In Vitro Studies on the Relative Sensitivity to Ivermectin of Necator americanus and Ancylostoma ceylanicum

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Abstract—Richards J. C., Behnke J. M. & Duce I. R. 1995. In vitro studies on the relative sensitivity to ivermectin of Necator americanus and Ancylostoma ceylanicum. International Journal for Parasitology 25: 1185–1191. Experiments were carried out to compare the sensitivity of Ancylostoma ceylanicum and Necator americanus to ivermectin (IVM) and pyrantel in vitro. Loss of motility and inhibition of ingestion by IVM were compared and A. ceylanicum was found to be approximately 40–50 times more sensitive to IVM than N. americanus. Both species showed a similar sensitivity to pyrantel. Uptake of \(^{3}H\)JIVM across the cuticle was compared and shown to be unlikely to account for the differences in sensitivity to IVM between the two species.

Key words: Ancylostoma ceylanicum; Necator americanus; hookworm; anthelmintic; ivermectin; pyrantel.

INTRODUCTION

There is much interest in the potential of ivermectin (IVM) as a microfilaricide for the treatment of onchocerciasis (Bennett, Williams & Dave, 1988), and mass trials against onchocerciasis are being implemented in 12 African and Central American countries. IVM has proven to be a very effective antiparasitic drug and many of the common gastrointestinal nematodes are highly susceptible to IVM at concentrations much lower than those at which other anthelmintics are effective. However, some parasites, including Heligmosomoides polygyrus, Trichuris trichiura, and hookworms (Wahid, Behnke & Conway, 1989; Whitworth, Morgan, Maude, McNicholas & Taylor, 1991) have been found to be tolerant to IVM.

In vivo studies on hookworm infections in hamsters revealed that Ancylostoma ceylanicum was 300 times more sensitive to IVM than Necator americanus (Behnke, Rose & Garside, 1993). Previous work (Rajasekeriah, Deb, Dhage & Rose, 1989) also indicated that in a hamster model, Necator was relatively insensitive to IVM.

The difference in IVM sensitivity is of interest in two respects. First, the drug may be used clinically in situations where N. americanus and Ancylostoma duodenale are both present in the population and its use may influence epidemiology. Second, if the differential sensitivity of these 2 species reflects a difference in the drug target site, these species of hookworm may provide a good model system to probe further the molecular action of the drug.

In this paper we report in vitro experiments which show that the difference in sensitivity of N. americanus and A. ceylanicum to IVM can be demonstrated in vitro and is therefore not attributable to the host–parasite relationship. We further show that the differential responsiveness cannot be explained by differences in the uptake of the drug.

MATERIALS AND METHODS

Parasites and hosts. Infective larvae of N. americanus were obtained from Dr Rajasekeriah of CIBA-GEIGY.
Hindustan Ltd, Bombay, India in 1983 and maintained by regular passage through hamsters as described by Sen (1972) and Behnke, Wells & Brown (1986). *A. ceylanicum* was also obtained from Dr Rajasekariah and passaged through adult hamsters as described by Garside & Behnke (1989). Worms of both species were recovered from the host (*N. americanus* 5 weeks post-infection, *A. ceylanicum* 14–21 days post-infection), following terminal anaesthesia in chloroform, by cutting open the small intestine and picking out the worms into dishes of warm Hanks's saline (pH 7.0). After several washes with Hanks's saline, approximately 10 μl/ml of penicillin–streptomycin (10,000 IU/ml, 10,000 μg/ml, respectively) were added and the worms were maintained in Hanks's saline at 37°C. For incubations of 24 h, worms were incubated in sterile conditions in Roswell Park Memorial Institute (RPMI 1640 Medium) (GIBCO), containing 10 μ/l/ml penicillin–streptomycin, 10 μ/l/ml L-glutamine, 1 μ/l/ml sodium pyruvate and 0.2 μl/ml monothioglycerol (pH 7.0).

