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How do the macrocyclic lactones kill filarial nematode larvae?

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Abstract

The macrocyclic lactones (MLs) are one of the few classes of drug used in the control of the human filarial infections, onchocerciasis and lymphatic filariasis, and the only one used to prevent heartworm disease in dogs and cats. Despite their importance in preventing filarial diseases, the way in which the MLs work against these parasites is unclear. In vitro measurements of nematode motility have revealed a large discrepancy between the maximum plasma concentrations achieved after drug administration and the amounts required to paralyze worms. Recent evidence has shed new light on the likely functions of the ML target, glutamate-gated chloride channels, in filarial nematodes and supports the hypothesis that the rapid clearance of microfilariae that follows treatment involves the host immune system.

Keywords

Ivermectin; moxidectin; lymphatic filariasis; onchocerciasis; heartworm; glutamate-gated chloride channel

Introduction

The filarial nematodes form an important group of human and veterinary parasites. Diseases caused by infection with these parasites include lymphatic filariasis (LF) and onchocerciasis of humans, and dirofilariasis (heartworm) in dogs and cats. The medical and economic impact of these infections is enormous (Table 1) and place a severe constraint on the development of affected communities. Filarial nematodes have an indirect life-cycle and use blood-feeding arthropods such as mosquitoes and blackflies as intermediate hosts and vectors. The biting insects take up the microfilariae (Mf), which either circulate in the blood (in the case of mosquito vectors) or are present in the skin (in the case of the biting flies), and these then develop into infective L3 larvae within the vector. When another blood meal is taken, the L3 are deposited on the skin of the host and enter through the bite wound before continuing their slow development towards adulthood, which takes several months. Intriguingly, the Mf of many mosquito-transmitted filarial parasites exhibit a nocturnal

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periodicity and are found in greater numbers in the bloodstream during the times of day when the host is most likely to be bitten by a competent vector (Hayasaki et al. 2003; Lammie et al. 1994). Their location when they are not present in the circulation has been proposed to be in the lungs (Hawking, 1967).

The macrocyclic lactone family of anthelmintic drugs (MLs) include ivermectin, the only member currently approved for use in humans, selamectin, moxidectin and milbemycin oxime. All of these are used to control diseases caused by filarial nematodes. In humans ivermectin plays a vital role in the control and elimination programmes for onchocerciasis and lymphatic filariasis, and all four of the compounds are components of various heartworm disease prevention products (Nolan and Lok 2012). In the African Programme for Onchocerciasis Control, annual dosing of at-risk populations with 150 to 200 µg/kg body weight ivermectin is achieved via community-directed treatments (Seketeli et al. 2002). The Global Programme to Eliminate Lymphatic Filariasis uses ivermectin in its mass drug administration strategy to stop the spread of infection. In this case a combination of 150 µg/kg of body weight ivermectin + 400 mg albendazole is used in areas that are also endemic for onchocerciasis (Molyneux and Zagaria 2002). The importance of ivermectin to global human health is immense and was recently recognized with the shared award of the 2015 Nobel Prize for Physiology & Medicine to William Campbell and Satoshi Ōmura. In order to prevent heartworm disease in dogs and cats the American Heartworm Society recommends year-round administration of chemoprophylactic drugs, all of which belong to the ML classes. They also recommend use of a macrocyclic lactone, together with doxycycline, prior to treatment with melarsomine for heartworm disease in infected dogs if a patent infection has developed (Anonymous 2005).

