EFFECT OF TREATMENT WITH BENZIMIDAZOLE ANTHELMINTICS ON THE VIABILITY OF *BRUGIA MALAYI*: *IN VITRO* AND *IN VIVO* STUDIES

by

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(Under the Direction of Ray M. Kaplan)

ABSTRACT

The concentration and time point at which lethal effects in *Brugia malayi* adult female worms are observed following treatment with different concentrations of thiabendazole were studied *in vitro* by measuring worm motility, microfilariae release and MTT assay. Results showed that- worm motility and microfilariae release assays are the best indicators of worm viability, still leaving worms usable for gene expression studies; and motility assay determines worm viability quicker than mf release assay for gene expression studies. The concentration and time points, at which *B. malayi* worms show varying level of susceptibility to albendazole (ABZ), were determined *in vivo*. Moderately active worms with increased drug susceptibility at 21 days post-treatment (DPT) and highly active worms with greater tolerance at 28 DPT were selected. RNA was extracted from these worms for microarray analysis to gain basic knowledge of gene expression in *B. malayi* that survived the treatment compared to worms susceptible to ABZ.

INDEX WORDS: Lymphatic filariasis, *Brugia malayi*, benzimidazole, worm viability, *in vitro* and *in vivo*

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B.V.Sc. & A.H., ANGRA University, India, 1999.

A Thesis Submitted to the Graduate Faculty of The University of Georgia in Partial Fulfillment of the Requirements for the Degree

MASTER OF SCIENCE

ATHENS, GEORGIA

2008

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December 2008

DEDICATION

To my wife, Prachi, our son, Saket and our parents for their love and support.

ACKNOWLEDGEMENTS

I would like to thank my major professor Dr. Ray M. Kaplan for his able guidance and endless patience. He has been a constant source of inspiration and challenge for me all through my graduate study. I would like to thank my committee members Drs. Julie M. Moore, David S. Peterson and Michael Dzimianski for their invaluable suggestions. I would also like to thank my lab colleagues Drs. Dzimianski, Supakorndej, Moorehead, Mr. Bob Storey and Ms. Sue Howell for their much-needed technical help in the laboratory during my research .

My time during graduate study would have been very tedious and monotonous without the friendship of Dhanu, Srujana and Krishna. Finally, I would like to thank my family for providing encouragement and moral support during the completion of this work.

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CHAPTER I

LITERATURE REVIEW

1.1 Parasite Biology and Disease

Lymphatic filariasis (LF) is a disease caused by vector borne, filarioid nematode parasites *Wuchereria bancrofti*, *Brugia malayi and Brugia timori* (Mark, 1986). The long and thin adult worms (macrofilariae) reside in the lymphatic system. Female worms measure 43-55 mm in length and 130-170 μm in width, and male worms measure 13 to 23 mm in length by 70 to 80 μm in width. Fertilized females produce millions of larval forms, called microfilariae (mf), measuring 177 to 230 μm in length and 5 to 7 μm in width, which are covered by an external cuticular sheath and have nocturnal periodicity. The mf migrate from the lymph to the blood stream, reaching peripheral blood vessels where they are ingested by female mosquitoes during a blood meal. Over an approximately 2 week period, the mf molt twice to become 2nd and 3rd stage larvae (called the 'L3'). The L3 is the 'infective stage' which are deposited onto the skin of an another host during a subsequent blood meal. The L3 then invades the subcutaneous tissues and migrates to the lymphatics where it matures to the adult stage which live there for seven or more years. These adult worms may find other adults of the opposite sex, and mate resulting in the production of mf, thus continuing the life cycle.

A number of mosquito genera and species are able to transmit filariasis, but the two main forms of the disease, bancroftian and brugian filariasis, have distinct primary vectors. The most prominent vector of bancroftian filariasis, caused by *W. bancrofti* and responsible for most

(90%) cases of human filariasis, is *Culex quinquefasciatus,* a mosquito that thrives in urban areas and villages of tropical and subtropical regions (Mak, 1986). Brugian filariasis is caused by *B. malayi* and its main vectors belong to the genus *Mansonia* (Evans et al., 1993). *B. malayi* is limited to Southeast and Eastern Asia (Melrose, 2002) and accounts for 10% of filariasis worldwide. Since, the vectors of *B. malayi* require freshwater plants for their larvae, they are confined to rural regions (Evans et al., 1993).

Traditionally, filarial-infected people are grouped into one of the following categories at a given time: (1) endemic normal- no mf present in the bloodstream and no clinical signs; (2) asymptomatic microfilaraemia-mf found in the blood, but no clinical signs; and (3) chronic filarial disease- mostly amicrofilaraemic, with or without acute attacks (Dasgupta, 1984; Partono, 1987; Ottesen, 1992; Evans et al., 1993; Ottesen, 1994). But people affected with any of these conditions may also suffer from acute filarial disease (Melrose, 2002).

'Endemic normal' refers to people who, despite constant exposure to filariasis and circulating filarial antibodies, are amicrofilaraemic and have no clinical evidence of disease (Ottesen, 1989). But, these individuals may have cryptic infection due to the presence of circulating filarial antigen (Weil et al., 1996). Asymptomatic microfilaraemia is the most commonly seen form of filariasis and despite initial absence of obvious clinical disease, damage to lymphatics and organs may occur in people suffering from it (Freedman et al., 1995a; Freedman et al., 1995b; Senarath et al., 1995). Chronic disease is often manifested as lymphedema (with swollen limbs) progressing to elephantiasis in chronic brugian lymphatic filariasis and hydrocele in chronic bancroftian filariasis (Evans et al., 1993; Witte et al., 1993). Uncommonly, chyluria and Tropical Pulmonary Eosinophilia (also known as 'occult filariasis') may be seen in chronic filariasis (Evans et al., 1993). Tropical Pulmonary Eosinophilia

symptoms may include cough dyspnea and may be similar to asthma symptoms (Pinkston et al., 1987). Acute filarial disease usually begins in late childhood or early adulthood and may be caused by dermatolymphangioadenitis (with the development of a plaque-like skin lesion and inflammation of lymphatics) or filarial lymphangitis (due to immune reactions to dead or dying adult worms killed by host immune response or chemotherapy) (Dreyer et al., 1996a; Dreyer et al., 1996b). This type of filariasis is systemically manifested as fever, headache, chills, nausea, vomiting or loss of appetite (Nanduri and Kazura, 1989; Evans et al., 1993).

1.2 The social and economic impact of lymphatic filariasis

Filarioid nematodes are among the most important parasites affecting both animals and human health worldwide. The World Health Organization (WHO) estimates that 120 million people are currently infected, with 1.3 billion people at risk of infection in 83 countries throughout the tropics and sub-tropics of Asia, Africa, the Western Pacific, and parts of the Caribbean and South America. The e estimated Disability Adjusted Life Years (DALY) burden due to LF is 5.55 million (Molyneux et al., 2003). WHO has considered LF as the second leading cause of permanent and long-term disability worldwide (WHO, 1997c, b).

Lymphatic filariasis is an endemic disease and a major cause of acute and chronic morbidity and incapacitation with devastating public health and socio-economic consequences, associated with conditions of poverty. In 2003, the World Bank classified eighty percent of LF endemic countries as low or lower-middle income countries (www.worldbank.org/data/countryclass/classgroups.htm). Rural to urban migration and urbanization, occurring increasingly in low income countries, facilitate the spread of LF. This is mostly due to inadequate waste disposal and sanitation facilities, which increase the number of breeding sites for the mosquito vectors (Hunter, 1992; Dhanda et al., 1996). It has been

calculated that LF causes billions of dollars in lost productivity in India (Ramaiah et al., 2000a) and Africa (Haddix and Kestler, 2000). Reduced productivity from LF disability has been documented in rubber tappers in Malaysia (Kessel, 1957) and agricultural workers in Guyana (Gigliolo, 1960). In endemic rural regions of India, three-fourths of chronic LF patients spend 2% of the average yearly wage on treatment for their conditions and approximately 45% of the total expenditure on medicines (Rao et al., 1982; Ramaiah et al., 2000b; Babu et al., 2002). Stigma and discrimination are byproducts of LF that lead to reduced prospects of both marriage and a normal sexual life (Kessel, 1957). Thus, research on filariasis will have tremendous social and economic benefits.

1.3 Chemotherapy and control programs

Two decades ago three barriers to LF elimination existed: lack of- a) tools necessary to interrupt transmission and halt disease progression; b) understanding of disease dynamics and pathogenesis; and c) public awareness of LF's impact and public commitment to overcome it (Ottesen, 2000). In response to this global health crisis, a public-private partnership was developed for the control of LF (WHO, 1997a). This led to the establishment of the Global Program to Eliminate Lymphatic Filariasis (GPELF), which is largely based upon the regular administration of community-wide mass chemotherapy of the entire at-risk population with the anthelmintic albendazole (ABZ) in combination with ivermectin (IVM) or diethylcarbamazine citrate (DEC) (WHO, 1997a, 1999).