**Chemicals.** Pyrantel (Pfizer) was taken from a stock solution of 10 mg/ml pyrantel pamoate in DMSO. Ivomectin (IVM), ivermectin phosphate (IVM-PO4) and [3H]ivermectin ([3H]IVM) were obtained from Merck and Co. (Rahway, U.S.A.). IVM and IVM-PO4 were taken from stock solutions of 1 and 2.5 mg/ml, respectively, in DMSO and the [3H]IVM was from a stock with a specific activity of 2.09 MBq/μg; [3H]inulin (specific activity 3.33 MBq/g) was purchased from ICN Biochemicals. All drugs were administered in a final DMSO concentration of 1% v/v.

**Toxicity of IVM and pyrantel.** Groups of 10 worms (mixed sexes) were incubated at 37-38°C in 1 ml of 1% DMSO Hanks's saline containing IVM, IVM-PO4 or pyrantel at a range of concentrations. Preliminary experiments showed that male and female worms of both species were similarly affected by all three anthelmintics under these conditions. Although in the control groups 100% of the worms remained active, some loss of motility was observed after 3 h incubation in Hanks's and the level of motility rapidly declined after periods of 6 h or longer. Sterile RPMI 1640 was therefore used as the medium for incubations >3 h as the control groups of worms incubated in RPMI retained much higher levels of motility after 6–24 h than in Hanks's saline. The activity of the worms at various times (1–24 h) was compared with controls incubated in 1% DMSO in Hanks's saline or RPMI 1640. Worms were classed as either active or inactive, and inactive worms were defined as those which showed no motility and did not respond to a mechanical stimulus (gently prodding and lifting the worms with fine watchmaker's forceps).

**Uptake of [3H]IVM.** Fine wire ligatures (resin-coated copper wire, approximately 100 μm diameter) were tied as near to the head and tail of each worm as possible. Damage to the cuticle by the ligatures was assessed microscopically, and visibly damaged worms were discarded. The worms were incubated in groups of 10 (mixed sexes) for 3 h at 37–38°C in 1 ml of incubation medium comprising Hanks's saline/1% DMSO; 11.44 μM IVM; 0.0333 MBq [3H]IVM. Each worm was washed 3 times in Hanks's saline and then placed on filter paper to remove excess moisture. As the [3H]IVM was found to be adsorbed by the wire ligatures, the ends (0.5 mm) of each worm (both ligatured and unligatured) were removed. After being left to dry overnight on pieces of foil, individual worms were placed in scintillation vials. The worms were solubilized in 100 μl of 2.5 M NaOH for 1–2 h. HCl, 100 μl 2.5 M, was then added, followed by 4 ml of scintillation fluid (Packard “Emulsifier Safe” liquid scintillation cocktail for aqueous samples). Radioactivity was measured on a Packard Liquid Scintillation Spectrometer. Results were corrected for quenching and expressed as d.p.m./worm. A number of individual worms were incubated without the radioisotope and then analyzed as above to provide background levels; the d.p.m./worm values given are corrected for background.

**Uptake of [3H]inulin.** As IVM is a highly lipophilic molecule, it readily crosses cellular barriers. Inulin, on the other hand, does not pass across cell membranes, and is not readily taken up by cells. [3H]Inulin uptake was therefore used to assess the amount of material taken up from the surrounding medium through ingestion. Ligatured and unligatured worms (mixed sexes) were incubated in 1 ml of Hanks's saline containing 1% DMSO; 0.0333 MBq [3H]inulin for 3 h then analyzed as above.

**Inhibition of [3H]inulin uptake.** Groups of 10 worms (mixed sexes) were incubated for 24 h in sterile conditions in either 1 ml of RPMI 1640 or HLaC (Hanks's saline containing 5 μg/ml lactalbumin, 0.2 M HEPES, 10 μ/l/ml penicillin–streptomycin, 0.3 mg/ml kanamycin, pH 7.0) and various concentrations of IVM-PO4 or pyrantel, then analyzed as above.