Anthelmintic effects of macrocyclic lactones in filarial infections

In the cases of LF and onchocerciasis, the ivermectin treatments rapidly reduce the number of microfilariae in circulation (LF) or in the skin (onchocerciasis), thus preventing any transmission to a subsequent insect vector that bites the treated person (Ottesen 2006). In addition, they cause a long-term sterility of the adult female worms, which suppresses the Mf population for several months. There is little information on their effects on the L3 and L4 larvae of the human parasites. By contrast, in dogs and cats the MLs prevent the development of infecting *Dirofilaria immitis* L3 to adulthood, and hence the establishment of a patent infection and presentation of clinical disease (Bowman and Atkins 2009). Most heartworm preventative formulations are intended to be given monthly, and their effect is to remove any developing L3 and L4 that have infected the host in the preceding periods. There is also an injectable formulation of moxidectin that provides protection for 6 months. Higher doses do have a microfilaricidal effect on *D. immitis*, similar to that seen in the human infections, and are sometimes used as an alternative to the use of melarsomine in treating heartworm disease – the so-called ‘slow kill’ approach. However, at the doses normally used for both human and animal filarial nematode infections the main action of the MLs is against larval forms of the parasites with limited effects on the adult worms other than long-term sterility. Certain of the MLs, such as moxidectin, may prove to have better activity against the adult stages of both human and animal parasites than ivermectin, but this is still under investigation (Awadzi et al. 2014; Tompkins et al. 2010; Chandrashekar et al. 2014).

At the molecular level, the MLs act as unconventional agonists or allosteric modulators at a variety of vertebrate and invertebrate ion channels, including glycine receptors (Shan 2001), some nicotinic acetylcholine receptors (Krause 1998) and P2X receptors (Silberberg et al. 2007). In nematodes it is widely accepted that the glutamate-gated chloride channels (GluCl_s) (Wolstenholme 2012) are the most important and biologically relevant target (Wolstenholme and Rogers 2005). This is based on multiple lines of evidence, including direct activation of recombinant GluCl expressed in *Xenopus* oocytes (Cully et al. 1994; Vassilatis et al. 1997; Horoszok et al. 2001; McCavera et al. 2009; Yates and Wolstenholme 2004; Dent et al. 1997), high-affinity binding to those receptors expressed in cell lines (Cheeseman et al. 2001), and drug resistance phenotypes in *Caenorhabditis elegans* strains with mutations and variations in the genes encoding the channels (Dent et al. 2000; Ghosh et al. 2012). This association was strengthened by the correlation between the expression patterns of the GluCl_s in *C. elegans* (Dent et al. 1997; 2000; Laughton et al. 1997; Pemberton et al. 2001) and *Haemonchus contortus* (Delany et al. 1998; Portillo et al. 2003) with the effects of ivermectin on the worms. These effects, which include paralysis and the inhibition of pharyngeal pumping (Avery 1990; Geary et al. 1993; Brownlee et al. 1997; J. A. Dent et al. 2000), are found on many nematode species and are readily explained by the expression of GluCl_s on motor neurons, interneurons and pharyngeal muscle cells (Adelsberger et al. 1997; Martin 1996; Kass et al. 1980). Direct proof of the binding of ivermectin with a recombinant GluCl was revealed by X-ray crystallography (Hibbs and Gouaux 2011); the binding site is within the plasma membrane, at the interface between two adjacent subunits. The affinity of radiolabelled ivermectin for recombinant *H. contortus* GluCl (25–70pM) is similar to that for *H. contortus* membrane fractions (~70pM) (Cheeseman et al. 2001), and the channels of at least one *H. contortus* GluCl are opened by drug concentrations as low as 100pM (McCavera et al. 2009). These concentrations are comparable to the peak plasma concentrations reported following administration of ivermectin to sheep (McKellar and Gokbulut, 2012). Even in *H. contortus*, however, the IC₅₀ values for ivermectin against larvae in in vitro assays can vary widely; those for the larval development assay compare well with those from binding and electrophysiology measurements, but the larval migration inhibition assay, which examines effects on motility, gives much higher IC₅₀ values. For example, Raza et al. (2015) found an IC₅₀ for ivermectin against a susceptible isolate of ~200pM in a larval development assay but ~1.3μM in a larval migration assay. At those higher concentrations other nematode ligand-gated chloride channels are also affected by the drugs, including those gated by GABA and dopamine (Holden-Dye and Walker, 1990; Accardi et al. 2012; Beech et al. 2010a; Rao et al. 2009), and it is possible that these channels affect performance in the migration assay in addition to, or instead of, the GluCl_s. It is worth emphasizing that for gastro-intestinal nematodes it is the adult worms rather than the larvae that are targeted by the drug and there is considerable evidence that inhibition of feeding, via paralysis of the pharyngeal muscle, may be the most important mechanism of action at this stage (Geary et al. 1993; Sherriff et al. 2005; Kotze et al. 2012).

