to six episodes performed by each worm. Relative crawling frequency and relative crawling speed were defined as previously described for relative swimming frequency.

**Electrophysiological testing**

Techniques for noninvasive electrophysiological recording were used as previously described (Drewes, 1984; Zoran and Drewes, 1987; Rogge and Drewes, 1993). Briefly, a worm was placed next to a smooth strip of Plexiglass (4 × 1 cm) on a printed circuit board recording grid. Excess water was removed, thus trapping the worm in surface tension along a narrow band of water between the Plexiglass and electrode grid. The worm’s giant fiber system was activated indirectly through sensory stimulation or directly through electric stimulation. Evoked spikes, as well as muscle potentials, were detected by two pairs of recording electrodes. Signals were amplified, filtered and displayed as two channels on a digital oscilloscope (TENMA, model 72-915 20MHz).

**Conduction velocity of giant fibers**

The worms’ medial and lateral giant fiber (MGF and LGF) systems were activated by tactile stimulation to the anterior and posterior ends of the worms, respectively. Giant fiber conduction velocity was measured at a mid-body location over a 10 mm conduction distance. To obtain velocity, conduction distance was divided by conduction time, as
indicated on the oscilloscope screen by the peak-to-peak interval between spikes in the two recording channels. Each worm was measured before and after the treatment (0 and 3 h). Mean velocity (five measurements per worm) was then converted to relative conduction velocity, which was defined as the ratio of the mean velocity at any time after treatment compared to the mean velocity in the same worm before treatment. Therefore, by definition, the relative velocity before treatment in each worm was 1.0.

**Muscle potentials**

Using one pair of metallic electrodes on the grid surface, twin pulses (duration 100 µs, inter-pulse interval 10 ms) from an electronic stimulator (Model SD9, Grass Medical Instruments, Quincy, MA) were applied to the anterior end of a worm. The voltage of the pulse was adjusted so that only MGF spikes, but not LGF spikes, were evoked. Peak-to-peak amplitude of the muscle potential associated with the second of the two evoked MGF spikes was measured on the oscilloscope screen. To account for variation in signal attenuation, muscle potential amplitudes were referenced to the average amplitudes of the all-or-none MGF spikes. Usually, the ratio between the amplitude of the muscle potential and the amplitude of the MGF spike is $0.72 \pm 0.08$ (n = 10). Duplicate measurements of this ratio from each worm were obtained before and after treatment (0 and 3 h), and then expressed as a relative value (mean ratio after treatment / mean ratio before treatment = relative muscle potential).

**Data analysis**

Mean lethal concentration ($LC_{50}$) and 95% confidence interval were calculated using the method described by Well (Well 1952). Mean inhibitory concentrations ($IC_{50}$) were calculated using a computer program (phrmcalc.bas).

In all cases, including figures, parametric data were expressed as means ± SEM and analyzed by ANOVA. The conservative $F$ value was used to establish significance for the treatment effect. Then the least significant difference test was used to determine significance of each concentration level. In picrotoxin and ivermectin antagonism experiments, data were analyzed using the SAS Proc General Linear Means (GLM) procedure. Significance of interaction between ivermectin and picrotoxin was
established using two-way factorial analysis. Simple effect comparisons were then used to evaluate significance of each concentration level of picrotoxin. $\chi^2$-test was used to evaluate significance of the non-parametric swimming pattern change. The significance level was set at $P < 0.05$.

**Results**

**Lethal effect**

Ivermectin concentrations of 560 nM or higher were lethal to worms (Fig. 3). LC$_{50}$ at 72 h post-ivermectin treatment was 560 nM (95% confidence interval: 440 – 720 nM). A characteristic of ivermectin’s lethal effect in *L. variegatus* was that the concentration range for 0 – 90% death was narrow, only representing a three-fold difference. It was also evident that onset of ivermectin-induced mortality in *L. variegatus* was rather delayed. At a concentration of 1000 nM and 1800 nM, only one and three out of ten treated worms, respectively, died within 24 h of exposure. Most worms (eight and six, respectively) died between 24 and 72 h after treatment. In another experiment, ten

![Figure 3. Lethal effect of ivermectin in *Lumbriculus variegatus*. Ten worms were treated in each concentration group.](image-url)
worms were treated with 2,400 nM (extrapolated 72 h LC90) ivermectin for 8 h, and then transferred to distilled water. All of them recovered.