Three events became critical building blocks of this program's foundation. First, in 1993, the International Task Force for Disease Eradication reviewed approximately 100 medical conditions (mostly infectious diseases) and six of these diseases, including LF, were categorized as eradicable or potentially eradicable with the tools then available (CDC, 1993). This created

awareness and interest in LF elimination and enhanced its plausibility among the scientific and public health communities. Second, in 1997 the World Health Assembly (WHA) called for the countries to strengthen activities toward eliminating LF as a public health problem and mobilize support for global and national elimination activities. The importance of this resolution was key in its collective recognition of both the achievements to date and the potential benefits to be gained from local and global LF elimination programs. Third, following on the heels of the WHA resolution (50.29), which called for the formation of a public health program focusing on elimination of LF, three additional events catalyzed an exponential increase in the number of persons treated for LF. These events were: 1) donation of ABZ globally by GlaxoSmithKline; 2) donation of Mectizan® (IVM) (Merck and Co. Inc.) for countries where LF and onchocerciasis (river blindness) are co-endemic, for as long as needed and; 3) a US $20 million grant from the Bill & Melinda Gates Foundation for LF elimination (Ottesen et al., 1997; Molyneux et al., 2000). This led to the establishment in early 2000 of the GPELF, which had two major goals: to interrupt transmission of the parasite and to alleviate and prevent both suffering and disability caused by the disease.

To interrupt transmission of infection, the entire 'at risk' population (WHO, 1997a, 1999) must be treated for a period long enough to ensure that levels of mf in the blood remain below those necessary to sustain transmission. Three drugs shown to be safe and effective in killing the mf are DEC,IVM and ABZ (Addiss et al., 1997; Eberhard et al., 1997; Ismail et al., 1998) and their yearly, single-dose regimens include combinations of- ABZ (400mg) and IVM (150-200 mg/kg/body wt) or ABZ (400mg) and DEC (6mg/kg/body wt) (http://www.filariasis.org).

The principal strategy to alleviate suffering and decrease the disability caused by LF is focused on decreasing secondary bacterial and fungal infections in LF. Secondary infections may worsen the lymphedema and elephantiasis in genitals and limbs-with lymphatic function compromised by filarial infection. Hygiene in treatment of affected areas and creating hope and understanding among the patients, caregivers and their communities are also used (Mathieu et al., 2004).

1.4 Benzimidazoles

Anthelmintics are drugs that expel helminths which include cestodes, nematodes and trematodes from the body, by either killing or paralyzing the organisms. Three major classes of anthelmintics include: benzimidazoles (BZ), tetrahydropyrimidines, and macrocyclic lactones. Benzimidazoles are widely used for the control of nematode infections in animals and humans, and include the drugs ABZ, mebendazole (MBZ) and thiabendazole. Benzimidazoles are commonly used because of their high therapeutic index, low price and the absence of toxic residues in food animals (Brown, 1961; McKellar and Scott, 1990). The first BZ to be developed and licensed for animals use in sheep in United States of America was thiabendazole in 1961(Brown, 1961). These drugs are believed to act by binding to the cytoskeletal heterodimeric protein- tubulin, and blocking the formation of the microtubule matrix-an essential component of all eukaryotic cells (Friedman, 1979; Lacey, 1988b). Microtubules play an important role in several important cell functions such as cell division, structure, motility and intracellular substrate transport. The tubulin dimer is comprised of two closely related soluble proteins, α and β-tubulins (Lacey, 1988b). When α-/ β-tubulins with bound BZ are incorporated into the growing end of the microtubule, further heterodimers are prevented from being associated. Since dissociation continues at the opposite end, the microtubules become

destabilized and finally degraded (Lacey, 1988b, 1990; Lacey and Gill, 1994a). β-tubulins consist of a highly conserved polypeptide framework, with regions of distinctly clustered amino acid substitutions, particularly at the carboxyl terminus (Lopata and Cleveland, 1987; Sullivan, 1988). β-tubulins are grouped into six isotype classes in birds and mammals based on differences in carboxy-terminal sequences and each isotype represents a structurally unique protein sequence encoded by a distinct member of the gene family (Sullivan, 1988).

Several nematodes, including *C. elegans* (Driscoll et al., 1989), *B. pahangi* (Guenette et al., 1991), *H. contortus* (Geary et al., 1992a) and *Trichostrongylus colubriformis* (Grant and Mascord, 1996), possess more than one β-tubulin isotype, but except for some differences in carboxy-termini, β-tubulin sequences are highly conserved in them (Driscoll et al., 1989; Guenette et al., 1991). Despite close sequence homology, nematodes and mammals are differentially sensitive to tubulin inhibitors like BZs, which bind differentially to nematode tubulin compared to mammalian tubulin (Lacey, 1988b; Geary et al., 1992a). This may at least partially account for the selective clinical utility of BZs in controlling nematode infections (Geary et al., 1992b).

Of all the BZ drugs, ABZ is the one most commonly used in LF elimination programs. The filariasis elimination strategy requires a community-wide treatment in endemic areas of all ‘at risk' for infection and not just those with documented filariasis (Ottesen et al., 1997). Albendazole, which is co-administered with either DEC or IVM, has micro- and macrofilaricidal effect (Ottesen et al., 1999) and is also effective in treating intestinal helminth infections (Albonico et al., 1994). Therefore, the inclusion of ABZ into a two-drug treatment regimen for the control or elimination of LF may result in a public health impact far greater than LF elimination alone (Ottesen et al., 1999).

1.5 Benzimidazole resistance

Drug resistance is defined as 'when a greater frequency of individuals in a parasite population, usually affected by a dose or concentration of compound, is no longer affected or a greater concentration of drug is required to reach a certain level of efficacy' (Wolstenholme et al., 2004). This is caused by a change in gene frequency of that population resulting from drug selection and alleles for resistance being inherited by the next generation (Prichard et al., 1980).

Molecular mechanisms of BZ resistance are very well studied compared to other major anthelmintic classes (Gilleard, 2006). It has been discovered that certain β-tubulin sequence polymorphisms that result in amino acid sequence changes and the loss of high affinity receptor binding sites cause resistance against the BZs (Lacey and Gill, 1994b). A single nucleotide polymorphism (SNP) at codon 167 or 200 of the β-tubulin isotype-1 results in the change to amino acid tyrosine (TAC) instead of phenylalanine (TTC) in resistant isolates as compared to the susceptible worms (Kwa et al., 1993b, 1994; Prichard, 2001; Silvestre and Cabaret, 2002), though substitution at position 200 is more common in parasitic nematodes (Pape et al., 1999; Prichard, 2001; Silvestre and Cabaret, 2002). This subtle mutation was repeatedly found to correlate with drug resistance in several nematodes including *H. contortus* (Kwa et al., 1993b, 1994; Kwa et al., 1995), *Caenorhabditis elegans* (Driscoll et al., 1989; Kwa et al., 1995), *Cooperia oncophora* (Njue and Prichard, 2003) and some equine small strongyles (Pape et al., 2003; Drogemuller et al., 2004b). However, there is increasing evidence that the situation is more complex and other loci may be involved (Lejambre et al., 1979; Kwa et al., 1993a; Kwa et al., 1993b; Roos et al., 1995; Anderson et al., 1998; Sangster et al., 1998; Prichard, 2001). SNPs, also resulting in phenylalanine to tyrosine exchange at codon 167 of the β-tubulin isotypes 1 and 2 in *H. contortus* (Prichard, 2001) and *T. circumcincta* (Silvestre et al., 2001), or only isotype 1

in equine cyathostomins (Drogemuller et al., 2004a), have been found to be closely linked with BZ-resistance. Furthermore, β-tubulin isotype 2 deletion has also been associated with high levels of BZ-resistance (Kwa et al., 1993a).

Several reports show an evidence of BZ resistance in human parasitic nematodes too. Evidence of reduced efficacy of BZ anthelmintics against geohelminths has been reported, but has yet to be confirmed by biological or molecular tests (Albonico et al., 2004). In the human filarial nematode, *W. bancrofti*, the Phe200Tyr SNP has been found in β-tubulin and its prevalence increased dramatically in microfilariae obtained from patients in Burkina Faso, after two yearly doses of combination treatment with ABZ and IVM (Schwab et al., 2005).

