An Overview of Anthelmintic Drugs in Ascaris suum Intestine

Katie Tharaldson

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Part 1

Abstract

In part 1 of this paper, I will discuss *Ascaris suum* and *Ascaris lumbricoides*. *Ascaris suum* acts as a model organism for *Ascaris lumbricoides*, a parasitic nematode that impacts roughly 1.2 billion people worldwide (de Silva *et al.* 2003). I will then go into the anthelmintic drugs currently being used to treat these infections, as well as the receptors they act on. In part 2 of this paper, I will discuss the research I did with levamisole, an anthelmintic drug, on nicotinic acetylcholine receptors (nAChRs) in the intestine of *Ascaris suum*. There were 5 control worms, as well as 5 levamisole treated worms. In the past, the nAChRs have been studied predominantly on the muscle in all nematode species. However, in previous work in the lab, although unpublished, there was promising expression of nAChRs in *Ascaris suum* intestine. After running PCR amplification to confirm expression of the putative subunits that make up the levamisole receptor, one of the three subtypes of a nAChRs, the samples were separated on a 1% Agarose gel. It was found that the subunits were present in the intestine of *Ascaris suum*.

Background

*Ascaris* is a parasitic nematode worm that infects both humans and pigs. *Ascaris lumbricoides* is the nematode that infects humans, where *Ascaris suum* is the nematode that infects pigs. It is not only a health problem, but economic as well. When humans are infected, they are at risk for a variety of symptoms, and even death. The problems are coming from not only the lack of vaccinations available for parasitic nematodes, but the lack of sanitary accommodations for people in third world countries. The problem goes even further when dealing with the plants and animals that parasitic nematodes infect. As an economic standpoint, the farmers lose potential income from the animals they lose to the parasitic nematodes. They also lose the food they would’ve had from the animals. With this drastic and widespread occurrence, it is a problem that is larger than most realize. Based on a study from (Fausto *et al.* 2015) it was shown that of the total number of pigs slaughtered during a year and a half time period that were infected with *Ascaris suum* was 9.75%. This gives reason to believe that the devastating impacts of these infections are being drastically overlooked, and undermanaged.

Life cycle

*Ascaris* live in the lumen of the small intestine and produce eggs which are released from the body inside feces. However, the eggs are only infective if they are fertilized. If a fertilized egg is swallowed the larvae will hatch and invade the intestinal mucosa. They are then released into the lungs following systemic circulation. The larvae then mature further in the lungs penetrating the alveolar walls. They then ascend the bronchial tree to the trachea, into the pharynx where they are swallowed. When they reach the small intestine, they develop into adult worms.
Symptoms

*Ascaris* can cause different symptoms depending on where they are in their life cycle. If they are present in lungs they can cause a variety of symptoms including coughing, shortness of breath, aspirating pneumonia, blood in mucus, and chest discomfort. If they are present in the intestine, they can cause different symptoms including nausea, vomiting, diarrhea, intestinal blockage, loss of appetite, abdominal pain, weight loss, and growth impairment in children due to malabsorption. *Ascaris* also causes damage in the liver caused by its larval migration (Fausto et al. 2015). The damage is identified by “milk spots” or patches of white on the surface.

Anatomy

As shown in Figure 1, *Ascaris* is a pinkish cylindrical worm ranging in size from about 15 to 35 centimeters. The male is a shorter, slenderer worm. There are two lateral lines on the body of the worm. These are present in a darker pink color on opposite sides of the body. Surrounding the inside of the cuticle are muscle bags, with the gut in the very center of the worm.

The nervous system of nematodes contains about 300 neurons (Wolstenholme 2011). However, the neurochemistry is more complex. They have receptors and neurotransmitter combinations that aren’t present in mammals, making them great targets for anthelmintics. However, the neurochemistry differs between nematodes, which makes a more universal drug harder to develop.

Figure 1

Diagram of the cross section of *A. suum* caudal to the pharyngeal muscle, showing the relative position of the intestine, muscle bags, lateral lines, and nerve cords. Drawn in reference to figure 2 from (Martin 1997).
History of research

There are many reasons for using Ascaris suum as the model organism for Ascaris lumbricoides. The main reason is simply because there isn’t a prevalence of Ascaris lumbricoides in the United States, and therefore, is much easier to obtain Ascaris suum than Ascaris lumbricoides (de Silva et al. 2003).

