An examination of the effects of ivermectin on *Brugia malayi* adult worms

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Abstract

Brugia malayi is one of the causative agents of the disabling and disfiguring disease known as Lymphatic Filariasis (LF). This infection is a well-established ailment in tropical and subtropical countries and recently the drug ivermectin has been introduced for the LF control programs. Ivermectin (IVM) is an excellent microfilaricide, but is not markedly macrofilaricidal. However, it causes a long-lasting reduction in the production of new larvae by female worms, suggesting that adult stages are affected. However, the mechanism by which IVM produces such effect in the adult worm is not well understood. One major reason is our incomplete understanding about the biological effect of IVM on adult stages. The present study was carried out to examine the *in vitro* effects of IVM on *B. malayi* adult worms using *Brugia*-gerbil animal model. And also to have some leads in understanding the drug-uptake and location of probable targets in the worm body by using fluorescent labeled IVM and confocal microscopy.

The antifilarial effects of IVM were examined using three parameters: mf release by female worms, and motility, and viability in both male and female worms. The results reported in this study demonstrate that although IVM did not kill the adult worm, but showed significant antifilarial effects on *B. malayi* adult stages when examined in an *in vitro* system. Confocal microscopy images of the worms incubated in bodipy FITC-IVM showed strong specific localization signal in the anterior cephalic region of both male and female worms. These observations suggest the early / initial interactions of the drug with its probable receptors that could be located specifically in the head region.

Abrégé

Brugia malayi est un des agents étiologiques d'une maladie débilitante, la filariose lymphatique. Cette infection est une maladie bien connue des régions tropicales et subtropicales, et tout récemment un nouveau programme de traitement par ivermectine a été introduit. L'ivermectine (IVM) est un excellent microfilaricide mais son effet sur les macrofilaires n'est pas aussi démarqué. Néanmoins, IVM réduit la production de larves des femelles adultes de façon durable, ce qui suggère que les vers adultes sont aussi atteints. Toutefois, le mécanisme par lequel l'IVM agit chez le ver adulte n'est pas très bien compris. Une des raisons principales est que les effets biologiques de l'IVM sur les stades adultes du parasite restent encore inexpliqués. Cette étude avait pour but d'examiner les effets in vitro de l'IVM sur les vers adultes de *B. malayi* à l'aide du modèle animal *Brugia*-gerbille. Nous étions aussi intéressés d'élucider certains indices du mécanisme d'absorption du médicament et la localisation de cibles potentielles chez le ver adulte à l'aide d'IVM marquée par une sonde fluorescente et la microscopie confocale.

L'action filaricide d'IVM a été analysée selon trois paramètres : la production de microfilaires par les femelles, la motilité et la viabilité des vers adultes mâles et femelles. Les résultats ont démontré que même si l'IVM ne tue pas les vers adultes, elle démontre une action antifilarienne importante sur les vers *B. malayi* lorsqu'ils sont étudiés en système *in vitro*. Un test MTT a démontré que les effets d'IVM sur la motilité et la viabilité étaient plus prononcés chez les vers femelles que chez les mâles. L'imagerie de microscopie confocale de vers incubés avec Bodipy IVM-FITC a démontré un signal de localisation précis dans la région céphalique des vers adultes femelles et mâles. Ces

observations suggèrent que les interactions initiales du médicament avec des récepteurs potentiels pourraient être localisées notamment dans la région céphalique du parasite.

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Chapter I

Introduction

Brugia malayi is one of the causative agents of the disabling and disfiguring disease known as Lymphatic Filariasis (LF). This infection is a well-established ailment in tropical and subtropical countries and recently the drug ivermectin has been introduced for the LF control programs (Brown et al., 2000). Ivermectin (IVM) is an excellent microfilaricide, but is not markedly macrofilaricidal. However, it causes a long-lasting reduction in the production of new larvae by female worms (Schulz-Key, 1986; Albiez et al., 1988a, 1988b; Duke et al., 1991a, 1991b; Chavasse et al., 1993; Klager et al., 1993).

The majority of information pertaining to the use of IVM against human filarial worms has been obtained for *Onchocerca volvulus*, a related species. IVM has shown outstanding results in the control of onchocerciasis in African and American countries because of its microfilaricidal activity and the profound sterilization effects in adult female worms for a long period of time (six to twelve months). The effects are long-lasting, despite the fact that the half-life of IVM is only a few hours in humans (Bennett et al., 1988; Vande Waa, 1991; Awadzi et al., 1995, 1999; Gardon et al., 2002). This effect on reproduction is most evident following multiple doses of IVM. However, the mechanism by which IVM produces this effect in the adult worm is not well understood (Chavasse et al., 1992, 1993; Brown et al., 2000; Horton et al, 2000). Research on this phenomenon may have important implications for control programs.

There is a substantial gap in our information on the long term effects of this drug on the adult stages of *B. malayi.* The present study was carried out to examine the *in vitro*

effects of IVM on *B. malayi* adult worms using *Brugia*-gerbil animal model. And also to have some leads in understanding the drug-uptake and location of probable targets in the worm body by using fluorescent labeled IVM and confocal microscopy.

Chapter I includes a review of what we know about IVM use in LF, its effects on the adult worms of *O. volvulus,* its mechanism of action in non-filarial nematodes and its anthelmintic effects, and discussion on bodipy fluorophores (section 3, 4, 5 and 6). Section 2 in chapter I gives a brief introduction to LF, the life cycle of filarial nematodes, their basic biological and morphological features, and the reproductive and nervous system. Morphological features of the nervous system of *O. volvulus*, a related filaria, are also briefly described.

This thesis also includes one manuscript. In chapter II (Manuscript), I describe the antifilarial effects of IVM on the male and the female worms of *B. malayi* examined in an *in vitro* system and the distribution / accumulation of fluorescent IVM in the worm, examined by fluorescent and confocal microscopy, thereby giving a lead to understand how the drug is taken in and where the possible target receptors located in the worm body.

Literature Review

2.1 Lymphatic Filariasis

Lymphatic filariasis (LF) is a mosquito-borne parasitic disease caused by infection with the filarial nematodes *Wuchereria bancrofti, Brugia malayi*, and *B. timori.* It is estimated that > 128 million people are infected in 80 countries and > 1 billion are at risk of getting infection worldwide. Of the estimated 128 million cases of LF, 90 % of the infections are caused by *W. bancrofti* and the remaining 10 % by *B. malayi* and *B. timori* (Scott, 2000; WHO, 2006). LF is a major public health problem in developing countries, in Southeast Asia, Africa, Western Pacific, Eastern Mediterranean and some regions in Central and Southern America. It was previously reported that two-third of those infected live in China, India, and Indonesia (Nanduri et al., 1989). LF causes disability, loss of work and marriage opportunity, and social stigmatization. It is also the world's second leading cause of long-term disability, with morbidity estimated at 5.5M disability adjusted life-years (DALYs) (Molyneux et al., 2003).

Depending upon the species of parasite, the disease exhibits a variety of clinical manifestations ranging from acute to chronic. These are also influenced by host immune status and geographical area. The various manifestations result primarily from lymphatic tract damage induced by the parasite (either adult worms or microfilariae) and subsequently by opportunistic fungal and bacterial infections. The most common symptoms include – fever, lymphangitis, lymphadenopathy, subacute or chronic oedema, elephantiasis (lymphoedema), genital damage (hydrocoele), and chyluria, caused by drainage of lymph into the bladder. Patients usually go through a prolonged asymptomatic period (with or without microfilaremia) before the onset of symptomatic disease (Kumaraswami, 2000).

Another related species of filarial nematode, *O. volvulus* causes River Blindness or Onchocerciasis and is prevalent in sub-Saharan Africa and Central and South America (Nanduri et al., 1989; Scott, 2000). Approximately 17 million people are infected, with a further 50 million people at risk. The disease is manifested by ocular inflammation, formation of nodules in the skin, severe dermatitis, depigmentation of the skin and pruritus. The majority of skin pathology is caused by the immune response against migrating microfilariae (Scott, 2000).

The lymphatic filariae are transmitted to humans by blood-feeding mosquitoes of the genera *Anopheles, Aedes, Culex,* and *Mansonia*. Black flies of the genus *Simulium* are the vector for *O. volvulus* (Nanduri et al., 1989).

2.2 The life-cycle of *Brugia malayi*

The lymphatic filariae have biphasic life cycles (Figure A) in which larval development takes place in the mosquito (intermediate) host, and larval and adult development occurs in the human (definitive) host. These parasites have no free-living forms.

Infection is initiated by deposition of third stage larvae (L_3) in the skin of the human host following the bite of an infected mosquito. The larvae enter the body through the skin, at the site of the bite, and undergo an additional molt to the fourth larval stage (L_4) between 9 and 14 days post-infection. L_4's undergo dramatic growth during the next 6 to 12 months as they mature into lymphatic-dwelling adult male and female worms. The adults tend to localize in the varices of the lymphatic vessels of the lower extremities. The lymphatic-dwelling filariae are dioecous and undergo ovoviviparous reproduction in

which females can release of thousands of fully formed, sheathed first stage larvae (L_1 or microfilariae) into the lymph circulation of the host. From the lymph, they enter the peripheral circulation of the blood of the human host where they are available for ingestion by the vector during a subsequent blood meal. Within a few hours of ingestion the microfilariae (mfs) penetrates the gut wall of the mosquito, migrate via the haemolymph to the thoracic flight muscles and molt to the second larval stage (L_2), between 6 and 10 days post-infection. After several days, the parasite undergoes an additional molt to the L_3 stage. L_3's migrate from the flight muscles to the mouthparts of the mosquito, where they then are passed on to the next vertebrate host during a subsequent blood meal (Scott, 2000). It is interesting to note that the adult worms of lymphatic filariae may live for 7-10 years and mfs for 1 year (Nanduri et al., 1989).

Other human filariae also undergo a similar life-cycle. However, the vectors vary and the adults reside either in subcutaneous tissues of the host, as in the case of *O. volvulus* and *Loa loa*, in the lumen of the lymphatics as seen in *W. bancrofti and B. malayi,* or in the body cavity as seen in *Mansonella ozzardi* (Nanduri et al., 1989, Scott, 2000).

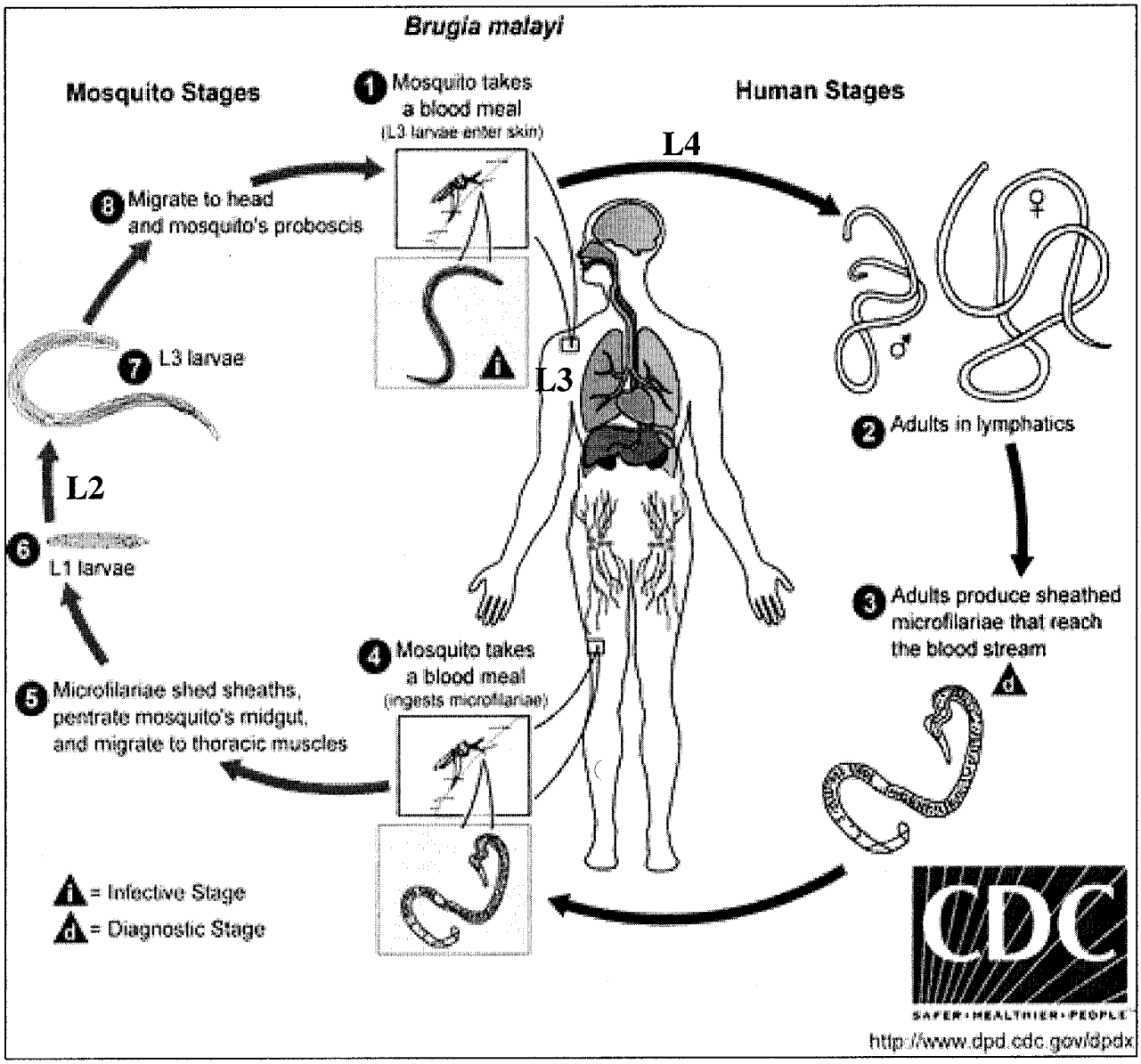


Figure A: Life cycle of *Brugia malayi* filarial nematode

2.3 Morphological features of filarial nematodes

The sexually mature adults are the largest of all stages. They are long and slender with blunt ends. *Brugia* male worms are distinctly smaller (~ 20 to 35 mm in length and 70 to 80 μm in diameter) than the females (~36 to 45 mm in length and 240 to 300 μm in diameter). Female worms of *W. Bancrofti* and *O. volvulus* are longer (6 to 10 cm and 33 to 50 cm, respectively) than *B. malayi* and *L. loa* females (36 to 45 mm and 20 to 70 mm, respectively). Male worms vary from 20 to 40 mm in length in the case of *W. bancrofti, L. loa,* and *O. volvulus* (Nanduri et al., 1989).