There is a large body of experimental data demonstrating that the β-tubulin isotype-1gene is strongly associated with BZ resistance in parasitic nematodes (Roos, 1990; Roos et al., 1990; Kwa et al., 1993a; Kwa et al., 1993b; Roos et al., 1995; Elard and Humbert, 1999). However, it is believed that BZ resistance is more complex than this, and is likely polygenic (involving more than one gene). Since worms gain higher levels of resistance with increased drug selection pressure, only a single gene point mutation could not be solely responsible for this phenotype. Benzimidazole resistance is semi-dominant and autosomal in character in *H. contortus,* and has been suggested to be inherited by more than one gene (Lejambre et al., 1979; Herlich et al., 1981; Anderson et al., 1998). Apart from such target gene (β-tubulin) changes, it has been shown that inhibitors of P-glycoprotein can increase the sensitivity of trichostrongylid nematodes to benzimidazoles suggesting that drug metabolism and drug efflux mechanisms could also play a role in BZ resistance (Gilleard, 2006).

Why should we be concerned about BZ resistance developing in human filarial nematodes?

Benzimidazole anthelmintics have been used extensively in veterinary medicine over the past few decades, but unfortunately resistance to these drugs has made them useless against most of the important nematode parasites of livestock (Kaplan, 2004).

Mass drug administration programs against human helminth parasites as seen in GPELF, APOC (African Program for Onchocerciasis Control) and other global programs can put strong selection pressure on those parasites to develop resistance (Prichard, 2007). Several reports suggest that BZ drugs have reduced efficacy against human geohelminthic parasites (Clercq et al., 1997; Reynoldson et al., 1998; Albonico et al., 2003; Albonico et al., 2004) and in *W. bancrofti* (Schwab et al., 2005), but enough data looking at anthelmintic resistance (AR) in human nematode parasites to understand the extent of existence of drug resistance, if any, is not available.

The extent of refugia (proportion of the parasitic population that is not exposed to a particular drug, thereby escaping selection for resistance) at the time of anthelmintic treatment also has a very important role in the selection of AR. In geohelminths, a high proportion of the total population at the time of treatment is on soil as eggs or free-living larval stages, leaving relatively high levels of refugia. Trichostrongylid nematodes of small ruminants and human filarial worms such as *W. bancrofti* have low levels of refugia at the time of treatment, believed to associate with the development of AR (Swan et al., 1994; van Wyk, 2001; Prichard, 2005). Thus, lower levels of refugia likely cause higher selection pressure for drug resistance in human filarial nematodes as compared to soil transmitted nematodes of humans or animals (Prichard, 2007).

BZ resistance in parasitic nematodes is recessive (Prichard, 2001) and if non-random mating is seen in these infections, the resistance will develop more rapidly as compared to random mating. Non-random mating(only with their own species), as seen in human filarial nematodes such as *W. bancrofti* and *O. volvulus,* increases the proportion of homozygous population, which in turn has been shown to have an increased rate of development of resistance as compared to the heterozygous population (Prichard, 2007).

Human filarial nematodes have very long life spans and generation intervals greater than livestock nematodes, which tends to slow the selection for AR. However, all anthelmintics used in mass chemotherapy are slightly/moderately macrofilaricidal (Eberhard et al., 1997; Ismail et al., 1998; Michael et al., 2004) and cause prolonged suppression of reproduction in the surviving adult worms. The susceptible worms are either removed or sterilized and surviving adult worms which can resume reproduction despite repeated treatments produce progeny for a long time-providing a very strong selection for resistance (Prichard, 2007).

A combination of drugs-ABZ, DEC and IVM used in mass chemotherapy should delay the development of resistance, if the resistance is entirely associated with changes in the drug receptor and the component drugs target different receptors. However, this is not always the case. Macrocyclic lactones such as IVM are potent substrates for P-glycoproteins and ATP-binding cassette (ABC) transporters. ABC transporters, which appear to be involved in IVM resistance (Prichard and Roulet, 2007) are also good receptors for ABZ, suggesting that a combination therapy (ABZ+IVM) could possibly result in enhanced selection of ABC transporters involved in resistance for both ABZ and IVM (Prichard, 2007). Similarly, in human filarial nematodes (*O. volvulus* and *H. contortus*) IVM selects β-tubulin (Eng and Prichard, 2005; Eng et al., 2006), which is known to be an excellent substrate for ABZ. A significantly higher

allele frequency of the resistance-associated 200 tyrosine genotype has been seen in worms collected from patients treated with ABZ in combination with IVM (Schwab et al., 2005). Thus, selection by two anthelmintics can potentially involve the same gene and it can be hypothesized that a combination chemotherapy with ABZ and IVM might actually increase AR selection rather than delay it (Prichard, 2007).

Frequent treatment has been portrayed as being important for the selection of AR, but rapid development of AR has been reported in parasites of sheep even in cases in which only two treatments per year were used (Besier and Love, 2003). This took place in a situation where low levels of refugia were present, the condition that prevails in LF when mass drug administration is applied. Furthermore, ABZ exerts a prolonged and continuous effect over several months and could be expected to exert a very high level of selection pressure for resistance (Prichard, 2007).

The spread of ABZ resistance is also strongly dependent on treatment coverage. Higher levels of therapeutic coverage would lead to faster mf reductions, but also to quicker spread of ABZ resistance. In small ruminants, where AR has developed rapidly, the practice has been to treat the whole herd. The LF control programs aim to cover 65-85% of the eligible population in a community, which is conducive to the selection for resistance (Prichard, 2005). The model proposed by Schwab et al indicates that increasing the treatment coverage by 10% would lead to an almost 4-fold increase in the frequency of ABZ-resistant mf population after the stopping of 10-yearly treatments. This is because the untreated hosts act as a refugia of susceptible parasites (Schwab et al., 2006).

1.6 Candidate gene approach

A major goal in current research is to identify specific genetic changes responsible for and/or associated with AR, which may serve as genetic markers of resistance. These markers

would allow the resistance to be monitored easily and be identified at an early stage, before genetic changes in the parasite reach a point of significant treatment failure. The major approach for identifying AR-conferring genes is to study candidate genes in susceptible and drug-resistant parasite isolates (Gilleard and Beech, 2007). Candidate genes have a known biological action involved with the development or physiology of a trait. Candidate gene study is based on understanding the drug effects and the physiological response to it. The study involves hypothesizing as to which genes might be involved in the resistance and then conducting experiments to test the hypothesis. Examples of such studies include investigating genetic and biochemical differences between susceptible and resistant gastrointestinal parasites of ruminants and horses and identifying associations of the resistance phenotype with polymorphism in candidate genes (Gilleard, 2006; Gilleard and Beech, 2007).

1.6.1 Candidate gene strategy applied to BZ resistance

Candidate gene approach in parasitic nematodes has elucidated the role of β-tubulin encoding genes which confer resistance to the BZ class of drugs (Gilleard, 2006; Gilleard and Beech, 2007). β-tubulin is an important target of BZs in a wide range of organisms like nematodes and fungi and mutations in this locus, including F200Y substitution, have been known to provide high levels of resistance to these organisms (Sheir-Neiss et al., 1978; Driscoll et al., 1989; Roos, 1990; Roos et al., 1995; Elard and Humbert, 1999). The candidate gene approach has identified several mutations in this gene and has been highly successful (Gilleard, 2006).

1.6.2. Limitations of candidate gene association studies and need of expression-based genomic technologies

The candidate gene or 'one gene at a time' approach was successful with BZ resistance since the hypothesis chosen was robust. But the same approach has not yet identified a locus that is clearly a major single determinant of IVM resistance in parasitic nematodes (Gilleard, 2006). One reason for this could be that the mechanism of IVM resistance is complex and multigenic, as seen in *C. elegans,* where a high level of resistance is dependent upon the co-occurrence of mutations in many genes. Other reasons could be that only one or two loci are important, but they have not yet been considered as candidate genes, demonstrating the limitation of this 'one-gene at a time' strategy.

The candidate gene approach established that mutations in the β-tubulin gene at amino acid positions 167 and 200 correlate with BZ-resistance phenotype. BZ resistance may involve more than one gene (polygenic) (Lejambre et al., 1979; Herlich et al., 1981; Anderson et al., 1998; Gilleard, 2006) and candidate gene strategy is not suitable for polygenic traits. This strategy does not identify novel genes which are responsible for resistance, but are not predicted by the current hypotheses (Gilleard, 2006). Furthermore, if wrong hypotheses or candidate genes are selected, the data generated do not help in understanding the genetics of resistance.

Consequently, detailed genetic and population genetic studies are essential to support on-going work with candidate genes and the future application of genome- wide approaches. Genomic and genetic approaches (gene microarrays, proteomics etc) not only identify the resistance genes, but also assess their relative importance to the resistance phenotype as seen in insecticide resistance (Gilleard, 2006). These approaches require development of good genetic tools and resources, including fully sequenced genomes of parasitic nematodes requiring detailed evaluation of their AR (Gilleard and Beech, 2007).