For the time being, chemotherapy is a strategic tool for the treatment of parasites. They act fast, are cheap and easy to administer (Clarke et al. 2017) Most drugs that are currently being used target the nervous system of the nematode. The reason for this is because the nervous system makes up 30% of their cells (Wolstenholme 2011). The nematode will need to be continuously moving to stay in the digestive tract while the animal is digesting food. Therefore, paralysis of the nematode will prevent them from staying in the digestive system. To test the anthelmintic, the locomotion of the worm is observed.

Most drugs target the ion channels located on nerves and muscles within the nematode. However, benzimidazoles are one of the drugs that do not target either nerves or muscles. They instead target b-tubulin.

Overview of anthelmintic drugs that act on nAChRs

Anthelmintic drugs such as levamisole, butamisole, pyrantel, etc. are used to treat infections caused by parasitic worms. There are no effective vaccines so anthelmintic drugs will be used until one develops or hygiene standards have been improved to the point where they are no longer needed. This leads to heavy reliance of anthelmintic drugs. Resistance is due to a variety of things such as misuse of the drug and receptor modification. Anthelmintic drugs that have the same mode of action as drugs that have resistance against them will most likely also be ineffective.

Studying the mode of action of anthelmintics will help to understand why parasites develop resistance to the drug. Anthelmintics have two major modes of action, including drugs that act on parasite membrane ion-channels and drugs that act on parasitic biochemical target sites. The drugs that act on parasitic membrane ion-channels have a faster effect than target sites other than ion-channels. This paper will focus more on the ion-channel target sites: namely excitatory nicotinic acetylcholine receptors on the muscle of nematodes. There are more and more companies trying to develop selective drugs that act on nAChRs (Jones and Sattelle 2004).

Nematodes have acetylcholine-, 5-HT-, dopamine-, and tyramine-gated chloride channels, where mammals do not have any of these. This means that there will be less side effects in mammals when using drugs to target these receptors. If the mammal has the same receptor there is a possibility for the drug to have interactions with the mammal receptor as well.

Structure of nAChR

Acetylcholine receptors (AChRs) as shown in Figure 2, are ligand-gated neurotransmitter receptors that consist of two subtypes: the metabotropic muscarinic receptors and the ionotrophic nicotinic receptors. Both types are activated by the endogenous neurotransmitter...
acetylcholine (Albuquerque et al. 2009). The ionotropic cationic nicotinic receptors are faster, with only four transmembrane domains, where the muscarinic receptors are G coupled proteins that are slower with seven transmembrane domains. The nicotinic acetylcholine receptors along with GluCls and GABA receptors, are called ‘cys loop’ transmembrane ligand-gated ion-channels. This is because they have a region of amino acids that are cross-linked by cysteine residues, forming a small loop. The nAChRs are important in survival of Ascaris because they mediate synaptic transmission at the neuromuscular junction and in the nervous system.

This receptor is made up of five homomeric or heteromeric subunits. A homomeric nAChR consists of five α subunits. A heteromeric nAChR consists of at least two α-, and three or less non-α subunits. Table 1 shows the α and non-α subunits of a putative levamisole receptor. These five subunits surround a central pore. Each subunit has a range of 437-501 amino acids that make it up (Martin, Robertson and Bjorn 1997). Unc-38, unc-29, unc-63, acr-8, are putative subunits of the levamisole receptor. Although we are unaware of what subunits make up a functional receptor for Ascaris suum, there are studies suggesting that an increase in receptors without ACR-8 are associated with levamisole sensitivity (Wolstenholme 2011). This means that ACR-8 might be the subunit that controls the sensitivity of the receptor to levamisole.

There is an extracellular N-terminal region, an intracellular loop, a transmembrane region, and a cytoplasmic loop. The second transmembrane region (TM2) of each subunit is what makes up the pore of the channel. The negatively charged amino acids in the TM2 region are what make the conductance of the channel as well as the cation selectivity (Martin et al. 2005). Sites for kinase activity are on the intracellular loop, suggesting that the intracellular loop is involved in the regulation of the channel opening.