In filarial species there have been few reported studies on GluCl_s or any ligand-gated chloride channel. Given the importance of the macrocyclic lactones in the control of heartworm and human filariases, together with the emergence of resistance to the drugs in *D.*

immitis (Bourguinat et al. 2015; Wolstenholme et al. 2015; Pulaski 2014; Geary et al. 2011) and reports of reduced drug efficacy in some *Onchocerca volvulus* patients (Osei-Atweneboana et al. 2011) it is essential that we understand how they work against these parasites. Bioinformatic analysis indicates that filarial species have lost many genes encoding ligand-gated ion channels, including many of the GluCl-encoding genes, when compared to other nematodes (Williamson et al. 2007; Beech and Neveu 2015). An ivermectin-sensitive GluCl, called Dim-AVR-14B according to the nomenclature proposed by Beech et al (2010b), has been reported from *D. immitis* (Yates and Wolstenholme 2004). In this case the application of 1 μ M ivermectin to the homomeric channel expressed in *Xenopus* oocytes caused a slow opening of the channels, but only very limited functional and pharmacological data could be obtained. For *Brugia malayi*, immunolocalization and in situ hybridization methods have been used to map expression of the Bma-AVR-14 subunits to the excretory/secretory pore of the Mf (Moreno et al. 2010) and to the reproductive tissues of adult male and female worms (Li et al. 2014).

Is motility a good read-out for ML action against filarial nematodes?

Motility is often used as a read-out of nematode health and of the effect of anthelmintics, whether via direct observation or by one of many systems which aim to automate, at least partially, its measurement (Storey et al. 2014; Marcellino et al. 2012; Smout et al. 2010; Buckingham et al. 2014; Buckingham and Sattelle 2008, 2009; Calahorro et al. 2015). Motility has the obvious advantage as a measurement of nematode health that it is quick and easy to obtain quantitative data and to generate dose-response curves. Motility-related assays, such as those that measure migration, usually of larvae, through a mesh have also been developed and used to determine the resistance status of parasite populations (Demeler et al. 2010; Kotze et al. 2006; McArthur et al. 2015; Wagland et al. 1992). The larval migration inhibition assay has been applied to infectious L3 of *D. immitis* and used to derive IC₅₀ values for ivermectin and eprinomectin (Evans et al. 2013). Similar measurements of adult and Mf thrashing have been made on *Brugia* spp. and *D. immitis* using Wormassay, the Worminator or direct observation (Marcellino et al. 2012; Storey et al. 2014; Bourguinat et al. 2011). In all cases however the concentrations of the drugs required for an obvious phenotypic effect in vitro were 2–3 orders of magnitude higher than the peak drug concentrations found in plasma (Table 2). Similar differences are also noted for the other MLs, moxidectin and doramectin, though it is worth noting that the data are less complete for these drugs. It is worth emphasizing that in vivo the drugs are essentially 100% effective, indeed this is required for them to be marketed as a heartworm preventative (Hampshire 2005), but the in vitro values shown are the concentrations at which they cause 50% inhibition of motility or migration. For comparison, in sheep the peak plasma concentrations following oral administration of 0.2 mg/kg ivermectin are in the range of 20–30 nM (summarized in McKellar and Gokbulut, 2012) whereas the IC₅₀ against susceptible isolates of *Haemonchus contortus* can be as low as 0.2 nM in some in vitro assays, though not those based on motility. The large discrepancy between the apparent concentrations of ivermectin that are effective in vivo and in vitro suggests that the anti-filarial activity of the MLs, especially the clearance of circulating and skin Mf observed in human infections, is not mediated via paralysis. The same is true for their ability to block the development of *D.*