**Sublethal effects**

Our subsequent experiments focused on ivermectin’s sublethal effects (0.03 nM – 300 nM). During the first hour after exposure to 300 nM ivermectin, worms became flaccidly paralyzed. The worms’ bodies became thinner and longer. Spontaneous movement was totally inhibited, as well as swimming, reversal and crawling. In worms exposed to lower concentrations of ivermectin, the effects were similar, but less extreme and more delayed in onset. In contrast to the ivermectin effects on slow locomotor movements, rapid escape reflexes appeared less affected by these exposures (see last section of results).

**Effect of ivermectin on locomotor behaviors controlled by non-giant-interneuron pathways**

**Swimming and reversal**

Ivermectin inhibited helical swimming and body reversal behaviors in a time- and concentration-dependent manner (Fig. 4). There were no observable behavioral changes within 10 min of exposure to 300 nM ivermectin. But within 60 min of ivermectin administration, swimming and reversal were abolished. Ivermectin at 30 nM also abolished swimming (within 3 h) but not reversal. The IC50 at 3 h for swimming and reversal were 1.1 nM and 16 nM, respectively.

**Swimming frequency and swimming pattern**

Normal swimming movements consist of a series of alternating left-handed and right-handed, rapid, and rhythmic waves of helical body bending. Each wave rapidly progresses from head to tail, thus propelling the worm forward (Drewes 1999a). The swimming frequency in untreated worms was remarkably consistent, 10.7 ± 0.1 Hz (n = 80). Ivermectin at 0.3 nM or higher significantly decreased the swimming frequency 3 h
Figure 4. Time- and concentration-dependent inhibition of ivermectin on helical swimming and body reversal behaviors in *Lumbriculus variegatus*. A: Effect of ivermectin on swimming during 8 h continuous treatment. B: Effect of ivermectin on reversal during 8 h treatment. C: Concentration-dependent inhibiting effects of ivermectin on swimming and reversal after 3 h treatment. Values are mean ± SEM (n = 10).
after treatment (Fig. 5). At a concentration of 30 nM, six out of 16 worms failed to swim. There were qualitative changes in the basic pattern of swimming in another three worms. In these worms, helical waves appeared to initiate from the middle of the body rather than from the anterior end. In the remaining seven worms, there was no qualitative change in swimming pattern, but the swimming frequency was further decreased (Fig. 5).

Picrotoxin, a Cl⁻ channel blocker, was used to assess its possible influence on ivermectin-induced decreases in swimming frequency and changes in swimming pattern. The worms were pretreated with picrotoxin (0, 1, 10, 100, 1000 or 10000 nM) for 60 min before ivermectin was added (vehicle only or 30 nM) for 30 min. Picrotoxin alone had no effect on swimming frequency (Fig. 6). Ivermectin decreased swimming frequency by 4% in picrotoxin 0 groups (the difference between the first dotted and open bars, P < 0.05). The differences were also significant at picrotoxin 1 and 10 nM groups, but not 100, 1000 and 10000 nM groups. However, when compared the differences of picrotoxin

![Figure 5: Inhibitory effect of ivermectin on swimming frequency in Lumbriculus variegatus. Six worms in ivermectin 30 nM group failed to swim, so ten out of 16 worms were used for calculation of swimming frequency. Values are means ± SEM (n = 16). * P < 0.05, compared to control group.](image-url)
Figure 6. Antagonism of picrotoxin on ivermectin-induced decrease in swimming frequency in *Lumbriculus variegatus*. A: Effect of picrotoxin. The worms were pretreated with picrotoxin for 60 min before ivermectin administration for 30 min. * P < 0.05, compared to vehicle only group at the same picrotoxin level. B: Effect of picrotoxin on ivermectin-induced decrease in swimming frequency. Data were derived from panel A (Bar heights are the differences between vehicle only and ivermectin groups at each picrotoxin concentration). * P < 0.05, compared to picrotoxin 0 level. Values are mean ± SEM (n = 20).
100, 1000 and 10000 nM groups to that of picrotoxin 0 groups, only 100 and 1000 nM groups were significantly different.