The channel of the receptor opens upon binding of a ligand to the extracellular loop of the receptor. When the channel opens, ions can pass through. The ligand binding sites are composed of 6 loops of amino acids. Three of the loops are from the α subunit, and three are from the non-α subunit. If the cytoplasmic loop has two adjacent cysteines, the subunit is an α subunit (Martin et al. 2005). With each receptor having at least two α subunits, there are at least two ligand binding sites on the receptor as well.

There have been three subtypes of nAChRs identified in Ascaris suum. The subtypes are formed from different combinations of α and non-α subunits. Therefore, they each have different conductance, calcium permeability, and pharmacology. The names of the receptors are based upon their sensitivity toward certain compounds. The ‘N’ subtype is based on their sensitivity toward nicotine, the ‘L’ subtype is based on levamisole, and the ‘B’ subtype is based on bephenium. When taking the ratio of the subtypes ‘N’ and ‘L’ it was shown that these receptors have different sensitivities to agonists and antagonists (Martin et al. 2005). Since nAChRs are so diverse with the presence of multiple subtypes, they are still the targets of new anthelmintic drugs such as derquantel, which acts on the ‘B’ subtype the most.
Figure 2
A diagram of functional nAChRs from (Jones and Sattelle 2004)

Table 1
A summary of the putative subunits of *Ascaris suum*, based off the table from (Holden-Dye et al. 2013)

<table>
<thead>
<tr>
<th>Subunit</th>
<th>α or non-α</th>
<th>Expression</th>
</tr>
</thead>
<tbody>
<tr>
<td>ACR-8</td>
<td>α</td>
<td>All body wall muscle, head and tail neurons, anal and vulval muscles, nerve ring, nerve cord, ventral nerve cord motoneurons</td>
</tr>
<tr>
<td>UNC-38</td>
<td>α</td>
<td>Body wall and vulval muscle, nervous system</td>
</tr>
<tr>
<td>UNC-63</td>
<td>α</td>
<td>Body wall and vulval muscle, ventral cord motoneurons</td>
</tr>
<tr>
<td>UNC-29</td>
<td>non-α</td>
<td>Body wall and head muscles, nervous system, ventral and dorsal cords, nerve ring</td>
</tr>
</tbody>
</table>

Mode of action for nicotinic agonists
Levamisole, pyrantel and morantel along with acetylcholine was shown in the experiments of (Martin 1982) to result in depolarization and an increase in input conductance of the muscle membrane to sodium and potassium. Levamisole opens ligand-gated, nematode AChRs that are non-selective cation-channels (Martin et al. 2005). The mode of action of levamisole is to mimic acetylcholine and act as an agonist of the post-synaptic nicotinic acetylcholine receptors located on nematode somatic muscle (Martin et al. 2005). This results
in spastic paralysis from the selective depolarization of the muscle cell, causing the nematode to be expelled from the body (Wolstenholme 2011).

The nicotinic receptor that the anthelmintic drug is targeting may also be present in the host. In this case, the drug may interact with both the host nicotinic receptor and the nematode nicotinic receptor, causing even more issues for the host. Therefore, it is imperative that the host nicotinic receptor differ from the nematode nicotinic receptor. Anthelmintic drugs with a mode of action that acts on a nicotinic receptor only found in the nematode are usually favored because they do not pose the risk of having the same effects on the host. If the drug were to stop the function of a human nicotinic acetylcholine receptor as well as the one of the nematode you would see profound effects including over-stimulation such as muscle spasms, and eventual muscle weakness and paralysis. According to (Robertson and Martin 1993) nicotinic anthelmintics open non-selective cation channels. Since conductance differs from channel to channel, it is possible to make sure the nicotinic anthelmintics are acting on receptors differing from the host.

Phosphorylation of the receptors can change the receptor properties, making them more or less likely to open in response to ligand binding (Martin et al. 2005).

Other targets for anthelmintics

Potassium ion channels are another target for anthelmintic drugs. There are no voltage-gated sodium channels that have been detected do far in nematodes. Therefore, potassium channels are the targets for new drugs. There are already pharmaceuticals that are blocking human potassium channels so the next step is to target nematode potassium channels. Nematodes action potentials are also calcium-dependent. If you were to inhibit the voltage-gated ion channels of the nematode, it would cause deficits to the neuronal signaling of the nematode. This would cause death of the parasite.