The body wall of nematodes consists of the cuticle, hypodermis, and body wall musculature. The surface of the filarial worm, as of other nematodes is entirely covered by a complex proteinaceous exoskeleton, the cuticle (Howells, 1987). The cuticle of the *B. malayi* female is approximately 2.5 μm thick and is comprised of basal, median, and cortical layers common to many nematodes. Variations in the absolute thickness of the cuticle and of its component sub-layers are noted in different regions of the body (Vincent et al., 1975). The major components of the cuticle are collagens (Kingston, 1991). The hypodermis lies just beneath the basement membrane of the cuticle. It consists of hypodermal cords that run longitudinally and divide the somatic musculature into four quadrants, - the dorsal, ventral, and the two lateral cords that contain nerve fibres and other organelles like mitochondria, nuclei, golgi bodies, and endoplasmic reticulum (Vincent et al., 1975). The somatic musculature and the rest of the body wall enclose a fluid-filled cavity, the pseudocoelom or pseudocoel. These two compartments, along with the cuticle, function together as a hydrostatic skeleton, which helps in nematode locomotion (Roberts et al., 2005).

Significant efforts have been made by Howells et al (1987) to advance our understanding of the dynamics of the filarial surface. The cuticle of filarial worms is adapted to the nutrient-rich environment of the host tissues by being freely permeable to a wide range of nutrients of low molecular weight. Comparison of an adult filarial nematode such as *O. volvulus* and a gastrointestinal species such as *Ancyclostoma duodenale* or *Ascaris lumbricoides* shows that filariae have much smaller intestines and less musculature in the body wall, whilst the lateral cords of hypodermal tissue are expanded to subtend a larger proportion of the subcuticular area. Experimental conditions have not yet been developed that permit quantification of the rate of ingestion of nutrients by filariae *in vitro*, but the slow appearance of dye in the worm intestinal lumen, after exposure *in vivo* to protein-dye complexes, indicates that the rate of ingestion is low.

2.4 Reproductive System in filarial nematodes

Male

The male reproductive system is composed of a single, telegonic testis that runs the length of the worm. The term telegonic represents the condition of a nematode gonad in which the germ cells proliferate only at the inner end and then must traverse the remaining length of the gonad before expulsion. Cells from all stages of spermatogenesis can be found within the testis. This consists of a distal zone of germ cell formation, a zone of maturation, and a proximal storage area for the immature sperm consisting of a seminal vesicle and vas deferens. The posterior muscular region of the vas deferens surrounds an ejaculatory duct, which opens into a cloaca. The accessory reproductive

organ in male worms is the spicule, the function of which is to dilate the vulva and vagina of the female during copulation (Scott, 2000).

Female

The female reproductive system consists of paired uteri, telegonic ovaries and oviduct. Each uterus is long and is coiled extensively within the body cavity, the combined length of which is over 1.5 times the length of the female worm.

The reproductive system can be split into a distal ovarian-like zone, where germ cell formation and egg maturation (oogenesis) take place and the proximal uterine-like zone, where the embryos develop (embryogenesis). At the junction of the two zones, the epithelium becomes modified to form a specialized structure to store sperm after copulation – the spermatheca or seminal receptacle. At the distal end of the uterus is located the vagina that opens through the vulva that appears as a small opening in the oesophageal region When an oocyte enters the seminal receptacle, fertilization takes place. Upon fertilization, the egg is released, undergoes meiosis and forms an eggshell around the new embryo. The embryo undergoes gastrulation and further differentiates into different embryonic stages, such as the comma-stage, the tadpole stage, and the pretzel stage. As embryogenesis is taking place, the embryos are propelled down the uterus towards the vulva. L_1 larvae hatch in utero and emerge from the female as fully formed, sheathed and motile microfilariae (Scott, 2000).

2.5 Morphology of Nervous System of Nematodes

The nervous system of nematodes is relatively simple. There are two main concentrations of the nerve elements in nematodes, one in the oesophageal region and one in the anal area, connected by longitudinal nerve trunks. The most prominent feature of the anterior concentration is the nerve ring, or circumesophageal commissure. The ring serves as a commissure for the ventral, lateral, and dorsal cephalic ganglia (Figure B1). Emanating from each ganglion posteriorly are longitudinal nerve trunks, which become embedded in the hypodermal cords.

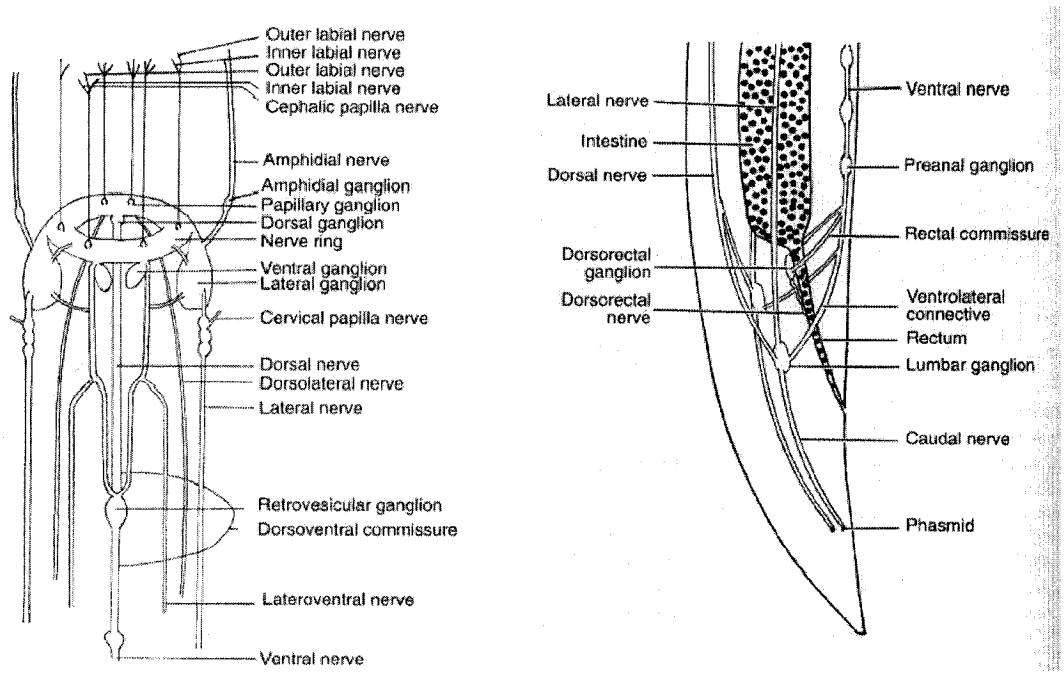


Figure B: Schematic representation of the nervous system of a nematode

(B1) Anterior end; (B2) Posterior end, (Image adapted from Roberts et al., 2005)

Proceeding anteriorly from the lateral ganglia are two amphidial nerves, which innervate the amphids. Six papillary nerves, which are derived directly from the nerve

ring, innervate the cephalic sensory papillae surrounding the mouth. The ventral nerve trunk runs posteriorly as a chain of ganglia, the last of which is the preanal ganglion. The preanal ganglion gives rise to two branches that proceed dorsally into the pseudocoel to encircle the rectum, thus forming the rectal commissure, or posterior nerve ring (Figure B2.). The peripheral nervous system consists of a network of nerves that interconnect with fine commissures and supply nerves to sensory endings within the cuticle. The main sense organs are cephalic and caudal papillae, amphids, and phasmids. The pattern of sensory papillae on the head of a nematode is a very important taxonomic character. The typical arrangement pattern is shown in Figure B3. There are four pairs of cephalic papillae arranged in an anterior and posterior row. The amphids are a pair of complex sensory organs that open on each side of the head at the about the same level as the cephalic circle of papillae. The amphidial opening, which is at the tip of a papilla, leads

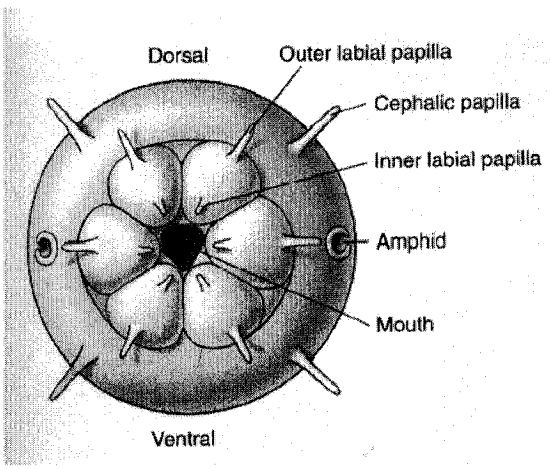


Figure B3: Diagram of the anterior end of a nematode showing arrangement of a sense organs

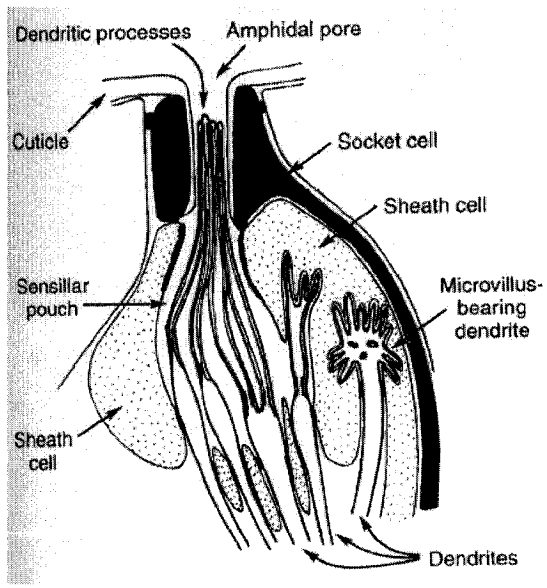


Figure B4: Diagram of amphid in *C. elegans*

(Images adapted from Roberts et al., 2005)

into a deep, cuticular pit, at the base of which is a nerve bulb with several nerve processes (Figure B4). The amphids are considered as chemoreceptors (Strote et al., 1996). Caudal papillae are more elaborated in males, aiding in copulation. Near the posterior end of many nematodes is a bilateral pair of cuticle-lined organs, the phasmids. The phasmids are similar in structure to the amphids except that they have fewer neural endings, and the glands, if present, are smaller (Figure B2) (Roberts et al., 2005).

2.6 The Ultrastructure of the anterior end of filarial worms

Strote et al. (1996) described the ultrastructure of the anterior end of male *O. volvulus*, which consists of the sensory organs (cephalic papillae and amphids), the nerve ring and a glomerulus-like structure resembling an excretory system in the adult filarial worms. The 8 head papillae are arranged in the common 4 + 4 pattern of most filarial worms in circles around the mouth opening. The amphidial openings are found between the circles of inner and outer papillae on both sides of the mouth (Figure C).

The structural and morphological characteristics of *B. malayi* adults observed under the scanning electron microscopy are well described by Zaman (1987). The anterior ends of the both male and female adult worms appear globose with a neck-like constriction. There are four pairs of cephalic papillae arranged in an anterior and posterior row. Two amphids are located between the papillae in the anterior row. In the centre is the mouth opening, which is circular and surrounded by a thin collar (Figure D).

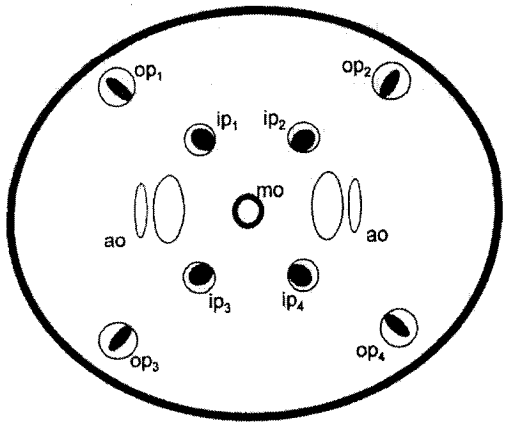


Figure C: Schematic diagram showing major structural components examined in serial sections of the anterior tip of male *O. volvulus*. Four inner (ip) and four outer (op) papillae are seen. The amphidial openings (ao) are split; papillae and paired amphids are arranged in a species-specific manner around the stoma (mo). (Image adapted from Strote et al. 1996).

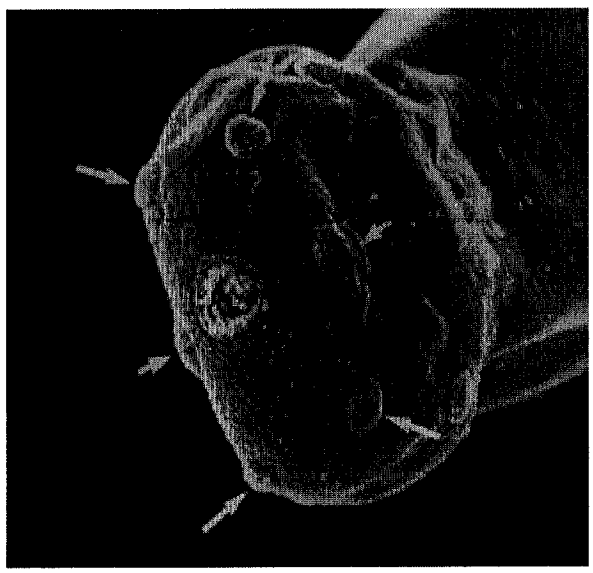


Figure D: Adult *Brugia malayi* male worm: longer arrows mark the anterior row of cephalic papillae. The two smaller arrows mark the amphids. (Image adapted from Zaman 1987)

3. Ivermectin use in lymphatic filariasis

Chemotherapeutic approaches to the control and treatment of filarial diseases currently rely on diethylcarbamazine (DEC) and ivermectin (IVM), the well-established microfilaricidal drugs. IVM has recently been introduced against LF after its successful role in the program of elimination of Onchocerciasis from African countries (Brown et al, 2000; Horton et al., 2000). Another drug, albendazole (ALB) is also being used along with DEC and IVM. An added benefit of ALB administration is that it is effective against the majority of intestinal nematodes, such as, *Ancyclostoma duodenale, Necator americanus, Ascaris lumbricoides, Trichuris trichuria* etc. (Horton, 2000; Crichley et al., 2005).