**Statistical analysis of results.** Non-parametric statistical procedures were used to analyse the data throughout because a normal distribution of data could not be assumed. The results shown in Figs 1a, b and Fig. 2 are presented as mean % of active worms ± standard error (S.E.M.). For each different concentration of drug, the mean number of active worms given is derived from the results of 3–6 experiments in which groups of 10 worms were incubated for 3 h and the number of active worms assessed after 1, 2 and 3 h. The results of the radioisotope uptake experiments are arranged into four groups: (i) uptake of [3H]IVM, (ii) effect of ligatures on uptake of [3H]inulin, (iii) and (iv) effects of IVM and pyrantel on uptake of [3H]inulin. Mean d.p.m. values given for each experimental group are derived from the pooled results from the analysis of individual worms. In order to avoid Type I errors arising from multiple comparisons within experiments, a maximum of two a priori hypotheses were examined within each of the different experimental groups. A non-parametric form of the ANOVA test (analysis of variance by ranks) was used throughout the analysis. A two-way ANOVA (Meddis, 1984) was employed to assess the results of the uptake of [3H]IVM experiments (Experiment 1) specifying species (*A. ceylanicum vs. N. americanus*) and treatment (ligatured vs. non-ligatured) as the two factors. The following hypotheses were predicted: non-ligatured > ligatured and *A. ceylanicums > N. americanus.* A one-way ANOVA was used to test the hypothesis: ligatured < non-ligatured (both species) for the results of the ligature/uptake of [3H]inulin experiments (Experiment 2). Similarly, in order to test the hypothesis that IVM and pyrantel inhibited the
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1.187

Fig. 1. Comparison of the effects of IVM (Fig. 1a) and IVM-PO4 (Fig. 1b) on % active A. ceylanicum and N. americanus. Worms were classed as either active or inactive (see Methods) and the data are presented as the mean % (± S.E.M.) of worms showing activity after 1, 2 and 3 h incubation in IVM or IVM-PO4 (n = 30–60 worms). IVM-PO4 was shown to immobilize N. americanus dose-dependently (Spearman rank rs = 0.898, n = 18, P < 0.0001, t = 3 h). Similarly IVM and IVM-PO4 both caused inactivity in A. ceylanicum dose-dependently (Spearman rank r, = -0.80, n = 55, P < 0.0001, t = 3 h; r, = -0.986, n = 13, P < 0.0001, t = 3 h, respectively).

uptake of [3H]inulin by A. ceylanicum and N. americanus (Experiments 3 and 4), one-way ANOVA was used to test the prediction that control > IVM/pyrantel treated for each species. For all a priori hypotheses examined, the test statistic z is given as appropriate. Probabilities (P) of 0.05 or less were considered significant.

RESULTS

Comparison of the effects of IVM and pyrantel on the activity of A. ceylanicum and N. americanus

Over a period of 3 h, A. ceylanicum was found to be equally sensitive to IVM and IVM-PO4 and was immobilized by IVM or IVM-PO4 dose-dependently with an approximate EC50 at 3 h of 1.14 µM, 2.0 µM (IVM, IVM-PO4, respectively, EC50s derived from Figs 1a, b).

N. americanus was not visibly affected by the standard therapeutic drug IVM at the range of concentrations used (0.11–11.44 µM) over a period of 3 h. IVM did not remain in solution above this concentration range for >8 h. However, experiments with the more soluble phosphate form of IVM (IVM-PO4) were carried out and IVM-PO4 was found to paralyse N. americanus dose-dependently, with an approximate EC50 of 55 µM after 3 h (Figs 1a, b).

Even after incubation periods of 24–48 h, N. americanus was not visibly affected by IVM-PO4 at concentrations of 11.44 µM compared with controls incubated in normal media. However, this concentration caused >90% inactivity in A. ceylanicum after 3 h.

Pyrantel was found to be similarly effective at paralysing A. ceylanicum and N. americanus dose-dependently, with approximate EC50s of 1.34 and 3.5 µM (A. ceylanicum and N. americanus, respectively) (t = 3 h) (Fig. 2).