immitis L3 and L4, which requires even lower doses than the clearance of Mf from circulation (Bowman and Atkins 2009; Bowman and Mannella 2011) (Table 2). This is consistent with the lack of reported expression of GluCl_s (assuming these are the relevant targets) in the motor nervous system of *B. malayi* (Li et al. 2014; Moreno et al. 2010) and suggests that paralysis may result from the drugs affecting other targets, possibly neuromuscular GABA-gated channels (Holden-Dye and Walker 1990; Blackhall et al. 2003). These results imply that Mf are not removed from the circulation or from the skin of treated animals and people by simple paralysis, and that other mechanisms may be involved. Filarial Mf do not feed, so inhibition of pharyngeal pumping cannot be the explanation in this case. In vitro application of 100nM ivermectin, which is similar to peak plasma concentrations recorded in humans (Table 2), to Mf caused a reduction in protein secretion which is consistent with expression of GluCl around the excretory/secretory pore (Moreno et al. 2010). It has long been known that ivermectin causes a long term reduction, of up to 6 months, in Mf load in humans (Cupp et al. 1986; Ottesen et al. 1990; Schulzkey et al. 1986). However, we do not understand how this long-term sterility is achieved, as the half-life of the drug following administration to humans is ~16–36 hrs (Baraka et al. 1996; Canga et al. 2008; Elkassaby 1991), nor do we understand how the Mf are removed so rapidly from circulation.

Is the host immune system involved?

This discrepancy between the concentrations of MLs required to exert an anthelmintic effect in vitro and in vivo has suggested that a host component, presumably the immune system, is required for full activity. Previous studies on other filarial nematodes, *Acanthocheilonema viteae* and *Litomosoides carinii*, have suggested that killing of Mf by host peripheral blood mononuclear cells (PBMC) or granulocytes *in vitro* is enhanced by ivermectin (Rao et al. 1987; Zahner et al. 1997). We have shown that the *in vitro* serum-dependent adherence of PBMCs and neutrophils from naïve dogs to *D. immitis* Mf is increased by incubation in 10nM and 100nM ivermectin respectively (Vatta et al. 2014) (Figure 1), concentrations that are comparable to those found in plasma following a microfilaricidal dose of the drug (Table 2). El-Sadr et al. (1983) showed that canine neutrophils and PBMCs would adhere to, and the neutrophils could kill, *D. immitis* Mf with the help of IgM from occultly infected animal serum. Parasitic nematodes, including the filaria, secrete a cocktail of immunomodulatory proteins, metabolites and microRNAs which have significant effects on the host immune system (Ashour 2013; Harnett 2014; Harnett and Harnett 2006; Hoerauf et al. 2005; McSorley et al. 2013; Pineda et al. 2014; Wang et al. 2014; Buck et al. 2014), so it is an obvious speculation that they act to inhibit granulocyte and PBMC binding and antibody-dependent killing of Mf in the infected animal. Another important drug used in the LF elimination programme, diethylcarbamazine (DEC), is believed to exert its anthelmintic effects via activation of the host immune system (Maizels and Denham 1992; Vickery et al. 1985), which is consistent with its limited direct effects on nematodes in vitro (Alves et al. 2005; Court et al. 1986; Gunawardena et al. 2005). Perhaps the simplest hypothesis, given the effects of IVM on protein secretion by Mf (Moreno et al. 2010), is that the MLs inhibit release of immunomodulatory signals by the parasites and thus allow the innate immune system to recognize and remove them. Ivermectin treatment does cause neutrophil activation