None of these drugs show profound macrofilaricidal activities against filarial nematodes and that is why there is no safe, effective, inexpensive, convenient, and well-tolerated drug for the elimination of filarial infections (Ottesen et al., 1987; Brown et al., 2000). For more than 40 years, DEC was the sole chemotherapeutic agent used to treat most forms of filariasis, since it rapidly killed microfilariae of *W. bancrofti, B. malayi, O. volvulus, L. loa* and *Mansonella streptocerca.* In addition, DEC has a recognized macrofilaricidal effect against some species (Ottesen et al., 1985; Addiss et al., 1993, 2000; Noroes et al., 1997; Ottesen et al., 2000). However, DEC, with a typically recommended course of 6-12 days, has significant disadvantages as the very rapid killing of microfilariae induces a number of post-treatment adverse reaction syndromes, especially in the areas where LF is co-endemic with other filarial infections. These adverse reactions include general malaise with headache, nausea, and vomiting, dermal and systemic effects. In patients with onchocerciasis, the adverse response has been

termed the Mazzotti reaction (Francis et al., 1985; Vande Waa, 1991; Kumaraswami, 2000). This is caused by an acute inflammatory response following the clearing/killing of microfilariae and is characterized by itching and swelling of the skin, fever, tachycardia, hypotension, and severe inflammatory reactions in both the anterior and posterior segment of the eyes in onchocerciasis patients with ocular infection. Encephalopathy may occur in patients infected with *Loa loa* after DEC treatment (Ottesen, 1987; Carme et al., 1991).

IVM, a macrocyclic lactone, is a broad-spectrum anthelmintic drug (Figure E), which was developed in 1975 from the fermentation product of filamentous bacterium, *Streptomyces avermitilis* (Bennett et al., 1988; Campbell, 1991). It is used commonly in veterinary medicine against gastrointestinal nematodes (GI nematodes), such as *Haemonchus, Ostertagia, Trichostrongylus, etc.* and also for human gastrointestinal nematodes, such as *Ascaris lumbricoides* (Kazura et al., 1993; Horton et al., 2000). In the late 1980's, IVM became the universally accepted drug of choice, both for individuals and for mass treatment programmes, to control onchocerciasis (Campbell, 1991; Ottesen et al., 1994). This fact, coupled with the excellent safety profile of IVM in onchocerciasis patients, and the simplicity of dosing led to the first studies of its potential effectiveness in LF (Awadzi et al., 1995). The rationale for using IVM was based on the known risks associated with the use of DEC in patients with LF who might also be infected with either *O. volvulus* or *L. loa.* In these patients, reactions to DEC treatment are severe and sometimes life-threatening. The expectation was that IVM might not induce the same degree of post-treatment complications, associated with the use of DEC in patients with LF (Brown et al., 2000). The goal of such treatment, in community settings, is the long-

term suppression of microfilaraemia that will result in the reduction of transmission and ultimately in a decrease in both incidence and prevalence of the clinical disease itself.

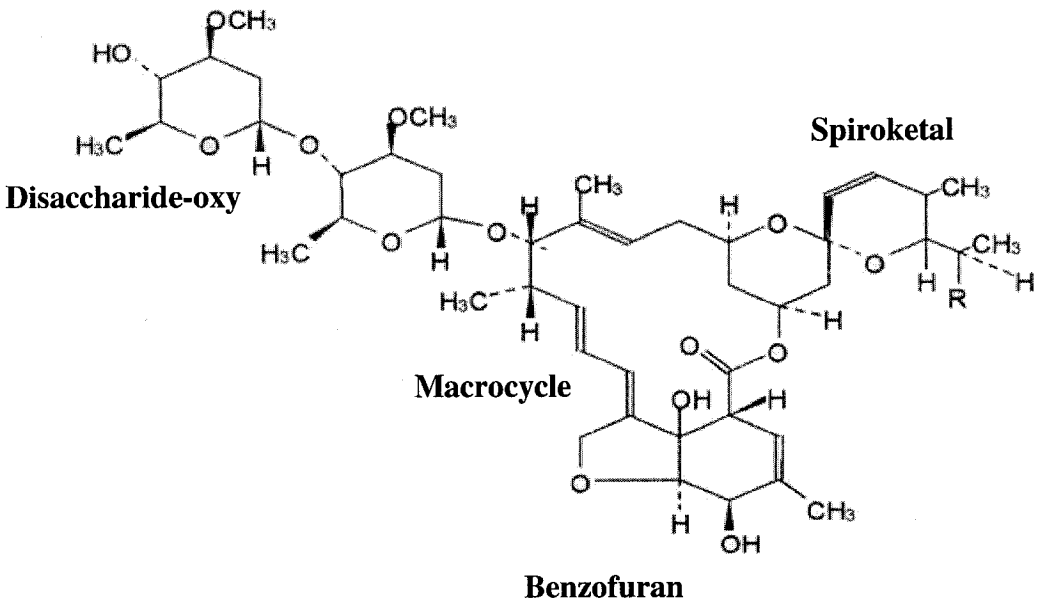


Figure E: Structure of Ivermectin

Most commonly, the effects of treatment have been assessed by measuring the change in Mf density over time. Based on efficacy assessments, the oral administration of a single dose of 200 μg/kg of *IVM* for the treatment of bancroftian and brugian filariasis was effective in decreasing blood microfilarial density by 90-95% of pre-treatment values. The density then rises to 10-25% of pretreatment value 6-8 month post-treatment (Bennett et al., 1988; Nanduri et al., 1989; Campbell, 1991; Brown et al., 2000).

It is important to note that IVM is a recently introduced drug for LF. It reduces the density of mf in the blood and also the intensity of side effects. The reduction in mf

production indicates that adult worms are affected, but the nature of this effect (e.g., death or sterilization of worms, reduced mf released from the female worm uterus) has been a subject of study. There is a substantial gap in our understanding of the effects of IVM treatment on the adult worms probably because the biological effects of the drug on adult worms are unclear. Such effects should be known because they may have important implications for control programs.

In contrast, the literature gives examples of studies which have examined the long-term effects of IVM on mf and adult worms of *O. volvulus*, a related pathogen. Since IVM has been a part of the onchocerciasis control programs (Onchocerciasis Control Program, OCP and now the African Program of Onchocerciasis Control, APOC) for the last two decades, there is a considerable amount of information on the effects of IVM. The studies were done on human subjects and the effects of IVM treatment were examined using criteria, such as skin microfilaria count, microscopic examination of adult worms (histology), and by the analysis of embryograms to examine the effects of IVM on reproduction of filarial worms.

4. Ivermectin: Effects on microfilariae and the adult worms of *O. volvulus*

Unlike the adulticidal effects of IVM on non-filarial nematodes such as *Haemonchus contortus, Ancyclostoma cervicalis, Strongyloides stercoralis* (Behnke et al., 1993; Geary et al., 1993; Richards et al., 1995; Sithithaworn et al., 1998), it acts as an efficient microfilaricide for *O. volvulus* (Awadzi et al, 1986; Schulz-Key 1986; Albiez et al., 1992, 1993). It was observed that a single dose of 150 - 200 μg/kg IVM reduced microfilariae in the skin by about 90-95% within 2-3 days of drug administration.

Although the drug does not kill adult worms (both male and female worms), the production of new mf by female worm is inhibited for 6-12 months. A closer look at this condition shows that the mf are not released, but degenerate in the uteri of female worms, resulting in a build up of normal and degenerating intrauterine mf. This effect is reversible, lasting about 9 – 12 months in the majority of worms (Duke et al., 1991a, 1991b).

To control the re-emergence of mf in the skin, multiple doses of IVM (150 μg/kg given at six monthly interval) were tested and it was demonstrated that repopulation of mf in the skin was slower after multiple doses of IVM, rather than after a single dose (Whitworth, 1992). An examination of the long-lasting effects of IVM on the adult worm after multiple dose treatment was made by direct microscopic examination. This showed a reduction in the proportion of female worms producing mf, and an increase in the proportion of unfertilized or uninseminated female worms with degenerating mf. The extent of any macrofilaricidal activity of IVM was not determined (Chavasse et al., 1992; Klager et al., 1993).

In one study in Sierra Leone (Chavasse et al., 1992), the effect of multiple doses (four or five six-monthly doses) of IVM on the adult female worms of *O. volvulus* was examined. It was observed that multiple dose treatment leads to almost complete cessation of embryogenesis with a significant decrease in the number of viable multicellular embryonic stages. Multiple doses showed no major effect on oogenesis because oocytes appeared to be produced normally. Similar studies suggest that multiple doses of IVM may have little direct effect on oogenesis in *O. volvulus* (Albiez et al.,

1988b; Duke et al., 1990, 1991; Klager et al., 1996) but have effects on younger embryonic stages (Lariviere et al., 1985).

Chavasse et al., 1993 conducted another study to determine the causes of embryogenic failure, due to multiple doses of IVM. The seminal receptacles were examined for the presence of sperm and mature primary oocytes. The presence of sperm in the seminal receptacles was postulated to indicate effective insemination of the female worms. Results showed that there was a significant reduction in the proportion of female worms containing sperm in seminal receptacles with increasing doses of IVM treatment. It has been suggested that the degeneration of oocytes could be caused by the lack of sperm in the seminal receptacle, leading to a failure of fertilization of the viable oocytes produced by the ovaries. This indicates that IVM may interfere with insemination.

The possibilities that the presence of degenerating mf in the lower uterus prevents sperm from moving up to the seminal receptacle to fertilize the oocytes, or that the lack of sperm is due to an effect of IVM on male spermatogenesis, or on mating behaviour of the adult worms, have been suggested (Chavasse et al., 1992). However, there was no observable effect of a single dose or multiple doses of IVM on spermatogenesis. Intact spermatozoa in the vas deferens were observed in the male worm even after single and multiple rounds of IVM treatment (Albiez et al., 1988a, 1988b; Chavasse et al., 1993). Duke et al. (1990) reported a significant reduction in female worms containing sperm, after multiple doses of IVM (five treatments at six-monthly interval).

In one study by Awadzi et al. (1999) in Ghana, the effects of high-dose IVM regimens (1600 μg/kg body weight) on *O. volvulus* in onchocerciasis patients were examined after six months of treatment. The aim was to determine whether high doses of

IVM kill adult worms, and whether cessation of microfilarial production after multiple treatments could be reproduced by an equivalent, single, high dose. The result showed that a single high dose of IVM was not superior in its antiparasitic effects and that it did not reproduce the marked inhibitory effects on embryogenesis, or the mild-to-modest effect on adult worm viability seen after multiple doses.

Recently, the effects of standard and high doses of IVM on adult worms of *O. volvulus* reproduction, as well as macrofilaricidal activity, have been analysed (Gardon et al., 2002; Duke 2005). In these community based trial studies, it was observed that treatment with 3-monthly IVM at dose of 150 μg / kg greatly reduced the number of female worms. There was a sustained decrease in overall production of mfs by adult female worms over 1 year, suggesting a greater effect on fertility of female worms following multiple doses of IVM than a single dose (Gardon et al., 2002). Upon further analysis of clinical trials, [1] suggested the involvement of two distinct killing mechanisms of *O. volvulus* adult worms, a direct anthelmintic action of IVM, and a potentially fatal pleomorphic ovarian neoplasm (PN), observed as small, roundish, basophilic cells of diverse sizes, often forming a 'rosette' pattern around amorphous eosinophilic centres (Duke et al., 2002).

A few reports cite examples where IVM affects the fertility of other nematode species such as *H. contortus* (Le Jambre, 1995) and *C. elegans* (Grant, 2000). In these cases, IVM appears to inhibit the release of eggs already *in utero* and suppresses the production of new eggs.

It is important to note that there are growing concerns about IVM resistance in onchocerciasis patients based on recent reports about persistent microfilaraemias

observed in some individuals (sub-optimal responders) who had received at least nine treatments with IVM. In one of the study by Awadzi et al. (2004), twenty-one of 'sub-optimal' responders (cases) were treated with IVM and the microfilarial and adult-worm responses to this treatment were assessed from skin microfilarial counts. The results based on embryogram studies indicated that the microfilaridermias that persist despite multiple treatments with IVM were mainly due to the non-response of the adult female worms. The possibility that some adult female worms have developed resistance to IVM has been pointed out. Certain genes of the parasite have been identified that could be under selection pressure due to repeated treatment with IVM, such as β-tubulin, PGP and ABC (ATP binding cassette) transport genes (Eng et al., 2005; Ardelli et al., 2006; Bourguinat et al., 2006).

5. Ivermectin

5.1 Mechanism of action and its Anthelmintic effects

IVM was initially introduced as a veterinary medicine in 1981 for the treatment of gastro-intestinal nematode infections and ectoparasite infestations; they are used in agriculture for the control of insect pests and, in humans for onchocerciasis and LF. Because of its extensive use as a veterinary medicine, there are certain reports of IVM resistance in parasites of sheep, cattle, goats, and horses (Prichard, 1994; Jackson et al., 2000). Much of the research on IVM resistance has focused on elucidating IVM's mode of action by using free-living nematode, *C. elegans* as a model organism. Based on molecular biological and electrophysiological experiments in *C. elegans*, it has been shown that IVM binds selectively and with high affinity to glutamate-gated chloride

(GluCl) ion channels, which occurs in invertebrate nerve and muscle cells. This leads to an increase in the permeability of the cell membranes to chloride ions with hyperpolarization of the nerve or muscle cell, resulting in paralysis and death of the parasite (Cully et al., 1994; Arena et al., 1995; Dent et al., 1997).

GluCls or receptors are the groups of intrinsic transmembrane ion channels which are opened in response to binding of a chemical messenger, glutamate neurotransmitter. More than five predicted subunits of GluCl genes in *C. elegans* have been identified as well as functionally characterised. GluCl consist of at least 5 predicted subunits (such as [GluClα3A, GluClα3B; two splice variants], [GluClα2A, GluClα2B; two splice variants], GluClα1, GluClβ, GluClα4) (Wolstenholme et al., 2005). Certain GluCl subunits have also been identified in other nematodes like, *Ascaris suum, Cooperia oncophora, Dirofilaria immitis, and O. volvulus* (Njue et al., 2004; Yates et al., 2003, 2004). These subunits are widely expressed in the nematode nervous system and are responsible for certain anthelmintic effects such as, paralysis or death of parasite (Wolstenholme et al., 2005).

5.2 Anthelmintic Effects in GI / free-living nematodes

When IVM is applied to non-filarial nematodes (such as *C. elegans, H. contortus*, or *Ascaris suum*) two main affects are observed: rapid paralysis of pharyngeal pumping and of movement. As a result, the treated worms are unable to either feed or to move and, for most infections, the paralysed parasites are consequently rapidly removed from the host (Wolstenholmes et al., 2005).