Picrotoxin also antagonized the ivermectin-induced changes in the qualitative pattern of swimming (Table 3). Picrotoxin alone did not cause significant changes. Ivermectin at 30 nM caused abnormal swimming pattern in six of 20 worms. Picrotoxin at 10 and 100 nM significantly reversed the effect of ivermectin. However, higher concentrations of picrotoxin (1000 and 10000 nM) did not significantly antagonize the pattern changes.

Table 3. Antagonism by picrotoxin of ivermectin-induced change of swimming pattern in *Lumbriculus variegatus*.

<table>
<thead>
<tr>
<th>Ivermectin</th>
<th>Picrotoxin (nM)</th>
<th>0</th>
<th>1</th>
<th>10</th>
<th>100</th>
<th>1,000</th>
<th>10,000</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vehicle</td>
<td></td>
<td>0/20</td>
<td>0/20</td>
<td>0/20</td>
<td>0/20</td>
<td>1/20</td>
<td>1/20</td>
</tr>
<tr>
<td>30 nM</td>
<td></td>
<td>6/20</td>
<td>3/20</td>
<td>0/20</td>
<td>1/20</td>
<td>2/20</td>
<td>7/20</td>
</tr>
</tbody>
</table>

The worms were pretreated with picrotoxin for 60 min before ivermectin administration for 30 min.

*a* number of worms out of 20 that showed abnormal swimming patterns

*b* P < 0.05, compared to the 30 nM ivermectin, 0 picrotoxin group

Crawling

Forward crawling movements consist of a series of rhythmic peristaltic waves of body contraction. Each wave begins at the anterior end and progresses toward the tail. In contrast to swimming frequency, there was a great variation in wave frequency and forward velocity of crawling. We attempted to reduce this variation by using only the most vigorous crawling episodes for each worm. We found that one way to minimize the variation in crawling frequency and speed was to stimulate the worms to crawl as fast as possible. To do this, we repetitively brushed the worms’ tails at a frequency of 3 – 4 strokes/second.

In untreated worms, the crawling frequency and crawling speed were $1.21 \pm 0.02$ Hz and $6.1 \pm 0.1$ mm/s, respectively (n = 127). Ivermectin decreased crawling frequency in
a time- and concentration-dependent manner (Fig. 7). Crawling was totally inhibited after 3 h exposure to 300 nM ivermectin. IC_{50} at 3 h for crawling frequency was 91 nM.

Picrotoxin antagonized the ivermectin-induced decrease in crawling frequency. The worms were pretreated with picrotoxin (0, 1, 3, 10, 30 or 100 µM) for 60 min before ivermectin co-exposure (vehicle only or 300 nM) for 30 min. Picrotoxin alone at all concentrations studied did not change crawling frequency (Fig. 8). Ivermectin at 300 nM significantly decreased crawling frequency by 47% in picrotoxin 0 group. Picrotoxin (3 and 10 µM) significantly antagonized the effect of ivermectin. However, picrotoxin at 1, 30, and 100 µM failed to do so (Fig. 8).

Ivermectin’s inhibitory effect on crawling speed was similar to its effect on crawling frequency (Fig. 9). IC_{50} for crawling speed at 3 h was 51 nM. Ivermectin-induced decrease in crawling speed was significantly reduced from 52% to 19% by 10 µM picrotoxin (Fig. 10).

**Effect of ivermectin on behavior controlled by giant-interneuron pathways**

The worms retained escape reflex function throughout the 3 h of treatment with 300 nM ivermectin. That is, while crawling on substrate, worms were capable of rapidly withdrawing head or tail in response to tactile stimulation. However, responses were different from those in normal worms in two ways. First, the escape response was not followed by any slower locomotor movements (swimming, reversal or crawling), which usually occurred immediately after escape responses in normal worms. Second, while normal worms had little difficulty in rapid withdrawing in response to repeated tactile stimulation, the escape withdrawal in treated worms was hardly noticeable after four or five repeated stimuli. When the worms were allowed to rest for 1 or 2 min, their escape reflex reinstated.