and increases in C-reactive protein and IL-6 in onchocerciasis patients and although this was associated with adverse effects of the treatment (Njoo et al. 1993; 1994), it could be an important part of the drug's mode of action. Nematodes release immunomodulatory molecules from a variety of places other than the excretory/secretory pore, including the gut and uterus (Chehayeb et al. 2014; Buck et al. 2014), however neither of these structures are present in Mf, and the ES pore is likely to be the main, if not only, site of release from this particular stage of the parasite life cycle. The presence of GluCl associated with the pore suggests that secretion may be under neuronal control or regulation. In *C. elegans* components of the ES system pass close to the nerve ring and are likely to receive synaptic inputs (Nelson et al. 1983). In *C. elegans* and *H. contortus* the structure has been reported to rhythmically pulsate in a manner similar to pharyngeal pumping (Nelson and Riddle 1984; Wharton and Sommerville 1984) and if this process is conserved in filaria it is conceivable that activation of ES-associated GluCl by ivermectin inhibits this process and thus secretion from the worm. There are 38 classes of glutamatergic neuron in *C. elegans* (Serrano-Saiz et al. 2013) and 11 of these are present in the nerve ring and so could potentially synapse onto the ES cells, including 7 classes of sensory neuron, 3 classes of interneuron and the RIM motoneurons. Again, if aspects of this arrangement are conserved in filarial Mf then it is tempting to speculate that excretion/secretion could be regulated by sensory inputs and this regulation would also be subverted by the drugs. Uterine fluid secretion from adult worms (Chehayeb et al. 2014) might also be inhibited by MLs acting on GluCls present on reproductive tissue and this could also potentially dampen the immune modulation mediated by adult parasites, and hence contribute to Mf clearance.

Conclusion

It has been stated (Wolstenholme and Rogers 2005) that “The known expression patterns of the GluCl explain most of the observed biological effects of treatment with the macrocyclic lactones”. The statement may still hold but at the time it was made this assertion really only applied to *C. elegans* and one or two gastro-intestinal parasites of livestock, notably *H. contortus*, as nothing was known about the expression or function of GluCls in filarial species. More recent data have indicated that the GluCl are present in the excretory/secretory pore of Mf and the reproductive tissue of adult filaria (Moreno et al. 2010; Li et al. 2014). The former finding is consistent with the long-standing but still developing belief that the drug enhances the immune response against the parasites, perhaps by inhibiting the secretion of immunomodulatory molecules, with the latter location providing a partial explanation for the long-term sterilization caused by ivermectin. There is obviously a lot more to discover about the functions of the GluCl in the filarial nematodes and how the macrocyclic lactones exert their anthelmintic activities. For the moment however, it is clear that these are not via paralysis and this may sound a warning about an over-reliance on this single phenotype in drug discovery.

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Figure 1.

Pharmacologically relevant concentrations of ivermectin promote peripheral blood mononuclear cell (PBMC) and polymorphonuclear leukocyte (PMN) attachment to *D. immitis* Mf. A). Proportion of *D. immitis* Mf with at least 1 PBMC attached after 40 hrs incubation in ivermectin. The bars represent the average values ($\pm$ SE) for 4 assays. B). Proportion of Mf with at least 1 PMN attached after 16 hrs incubation in ivermectin. The bars represent the average values ($\pm$ SE) for 4 assays. The greater variability seen with the PMN experiments compared to those with PBMC is most likely due to the greater viability of the latter cell population in culture. C). A representative image of a Mf incubated in 100nM ivermectin together with PBMC isolated from an uninfected dog. The preparation was stained with Modified Wright's Stain. D). The image shows an example of a Mf with multiple PMN attached after incubation in 100nM ivermectin, stained with Modified Wright's Stain. Reprinted from Veterinary Parasitology, Vol 206, Vatta, A.F. et al., Ivermectin-dependent attachment of neutrophils and peripheral blood mononuclear cells to *Dirofilaria immitis* microfilariae in vitro, Copyright (2014), with permission from Elsevier.