In these nematodes ingestion takes place by mouth, and pharynx (a muscular organ associated with a nerve and a gland cells) (Roberts et al., 2005). Pharyngeal pumping in GI nematodes is extremely sensitive to ivermectin. Evidence for this comes from *in vitro* studies by Avery et al. (1990) on *C. elegans,* Geary et al. (1993) on *H. contortus*, Sheriff et al. (2002) on *Trichostrongylus colubriformis* that IVM at 5 nM, ≥ 1nM, and 0.251 nM respective concentrations inhibited the uptake of inulin (> 5000 molecular weight) by pharyngeal pumping. On the basis of localization of IVM-sensitive GluCl channels, it has been suggested that the inhibition of pumping is due to presence of GluCls on pharyngeal muscle cells (Martin, 1996; Laughton et al., 1997) as shown in Figure F (adapted from Wolstenholmes et al., 2005). Since most of the subunits of these channels were widely distributed in the nervous system, it suggests their possible role in controlling not only pharyngeal function, but also locomotion, and possibly sensory processing in parasitic nematodes (Portillo et al., 2003).

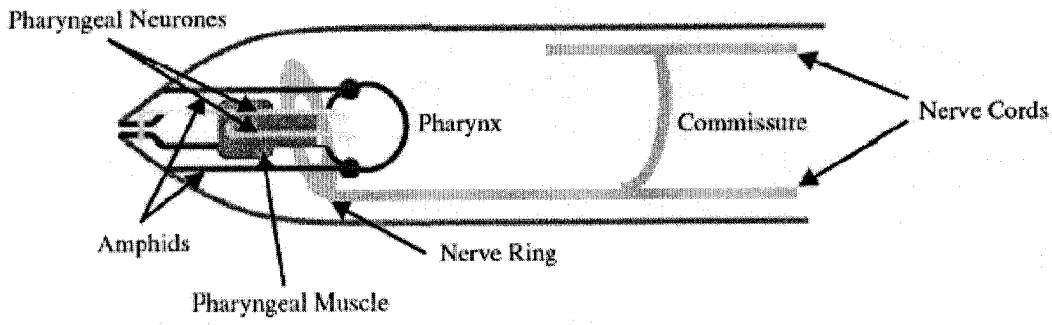


Figure F: Schematic representation of the distribution of GluCl in nematodes. The cuticle is outlined in grey and the pharynx in black. Structures reported to express GluCl are indicated by arrows (Image adapted from Wolstenholme et al., 2005)

The second major effect of IVM on nematodes is paralysis of body-wall muscle, rendering them immobilised. Locomotion in nematodes is controlled by both excitatory and inhibitory motor neurons, organized into ventral and dorsal nerve cords, each of which innervates body-wall muscle. Studies on *C. elegans* showed that the motor neurons in turn are controlled by interneurons in the head of the worm that regulate the rate of locomotion and also the frequency with which the worm reverses and moves backwards. Geary et al., 1993 showed that IVM at concentrations $\geq 10^{-8}$ M reduced *H. contortus* motility of adult worms by ~75 % when monitored using a motility meter for the time 1 hr to 30 hrs which were detected by the motility meter. Careful analysis also showed that the drug-induced paralysis was restricted to the midbody region of the parasites, while the head and tail sections had apparently normal motility. Since the concentration of $> 10^{-10}$ was sufficient enough to paralyze the pharynx, this suggested that the paralysis of pharynx was more sensitive than paralysis of movement by the action of IVM. Recently, Cook et al. (2006) studied the role of GluCl channels in locomotor behaviour in *C. elegans*. They examined the effects of mutations in several GluCl genes on this behaviour and found the opposite phenotype; that is, the worms reverse more frequently and the durations of the forward movements are reduced. These observations suggest that GluCl have a major role in regulating nematode feeding and locomotion that leads to their paralysis.

5.3. Anthelmintic Effects in filarial nematodes

The anthelmintic effects of IVM observed in filarial nematodes are different from those in non-filarial nematodes. In contrast to gut parasites, for which IVM is adulticidal,

adult filariae of *O. volvulus, W. bancrofti, B. malayi* are relatively resistant i.e., they are not killed by IVM and the main effects are against microfilariae (L_1s or mfs) and on female worm production of mfs.

The molecular basis of the anthelmintic action of IVM and the basis for differences in sensitivity to the drug between adult and larval stages remain largely unknown. One possible explanation could be that there are differences in GluCl expression between the adults and larvae (Yates et al., 2004; Wolstenholmes et al., 2005). Culley et al. (1996) reported a *Ov*GluClα3 subunit from *O. volvulus* and Yates et al. (2004) reported *Di*GluClα3 subunits from *Dirofilaria immitis* (filarial nematode infecting dogs). Yates et al. (2004) reported the presence of GluClα/γ subunit in *B. malayi* which can be amplified from *D. immitis* cDNA. However, functional characterization of these subunits has not been reported (Wolstenholmes et al., 2005). Li et al., 2004 reported quantitative analysis of gender-regulated transcripts in the filarial nematode, *B. malayi* by real time RT-PCR to have a better understanding of the biology of reproduction in filarial worms. It is interesting to note that the gene expression of glutamate-gated chloride channels in *Brugia* female worms are up-regulated compared to male worms. It is not clear what roles GluCl proteins play in filarial reproduction (Li et al., 2004; Wolstenholmes et al., 2005).

There are no reports on paralysis of the pharynx in filarial nematodes as both adults and microfilariae have atrophied pharynx/intestine, and most nutritional exchange is across the cuticle. There are no reports on cuticular damage to adult filarial worms caused by IVM treatment. However, IVM appears to inhibit the intrinsic exsheathing process of microfilariae in the mosquito host, thereby blocking their development and

further transmission (Rao et al., 1992). The adult filarial nematodes are not killed and the extent of paralysis of body musculature in has been based on the subjective description of worm motility described by visual microscopic observation.

5.4 Drug uptake by nematodes

Studies with the free-living nematode *C. elegans* and *H. contortus* have yielded insight into how IVM acts. In one experiment Smith et al. (1996) demonstrated that, in the absence of pharyngeal pumping, first stage larvae of *C. elegans* submerged in IVM became irreversibly paralysed suggesting that ingestion is not the only means of IVM uptake and that the other means of entry, such as trans-cuticular penetration could be taking place as well.

One possible route of uptake of the macrocyclic lactone (ML) is absorption through the amphids located on the head of the nematodes. Amphids are the sensory organs present in the nematode head that contain sensory neurons. It has been proposed that nematode amphids are involved in the process of MLs action and strains of *C. elegans* displaying low-level resistance to IVM have an amphid defect called Dyf (dye-filling negative) (Blaxter et al., 1997).

In another study, Freeman et al. (2003) described the amphidial structure of macrocyclic-lactone-resistant *H. contortus*. In this study, it was proposed that the critical point of entry for these drugs may be through amphidial neurons, located in the amphids which are exposed to the external environment via pores at the anterior tip of the worm. Through these neurons, important chemical and thermal cues are gathered by the parasite. On microscopic examination of the amphidial regions of ivermectin-susceptible and

ivermectin-resistant *H. contortus,* it was shown that the ivermectin-resistant worms examined, had markedly shorter sensory cilia than their ivermectin-susceptible parental counterparts. Additionally, the amphidial neurons of ivermectin-resistant worms were characterized by generalized degeneration and loss of detail, whereas other neurons outside of the channels, such as the labial and cephalic neurons, were normal in structure. These findings highlight the possible relationship between amphidial structure and macrocyclic lactone resistance as well as the role of amphids as a means of entry for these molecules.

6. Confocal microscopy approach to investigate the distribution / localization of target receptors.

A full understanding of the effects of IVM and its mechanism requires that we know where the possible target receptors are located. One of the possible approaches could be to observe the distribution pattern of the fluorescent labelled IVM, such as bodipy FITC-Ivermectin (bodipy FITC-IVM; two fluorophores linked to IVM), in the whole worm body.

Bodipy fluorophores (4,4-difluoro-4-bora-3a,4a-diaza-s-indacene; Figure G) are the patented dyes supplied by 'Molecular Probes' and have spectral characteristics that are often superior to those of fluorescein, Texas Red and other longer-wavelength dyes and may be substituted for these dyes in some applications. For example, bodipy FITC have an advantage over FITC (fluorescein isothiocyanate) because they are nonpolar and electrically neutral, and they have narrow emission bandwidth, resulting in higher peak

intensity than that of FITC (Figure G). They also show greater photostability than FITC in some environments (Kambara et al., 1992).

Bodipy FITC-IVM has allowed researchers to investigate epithelial transport in isolated proximal kidney tubules (Fricker et al., 1999). Similar fluorescent derivatives of ivermectin have been used to measure cellular uptake of ivermectin (Audus et al., 1992), to study ivermectin-sensitive Cl^- channels (Adelsberger et al., 1997; Dent et al., 1997), and to measure the concentration, mobility and distribution of ivermectin in the plasma membrane (Martin et al., 1990, 1992).

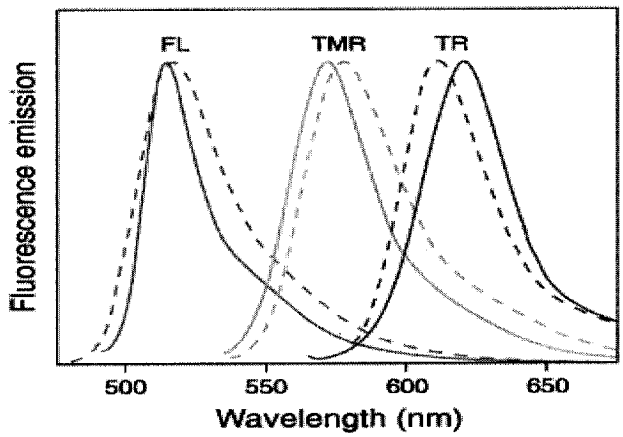
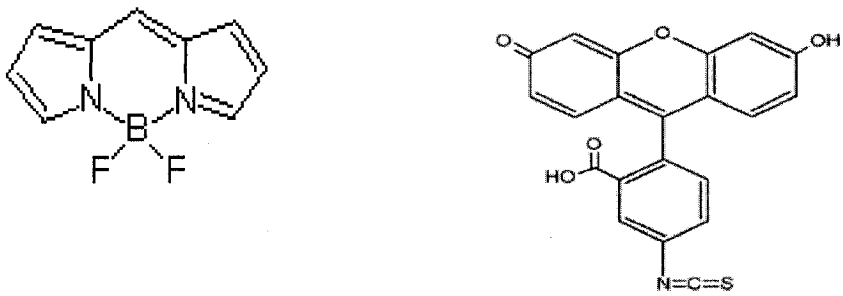


Figure G: Schematic graph of fluorescence emission spectra of regular fluorescein (FL), tetramethylrhodamine (TMR), and texas red (TR) (represented with ----------) comparable to emission spectra of bodipy FL, bodipy TMR, and bodipy TR (represented with ———)



(a) 4, 4-difluoro-4-bora-3a, 4a-diaza-s-indacene and (b) fluorescein isothiocyanate (FITC)

Figure H: Structure of Bodipy dye (a) and FITC dye (b)

In one study by Martin et al. (1992), a fluorescent derivative of the IVM (4"-5, 7-dimethyl bodipy proprionylivermectin, referred to as bodipy IVM) was synthesized for an investigation of the distribution of avermectins (e.g., IVM) based on the fact that the active site of avermectins lies within the area of macrocyclic lactone ring and spiroketal subunit, and the disaccharide substituent is not essential for biological activity (refer Figure E). Bodipy IVM at doses greater than 0.1 μg per worm was injected into adult *Ascaris suum*. Fluorescent microscopy of frozen sections revealed the distribution of the probe in the whole nematode. Most of the bodipy IVM was found at the injection site. The fluorescent probe accumulated in the muscle membranes and within the nerve cord, consistent with the view that avermectins act at these sites. However, they did not consider the distribution of bodipy IVM being reflected as the distribution of putative receptor sites for IVM in this nematode. They also described autofluorescence being always detected in the medullary region of muscle spindles, in the intestinal cells, and in the outer cuticle layer. The fluorescence seen in these regions was thus not considered to represent a positive result (Martin et al., 1992). They also observed the uptake of bodipy IVM by the lipid rich regions of hypodermal layer. In addition, since bodipy IVM was chemically synthesized, its stability as well as biological activity were examined by thin-

layer chromatography and by observing the paralysing effects in the worms. There were no changes in the chemical structure that would disturb the biological activity of bodipy IVM.

There is no study reporting the use of bodipy FITC-IVM in filarial nematodes to understand the drug distribution / localization patterns.

It is clear that IVM is an important anthelmintic drug for veterinary as well as human use. It has recently been introduced for LF control program and therefore it is important to understand the effects of this drug on the filarial worms, such as *B. malayi.* Thus, this project aims at understanding the effects of IVM on the adult stages of filarial worm, *B. malayi.*

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Chapter II

Manuscript I

An *in vitro* examination of the effects of ivermectin on *Brugia malayi* adult worms

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In Preparation

ABSTRACT

Background

Brugia malayi is one of the causative agents of Lymphatic Filariasis (LF). Recently ivermectin (IVM) has been introduced for use in LF control programs. There is a substantial gap in the information on the effects of this drug on the adult stages of filarial nematodes. The present study was carried out to examine the effects of IVM on adult worms of *B. malayi* in an *in vitro* culture using the *Brugia*-gerbil animal model.

Method

The effects of IVM at concentrations 1, 0.5, 0.1, and 0.05 μM on *B. malayi* adult male and female worms were studied in 7-day cell-free culture. The anti-filarial effects of the drug were measured in terms of parasite motility, by using grid analysis and an MTT assay for parasite viability. The release of microfilariae per day was also studied in female worms by using a McMaster counting chamber. For drug-localization studies, male and female worms were incubated in bodipy FITC-IVM / bodipy FITC (at concentrations 0.5, 0.05, and 0.005 μM) for two hours followed by washing in PBS, and examined by confocal microscopy.

Results

The female worms of the control group showed a relatively stable release of microfilariae for 7 days. Compared with the controls, the 1.0 μM and 0.5 μM concentrations of IVM showed significant effects on the release of mfs after day 2 of treatment. The motility was markedly reduced at day 4 in both male and female worms. The reduced motility effect was more severe in the female worms than in the male

worms, when compared with the untreated group. The MTT assay results were generally consistent with the results of the motility study. Worms incubated in bodipy FITC-IVM showed a strong accumulation of the fluorescent drug in the anterior head region compared with worms of control group, incubated in bodipy FITC alone.

Conclusions

These results show effects of IVM on mf release, motility and viability of the adult worms. Localization of bodipy FITC-IVM in the head region suggests interaction of the drug with probable neuronal receptors.