MGF and LGF spiking, recorded noninvasively, was used as an indicator of the function of giant-interneuron pathways. In untreated worms, conduction velocity of MGF and LGF was 8.8 ± 0.1 m/s and 6.4 ± 0.1 m/s (n = 60), respectively. Ivermectin up
Figure 7. Time- and concentration-dependent inhibition of ivermectin on crawling frequency in *Lumbriculus variegatus*. A: Effect of ivermectin on crawling frequency 3 h after treatment (n = 21). *P < 0.05, compared to controls. B: Effect of ivermectin on crawling frequency during 3 h of exposure (n = 10 - 21). *P < 0.05, compared to the vehicle only groups at the same time point. Values are mean ± SEM.
Figure 8. Antagonism of picrotoxin on ivermectin-induced decrease of crawling frequency in *Lumbriculus variegatus*. A: Effect of picrotoxin. The worms were pretreated with picrotoxin for 60 min before ivermectin administration for 30 min. *P < 0.05, compared to vehicle only group at the same picrotoxin level. B: Effect of picrotoxin on ivermectin-induced decrease of crawling frequency. Data were derived from panel A (Bar heights were the differences between vehicle only and ivermectin groups at each picrotoxin concentration). *P < 0.05, compared to picrotoxin 0 level. Values are mean ± SEM (n = 10).
Figure 9. Time- and concentration-dependent inhibition of ivermectin on crawling speed in *Lumbriculus variegatus*. A: Effect of ivermectin on crawling speed 3 h after treatment (n = 21). * P < 0.05, compared to controls. B: Effect of ivermectin on crawling speed during 3 h of exposure (n = 10 - 21). * P < 0.05, compared to the vehicle only groups at the same time point. Values are mean ± SEM.
Figure 10. Antagonism of picrotoxin on ivermectin-induced decrease of crawling speed in *Lumbriculus variegatus*. A: Effect of picrotoxin. The worms were pretreated with picrotoxin for 60 min before ivermectin administration for 30 min. * P < 0.05, compared to vehicle only groups at the same picrotoxin level. B: Effect of picrotoxin on ivermectin-induced decrease of crawling speed. Data were derived from panel A (Bar heights were the differences between vehicle only and ivermectin groups at each picrotoxin concentration). * P < 0.05, compared to picrotoxin 0 level. Values are mean ± SEM (n = 10).
300 nM did not change either MGF or LGF conduction velocity (Fig. 11A and 11B). There were also no noticeable changes related to the function of sensory inputs and motor output associated with the giant fiber pathways, i.e., no changes were observed in the sensitivity to tactile stimulation, or muscle potentials associated with multiple spiking in the MGF (Fig. 11C).

**Discussion**

Numerous environmental fate and effect studies on non-target organisms have been carried out in the development of ivermectin as an antiparasitic agent (Campbell 1989; Halley *et al.* 1993). Lethal level has been determined in some non-target organisms. Among them, *Daphnia magna* was most sensitive with 48 h LC$_{50}$ of 0.025 ppb ($= 0.03$ nM, Halley *et al.* 1993). Fish were less sensitive (48 h LC$_{50}$ for bluegill and rainbow trout were 4.8 ppb and 3.0 ppb, respectively), and earthworms (28 d LD$_{50}$ 315 ppm in soil) were relatively insensitive to ivermectin (Halley *et al.* 1993). Our results showed that *L. variegatus* was much less sensitive to ivermectin than *Daphnia magna* and fish (72 h LC$_{50}$ of 560 nM $\approx$ 490 ppb), but much more sensitive than earthworms. However, there were differences between the exposure method (aqueous solution vs. soil) and time scale (3 d vs. 28 d), which makes the comparison to earthworms less meaningful.