1.7 *Brugia malayi* as a model for parasitic nematodes for gene expression

Wuchereria bancrofti is the main species responsible for 90% human lymphatic filariasis (Mak, 1986) whereas *B. malayi* accounts for 10% of infections worldwide (Evans et al., 1993; Melrose, 2002). Despite its importance, the biology of *W. bancrofti* is difficult to study due to a lack of an animal model, difficulty in obtaining worm material (Unnasch, 1994) and experimental and ethical limitations of studies of human patient populations. Therefore, *B. malayi* is generally used as a surrogate in LF research (Unnasch, 1994).

Brugia malayi, like other filarial nematodes, develops through five larval stages into an adult male or female, entirely within two host species- a mosquito vector (*Culex, Aedes*, and *Anopheles*) and a human host. *B. malayi* was chosen for sequencing of its entire genome because it can be easily maintained in small laboratory animals, and therefore can be easily manipulated in order to conduct biological experiments and is extremely well characterized biologically (Ghedin et al., 2004a). Several animal models have been used to study *Brugia* species including dogs, cats, ferrets, rats, mice and gerbils (Schacher and Sahyoun, 1967; Ah and Thompson, 1973b; Ash, 1973; Crandall et al., 1982; Lawrence, 1996; Bell et al., 1999). Each of these models has its own value, and has greatly contributed to understanding the biology of this parasite and the disease it produces. However, Mongolian gerbil (*Meriones unguiculatus*), models are preferred over others due to maintenance of all parasite life cycle stages, ease of availability, low maintenance costs, and less societal attention to animal experimentation. Hence, gerbil, because of its many characteristics, is a widely used rodent model for *Brugia* species to study lymphatic filariasis (Ah and Thompson, 1973a; McCall et al., 1973a; Chirgwin et al., 2005). As compared to other animal models, the gerbil model is cheaper, quicker and more efficient in intraperitoneal worm implantation procedures used to study the effects of

antifilarial drugs (McCall et al., 1973a). In addition, a high percentage (90-95%) of implanted worms can be recovered from this model, reducing the number of gerbils required for the study.

1.7.1 Brugia malayi draft genome

A large expressed sequence tag (EST) effort (over 26,000 ESTs of the significant *Brugia* life cycle stages have been sequenced) was followed by complete sequencing of the genome of *B. malayi* (Williams et al., 2000; Ghedin et al., 2004b). The WHO-sponsored Filarial Genome Project (FGP), which was organized in 1994, completed the draft genomic sequence of *B. malayi* in September 2007. Like most other filarial nematodes, *B. malayi* has three genomes: nuclear, mitochondrial and that of an endosymbiont bacteria, Wolbachia. The nuclear genome of *B. malayi* is organized into five pairs of chromosomes (4 pairs of autosomes and one pair of sex chromosomes) (Sakaguchi et al., 1983). Using a whole-genome shotgun method, the *B. malayi* nuclear genome was sequenced at nine fold (9x coverage) redundancy (Ghedin et al., 2004b). The genome has been estimated to be 80 to 100 megabases (Mb) (McReynolds et al., 1986; Sim et al., 1987) and was assembled into scaffolds totaling ~71 Mb of data. *B. malayi* is estimated to have ~ 11,500 protein coding genes from ~ 71 Mb of assembled sequence and occupy ~32% of the sequence at an average density of 162 genes/ Mb (Ghedin et al., 2007).

1.7.2 Brugia malayi microarray

Complete sequencing of the genome of *B. malayi* was followed by the construction of oligonucletoide microarrays for expression profiling (Li et al., 2005; Li et al., 2006). With microarray technology, the expression levels of thousands of genes in a single experiment can be measured unlike with Northern blot analysis or PCR-based techniques (Gobert et al., 2005). Microarrays are specially produced slides that have thousands of individual DNA probes attached in an ordered array to the surface. The most commonly used microarrays are (1)

Complementary DNA (cDNA) array: cDNA probe is transferred to a glass slide by an array-printing machine and stored until use. (2) Oligonucleotide array: oligos are manufactured separately and then chips are facilitated by simple array printing machines. This makes this method inexpensive compared to others (Street, 2002). In both types, a known gene set is gridded onto a solid support medium in an ordered manner and probed with mRNA/cDNA from parasites exposed to different conditions of interest (Knox, 2004). Technological advances in robotics allow thousands of genes to be spotted onto glass microscopic slides. Nucleic acid probes can carry fluorescent labels (Cy3 and Cy5 dyes) which allow the direct analysis of differential gene expression with the aid of sophisticated image analysis software. Experiments can be repeated in a highly reproducible manner, which allows statistical validation of the output.

New version 2 (V.2) microarray chips are available for *B. malayi* at Washington University School of Medicine, Microarray Core Facility. These chips are spotted with 18,153 oligos in total and each oligo is unique. The 18, 153 oligonucleotides on the version 2 (V.2) array include:

1. 15, 455 *B. malayi* oligos (accounts for 85% of total spots)
2. 1,016 *O. volvulus* oligos (6%; those not already represented above)
3. 878 oligos based on EST clustering of *W. bancrofti* data (5%; those not already represented above)
4. 804 endosymbiont bacteria *Wolbachia* oligos (4%; those not already represented above) (Dr. S.A. Williams, personal communication, 2008 and http://www.filariasiscenter.org/index.htm).

1.7.3 Identifying genes in B. malayi that may be important in the development of resistance to albendazole

To develop more effective strategies to monitor and prevent the development of resistance, a better understanding of the genetic and molecular basis of anthelmintic resistance in parasites is required. Gaining basic knowledge of gene expression in *B. malayi* and identifying differentially expressed genes in worms that are able to survive levels of ABZ that kill a majority of the worms exposed to the drug and comparing their gene expression levels may give a clearer picture about genes disregulated due to treatment with ABZ. It should also help to elucidate mechanisms involved in the action of ABZ on worms, and at the same time help determine, what allows some worms to survive doses of drugs that kill the majority of worms. Such knowledge may generate important insights into possible mechanisms of drug resistance.

With the aid of microarray technology, identifying differentially expressed genes becomes feasible. Further characterization of those identified genes will be possible by other advanced molecular techniques like Real-time RT-PCR and RNA interference (RNAi). Having such powerful molecular tools to use on an important human nematode parasite that is so easily maintained in a rodent model provides an excellent and unique system for studying the biology of parasitic nematodes. This study will be an important step in gaining increased understanding of anthelmintic resistance at the molecular level. Furthermore, it is expected that findings will be applicable to other human filarial nematodes including *W. bancrofti* and *O. volvulus* and parasitic nematodes of livestock where anthelmintic resistance is now reaching critical and alarming levels.

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CHAPTER II

EFFECT OF TREATMENT WITH BENZIMIDAZOLE ANTHELMINTICS ON THE VIABILITY OF *BRUGIA MALAYI*: *IN VITRO* STUDY

Abstract

Human lymphatic filariasis (LF) is caused primarily by *Wuchereria bancrofti* and *Brugia malayi*. Thiabendazole (TBZ) belongs to the benzimidazole (BZ) class of drugs and is used against helminths because of its broad spectrum and low toxicity. The concentration and time point at which lethal effects in *B. malayi* adult female worms are observed following treatment with different concentrations of thiabendazole were studied by measuring worm motility, microfilariae (mf) release and MTT assay. Four drug concentrations (0, 200, 700 and 1200μM) were used in a 15-day cell-free culture system. Worms in control wells remained highly active throughout the experiment and there were no significant differences in motility between days. In treated groups, the motility of worms decreased in a concentration-and time-dependent fashion and there were significant differences in motility between drug concentrations ($p<0.0001$). For 200μM, 700μM and 1200 μM, significantly lower motilities were seen between days 7-15, 4-15 and 3-15 respectively as compared to day 0. It took 15, 12 and 10 days at 200μM, 700μM and 1200μM respectively for the adult female worms to be completely immobilized. In treated groups, there were significant differences in mf release between drug concentrations. All drug concentration pairs were significantly different from each other ($p<0.0001$) except for 700μM and 1200μM ($p=0.0652$). In the treated groups all days had significantly lower mf release than

day 0, and there were significant differences in mf release between doses ($p<0.0001$). MTT assay carried out on TBZ-treated worms with different motility scores revealed that there was a direct correlation between motility and viability. Our results show that worm motility and mf release assays are the best indicators of worm viability, still leaving worms usable for RNA extraction and gene expression studies. Motility assay determines worm viability quicker than mf release assay for gene expression studies and thiabendazole can have macrofilaricidal effects *in vitro*.