Background

Lymphatic filariasis, a mosquito-borne parasitic disease, is a major public health problem in tropical and subtropical countries in Asia, Africa, and South America. It is caused by infection with the filarial nematodes *Wuchereria bancrofti, Brugia malayi*, and *Brugia timori* - afflicting over 128 million people. About 90 % of LF is caused by *W. bancrofti* and the rest by *B. malayi* and *B. timori*. It is estimated that there are nearly 1.3 billion people who are at risk of developing the disease (WHO, 2006; Addiss et al., 2000; Ottesen, 2000). Infective larvae (L_3) from mosquitoes enter the mammalian host and develop into male and female adult stage parasites in the lymphatics. Depending upon the species of parasite, host immune status, and geographical area, the disease exhibits the following most common symptoms – lymphoedema (elephantiasis), genital damage (hydrocoele), and chyluria, caused by drainage of lymph into the bladder. These pathologies occur mostly due to the presence of adult stages of the parasite in the lymphatics, whereas the L_1 larvae (microfilariae, mfs) reside in the peripheral blood (Scott, 2000; Nanduri et al., 1989; Dreyer et al., 1997; Kumaraswami, 2000).

Chemotherapeutic approaches to the control and treatment of filarial diseases currently rely on diethylcarbamazine (DEC) and ivermectin (IVM), the well-established microfilaricidal (Addiss et al., 2000, Brown et al., 2000, Horton et al., 2000; Kazura, 1993), but not markedly macrofilaricidal drug (Brown et al., 2000; Horton et al., 2000). Another drug, albendazole (ALB) is also being used along with DEC and IVM. An added benefit of ALB administration is that it is effective against the majority of intestinal nematodes, such as, *Ancyclostoma duodenale, Necator americanus, Ascaris lumbricoides, Trichuris trichuria* etc. (Horton, 2000; Critchley et al., 2005; Behnke et al.,

1993; Richards et al., 1995). IVM, a macrocyclic lactone, is a broad-spectrum anthelmintic drug. It was developed in 1975 from the fermentation product of filamentous bacterium, *Streptomyces avermitilis* (Bennett et al., 1988; Campbell, 1991). It has been widely used in veterinary medicine for the treatment of the gastro-intestinal (GI) nematode infections (e.g. *Haemonchus contortus*) (Wolstenholme et al., 2004; Prichard, 1994; Geary et al., 1993) and in human medicine for the treatment of filarial nematode infections (e.g. *Onchocerca volvulus*) (Campbell, 1991; Awadzi et al., 1984; Ottesen et al., 1994). In addition, it is effective against certain ectoparasites that figure in public health (it has well-known effects on lice and scabies) (Dourmishev et al., 2005). IVM has been recently introduced in the LF control program, the Global Program to Eliminate Lymphatic Filaraisis by 2020 (Addiss et al., 2000; Brown et al., 2000; Horton J et al., 2000; Molyneux et al., 2003; Gyapong et al., 2005). The rationale for using IVM against LF was based on the known risks of severe side effects (Mazzotti reaction) associated with the use of DEC in patients with LF who might also be infected with either *O. volvulus* or *Loa loa* (other related filariae) (Kumaraswami, 2000; Francis et al., 1985). It has also been part of the Onchocerciasis (caused by *O. volvulus*) control program from last two decades in African and American countries (Campbell, 1991; Awadzi et al., 1984, 1995, 1999; Albiez et al., 1988a, 1988b; Duke et al., 1990, 1991, 1992; Geary, 2005). Since a single oral dose of IVM, about 200 μg / kilogram of body weight is effective in reducing about 90-95 % of blood microfilariae density, it is regarded as safe and well-tolerated drug for the treatment of filarial infections in community settings (Addiss et al., 2000; Brown et al., 2000; Horton et al., 2000; Kazura, 1993). However, it is unclear how many treatment rounds of currently administered drugs will be required to

achieve the goal of elimination (Stolk et al., 2005; Michael et al., 2004). One of the major problems is our incomplete understanding of the biological effects of IVM treatment on the adult worms (Grant, 2000; Stolk et al., 2005).

What the biological effects and molecular targets of IVM are in nematodes has been a subject of study from a long time. Most of the research pertaining to understand the mechanism of action of IVM has been done in the free-living nematode, *Caenorhabditis elegans* (Cully et al., 1994; Arena et al., 1995; Dent et al., 1997). The most accepted explanation of its mode of action is that it selectively binds to glutamate-gated chloride channels (GluCls) in invertebrate nerve and muscle cells. This binding leads to an increase in cell membrane permeability to chloride (Cl^-) ions. Hyperpolarization of the cell results in paralysis and death of the parasite. Two main anthelmintic effects observed in free-living and GI nematodes are: rapid paralysis of the movement and of pharyngeal pumping. As a result the treated worms are unable to either move or to feed and, such paralysed parasites are then consequently removed from the host (Bennett et al., 1988; Geary et al., 1993; Yates et al., 2003; Portillo et al., 2003; Wolsteholme et al., 2005). In filarial nematodes, IVM acts as an excellent microfilaricide. However, it is not markedly macrofilaricidal, but shows a long-lasting reduction (of several months) in the production of new larvae (mfs) by female worms (Campbell, 1991; Albiez et al., 1988a, 1988b; Schulz-Key, 1986, 1990; Klager et al., 1993; Chavasse et al., 1992, 1993). The reduction in mf production indicates that adult worms are affected, but the nature of this effect (e.g., death or sterilization of worms, reduced mf released from the female worm uterus) has not been clearly understood. There is a substantial gap in our understanding of the effects of IVM treatment on the adult worms because the biological

effects of the drug on these stages are not clearly known (Brown et al., 2000; Bennett, 1988; Chavasse et al., 1992, 1993; Stolk et al., 2005). Such effects should be known because they will help in understanding what are the possible target receptors of IVM in filarial worm and where they are located. Any information pertaining to the mechanism of action of IVM will also have important implications for control programs.

The present study was carried out to examine the *in vitro* effects of IVM on *B. malayi* adult worms using a *Brugia*-gerbil animal model and also to have some leads in understanding the drug-uptake and location of probable receptors in the worm body by using fluorescent labeled IVM and confocal microscopy.

Materials and methods

1. Chemicals

RPMI 1640 (with L-Glutamine), Phosphate buffer saline (pH 7.2), and fetal bovine serum were purchased from GIBCO (Grand Island, NY, U.S.A). HEPES, antibiotics, IVM, tetrazolium salt, and mounting medium were purchased from Sigma-Aldrich (St-Louis, MO, U.S.A). Bodipy FITC IVM (100 µg) and Bodipy FITC (5 mg) were purchased from Molecular probes (U.S.A). The 24 well-plates were purchased from costar (New York, U.S.A) and Triton-X from LKB Produckter (Sweden).

2. Animals and parasites

Jirds (*Meriones unguiculatus*), also known as Mongolian gerbils, are a suitable animal model for the study of lymphatic filariasis as both *B. malayi* and *B. pahangi* develop from the infective L_3 stage to reproductive adult stages. For this study, jirds infected with *B. malayi* were obtained from Dr. John McCall of the University of

Georgia. At this institution, male jirds were injected intraperitoneally with 200 to 300 L_3 stage of *B. malayi*. Adult male or female worms of *Brugia malayi* were obtained at necropsy from the peritoneal cavities of infected male jirds at > 120 days post infection. These adult worms were first washed 3-4 times in RPMI medium containing 20 mM HEPES and, 100 μg / ml penicillin and 100 units / ml streptomycin, to eliminate host cell contamination. They were then sexed and maintained in RPMI medium containing antibiotics, HEPES, and 10 % heat inactivated FBS (referred as complete medium unless otherwise stated) at 37°C, 5 % CO_2, and 95 % humidity until required.

3. An *in vitro* examination of the antifilarial effects of IVM on adult *Brugia* worms

a. *Drug preparation and the assay*: 1 mM stock solution of IVM was prepared in dimethylsulphoxide (DMSO). The working concentrations of the drug, 1 μM, 0.5 μM, 0.1 μM, and 0.05 μM, were prepared from the stock solution in final concentration of 1 % DMSO. Six to eight worms of each sex, at each concentration of IVM, were incubated in 24 well-plates with one worm placed in each well containing 1.5 ml of complete medium and the drug. Controls were established by incubating worms in drug-free RPMI medium and DMSO (1%). Day 0 is regarded as an over night incubated worms in the drug-free medium. The incubation of drug-exposed and control parasites were carried out at 37°C, 5 % CO_2 and 95 % humidity for 7 days. Each worm was transferred daily, for up to 7 days (day 1 to day 7), into a new well containing medium and drug. The antifilarial effects were studied using three parameters that are described in section b, c, and d.

b. *Daily release of mf by Brugia female worms under different concentrations of IVM* - McMaster counting chamber (Hawksley, England) was used to calculate the total number of mf released by the female worms. Briefly, the mf released by each female worm in each wells were diluted ten times and counted twice using the cell counter (Laboratory Counter, Clay Adams) by observing the mf under the fluorescent microscope at 4X (Nikon, Japan). The average number of mf was multiplied by the dilution factor of 10. The average of mf count at each drug concentration was measured daily for 7 days. Results are expressed as percentages of the pre-treatment number of mf released at day 0 by female worms.

c. *Assessment of motility using a grid analysis method*: Motility was analyzed under a light microscope (Nikon, Japan) at days 1, 4, and 7 of IVM treatment. To have a quantitative understanding of the effects on motility (and thus, the extent of paralysis), images were recorded using a digital camera (Nikon SMZ1500, Japan) during a time course of 0 to 40 seconds, at an interval of 2 seconds. These images covered 20 frames. By keeping the head and the tail of the worm as a reference point, motility was scored using a grid (0.5 cm x 0.5 cm) that covered all 20 frames (figure 1). While moving from one frame to another, the preceding frame acting as a reference frame for the next one. The average value of the motility score (referred as average grid score per 2 sec., which is the index of motility) was calculated for days 1, 4 and 7 of IVM exposure.

d. *Assessment of viability by MTT reduction assay:* The MTT (3-(4, 5 dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide) reduction assay is commonly used to assess the viability of nematodes. The main principle is that

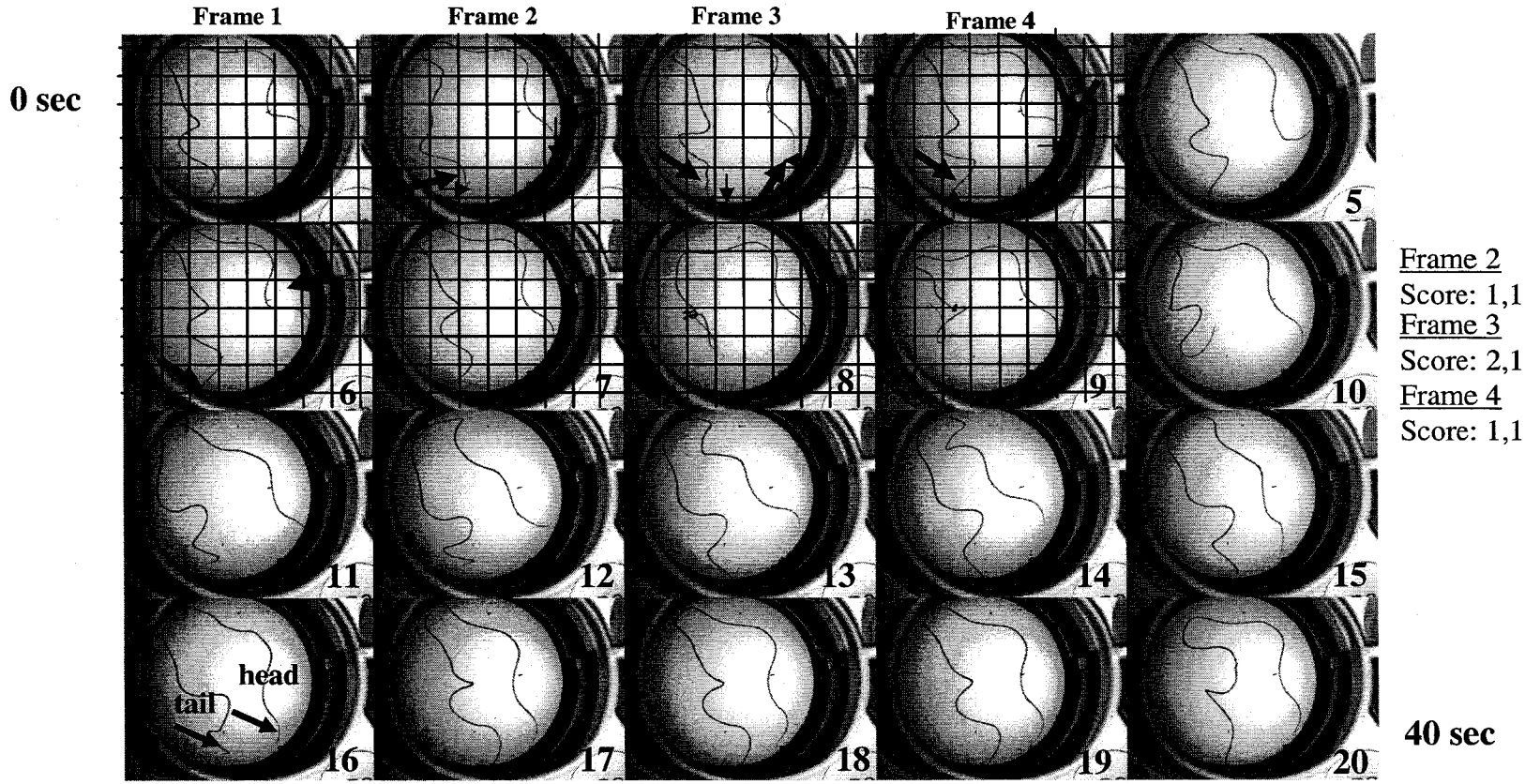


Figure 2: Motility scoring using 'grid analysis' method. Images of the worms were taken from time 0 second to 40 seconds at an interval of 2 seconds, which covered 20 frames. Scoring was done by taking the head and the tail as a reference point while moving from one frame to the next frame. Bold arrows represent the head and the tail where as the small arrows represent the grid scoring and the direction in which the head or the tail moved.

living (but not dead) filariae take up MTT and rapidly reduce it to formazan, so staining them dark blue. On completion of 7-day incubations, drug-treated and control worms were removed from the wells, blotted and transferred into 1.5 ml of PBS, containing 0.5 mg MTT /ml for 1 hour at 37°C (MTT reduction). Each worm was carefully transferred into a separate well of a microtitre plate containing 600 µl of DMSO (to solubilize formazan from the worm) and was allowed to stand at room temperature for 1 hr. The DMSO was removed and its absorbance was determined at 490 nm in a multi-well scanning spectrophotometer and compared with DMSO blank and heat-killed worms as controls. The absorbance per mm of worm length was calculated and the results were expressed as total percentage of MTT reduction (index of viability).