Uptake of [3H]IVM by A. ceylanicum and N. americanus

There was no significant effect of ligaturing the worms on the uptake of IVM; however, there was a significant difference between the species with A. ceylanicum taking up more [3H]IVM than N. americanus (Table 1a). There was no difference in the uptake by male and female worms.

Uptake of [3H]inulin by A. ceylanicum and N. americanus

The ligatures had a significant effect on the uptake of [3H]inulin by both A. ceylanicum and N. americanus (ratio non-ligatured vs. ligatured 7:1, 9:1, respectively, Table 1b), proving the effectiveness of the ligatures in preventing the entry of media via anatomical openings (Ho, Geary, Barshun, Sims & Thompson, 1992). N. americanus was more active in
Table 1a—The uptake of [3H]IVM by A. ceylanicum, N. americanus with and without ligatures

<table>
<thead>
<tr>
<th>Species</th>
<th>Ligatured Mean ± S.E.M. (n)</th>
<th>Non-ligatured Mean ± S.E.M. (n)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A. ceylanicum</td>
<td>126 ± 11.7 (33)</td>
<td>111 ± 11.0 (53)</td>
</tr>
<tr>
<td>N. americanus</td>
<td>78 ± 9.1 (39)</td>
<td>73 ± 17.2 (28)</td>
</tr>
</tbody>
</table>

Statistical analysis for predictions

\[
\text{A. ceylanicum} < \text{N. americanus (both species)}
\]

\[
\begin{array}{lll}
\text{z} & \text{P} & \\
3.9 & 0.0008 & -0.88 > 0.05
\end{array}
\]

For statistical analysis see text. d.p.m. = disintegrations per min.

Table 1b—the uptake of [3H]inulin by A. ceylanicum, N. americanus with and without ligatures

<table>
<thead>
<tr>
<th>Species</th>
<th>Ligatured Mean ± S.E.M. (n)</th>
<th>Non-ligatured Mean ± S.E.M. (n)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A. ceylanicum</td>
<td>1.05 ± 2.86 (22)</td>
<td>7.17 ± 2.96 (21)</td>
</tr>
<tr>
<td>N. americanus</td>
<td>5.52 ± 3.16 (23)</td>
<td>51.2 ± 7.4 (51)</td>
</tr>
</tbody>
</table>

Statistical analysis for predictions

\[
\text{Ligated} < \text{Non-ligated}
\]

\[
\begin{array}{lll}
\text{N. americanus} & \text{z} & \text{P} \\
4.74 & < 0.01 & \\
A. ceylanicum & 1.86 & < 0.05
\end{array}
\]

For statistical analysis see text. d.p.m. = disintegrations per min.

Comparison of the effect of IVM-PO4 on the uptake of [3H]inulin by A. ceylanicum and N. americanus

Although N. americanus fed more actively than A. ceylanicum over a period of 24 h (RPMI & HLac medium, Tables 2a, b) a significant inhibition of oral uptake of [3H]inulin by IVM-PO4 was demonstrated for both species (Tables 2a, b).

There was a highly significant difference between the two species with inulin uptake by A. ceylanicum being inhibited by much lower concentrations of IVM-PO4 than N. americanus (approximate EC50s 4 and 0.1 μM, N. americanus and A. ceylanicum, respectively, Tables 2a, b, Fig. 3).

Pyrantel was found to inhibit the uptake of [3H]inulin by N. americanus, A. ceylanicum dose-dependently (Tables 2c, d, Fig. 3), with both species showing similar sensitivity (EC50s approximately 0.6 and 0.4 μM, N. americanus and A. ceylanicum respectively).