1. Introduction

Filarioid nematodes are among the most important parasites affecting both animal and human health worldwide. Human lymphatic filariasis (LF), caused primarily by *Wuchereria bancrofti* and *Brugia malayi*, is a serious debilitating disease affecting over 120 million people, and another 1.37 billion people are at risk of infection in over 80 countries (WHO, 2006). This disease has been ranked by the World Health Organization (WHO) as the second leading cause of permanent and long-term disability (Melrose, 2002; WHO, 2002), and is a significant cause of major public health and socio-economic problems (Evans et al., 1993). In 1993, the International Task Force for Disease Eradication reviewed almost 100 medical conditions and categorized six as eradicable or potentially eradicable with the tools then available and one of them was lymphatic filariasis (CDC, 1993). In 1997, the World Health Assembly passed a resolution (WHA 50.29) that called for elimination of LF globally. In response to this, a public-private partenership was developed for the control of LF which led to the establishment of the Global Program to Eliminate Lymphatic Filariasis (WHO, 1997a). This global program currently relies on community-wide mass distribution of albendazole (ABZ) and ivermectin (IVM), either individually or in combination with diethylcarbamazine (DEC) in filariasis-endemic areas (WHO, 1997a, 1999). Of the three drugs, only ABZ and DEC have been shown to be both macro- and microfilaricidal (McCarthy et al., 1995; Norões et al., 1997; Ottesen et al., 1999) where as IVM is only microfilaricidal (Dreyer et al., 1996a). A further benefit of addition of ABZ to IVM or DEC in these regimens results in a significant reduction in prevalence and intensity of intestinal nematode infections (Ottesen et al., 1999).

Albendazole belongs to the benzimidazole (BZ) class of drugs and is widely used against human intestinal helminths and in veterinary practice because of its broad spectrum and low

toxicity (Horton, 1990). The detailed mechanism of action of ABZ is not clear, but experimental evidence with several important veterinary nematodes shows that ABZ, like other benzimidazoles, acts by binding β-tubulin, thereby inhibiting tubulin polymerization and formation of microtubules (Lacey and Prichard, 1986; Horton, 2000).

Identifying changes in gene expression in *B. malayi* that survive treatment with BZ is a critical step in understanding the molecular mechanisms of BZ resistance. For this, determining the viability of *B. malayi* following treatment with BZ is essential. Faulty gene expression from prolonged exposure to the undesirable environment in worm can happen while assessing its viability by all the available key parameters. An *in vitro* study was done to identify a parameter to quickly determine the worm viability and thus avoid spurious changes in gene expression. Viability of the worm, after exposure to a BZ drug was evaluated in terms of parasite motility and microfilariae (mf) release. Results of this *in vitro* study were subsequently applied to an *in vivo* study to identify the adult female *B. malayi* worms that survive treatment with ABZ (described in Chapter III)

To quickly determine the lethal effects produced by ABZ in *B. malayi* and avoid their prolonged exposure to the environment, the highest possible concentration of ABZ is required. Albendazole is highly insoluble in water and only slightly soluble in dimethylsulfoxide (DMSO). Thiabendazole (TBZ), also belonging to BZ class of drugs, has relatively better solubility in DMSO, leading to higher drug concentrations without affecting pH of the media or causing any precipitation problems. Therefore, TBZ was used in our *in vitro* study to determine the drug concentration and time response in observing the viability of adult female *B. malayi* worms.

2. Materials and Methods

2.1 Chemicals and drugs

NCTC135 medium, Iscove's modified Dulbecco's medium (IMDM), thiabendazole, Dimethylsulfoxide (DMSO), and 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl-tetrazolium bromide (MTT) were purchased from Sigma Chemical Co., St. Louis. The culture medium used in this study was a 1:1 mixture of NCTC135 and IMDM (NI) which was adjusted and maintained at pH 7.4 using 1M NaOH.

A stock solution of TBZ (200mM) was prepared by dissolving 201.26 mg (in 4 ml of DMSO. Then the total volume was made up to 5 ml with DMSO. The solution was passed through a 0.2µm filter (Corning Inc, Ithaca, NY), covered with aluminum foil to protect from light and the stored at 4°C. Fresh solution was prepared on every other day. Varying amounts of DMSO were used to make3 final TBZ concentrations: 200, 700 and 1200µM. The final pH of the media was adjusted to 7.4 using 1M NaOH.

2.2 Parasite

Brugia malayi infected Mongolian gerbils (*Meriones unguiculatus*) were obtained from the Filariasis Reagent Resource Center (FR3) at University of Georgia College of Veterinary Medicine. Adult worms (>100 days post-infection) were obtained from the peritoneal cavities of male gerbils at necropsy, and rinsed with NI medium. Female worms were separated and transferred singly into a well of a 24-well plate (Corning Inc., Ithaca, NY), containing two ml of NI medium supplemented with 10% heat-inactivated fetal bovine serum (HiFBS). The plates were incubated for 24 hours at 37°C in a humidified incubator in the presence of 95% air and 5% CO2. Only those females, which remained fully active and released more than 1000 mf/ml into the medium over 24 h of incubation, were selected for subsequent study.

2.3 Effect of different TBZ dose levels on adult female worms

Six female worms were allocated to each of the four dose levels of TBZ. Each worm was transferred daily to a new well containing fresh culture medium and TBZ. The motility, numbers of mf, and viability of worms were examined. Parasite motility and death were assessed visually each day for 15 days using an inverted compound microscope (until the death of last worm). The worm's motility was recorded using arbitrary scores as previously described (Khunkitti et al., 2000): score 3= rapid sinusoidal thrashing movements with over 30 movements/10 sec (highly active); score 2= slow sinusoidal movements with 10-30/10 sec (moderately active); score 1= no thrashing movements and occasional twitching at only one end of the body(less active); and score 0= immobile for at least 10 sec (dead). Immediately after a daily motility score was recorded, female worms were transferred into NI fresh medium containing freshly prepared drugs and the number of mf released into the medium over a 24 h period were counted. The number of mf in four 20µl aliquots of medium were counted, and the total number of mf released into 1ml medium calculated by multiplying the average of these values by 50. To determine the correlation between motility and viability, MTT assay as described by Comley et al.(Comley et al., 1989b). was performed. Briefly, after microscopic assessment of parasitic motility, each worm was transferred to a well of the 96-well plate with 200 µl of Hank's Buffered Salt Solution (pH 7.4) and 20µl of 5mg MTT/ml and incubated for 2 h at 37°C. The worm was then transferred to a respective well of a new 96-well plate containing 200µl of MTT solubilization solution (MTTSS) and kept at 37°C with shaking for 1 h. The quantity of formazan dissolved in MTTSS was measured at 570nm in a multi-well scanning spectrophotometer relative to a MTTSS blank. Heat killed (65°C) adults were separately incubated as controls for background absorbance.

2.4 Statistical analysis

All analyses were performed using SAS V 9.1 (Cary, NC). Motility and mf release were analyzed as raw values and as percent reduction compared to baseline. A repeated measures model that recognized multiple observations as belonging to the same parasite was used to test for differences in motility and mf release between drug concentrations. The full model included fixed factors of dose, day and a dose*day interaction term and a random parasite factor. Multiple comparisons were adjusted for using Tukey's test. A repeated measures model was also used to test for differences in motility and mf release between different days and baseline values for each drug concentrations separately. The model included a fixed factor of day and a random factor of parasite. Multiple comparisons were adjusted for using Dunnett's test. An unstructured covariance structure was used in all repeated measures models. All hypothesis tests were 2-sided and the significance level was $\alpha = 0.05$. The repeated measure analysis was performed using PROC MIXED and the correlation between motility and MTT assay was analyzed using PROC CORR in SAS.

3. RESULTS

3.1. Motility of adult female worms

The drug concentrations used in this study were 0μM, 200μM, 700μM, and 1200μM. These drug concentrations were selected based on initial trials (data not shown). The lowest concentration at which death was observed over a 2 week incubation was 200μM. The maximum drug concentration that could be obtained due to limits of drug solubility at neutral pH was 1200μM. Adult female worms in control wells remained highly active (motility score 3) throughout the experiment and there were no significant differences in motility between days ($p= 1.000$ for days 1-13 and $p=0.0419$ for days 14 and 15) (Fig 2.1). In treated groups, the motility

of female worms decreased in a concentration-and time-dependent fashion and there were significant differences in motility between drug concentrations ($p<0.0001$). All dose pairs (0μM and 200μM, 0 μM vs. 700μM, 0μM vs. 1200μM, 200μM vs. 700μM, 200μM vs. 1200μM, 700μM vs. 1200μM) were significantly different from each other ($p<0.0001$). At drug concentration 200μM, days 7-15 had significantly lower motility than day 0, at 700μM, days 4-15 had significantly lower motility than day 0, and at 1200μM, days 3-15 had significantly lower motility than day 0. It took 15, 12 and 10 days at 200μM, 700μM and 1200μM respectively for the adult female worms to be completely immobilized.