4. Examination of the localization of the fluorescent IVM in adult worms using confocal microscopy

a. *Preparation of the drug dilutions:* Bodipy FITC-IVM was reconstituted in 1 ml DMSO (86.96 µM). Similarly, bodipy FITC was reconstituted in 100 µl of DMSO (171.22 mM). From these stock solutions, the following concentrations for working solutions were made - 0.5, 0.05, and 0.005 µM.

b. *Incubation of worms in Bodipy FITC-IVM / Bodipy FITC*: Male and female worms extracted after necropsy of the infected jird, were washed 4 times in RPMI medium containing HEPES and antibiotics (serum free medium) to remove host cell contamination. Male and female worms were divided into 2 groups, the experimental and the control groups. 4 worms, at each concentration, in both

groups were incubated in serum free medium containing either bodipy FITC-IVM or bodipy FITC, for 2 hours at 37°C, 5 % CO_2, and 95 % humidity. This was followed by washing in PBS (pH 7.2) containing 0.01 % Triton-X 100 for a half hour and further in PBS for 1 1/2 hrs. Worms were then mounted in the mounting medium and slides were observed in a fluorescent microscope (Nikon Eclipse 800) and confocal microscope (Nikon Eclipse 800, Confocal Biorad Radiance 2100) at 40X magnification. (All the confocal images were taken at the same settings.

5. Statistical Analysis

The statistical analyses were performed using the Sigma Prism (version 4) statistical program (GraphPad software program). *Two-way analysis of variance* was used to evaluate the effect of concentration and days after IVM treatment on daily release of mf by female worms. *Two-way repeated measures analysis of variance* was used to evaluate the effect of concentration over time on the motility (grid scoring) in both male and female worms. The Bonferonni post hoc test was used to compare different groups. *One-way analysis of variance* was used to analyse data for the MTT assay. Dunnet's post-hoc test was performed to compare different groups. A P value of ≤ 0.05 was considered significant.

Results

1. *Microfilariae released by female worms*

The females of control group worms showed a relatively stable release of mfs for 7 days (no significant effect of incubation time). Two-way ANOVA results showed a significant effect of IVM and days of IVM exposure on release of mfs ($P<0.0001$). Concentration of IVM accounted for 56.4 % and days of IVM exposure had 29.7 % effect on the daily release of mf (Table 1). Compared with the controls, the 1.0 μM and 0.5 μM concentrations of IVM showed significant effects on the release of mfs after day 2 of treatment ($P < 0.05$ and < 0.01 respectively) (Fig. 2).

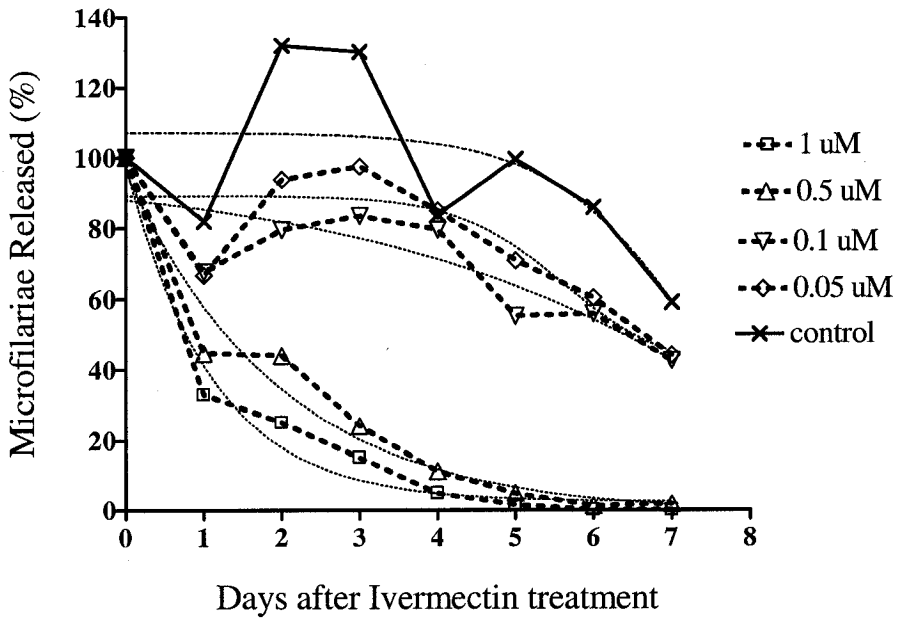


Figure 2– Daily release of microfilariae in a 7-day culture of *B. malayi* female worms under different concentrations of ivermectin, expressed as percentages of the number of pre-treatment (day 0) release. Each data point is an average of microfilariae output by six worms. Lines (……) represent non-linear fit of curve.

Factor	d.f.	SS	MS	*F* ratio	% of total variation	*P* value
Concentration	4	32860	8216	28.32	56.4	<0.0001
Days after IVM treatment	7	17310	2473	8.523	29.7	<0.0001
Residual (Error)	28	8124	290.1			

Table 1: Two-way ANOVA test for the daily release of mfs by *Brugia malayi* female worms (d.f. = degree of freedom, SS = sum of squares, MS = mean of squares). Results show concentration accounts for 56.4 % and days after IVM treatment accounts for 29.7 % of the effect on the mf release by female worms when compared with controls.

2. *Assessment of motility using grid analysis method*

The control worms had average motility scores of 3.25 – 3.5 grids per 2 seconds, in the case of female worms, and scores of 2 to 2.25 grids per 2 seconds in the case of male worms at days 1, 4, and 7 when motility was examined microscopically. Motility did not change significantly over the 7 day study period in the control worms. Since the motility score was consistent between different batches of untreated worms at all days of motility assessment, it was considered a reliable method of assessing motility. In the case of female worms, two-way repeated measures ANOVA results showed significant effects of IVM concentration, time and interaction, with concentration accounting for 18.2 % ($P < 0.0001$), time (days after IVM treatment) for 32.1 % ($P < 0.0001$), and interaction accounted for 20.6 % ($P < 0.0001$) of the total effect on motility (Figure 3a, Table 2). In the case of male worms, concentration had 12.9 % ($P=0.022$), time had 18.6 % ($P < 0.0001$), and interaction had 10.4 % ($P = 0.0032$) overall affect on the motility (Figure 3b, Table 3). Compared with the controls, concentrations of IVM $\geq$ 0.1 μM showed significant effects on the motility ($P < 0.001$ in case of female worms, and $P < 0.001$ – $P < 0.05$ in case of male worms). Compared to day 1, the affect on motility was more prominent at day 4 and 7, in both female and male worms. Overall, the effect of IVM on motility was greater in female worms than in male worms. In both female and male worms, the effect of IVM on motility accumulates with the length of exposure to the drug and the drug concentration.

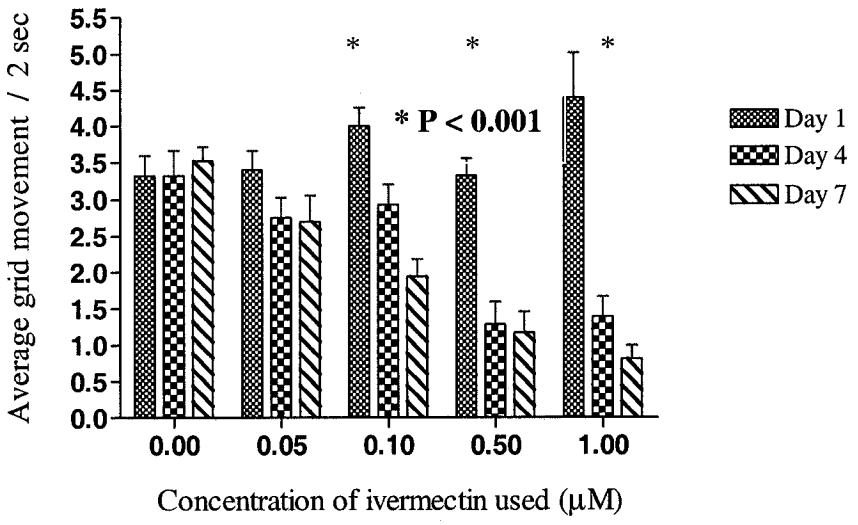


Figure 3a– Motility score of *B. malayi* female worms at days 1, 4, and 7 after exposure to different concentrations of ivermectin. Each bar represents the average grid score of 6 female worms, scored on that particular day in each treatment group. (Mean ± SEM; *, P < 0.001 from the post-hoc test value for ≥ 0.1 μM IVM treatment groups compared with the control incubations in the absence of drug).

Factor	d.f.	SS	MS	*F* ratio	% of total variation	*P* value
Concentration	4	26.05	6.51	11.43	18.2	<0.0002
Days after IVM treatment	2	46.63	23.32	40.93	32.1	<0.0001
Interaction	8	29.94	3.7	6.57	20.6	<0.0001
Residual (Error)	28	42.72	0.6			

Table 2: Two-way repetitive measures ANOVA test for the motility score of *Brugia malayi* female worms (d.f. = degree of freedom, SS = sum of squares, MS = mean of squares). Results show concentration accounts for 18.2 %, days after IVM treatment accounts for 32.1 %, and interaction accounts for 20.6 % of an effect on the motility of female worms when compared with controls.

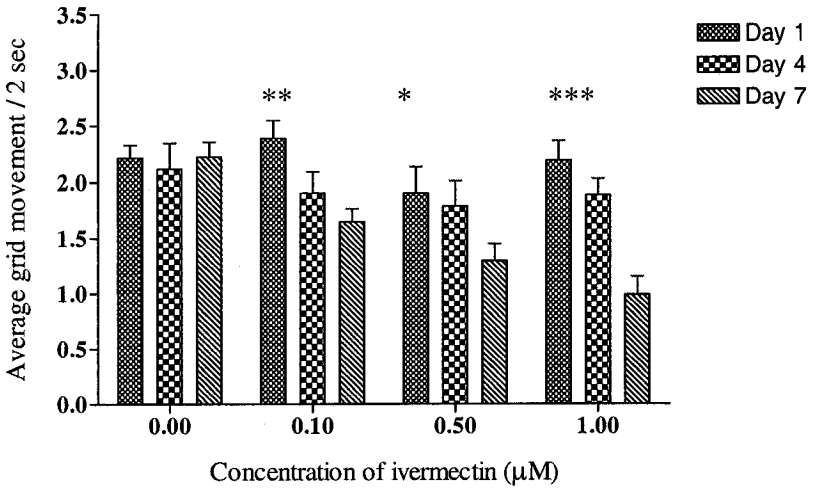


Figure 3b– Motility score of *B. malayi* male worms at days 1, 4, and 7 after exposure to different concentrations of ivermectin. Each bar represents average grid score of 8 male worms, scored on that particular day in each treatment group (Mean ±SEM). Post-hoc test P value are * 0.05, ** 0.01, and *** 0.001 for the corresponding treatment groups compared with the control incubations in the absence of drug.

Factor	d.f.	SS	MS	*F* ratio	% of total variation	*P* value
Concentration	3	4.5	1.5	3.7	12.9	<0.0227
Days after IVM treatment	2	6.5	3.3	20.21	18.6	<0.0001
Interaction	6	3.6	0.6	3.77	10.4	<0.0032
Residual (Error)	28	9	0.2			

Table 3: Two-way repetitive measures ANOVA test for the motility score of *Brugia malayi* male worms (d.f. = degree of freedom, SS = sum of squares, MS = mean of squares). Results show concentration accounts for 12.9 %, days after IVM treatment accounts for 18.6 %, and interaction accounts for 10.4 % of the effect on the motility of female worms when compared with controls.

3. *Assessment of viability by MTT reduction assay*

The effects of IVM on adult worm viability (as measured by the MTT test) are summarized in Figure 4 and Figure 5. Compared with the controls, drug treatment reduced the viability of both male and female worms ($P < 0.01$). Overall, the effect was concentration dependent and was greater in female worms than the male worms. In 0.5 μM and 1.0 μM of IVM, viability of female worms was decreased to 65 % and 52.5 %, respectively (Figure. 4a; $P < 0.05$ and $P < 0.01$, respectively) and to ~ 63 % in the case of male worms (Figure. 4b).

4. *Fluorescent and Confocal microscopy*

Confocal microscopy images of the worms are shown in the Figure 5, 6, and 7. Worms incubated in bodipy FITC-IVM showed a strong specific localization signal in the anterior cephalic region compared with the control groups incubated only in bodipy FITC. Comparison with the scanning electron microscopy image (Image adapted from Zaman, 1987, Figure 8b) of the head region, suggests that the drug is interacting with the cephalic papillae and amphids, which are the sense organs of the worms. These sense organs are associated with sensory neurons and fibres. The staining of the fluorescent drug could also be seen in the lateral nerve fibres, which runs along the lateral bodyline. There was no staining in the gut region of these filarial nematodes.