Glutamate-gated chloride channels (GluCls) are a part of the ‘cys loop’ ligand-gated ion channel family. GluCls are inhibitory ion channels, which are the targets of the macrocyclic lactones, a group of anthelmintics. They open and close in response to ligand binding. They are expressed on pharyngeal muscle and motor neurons, among other cells (Wolstenholme 2011). Inhibitory GABA-gated chloride channels are another target for anthelmintics. They increase the opening of the muscle membrane chloride channels, hyperpolarizing the membrane potential, resulting in spastic paralysis of the nematode (Martin, 1997).

Benzimidazoles

Benzimidazole derivatives, such as albendazole and mebendazole, act on b-tubulin in nematodes. Mebendazole act on the pharyngeal and intestinal cells of Ascaris suum, causing them to lose their cytoplasmic microtubules (Martin et al. 1997). This causes an inability to take up glucose, resulting in the death of the worm. Microtubules have many functions in animals and plants, including cell migration and neuronal pathfinding (Lüders 2016). They are made of amino acid proteins α-tubulin and β-tubulin. To form the microtubule, the tubulin must be polymerized at the positive pole and depolymerized at the negative pole. Microtubule formation can be prevented by depolarizing substances at the positive pole. This is done by benzimidazoles binding selectively to the β-tubulin molecules of nematodes. These drugs are
slower acting than those that target the neuromuscular junction, because of the slow starvation of the nematode and the inhibition of egg production.

### Macrocyclic lactones

Macrocyclic lactones are the largest selling class of drugs in veterinary medicine. Macrocyclic lactones such as ivermectin and avermectins which act on glutamate-gated chloride channels and increase the Cl- permeability of nerve and muscle membranes of *Ascaris suum*. They bind nematode GluCl with extremely high affinity. When they are bound, the drug will prevent the α-helixes from rotating, which will hold the channel in the open state (Wolstenholme 2011). The channel is thought to be a pentamer like that of nAChR. However, the center is lined with positively charged sites, therefore making the channel slightly permeable to anions such as Cl-, hyperpolarizing the cell. The pentamer is thought to be composed of GluCl2-α subunits that contain the glutamate binding site and GluCl-beta subunit that contains the ivermectin binding site (Martin et al. 1997). When the cell is hyperpolarized it is no longer excitabile, which impacts not only locomotion, but also feeding.

It was shown in (Martin et al. 1996) that the glutamate receptor gates the chloride channels. These are then increased in potential by the ivermectin analogs. Ivermectin can directly activate some GluCls very slowly, however, it is irreversible. Ivermectin does not bind to a normal agonist binding site.

Macrocyclic lactones are limited to two phyla including Nematode and Arthropoda. This is crucial because the drugs will not target the receptors of the host when getting rid of the nematode because GluCls are usually not present in the genes of other species (Geary & Moreno, 2010). Macrocyclic lactones are hydrophobic and dissolve in the plasma membrane of the cell.

The studies on macrocyclic lactones show that the concentrations that are necessary to paralyze or inhibit the movement of the nematode are higher *in vitro* than *in vivo*. It is hypothesized that the activation of GluCls inhibit protein secretion, causing the parasite to be more susceptible to an attack from the innate immune system, and therefore have a lower concentration amount for the *in vitro* studies (Wolstenholme 2011).

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**Table 2**

A summary of widely used anthelmintics drugs and their targets based off table 1 from (Holden-Dye et al. 2013)

<table>
<thead>
<tr>
<th>Drug</th>
<th>Target</th>
</tr>
</thead>
<tbody>
<tr>
<td>Imidazothizoles (levamisole)</td>
<td>Nicotinic acetylcholine receptors</td>
</tr>
<tr>
<td>Tetrahydropyrimidine derivatives (pyrantel, oxantel, morantel)</td>
<td>Nicotinic acetylcholine receptors</td>
</tr>
<tr>
<td>Macrocyclic lactones (ivermectin)</td>
<td>Glutamate-gated chloride channels</td>
</tr>
<tr>
<td>Benzimidazoles</td>
<td>β-tubulin</td>
</tr>
</tbody>
</table>
Part 2

Effects of levamisole on A. suum gut

In previous research the main target is to observe the effects that levamisole has on muscle cells within Ascaris Suum. However, the effects of levamisole on the gut are not very well known. In this section, the paper is exploring the effect of levamisole on the intestines of Ascaris Suum in comparison to a control.