3.2. Microfilariae release by adult female worms

Female adult worms in the control group showed a relatively stable release of mf (>1000mf/day/ml) for 15 days but all days had significantly lower mf release than day 0 (Fig 2.2). In all treated groups all days had significantly lower mf release than day 0, and there were significant differences in mf release between doses ($p<0.0001$). All dose pairs were significantly different from each other ($p<0.0001$) except between 700μM and 1200μM ($p=0.0652$). The percent mf release reduction compared to day 0 were 71.7, 97.5, 99.8 and 100.0 by day 1, 2, 3 and 4 respectively (for 1200 μM); 61.2, 88.0, 95.9, 99.6 and 100.0 by day 1, 2, 3, 4 and 5 respectively (for 700 μM); and 42.1, 57.2, 84.4, 92.6, and 97.7 by day 1, 2, 3, 4 and 5 respectively (for 200 μM). A 100% reduction happened by day 9 for 200 μM.

3.3. MTT assay

To determine the correlation between motility and viability, the MTT assay was performed. Results of this assay are shown in figure 2.3. Highly motile worms had high absorbance readings (suggestive of increased viability) compared to less active worms. The

results showed a positive correlation between motility and viability of these worms (Pearson Correlation Coefficient value is 0.97162).

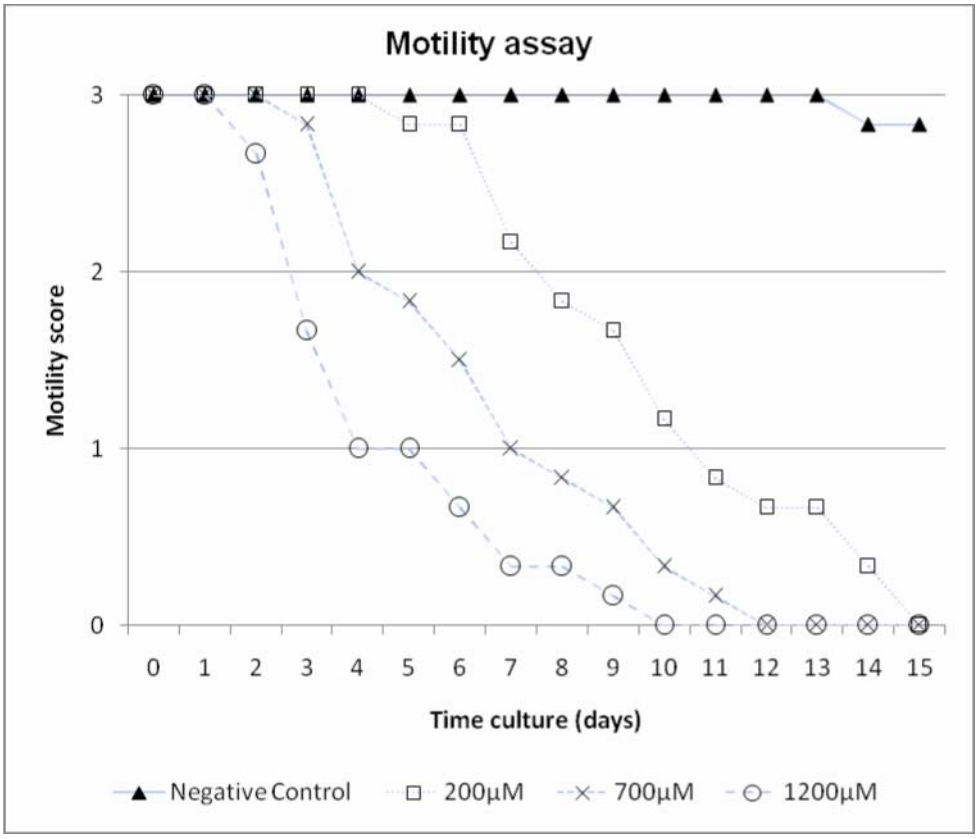

Fig. 2.1. Change in motility of B. malayi adult female worms in a culture medium with different drug concentrations (n=6 for each concentration) of TBZ. Motility scores used are: 3 (highly active), 2 (moderately active), 1 (less active), and 0 (inactive for at least 10 s). Motility was significantly reduced at 7, 4, and 3 days for 200 µM, 700 µM, and 1200 µM, respectively.

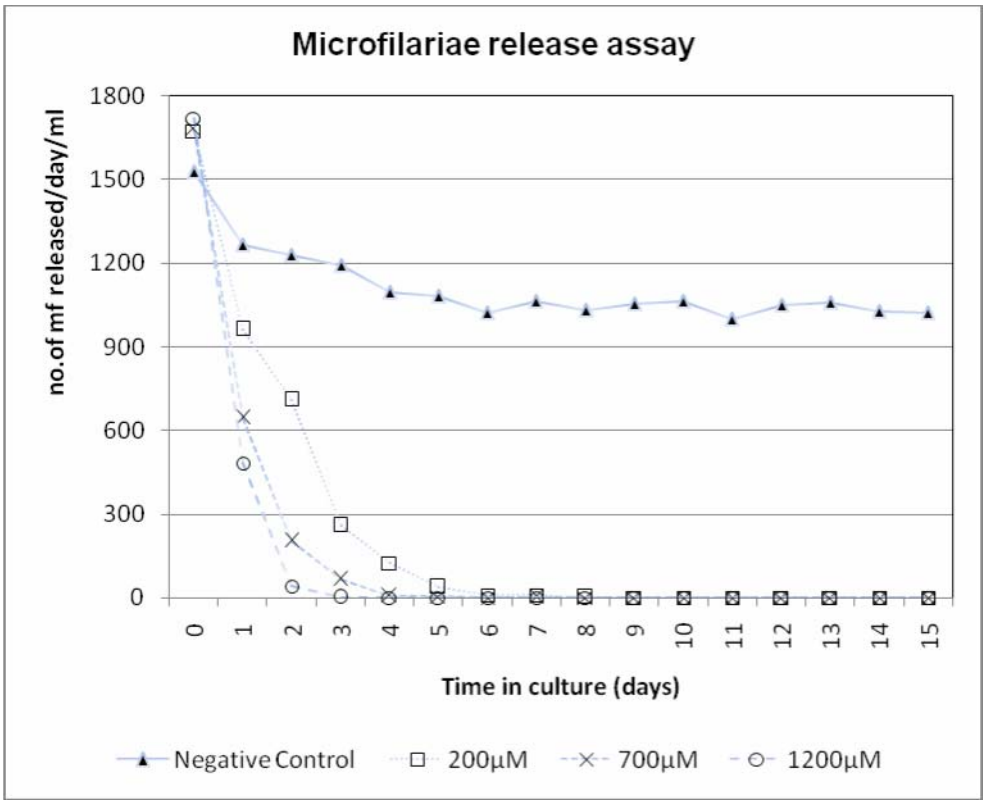

Fig. 2.2. Daily release of microfilariae by each B. malayi adult female worm in 1 ml of culture medium with different drug concentrations of TBZ. Each data point is the mean of 6 worms. Mf release was significantly reduced at day 0 for all the concentrations.

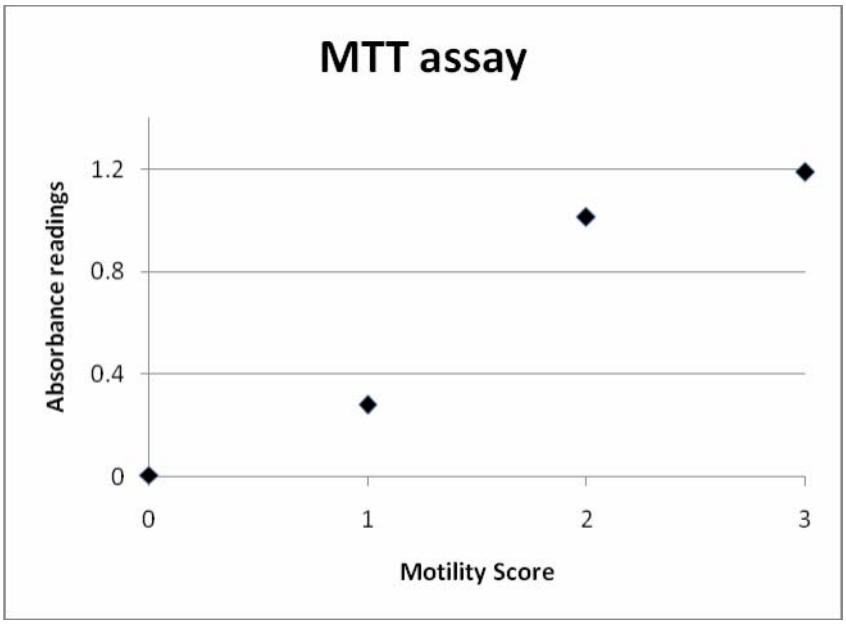

Fig. 2.3. Association between motility and formazan formation in vitro. Each data point is the mean of 8 worms. Direct correlation exists between motility score and MTT absorbance readings (Pearson Correlation Coefficient value is 0.97162).

4. Discussion

In this study, the TBZ concentration and time points at which lethal effects produced in *B. malayi* adult females were determined by measuring worm motility and mf release. Thiabendazole showed increased effects on motility with higher drug concentrations, and the effects increased over time. Statistically significant differences in motility were seen between drug concentrations.