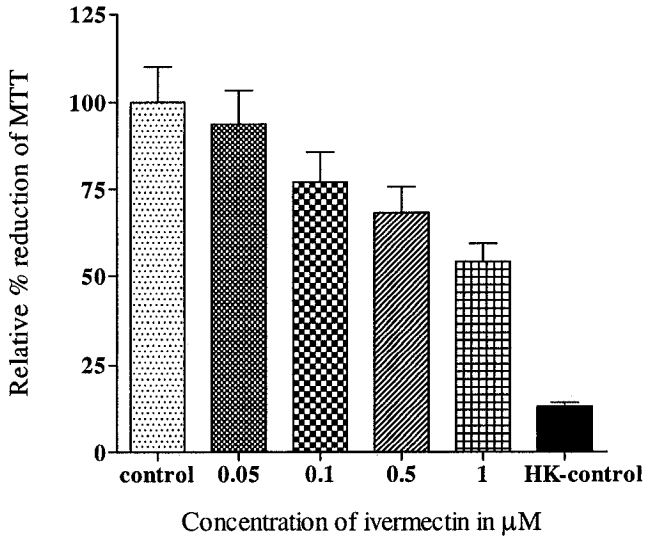


Figure 4a: Relative % reduction of MTT to formazan by *B. malayi* female worms after 7 day of culture with different concentrations of ivermectin. Each column is the average of 6 worms. (Mean ± SEM; HK = heat killed worms)

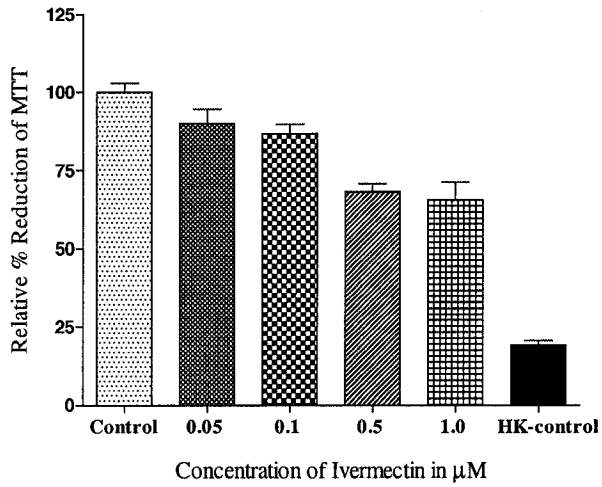
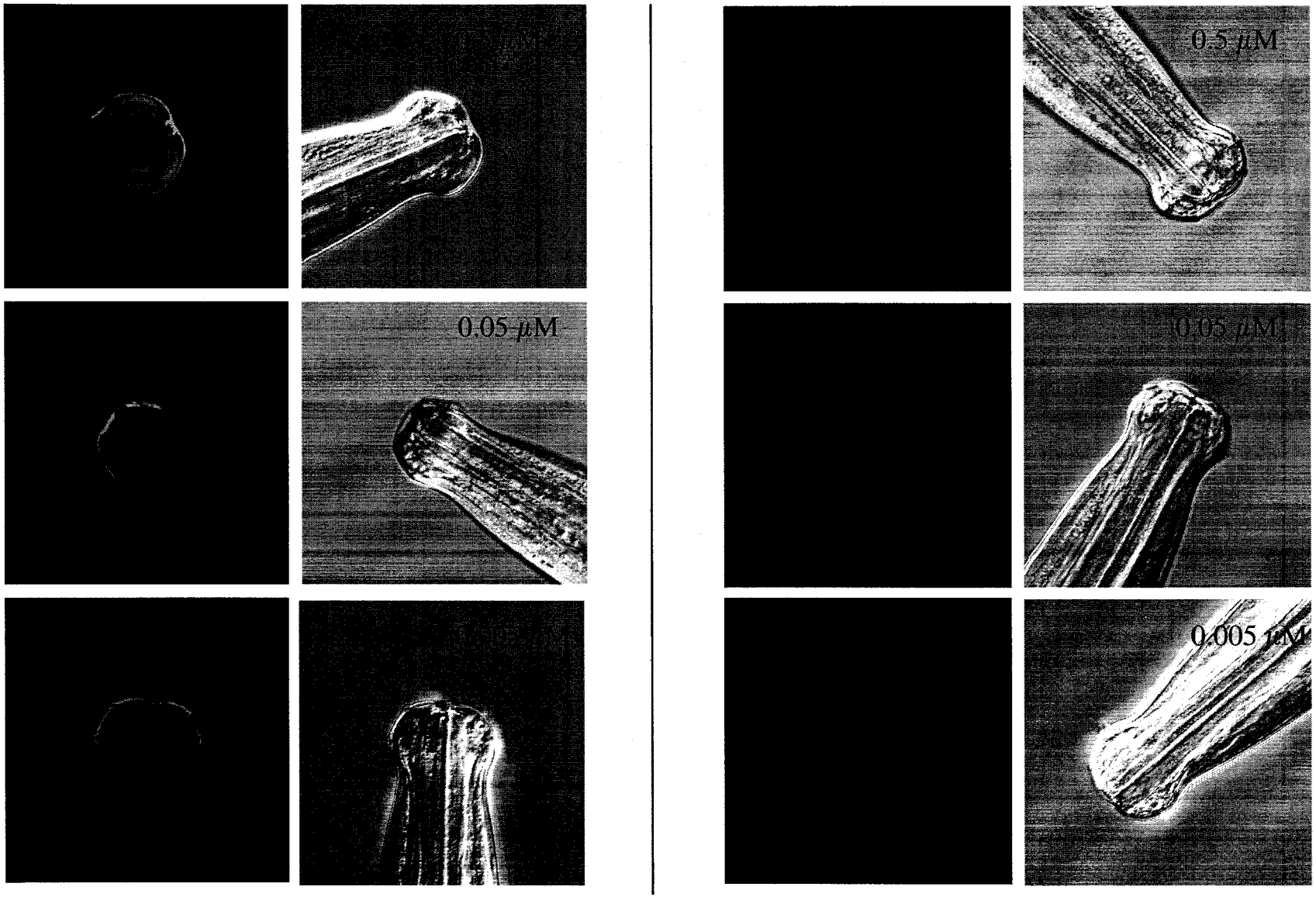
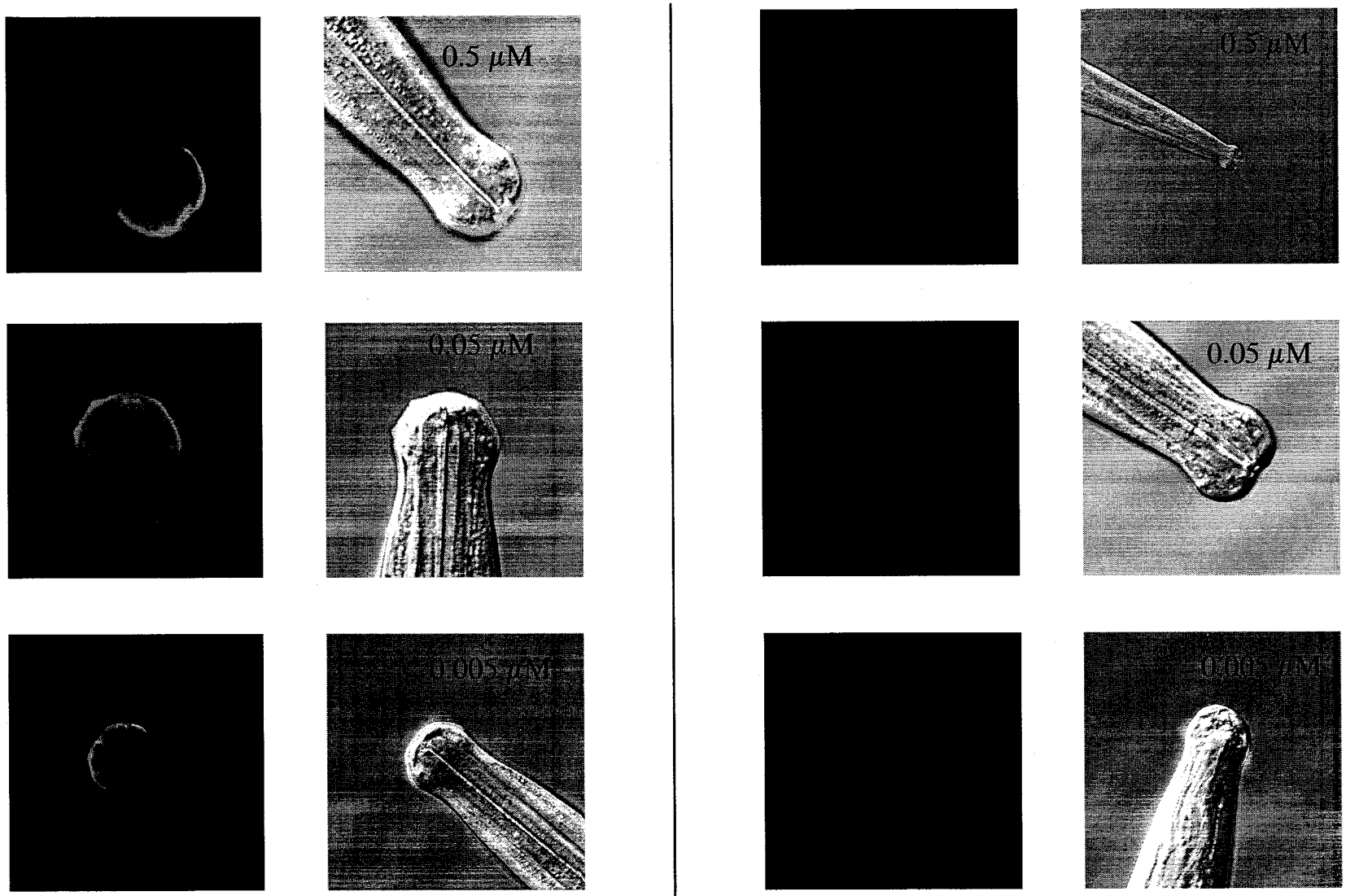


Figure 4b: Relative % reduction of MTT to formazan by *B. malayi* male worms after 7 day of culture with different concentrations of ivermectin. Each column is the average of 6 worms. (Mean ± SEM; HK = heat killed worms)



FITC-IVM **FITC only (control)**

Figure 5: Confocal microscopy images of the anterior region of *Brugia malayi* female worms



FITC-IVM **FITC only (control)**

Figure 6: Confocal microscopy images of the anterior region of *Brugia malayi* male worms

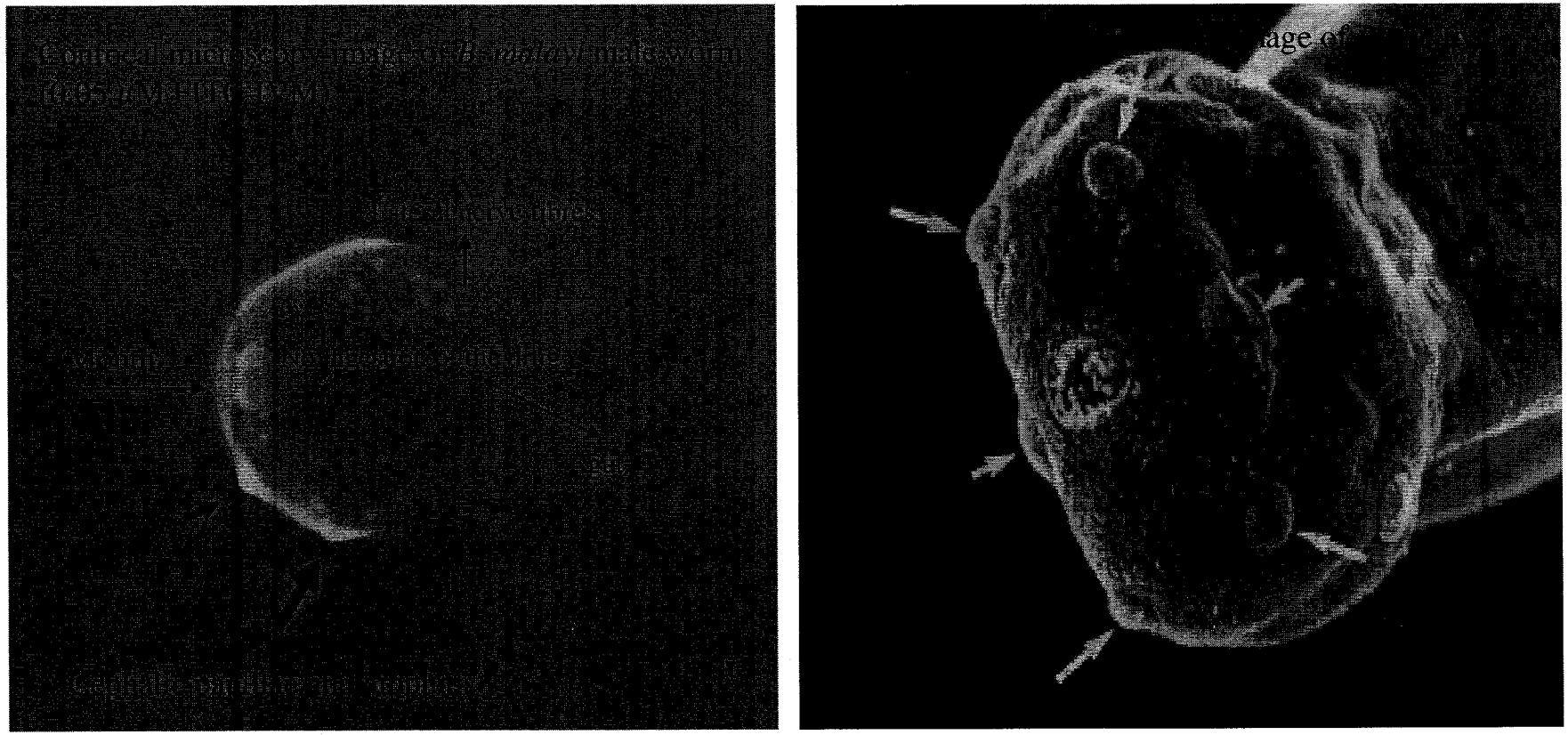


Figure 7: Interaction of bodipy FITC-IVM with sense organs of *B. malayi* worms (a) Confocal microscopy image (b) Scanning electron microscopy image (adapted from Zaman (1988)

Discussion:

1. Antifilarial effects of IVM on adult worms:

The *in vitro* effects of IVM against *B. malayi* adult male and female worms were studied in 7-day cultures of worms. The concentrations of IVM used were 1.0, 0.5, 0.1, and 0.05 μM. These concentrations were chosen because the *in vitro* experiments required a concentration of IVM that would have an observable drug effect, resulting in a single endpoint (eg. death, or loss of motility) being demonstrated within the limited timescale of 7 days. The antifilarial effects of IVM were examined using three parameters: mf release by female worms, and motility and viability in both male and female worms. The results reported in this study demonstrated that, although IVM did not kill the adult worm, it showed significant antifilarial effects on *B. malayi* adult worms when examined in an *in vitro* system.