Table 1

Diseases caused by filarial parasite infections

	Lymphatic Filariasis	Onchocerciasis (River Blindness)	Loiasis	Heartworm
Causative Agent(s)	<i>Wuchereria bancrofti</i> ; <i>Brugia malayi</i> ; <i>Brugia timoni</i>	<i>Onchocerca volvulus</i>	<i>Loa loa</i>	<i>Dirofilaria immitis</i>
Definitive Host	Human	Human	Human	Dogs and other canids
Intermediate Host	Mosquito	Blackfly (<i>Simulium</i> spp)	Deerfly (<i>Chrysops</i> spp)	Mosquito
Adult Predilection Site	Lymph vessels	Subcutaneous nodules	Subcutaneous tissue	Pulmonary artery
Microfilariae found in	Blood (often with nocturnal periodicity) & Lymph.	Skin	Skin	Blood (with nocturnal periodicity).
Disease Symptoms	A broad range from no evident disease to lymphedema and/or severe disfigurement of the extremities and genitalia.	Intense itching. Nodules under the skin, and vision changes. Eye lesions after years of severe infections.	Usually asymptomatic. Itchy swellings known as Calabar swellings and eyeworm.	Mild persistent cough, reluctance to exercise, fatigue after moderate activity, decreased appetite, and weight loss.
Treatment of infection	Diethylcarbamazine (DEC), 6mg/kg/day for 12 days.	Ivermectin, every 6 months for the life span of the adult worms or for as long as evidence of skin or eye infection.	DEC or albendazole.	Melarsomine dihydrochloride (2.5 mg/kg IM) three times. ML for two months prior to melarsomine. Doxycycline (10mg/kg) twice daily for 4 wks prior to melarsomine.
MDA/Prevention of Infection	Albendazole + DEC or ivermectin annually	Ivermectin annually or every 6 months		Macrocytic lactone – usually monthly
Economic and Social Impact	4,832,426 DALYs	598,270 DALYs	11–12 million people infected	Heartworm prevention market = ~US \$6 billion

Table 2

In vitro and *in vivo* concentrations of macrocyclic lactones.

Parasite	Host	Target Stage	Drug	Dose Given	Peak plasma concentration	<i>In vitro</i> IC ₅₀ for motility	Ratio <i>in vitro</i> IC ₅₀ :peak plasma concentration
<i>Brugia malayi</i> [*]	Human	Mf	Ivermectin	150–200 µg/kg	~50nM (Canga et al. 2008)	6.1 ± 1.1 µM (Storey et al. 2014)	~120
<i>Dirofilaria immitis</i>	Dog	Mf	Ivermectin	200µg/kg	~150nM (Al-Azzam et al. 2007; Gokbulut et al. 2006)	28.2 ± 2.9 µM (Storey et al. 2014)	~190
<i>Dirofilaria immitis</i>	Dog	L3	Moxidectin [†]	200µg/kg (oral) 2.5mg/kg (transdermal)	~200nM (Lallemond et al. 2007) ~55nM (Bowman et al. 2016)	9.3 ± 2.6 µM (Storey et al. 2014)	~46.5 ~170
<i>Dirofilaria immitis</i>	Dog	?	Doramectin [†]	200µg/kg	~100nM (Gokbulut et al. 2006)	2.78 ± 1.6 µM (Storey et al. 2014)	~28
<i>Dirofilaria immitis</i>	Dog	L3	Ivermectin	6µg/kg	3.4nM (Daurio et al. 1992)	4.6 µM (95% CI = 1.3–16.4 µM) (Evans et al. 2013)	~1350

^{*} Ivermectin is not normally used for Mass Drug Administration in areas endemic for Brugian filariasis, but it is not possible to maintain *Wuchereria bancrofti* in the laboratory.

[†] For doramectin and moxidectin the IC₅₀ values are for Mf as no data are available for the L3 stage. Doramectin is not currently used as a heartworm disease preventative.