The ultimate goal of our study is to gain basic knowledge of gene expression in *B. malayi* that survive treatment with BZ and understand the molecular mechanisms of BZ resistance. We wanted to identify a parameter, which would quickly reveal the worm viability and thus avoid faulty changes in gene expression that may be induced by dying worms. The viability was evaluated in terms of parasite motility and mf release after exposure to TBZ. We could have

used an *in vivo* model for this purpose, but it would have been expensive, laborious and requiring many animals, when the same objective was accomplished using *in vitro* experiments.

Several *in vitro* studies on filarial worms have been published where DEC or antibiotics were used (Court et al., 1986; Rao and Weil, 2002; Gunawardena et al., 2005). However, we could not identify any earlier publications where in vitro studies were performed on filarial worms using a BZ drug.

To quickly determine the lethal effects produced by ABZ in *B. malayi* and avoid their prolonged exposure to the environment so as it brings faulty changes in gene expression, the highest possible concentration of ABZ is required. However, ABZ is highly insoluble in water and only slightly soluble in dimethylsulfoxide (DMSO). The solubility of ABZ in DMSO is 25mg/ml and therefore higher drug concentrations (>100uM) could not be achieved in our study. A similar problem has been reported while assessing anthelmintic sensitivity for human hookworms and *Strongyloides* parasites (Kotze et al., 2004). Compared to ABZ, TBZ has a relatively better solubility (50mg/ml) in DMSO, which leads to higher drug concentrations and does not affect the pH of the medium or cause any precipitation problems. Therefore, TBZ instead of ABZ was used in our study.

Though current global programs to eliminate lymphatic filariasis are based on mass chemotherapy with the use of ABZ in combination with DEC or IVM, using TBZ instead of ABZ is acceptable in our study since these drugs are closely related and side-resistance is known to exist within the BZ class of anthelmintics (Colglazier et al., 1975; Martin et al., 1985). Resistance to one drug within this class indicates the existence of resistance to members of the class in general.

The MTT assay evaluates the viability of parasites and yields objective quantitative data. Filariae are unique amongst nematodes in their ability to take up MTT (Comley et al., 1989a). In this assay, worm viability was measured based on the ability of various NADH-dependent dehydrogenase enzymes in viable worms to reduce MTT, a pale yellow, to formazan-a dark blue crystalline deposit. Inhibition of MTT reduction on less active worms indicates damage and provides an estimate of the degree of impairment (Comley et al., 1989a; Comley et al., 1989b). In the present study, this assay was carried out on TBZ-treated worms with different motility scores to examine the relationship between motility and viability. The assay revealed that there was a direct correlation between activity (measured by motility) and viability (measured by MTT assay). Even though MTT assay yields quantitative data on the viability of the parasite, further analysis like extracting RNA from worms could not be done. Direct correlation between motility and MTT reduction proved that worm motility assay can be used alone in gauging macrofilaricidal drug effects where MTT assay cannot be employed.

Female adult worms in the untreated group released fewer mf on subsequent days as compared to day 0. However, there was a relatively stable release of mf (>1000mf/day/ml) throughout the experiment (till day 15). This sudden decrease in mf release from worms in control wells could be due to some unknown mechanisms, but the most probable cause could be sudden change in environment. Culture medium used in this study may have simulated but not completely replaced the *in vivo* environment. Microfilariae release from worms in treated wells was significantly lower than controls at all concentrations by day 1 and complete inhibition of mf release occurred by day 5 even in lowest dose (200µM) group. In the highest concentration group (1200µM), only 2 days were required to see over 95% reduction in mf release as compared to day 0, suggesting that mf release assay would be a quick indicator of viability as compared to

motility assay. In contrast, delayed microfilaricidal effects have been observed in *in vivo* studies, flubendazole-a BZ drug has been shown to have a negligible immediate microfilaricidal effect but a delayed dose dependent reduction in mf density in Mongolian gerbils (Zahner and Schares, 1993). A negligible effect of ABZ on mf release has been shown in humans also (Critchley et al., 2005). Filarial nematodes have been shown to often succumb to drug treatments when cultured *in vitro* as compared to *in vivo* (Townson et al., 2006). Possible reasons include differences in the physical properties of the drugs resulting in different bioavailability, and many biochemical pathways (Kotze et al., 2004).

It has been reported that BZs also affect secretory and absorptive functions of intestinal cells in *Ascaris suum* (Borgers and Nollin, 1975), *Hemonchus contortus* and *Trichuris globulosa* (Manpreet and Sood, 1996). This mechanism could induce exhaustion and consequently death of parasite. It has been reported that these drugs at higher doses could be potentially macrofilaricidal (Jayakody et al., 1993). Albendazole can affect the muscle cells and embryogenesis of the parasite causing reduced motility and decreased mf release respectively. Further studies are needed to understand the mechanism of action of BZs at genetic level in nematode parasites.

Based on our study, worm motility and mf release assays appear to be the best available indicators of worm viability, still leaving worms usable for RNA extraction to study gene expression. Despite the fact that mf release assay, as shown in this study, is a quick indicator of viability, the motility assay is preferred over it. We conducted a small experiment involving 20 adult female worms, isolated from an untreated gerbil. These worms were cultured in NI medium and checked for mf release at various time points (0.5, 1, 2, 4, 6, 12, and 24 h). No uniform mf release was observed at any specific time point, though about 40% of the daily

release occurred at 6 hours (results not shown). Two of the 20 worms did not release any mf during the entire 24 hours, but did so at 28 hours. This suggests that the worms need to be cultured for at least 24 hours post isolation from the gerbil to measure viability using mf release assay, whereas, viability can be measured in a few minutes using the motility assay. The rapidity of the motility assay would prevent the worms from being exposed to the environment other than tested and thus avoiding any faulty changes in gene expression.

Our work is the first *in vitro* study to determine the viability of adult *B malayi* worms following exposure to TBZ. This study shows that TBZ can have macrofilaricidal effects *in vitro* (similar to a previous *in vivo* study using ABZ in humans (Jayakody et al., 1993)) and both worm motility and mf release assays are good indicators of worm viability, but motility assay should be preferred.

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CHAPTER III

EFFECT OF TREATMENT WITH ALBENDAZOLE ANTHELMINTIC ON THE VIABILITY OF *BRUGIA MALAYI*: *IN VIVO* STUDY

Abstract

Previous studies have expressed concern that mass chemotherapy approach using albendazole (ABZ) employed by the Global Program to Eliminate Lymphatic Filariasis may lead to the development of benzimidazole (BZ) resistance. Available genetic data suggest that BZ resistance is a polygenic trait and the candidate gene strategy is not a suitable approach for understanding the molecular mechanisms of resistance. In this study, the concentration and time points, at which adult female *Brugia malayi* worms show varying levels of susceptibility to ABZ, were determined. Two sets of worm populations: moderately active worms (motility score-2) showing increased susceptibility to the drug at 21 days post-treatment and highly active worms (motility score-3) which tolerated well or showed least susceptibility to ABZ at 28 days post-treatment and were selected based on their motility scores. All but one worm in the control group were highly motile throughout the length of study. Macrofilaricidal effects of a single dose of ABZ administered subcutaneously in gerbils were also seen. RNA was extracted from these worms for future microarray analysis to gain basic knowledge of gene expression in *B. malayi* that survived the treatment compared to worms susceptible to ABZ. This gives insight into genes involved in drug action or tolerance which may give knowledge about genes that are involved in resistance.

1. Introduction

Human lymphatic filariasis, caused primarily by the nematode parasites *Brugia malayi* and *Wuchereria bancrofti,* is a serious debilitating disease affecting over 120 million people worldwide (WHO, 2006). In response to this global health crisis, a public-private partenership was developed for the control of lymphatic filariasis (LF) (WHO, 1997a) that led to the establishment of the Global Program to Eliminate Lymphatic Filariasis (GPELF). This global program is largely based upon the regular administration of community-wide mass chemotherapy of the entire at-risk population with the anthelmintic albendazole (ABZ) in combination with ivermectin (IVM) or diethylcarbamazine (DEC) (WHO, 1997a, 1999). Albendazole has both micro- and macrofilaricidal effects and is also a potent anti-geohelminthic agent (Ottesen et al., 1999).