DISCUSSION

The results reported in this paper provide the first direct in vitro comparisons of the sensitivities of two species of hookworm from the important genera Ancylostoma and Necator to IVM. Previous studies (Behnke et al., 1993) showed that in the hamster host, A. ceylanicum was on average 300 times more sensitive to IVM than N. americanus. Studies by Rajasekeriah et al. (1989) also reported the relative insensitivity of N. americanus to IVM in the hamster model. The results of the above in vitro experiments in which the effects of IVM and pyrantel on motility were assessed support the results of these previous in vivo experiments (Behnke et al., 1993; Rajasekeriah et al., 1989) as A. ceylanicum and N. americanus were shown to have similar sensitivities to pyrantel, whereas A. ceylanicum was found to be approximately 50 times more sensitive to IVM (t = 3 h) than N. americanus. The difference in responsiveness to IVM does not appear to be related to response time as concentrations of IVM-PO4 that immobilized A.
Sensitivity of hookworms to ivermectin in vitro

Table 2a—The effect of IVM-P0₄ on the uptake of [³H]inulin by N. americanus (t = 24 h)

<table>
<thead>
<tr>
<th>IVM (µM)</th>
<th>Mean d.p.m. ± S.E.M.</th>
<th>No. worms (n)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>279 ± 17</td>
<td>48</td>
</tr>
<tr>
<td>0.57</td>
<td>242 ± 21</td>
<td>15</td>
</tr>
<tr>
<td>1.43</td>
<td>146 ± 19</td>
<td>14</td>
</tr>
<tr>
<td>2.86</td>
<td>153 ± 16</td>
<td>16</td>
</tr>
<tr>
<td>5.72</td>
<td>73 ± 21</td>
<td>6</td>
</tr>
</tbody>
</table>

Statistical analysis for prediction
Control > 0.57 µM > 1.43 µM > 2.86 µM > 5.72 µM

z
5.72 < 0.0003

For statistical analysis see text. d.p.m. = disintegrations per min.

Table 2b—The effect of IVM-P0₄ on the uptake of [³H]inulin by A. ceylanicum (t = 24 h)

<table>
<thead>
<tr>
<th>IVM (nM)</th>
<th>Mean d.p.m. ± S.E.M.</th>
<th>No. worms (n)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>92 ± 9</td>
<td>26</td>
</tr>
<tr>
<td>28.6</td>
<td>60 ± 11</td>
<td>7</td>
</tr>
<tr>
<td>143</td>
<td>53 ± 8</td>
<td>14</td>
</tr>
<tr>
<td>286</td>
<td>60 ± 7</td>
<td>23</td>
</tr>
</tbody>
</table>

Statistical analysis for prediction
Control > 78 nM > 143 nM > 286 nM

z
2.59 < 0.0045

For statistical analysis see text. d.p.m. = disintegrations per min.

ceylanicum in 3 h (1.14 µm, 50% worms immobilized) had no detectable effect on N. americanus over 24 h.

The experiments reported here used concentrations ≥ 1 µM, which have been previously criticized as being unrepresentatively high compared to effective in vivo concentrations (Geary, Klein, Vanover, Bowman & Thompson, 1992; Geary, Sims, Thomas, Vanover, Davis, Winterrowd, Klein, Ho & Thompson, 1993). The difference in sensitivity between the two species in vitro is also relatively lower than the difference in sensitivity shown in vivo (A. ceylanicum 40–50 times more sensitive than N. americanus in vitro, compared to a 200–300-fold difference in vivo).

When comparing these in vitro experiments with experiments carried out in vivo, it is important to take into account a number of factors. For example, the in vitro experiments required a dose of IVM that had an acute physiological effect, resulting in a single endpoint (e.g. loss of motility) being demonstrated within the limited timescale of a few hours. In comparison, in vivo it is likely that a given drug affects worm viability by inhibiting or interfering with a range of processes in the parasite, e.g. inhibition of egg production, feeding, or movement.

In vivo experiments with IVM also tend to involve much longer periods of exposure to the drug. For example, Campbell & Benz (1984) found that the anthelmintic efficacy of IVM in cattle persisted for 2 weeks after treatment. Similarly, the microfilaricidal activity of IVM was shown to persist in rodents for at least 30 days after a single dose (Zahner, Sanger, Lammier & Muller, 1987).