Although the most predominant effects shown in target organisms are reduced motor activities, there are few studies in the literature examining sublethal effects of ivermectin on non-target organisms. Among a few studies concerning sublethal effects of ivermectin on non-target organisms, most have examined development and reproduction in dung-dwelling insect populations (Halley *et al.* 1993). The present study was the first attempt to examine the effects of ivermectin on locomotor behaviors of a non-target invertebrate, *L. variegatus*.

We developed a set of methods to measure the locomotor behaviors in *L. variegatus*. These worms are benthic inhabitants in ponds, lakes, and marshes of North America and
Figure 11. Effects of ivermectin on giant-interneuron pathways in Lumbriculus variegatus. A and B: Effect of ivermectin on conduction velocity of medial and lateral giant fiber (MGF and LGF). C: Effect of ivermectin on muscle potentials associated with repetitive MGF spikes. Values are mean ± SEM (n = 10).
Europe. They freely crawl within submerged and decaying vegetation, such as rotting leaves and logs (Drewes and Fourtner 1989). When touched or threatened, these worms use a variety of locomotor responses to move to safety. Their responses are context specific. When the worm's tail is extended above the sediments, it responds to the tactile stimulation or shadow by a rapid withdrawal (escape response). On wet surfaces or in confined spaces under water, the worm crawls forward or backward when touched in tail or head regions, respectively. In open spaces under water, however, tail stimulation evokes helical swimming, while head stimulation evokes body reversal (Drewes 1999a). These locomotor behaviors are highly stereotyped, thus making them ideal for sublethal toxicological tests (see Materials and Methods). The presence of giant nerve fibers that mediate escape response (Drewes and Fourtner 1989; Drewes and Brinkhurst 1990) and the capability of noninvasive electrophysiological testing (Drewes 1984; Zoran and Drewes 1987; Rogge and Drewes 1993) provide us with additional advantages in accessing sublethal effects of neurotoxicants, such as ivermectin.

Our results showed that ivermectin impaired the worm's locomotor behaviors. The behavioral endpoints were much more sensitive than LC50. LC50 at 72 h was 560 nM, while 3 h IC50s for swimming, reversal, crawling speed and crawling frequency were 1.1, 16, 51 and 91 nM, respectively. At 0.3 nM (lower than LC50 by more than three orders of magnitude), ivermectin decreased swimming frequency significantly. The differences in sensitivity to ivermectin suggested that motor behaviors in *L. variegatus* are probably independent. Therefore, it is important to have multiple measurements to obtain an overall picture of the effect of ivermectin. Our results demonstrated that *L. variegatus* is a sensitive model for assessing sublethal locomotor behavioral effects of environmental toxicants on aquatic invertebrates.

It was noted that the onset of paralytic effect of ivermectin in *L. variegatus* was rather delayed. Within 10 min of exposure to 300 nM ivermectin, there was no observable behavioral change. It is in sharp contrast to some other neuroactive chemicals, e.g. 4-aminopyridine, cadmium chloride, carbofuran, chloroform and diazinon, which caused behavioral changes in *L. variegatus* almost immediately upon exposure (< 1 min) (Rogge and Drewes 1993). It was evident that the onset of ivermectin-induced mortality
in *L. variegatus* was also delayed. A possible explanation for the slow actions of ivermectin is the availability of the chemical to the worms. Although ivermectin is lipophilic, it is a relatively large molecule (MW ≈ 870) compared to 4-aminopyridine, cadmium chloride, carbofuran, chloroform and diazinon (MW: 94, 183, 221, 119 and 304, respectively). Therefore, it might be difficult for ivermectin to penetrate the worm’s cuticle. Nevertheless, it is also likely that some of the biological processes involved in ivermectin’s paralytic and lethal effects are delayed or slowly developing. These processes are still elusive.