Mf release by female worms

There was a significant effect of IVM on the release of mf by female worms upon exposure to IVM for 7 days. The effect on mf release was considerable after day two of IVM exposure at higher concentrations ($\geq$ 0.5 μM), suggesting that the drug did enter the worm, and that it takes at least 2 days for the reduction of mf to become apparent. This observation is similar to what is observed in filariasis patients after treatment with a single oral dose of 150 – 200 μg / kg of IVM. It is seen that the blood mf level goes down within 3-6 days after IVM treatment (Kazura, 1993; Campbell, 1991; Schulz-Key, 1986, 1999; Grant, 2000). The number of mf released by female worms remained consistently low till the end of 7 days of culture. The decrease of mf release by female

worms is likely due to IVM (known to act on muscle and nerve cells in nematodes) which might be causing the paralysis of muscles associated with vulva, thereby preventing the mf ejection or release through the vulva (Wolstenholme et al., 2005; Schulz-Key, 1986, 1990; Grant, 2000; Stolk et al., 2005). Such effects are also observed in *O. volvulus,* a related filariae (Mossinger et al., 1988; Albiez et al., 1988a, 1988b). Low concentrations (≤ 0.1 μM) had no significant effect on mf release. This observation gives rise to the question whether low concentrations of IVM do not have any effect on adult worms? We speculate that there might be an affect which was difficult to discern from the present 7-day *in vitro* assay. An extended time of more than 7 days may be required to observe the affect on mf release by *B. malayi* female worms at low concentrations. However, filarial worms can be maintained *in vitro* for limited periods only (Engelbrecht et al., 1984). Since the intrauterine contents of the female worms were not examined, it is difficult to say what happens to unreleased mfs and if there was any affect on the development of embryos, due to the effect of IVM. However, we observed that the anterior regions of female worms became clear by the end of the 7 days of exposure, suggesting that unreleased mfs present in the anterior region might degenerate during this period. The mechanism by which IVM prevents the release of mfs by female worms remains unclear.

Motility

The extent of paralysis caused by IVM is an important measure of the effect of the drug on adult filarial worms. The effect of IVM on motility (extent of paralysis) of filarial worms is not well described. Some of the objectives that we addressed in the

present *in-vitro* system included developing a quantitative assay to measure drug effects on filarial motility and the determination of sex-specific effects on this parameter.

Most of the *in vitro* studies, reporting the effect of IVM (or effect of any antifilarial drug *per se*) on motility of adult worms or mf, have been based on scoring the motility (such as 0, non-motile; 1, motile; 2 highly motile). This classical method of scoring is based on subjective description of the worm motility through simple visual inspection (Townson et al., 1989, 1990; Rao et al., 2002). However, in the present study it was difficult to discern the motility effects with visual inspection of the culture wells, except at the drug concentration $\geq$ 0.5 μM. Motility is an index of paralysis and an important factor for understanding the extent of damage caused by IVM. We wanted to gain a quantitative understanding of the effect of IVM on motility. Therefore, we attempted to develop a different means of motility scoring, using a grid analysis method as described in the Materials and Method section. Since the motility score was consistent between different batches of untreated worms, at all days of motility assessment, it was considered a reliable method of assessing motility.

We examined motility microscopically on days 1, 4 and 7 of IVM exposure to reflect changes taking place in motility over time. By using the grid analysis method it was observed that in the presence of IVM, there was a significant decrease in the motility of both male and female worms, implying that IVM did affect the motility of adult stages *in vitro*.

There are very few reports concerning the pharmacokinetics of IVM in human; the peak plasma concentration of IVM measured in non-infected humans after a conventional treatment with IVM (200 μg / kg) is 60 nM at 3 h (Bennett et al., 1988;

Campbell, 1991). If we take into account the plasma concentration of IVM, the motility observations are comparable to studies carried out by Dreyer et al. (1995, 1996) in one of the clinical trial studies for Bancroftian filariasis. Dreyer el al. examined the adulticidal effects of IVM given at single oral dose of 400 μg / kg (~120 nM) (Dreyer et al., 1995), or at a 2 week interval, by using the ultrasound method (Dreyer et al., 1996) monitoring the movements of adult worms (termed 'the filaria dance sign'; Amaral et al., 1994). They did not detect any significant effect of IVM treatment on adult *W. bancrofti* as movements characteristic of the adult worm on ultrasound recording remained unchanged. In another study Ismail et al. (1996) reported that high doses of IVM (12 fortnight doses at 400 μg / kg; ~ 4.8 μM) showed a significant macrofilaricidal activity against *W. bancrofti.* These observations suggested that IVM might have adulticidal effects on filarial nematodes at higher IVM concentrations. We observed that $\geq$ 0.1 μM had significant effects on the motility of both male and female worms and that this effect was more prominent after day 4 of IVM exposure in both groups. These observations suggested that the effect of IVM on locomotion, in adult stages, accumulates over time. Another important observation was that the effect of IVM on motility was greater in female worms than in male worms. Why IVM is less effective in male worms than in female worms and how IVM interferes with motility / locomotion in filarial worms are not clearly understood. A possible explanation for the differences in the motility of male and female worms could be due to differences in the number of receptors for IVM. For example, GluCls are known to be involved in locomotion/ paralysis in non-filarial nematodes such as *Caenorhabditis elegans* and *Haemonchus contortus* (Geary et al.,

1993; Arena et al, 1995; Wolstenholmes et al., 2005; Cook et al., 2006) and the GluCls might be differentially expressed in male and female worms (Li et al., 2004).

MTT Assay

The MTT assay quantitatively evaluates the viability of parasites in the presence of a drug (Comley et al., 1989a, 1989b). The assay carried out at day 7 revealed that IVM caused a significant decrease in the viability of both male and female worms; the decrease was greater in female worms than in male worms. This could be due to degenerating mfs in the uterine tube of female worms that were not released by female worms due to IVM exposure. We examined motility on days 1, 4, and 7, where as the MTT assay was done at the end of day 7. Although the MTT assay results were in accordance with motility results, it is important to understand that, while motility reflects the muscular coordinated activity, viability reflects irreversible degenerative changes caused by drug exposure.

These results clearly showed that there was a significant decrease in the motility as well as viability of the adult worms in the presence of IVM at concentrations ≥ 0.1 μM and that there was a considerable decrease in mf release by female worms upon IVM exposure. These effects reflect the signs of drug activity in *B. malayi* adult worms *in vitro*. Overall, we observed that 0.5 μM was a threshold limit in the present *in vitro* system. This is a high concentration if it is compared to the concentration of IVM needed to paralyse a non-filarial nematode, such as *H. contortus* (~ 1 nM) (Geary et al., 1993), in an *in vitro* system. However, it is important to note that there are relevant differences between the two parasites, such as biological features of the parasite, location within the

host (*H. contortus* is located in the abomasum and the filarial nematodes in the lymph nodes), and host-parasite relationships. On the other hand, it is also a high concentration when it is compared to the peak plasma concentration of IVM in humans (60 nM) (Bennett et al., 1989). However, due to the limitation of the culture conditions in the *in vitro* system, we chose a high concentration of IVM. Also, the duration of exposure was an important factor for the antifilarial effect of IVM on the nematodes.

2. Confocal microscopy

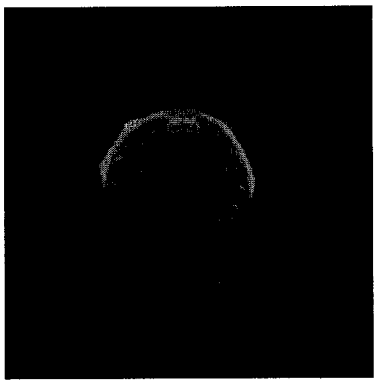
We wanted to observe the localization of IVM in adult worms which could reflect light on the probable target receptors located in the worm body. The concentrations used were 0.5 μM, 0.05 μM, and 0.005 μM of bodipy FITC-IVM and of bodipy FITC. The first two concentrations were also used in the *in vitro* experiments, where 0.5 μM IVM showed a significant antifilarial effect on the adult stage in comparison to 0.05 μM IVM. Confocal microscopy images of the worms incubated in bodipy FITC-IVM showed a strong specific localization signal in the anterior cephalic region of both male and female worms. The localization was observed at all concentrations of bodipy FITC-IVM used. We did not observe such localization in any other regions of the worm body, such as cuticle, reproductive tissues, or in the gut region. These observations suggest the early / initial interactions of the drug with its probable receptors, and that these could be located specifically in the head region. Further understanding comes from the fact that the cephalic region is associated with the sense organs, such as cephalic papillae and amphids. These sense organs are associated with sensory neurons and fibres which are lipid rich regions. The staining of the fluorescent drug was also observed in the lateral

nerve fibres, which run along the lateral bodyline. It could be possible that the drug is taken in by amphids which are considered as chemoreceptors (Strote et al., 1996, 1997). We report for the first time evidence of an interaction of bodipy FITC-IVM with the sense organs, the cephalic papillae and amphids in *B. malayi.*

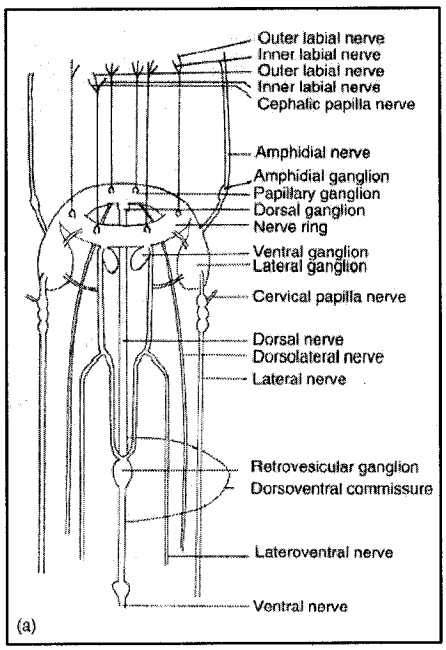
3. Correlating the antifilarial effects observed and confocal microscopy results

The present study reflected light on two very important observations that IVM affects the adult worms of *B. malayi* and the possibility of drug uptake through amphids and the location of probable drug receptors in the head region. How can we correlate the drug uptake observation with the *in vitro* antifilarial effects? This is explained in Figure 8.

IVM could be taken in or absorbed through amphids located in the cephalic region [Figure 8(a)]. Amphids are associated with neurons and nerve fibres from the nerve ring or circumesophageal commissure (Strote et al., 1996, 1997; Roberts et al., 2005). The nerve fibres originating from the nerve ring might be involved in the transmission of signals from the neurons located in the anterior region of the worm to the posterior regions via nerve and muscle junctions [Figure 8(b)] (Roberts et al., 2005). This might enervate other receptors sensitive to IVM, such as GluCls, GABA, or hypothetical reproductive target etc., located on the nerve and muscle cells. The combination of these targets, working synergistically or independently, might be responsible for effects on motility, viability, and reproduction in female worms. It could also be possible that such receptors are differentially expressed in male and female worms that could be responsible for the differences in the anthelmintic effects in male and female worms.



(a) Confocal microscopy image of the head region of *B. malayi* worm depicting localization of fluorescent labelled IVM



(b) Schematic diagram of the nervous system of a nematode (Anterior end), possessing probable target receptors of IVM

Figure 8: Correlation between drug uptake and antifilarial effects observed in *B. malayi* adult worms

There are certain observations that support this hypothesis and the interpretation of our results. First, the involvement of amphids in the process of IVM uptake has been well observed in *C. elegans* and *H. contortus.* It has been reported that the *strains* of *C. elegans* displaying low-level resistance to IVM have an amphid defect called Dyf (dye-filling negative) (Blaxter et al., 1997). Second, Freeman et al., (2003) described the amphidial structure of macrocyclic-lactone-resistant *H. contortus.* On microscopic examination of the amphidial regions of IVM-susceptible and IVM-resistant *H. contortus,* it was shown that the IVM-resistant worms had markedly shorter sensory cilia than their IVM-susceptible parental counterparts. Additionally, the amphidial neurons of IVM-resistant worms were characterized by generalized degeneration and loss of detail, whereas other neurons outside of the channels, such as the labial and cephalic neurons, were normal in structure. These findings highlight the possible relationship between amphidial structure and macrocyclic lactone resistance as well as the role of amphids as a means of entry for these molecules (Freeman et al., 2003). Third, GluCls and their subunits are known to be involved in the mechanism of IVM action and these subunits are widely distributed along the nervous system of nematodes, such as *C elegans*, *H. contortus* (Cully et al., 1994; Arena et al., 1995; Dent et al; 1997; Yates et al., 2003; Portillo et al., 2003; Wolstenholmes et al., 2005; Cook et al., 2006). Portillo et al. (2003) have shown the distribution of GluCl subunits (HcGluClα, HcGluClβ, HcGluClα3a, and HcGluClαb) in *H. contortus* nervous system. Most of these subunits were expressed on the motor nervous system, especially motor neuron commissures, amphid neurons, lateral and sublateral, and ventral nerve cords and have critical roles controlling locomotion, pharyngeal pumping, and sensory processing in nematodes. Recently, Cook et al. (2006)

have reported the involvement of *C. elegans* IVM receptors (GluClα subunits) in locomotor behaviour and have suggested that IVM exerts its paralytic action on parasitic nematodes through activation of GluCl channels in the motor nervous system. Although some GluCl subunits have been reported in filarial nematodes, e.g. Cully et al, 1994 reported *Ov*GluClα3 subunit from *O. volvulus* and Yates et al. 2004 reported *Di*GluClα3 subunits from *Dirofilaria immitis* (filarial nematode infecting dogs). However whether they are involved in controlling locomotion in the presence of IVM is not known. Fourth, Li et al., (2004) reported quantitative analysis of gender-regulated transcripts in the filarial nematode, *B. malayi* by real time RT-PCR to have a better understanding of the biology of reproduction in filarial worms. It is interesting to note that the gene expression of glutamate-gated chloride channels in *B. malayi* female worms was expressed at a higher level than in male worms. However, it is not clear what roles GluCl proteins play in filarial reproduction or locomotion (43). Is the effect of IVM on reproduction also observed in non filarial nematodes? A few reports cite examples where IVM affects the fertility of other nematode species such as *H. contortus* (50, 71) and *C. elegans* (50). In these cases, IVM appears to inhibit the release of eggs already *in utero* and suppresses the production of new eggs. Grant, 2000 suggested reproduction by female nematodes is another relevant target of IVM, i.e., a target where acute exposure to the drug results in a biological effect on the worm.

Summary and Conclusion

This study makes an attempt to understand the biological effects of IVM on the adult stages of *B. malayi*, a filarial worm. The present *in vitro* studies have shown that

IVM has significant effects on the adult stages thereby the release of mf by female worms, motility, and viability are affected. The accumulation of fluorescent labelled IVM in the head region suggests that IVM may be interacting with the probable receptors located in the cephalic region which could lead to the significant effects on mf release by female worms, motility, and viability. These observations are consistent with the hypothesis that we have proposed to account for the results that we have observed from the *in vitro* effects of IVM on *B. malayi* adult worms.

These initial *in vitro* studies on the effects of IVM on adult *B. malayi* provide a platform for further studies to unravel the effects of IVM on filarial worms at the molecular level. It will be interesting to look at the interaction of IVM with these receptors at molecular level.

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Appendix

Environmental Safety Documentation