Levamisole is a nicotinic anthelmintic drug. This drug will act as an agonist for nicotinic acetylcholine receptors on the muscle cells of Ascaris. It is more selective than the other drugs. You can give it as an injection so it will be distributed better. With pyrantel it is toxic if injected so it must be given orally, and is therefore limited to the gut and will not get rid of worms in the lungs.

Methods and Materials

Five adult female Ascaris suum worms were obtained from JBS Swift and Co. processing plant in Marshalltown, Iowa. From there, the worms were brought to Iowa State University to research the effects of levamisole on Ascaris suum intestine. The worms were maintained in Ascaris Ringers Solution at 32°C for 24 hours. The worms were then dissected by cutting three, two centimeter regions of the worm, two to three centimeters caudal to the head. One region was further dissected into intestine and muscle sections, and stored at -80°C for further PCR experiments. The other two sections were incubated at 37°C for six hours in a 12-well culture plate. One was in two mL RPMI 1640 medium as a control, and the other was in two mL RPMI 1640 medium treated with 30μM levamisole. After the six hours of incubation, the intestinal tissue was dissected from each section and snap frozen into liquid nitrogen, and then stored at -80°C.

In preparation for cDNA synthesis, each worm was homogenized separately in 1 ml of Trizol reagent using mortar and pestle, followed by phase separation by adding 200μl of Chloroform and removing the aqueous phase. Following phase separation was RNA extraction, where 500μl of Isopropanol was added and centrifuged. The supernatant was removed and the pellet was resuspended in 1 ml of 75% Ethanol, and centrifuged. Once again, the supernatant was removed and the pellet was resuspended in nuclease-free water, and quantified for total RNA using Nanodrop. Subsequently, one microgram (1μg) of total RNA from each sample was treated with DNase I for removal of residual genomic DNA and subsequently reverse transcribed to cDNA using qScript Flex cDNA Synthesis kit.

PCR amplification was performed to confirm the expression of each subunit with the use of primers targeting the TM1-TM4 regions. This region is highly conserved among nAChR subunits at the amino acid level, but less conserved at the DNA nucleotide level, thus eliminating overlaps and increasing the specificity among subunits. The PCR products were then separated on a 1% Agarose ethidium bromide gel, followed by visualization and sequencing to confirm the identity of the subunits.

After PCR amplification was performed, quantitative real-time PCR was conducted to compare the expression levels of each subunit in control versus 30μM treated Ascaris suum
intestinal samples. Primer sets were designed to amplify fragments of approximately 150-170 bp between the TM3 and TM4 region. The subunits unc-38, unc-29, unc-63, acr-8 and Gapdh were serially diluted for the generation of a standard curve for further data analysis. Target and reference (Gapdh) genes were amplified from each cDNA sample in triplicate, by qPCR. The relative quantification of target genes was estimated using the Pfaffl Method, followed by Paired T-Test to determine the significant difference.

**Data Analysis**

As shown in Figure 3, the section of each worm that was treated with levamisole had a lower relative expression in subunits `Asu-unc-38` and `Asu-acr-8`. However, in subunits `Asu-unc-63` and `Asu-unc-29` this was not the case.

![Graphical results of experiments done in Dr. Martin's lab.](image)

**Figure 3**

Graphical results of experiments done in Dr. Martin’s lab.

**Results**

After running a T-Test to find the statistical significance, the data of my experiments show that there was no statistical significant difference between the control and levamisole treated sections of each worm for subunits `Asu-unc-38`, `Asu-acr-8`, `Asu-unc-63`, and `Asu-unc-29`. Although there wasn’t a statistical difference, the sections of the worms treated with 30μM of levamisole had a lower relative expression. Therefore, in future studies we could increase the concentration of levamisole being used to treat the worms to 100μM, or even lower the concentration to 10μM, to see if this would increase the difference between the control and treated. There is no evidence to suggest this would work, however, it is something that could be tested out.
References