Although the mode of action of ivermectin is not fully understood, many studies have demonstrated that Cl⁻ channels are involved in its antiparasitic effects (Arena *et al.* 1995; Brownlee *et al.* 1997; Duce & Scott 1985; Kass *et al.* 1980; Martin *et al.* 1997). In the present study we used picrotoxin, a Cl⁻ channel blocker, to antagonize ivermectin’s effects on locomotor behaviors. Our results were consistent with the involvement of Cl⁻ channels. However, the antagonism of picrotoxin to ivermectin was only effective in a limited range of concentration (Fig. 6, 8, 10 and Table 2) and time scales. In our preliminary experiments, picrotoxin failed to reverse ivermectin-induced effects on swimming and crawling if the exposure to ivermectin lasted 3 h. A possible explanation is that picrotoxin at high concentrations has some additional effects mediated by action sites other than Cl⁻ channels. Ivermectin (≤ 300 nM) had no effects on MGF-evoked muscle potentials (Fig. 8C), but picrotoxin at 100 µM alone decreased the muscle potentials (unpublished data). Picrotoxin (5 mM) potentiates contraction while inhibiting voltage-dependent tubular Ca²⁺ current in frog skeletal muscle fibers (Jacquemond *et al.* 1996); it (10 µM) decreases the intensity of methylation of phospholipids (phosphatidylethanolamine) in rat olfactory cortex (Gerasimova *et al.* 1993). Another possibility is that ivermectin has action sites other than Cl⁻ channels (Ellis *et al.* 1987; Sani and Vaid 1988; Ahern *et al.* 1999), thus a Cl⁻ blocker can only reduce but not abolish the effect of ivermectin.

Ivermectin-sensitive Cl⁻ channels are present in nerve and/or muscle cells in many invertebrates, such as nematodes, insects, crustaceans, and mollusks (Cleland 1996). Although their physiological role has not been fully determined, they are reported to be
involved in generating rhythmic firing of the neurons within the crustacean stomatogastric ganglion (Cleland and Selverston, 1995, 1998). Our results showed that ivermectin decreased swimming frequency and crawling frequency, suggesting that ivermectin-sensitive Cl\(^-\) channels are involved in the neuropathways that control swimming and crawling.

It was interesting to observe that the escape reflex behavior controlled by giant interneuron pathways was still intact even after treatment with 300 nM ivermectin. Electrophysiological studies confirmed that ivermectin had no effects on the conduction velocity of MGF or LGF, or the muscle potentials evoked during multiple firing of MGF action potentials, suggesting that ivermectin-sensitive Cl\(^-\) channels are not crucially involved in the escape reflex functions of giant interneurons. This is not surprising if one takes a closer look at the giant interneuron system. The MGF and LGF pathways are derived from the electrically connected large axons of interneurons in each segment. They function as a syncytium, rapidly conducting nerve action potentials, without interruption, along their length (Drewes 1999b). In such a straightforward system, the main emphasis seems to be speed and reliability; negative feedback via inhibition may be unnecessary or inconsequential. On the other hand, some locomotor behaviors controlled by non-giant interneuron pathways (swimming and crawling) are slower, rhythmic, and probably subject to modulatory influence. Specific networks of neurons in an animal’s central nervous system, which control coordinated (and often rhythmic) pattern of movements, are termed central pattern generators (Young 1989). Negative feedback and other modulatory controls are usually utilized in such networks.

It has been reported that ivermectin has inhibitory effects on the pharyngeal muscle through opening Cl\(^-\) channels in the parasitic nematode *Ascaris suum* (Adelsberger et al. 1997; Brownlee et al. 1997). In *Lubriculus*, muscle potentials are normally associated with repetitive MGF spikes, but our results showed no significant changes in the appearance of these potentials following ivermectin treatment. However, our observations were based on muscle activities recorded from the body surface. This method is indirect, and may not sufficiently sensitive to detect subtle changes in membrane potential of muscle fibers.
CONCLUSIONS

In conclusion, our results demonstrated that (1) sublethal behavioral effects were much more sensitive endpoints than was mortality in assessing ivermectin’s potential neurobiological and ecological impacts; (2) locomotor behaviors controlled by non-giant interneuron pathways were sensitive to ivermectin whereas those controlled by giant interneurons did not appear to be affected at the concentrations studied; and (3) Cl⁻ channels appeared to be involved in ivermectin’s inhibitory effects.
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