Using a micromotility meter, Geary et al. (1993) demonstrated that the motility of Haemonchus contortus was affected by concentrations of IVM ≥ 10⁻⁸ M, much lower than the concentrations of IVM at which loss of motility could be determined by observation (10⁻⁶ M). It is likely that IVM affects motility in A. ceylanicum and possibly N. americanus at much lower concentrations than those at which the end-point was determined in these experiments. Nevertheless, in these acute in vitro experiments both motility and ingestion showed a significantly greater sensitivity to IVM in A. ceylanicum than in N. americanus.

The cuticle of nematodes can act as an important permeability barrier and it is possible that the differences in sensitivity to IVM between the two
species may reflect differences in the ability of the drug to cross the cuticle. This possibility was investigated by measuring the uptake of \[^{3}H\]IVM in worms which were ligatured to prevent the entry of material via anatomical openings (Ho et al., 1992). The results of our experiments suggest two things; first, the rate of IVM uptake across the cuticle is higher for \textit{A. ceylanicum} than \textit{N. americanus}, although the difference in uptake (resulting in an approximate internal IVM concentration \(1.6 \times\) greater) is unlikely to be large enough to account for the differences in sensitivity (50-fold) between the two species. Second, as ligatured worms of both species took up considerable amounts of the radio-labelled ivermectin, the results also suggest that the cuticle is an important route of IVM uptake in hookworms. This is supported by studies in which the rate of absorption of drugs across the cuticle was postulated to depend on their lipophilicity (Thompson, Ho, Sims & Geary, 1993; Ho, Geary, Raub, Barshun & Thompson, 1990).

Geary et al. (1993) showed that at lower IVM concentrations than those at which motility is affected, IVM caused the paralysis of pharyngeal muscles in \textit{H. contortus}, thus reducing the ingestion of erythrocytes. These authors proposed that this action of IVM may be important in the ability of IVM to control \textit{H. contortus}.

We carried out experiments to establish if IVM caused reduction of ingestion of \[^{3}H\]inulin in \textit{N. americanus} and \textit{A. ceylanicum} and also to determine if there was any difference in the sensitivity to IVM of the ingestion process between the two species. Our results indicate that IVM-PO\(_4\) causes a dose-dependent inhibition of ingestion in both \textit{N. americanus} and \textit{A. ceylanicum} and that the ingestion process in \textit{A. ceylanicum} is approximately 40 times more sensitive to IVM than in \textit{N. americanus} (EC\(_{50}\) approx. 0.1 \(\mu\)M, 4 \(\mu\)M, respectively, \(t = 24\) h). In contrast pyrantel inhibited ingestion in both species at the same concentration. The effect of IVM on ingestion has an EC\(_{50}\) approximately 1 order of magnitude less than for loss of motility. This implies some process involved in ingestion; possibly the action of the pharyngeal muscle is differentially affected by IVM.

It has been determined that the primary physiological effect of ivermectin is an increase in permeability of cell membranes to chloride ions (Turner & Schaeffer, 1989). Specific high affinity avermectin binding sites have been identified and characterized in \textit{Caenorhabditis elegans} (Schaeffer & Haines, 1989; Cully & Paress, 1991; Cully, Vassilatis, Liu, Paress, Van Der Ploeg, Schaeffer & Arena, 1994) and a clear correlation has been shown between the binding affinity of a series of avermectin analogues for \textit{C. elegans} membranes and in vivo efficacy, suggesting that the binding site is physiologically important (Rohrer, Meinke, Hayes, Mrozik & Schaeffer, 1992).

It is therefore possible that the differential sensitivity to ivermectin observed between \textit{A. ceylanicum} and \textit{N. americanus} may reflect differences in chloride channels or chloride channel binding sites between the two species. This intriguing possibility is currently under investigation in our laboratory using electrophysiological techniques.

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**REFERENCES**


of the cuticle of *Ascaris suum*. Molecular and Biochemical Parasitology 41: 153–166.