The detailed mechanism of action of ABZ in parasites is unclear and experimental evidence with several intestinal helminths shows that ABZ, like other benzimidazoles (BZs), acts by binding to β-tubulin, thus inhibiting the polymerization of tubulin and the formation of microtubules. The lack of microtubules inhibits many cellular functions ultimately leading to cell death (Lacey, 1988a, 1990; Robinson et al., 2002). The mass chemotherapy approach, aimed at a high level of coverage, typically 65-85 % of the eligible population in a community, will impose a significant drug selection pressure on virtually the entire parasite population, leaving few parasites in refugia. It is believed that lack of refugia is the single most important factor promoting the selection of anthelmintic resistance as seen in important nematode livestock parasites and as predicted in human filarial nematodes (Sangster, 1999; Wyk, 2001; Kaplan, 2004; Prichard, 2005). The candidate gene approach has successfully determined that a β-tubulin gene is responsibile for at least part of the BZ resistance in veterinary parasitic and

human filarial nematodes (Roos et al., 1990; Kwa et al., 1993b; Lubega et al., 1994; Grant and Mascord, 1996; Silvestre and Humbert, 2002; Winterrowd et al., 2003; Schwab et al., 2005). However, it is believed that BZ resistance is more complex and polygenic, since it is clear that the worms gain higher levels of resistance with increased drug selection pressure independent of further changes in β-tubulin. Such a phenotype is inconsistent with a single-gene point mutation. Apart from such target gene (β-tubulin) changes, it has been shown that inhibitors of P-glycoprotein and cytochrome-P450 can increase the sensitivity of trichostrongylid nematodes to BZs suggesting that drug metabolism and drug efflux mechanisms could also play a role in BZ resistance (Kerboeuf et al., 2003; Gilleard, 2006). Given that, available genetic data suggest that BZ resistance is a polygenic trait, candidate gene strategy is not a suitable approach for understanding the genetics of BZ resistance.

Although *W. bancrofti* is the main species responsible for 90% of LF, the biology of this parasite is difficult to study because there is no animal model available and worm material is difficult to obtain. Hence, *B. malayi* is often used as a surrogate in LF research. The Mongolian gerbil (*Meriones unguiculatus*) has proven to be a successful rodent model for *Brugia* species and is widely used to study LF (Ah and Thompson, 1973a; McCall et al., 1973b; Chirgwin et al., 2005).

Understanding the molecular mechanisms of anthelmintic resistance in *B. malayi* is very important to monitor the occurrence of resistance. Phenotypic detection of resistance in the form of treatment failure is not effective in managing or controlling the development and spread of anthelmintic resistance, and not much progress has been made in evaluating the resistance at a genotypic level. Gaining a basic knowledge of gene expression in *B. malayi* that survive treatment with ABZ will be a critical step in this direction. We hypothesize that ABZ resistance

is a complex process involving multiple genes and we believe that it is highly likely that a set of important genes are dysregulated in worms able to survive levels of ABZ which kill the majority of worms exposed to the drug. Direct comparisons between susceptible and resistant worms cannot be made since no ABZ resistant worms are present and hence we need a surrogate system. Since filarial nematodes are known to have extremely high genetic diversity and that they demonstrate differential susceptibility to drugs, we hypothesize that by comparing gene expression levels between these worms may lead to a better understanding of mechanisms involved in the action of ABZ on worms. At the same time it will allow us to understand what is responsible for some worms surviving doses of drugs that kill the majority of worms. Such knowledge may generate important insights into possible mechanisms of ABZ resistance.

Through this study, we attempted to determine the viability of adult female *B. malayi* worms following treatment with ABZ in a gerbil model and isolate two sets of worms with variable motility, in response to ABZ treatment. The factor that led us to select the motility assay to determine the viability was our finding in an in vitro study that motility is a quick and reliable indicator in determining worm viability (manuscript in preparation). We believe that a quick determination of worm viability after isolation from the gerbil model would minimize the possibility of exposure to environmental contamination that could lead to additional gene expression changes other than those imposed by the drug. For this, we infected Mongolian gerbils with live adult female worms by surgical transplantation. Then these gerbils were treated with ABZ and the worms were isolated. Isolated worms were separated into two groups based on their motility scores -worms which tolerated the drug well and the worms which succumbed to the drug effects and neared death.

2. Materials and methods

Chemicals

NCTC135 medium, Iscove's modified Dulbecco's medium (IMDM), Dimethylsulfoxide (DMSO) and ABZ were purchased from Sigma Chemical Co., St. Louis. Trizol LS reagent was obtained from Invitrogen (Carlsbad, CA). The culture medium used in this study was a 1:1 mixture of NCTC135 and IMDM which was adjusted and maintained at pH 7.4 using 1M NaOH (NI hereafter). A drug solution of 20mg/ml ABZ prepared in DMSO was used.

Experimental models

The male Mongolian gerbils, obtained from Charles River (Wilmington, Massachusetts) at approximately 8 weeks of age were used in this study. All the experiments involving animals were conducted in compliance with the Institutional Animal Care and Use Committee (IACUC) guidelines. Throughout the study, the animals were housed, organized and monitored by Animal Resources (AR) unit, College of Veterinary Medicine, University of Georgia, Athens, GA. They were provided with standard rodent chow and free access to drinking water.

Adult female *B. malayi* worms used in this study were obtained from the NIH/NIAID Filariasis Research Reagent Repository Center, University of Georgia, Athens GA. After isolation, worms were washed with NI medium and placed in sterile Petri dishes, containing filtered, fresh NI medium (37°C). Groups of 17-36 intact and highly active female worms were placed in Petri dishes containing sterile Phosphate Buffered Saline (PBS) solution.

For the establishment of adult parasite-transplantation-induced infection in gerbils, previously used surgical methods (Suswillo and Denham, 1977) and Sadanaga *et al*. (Sadanaga et al., 1984) were adopted but developed independently. Briefly, the recipient gerbils were anesthetized with intraperitoneal administration of xylazine-ketamine mixture in sterile water

(xylazine 2mg/kg BW and ketamine 50mg/kg BW). The abdominal fur was shaved with electric clippers and scrubbed with Betadine and alcohol. A 1-2 cm incision was made in the skin and a 1-2mm aperture cut ventro-laterally in the body wall of the posterior abdomen. Female worms were aspirated into a sterile-glass pipette and inserted through the opening in the body wall. The pipette was gently manipulated into the opposite side of the peritoneal cavity and worms were expressed out. The pipette was gently withdrawn and the muscle layers were sutured using 3-0 vicryl absorbable suture. Skin layers were closed with 9mm EZ clips, which were removed 5 days later. Post-operative analgesia was provided by once daily administration of Carprofen 5mg/kg BW S.C. as required.

Preparation of ABZ drug

A 20 mg/ml solution of ABZ was prepared by dissolving 200mg of ABZ powder in 9 ml of DMSO. Then the total volume was made up to 10 ml with DMSO and the solution was passed through 0.2 micron filter (Corning Inc, Ithaca, NY). Fresh drug solution was prepared every time on the day of drug administration.

Assessment of worm viability by worm motility

The viability of adult female worms was evaluated based on the level of worm motility. Following recovery at necropsy, worm motility was recorded using arbitrary scores as previously described (Khunkitti et al., 2000): score 3= rapid sinusoidal thrashing movements with over 30 movements/10 sec (highly active); score 2= slow sinusoidal movements with 10-30/30 sec (moderately active); score 1= no thrashing movements and occasional twitching at only one end of the body(less active); and score 0= immobile for at least 10 sec (dead). Mortality was determined by adding the number of dead and unrecovered worms. Percentage of mortality was

calculated by comparing the number of live worms recovered from the treated animals with those recovered from matched untreated gerbils.

Pilot study for this project

Experimental design

A pilot study was conducted to determine the drug concentration and optimum time points for isolating two sets of worm populations with varying levels of drug susceptibility. Eight days post worm implantation, 18 gerbils were divided into six groups of 3 gerbils each based on various time point post treatment at which they were euthanized (D-7, 10, 14, 21, 28 and 35). Albendazole was administered subcutaneously (150mg/kg BW). The volume of ABZ solution administered to gerbils weighing between 70 and 100 g was 0.53 – 0.75 ml and contained the required dose of 10.5-15mg/70-100 g animal. Similarly another set of 18 gerbils was divided into six groups but administered ABZ (250mg/kg BW) subcutaneously at two different sites. The volume of ABZ solution administered to gerbils weighing between 70 and 100 g was 0.0.875 – 1.25 ml and contained the required dose of 17.5-25mg/70-100 g animal. These animals were euthanized and all the worms contained within the peritoneal cavity were recovered and counted.

Results and conclusion

Results documenting the effect of ABZ on adult female worm motility are presented in tables 3.1 and 3.2. No considerable mortality percentage was seen in day 7, 10 and 14 groups with mean mortality percentages being 16.3, 11.8 and 6.3 respectively. The worms isolated at these time points were highly active (MS-3) and did not have noticeably different responses to drug. The mean mortality percentages in 21 and 28 DPT groups were 52.8 and 80.0 respectively and worms had variable motility in response to the drug. In 21 DPT group, 22.6% of the

recovered worms were moderately active while majority of the worms were highly active (25.8). In 28 DPT group, 7.9% of the recovered worms were highly active while a majority of worms was either dead and/or unrecovered (92.5%) or nearing death (10.5%). In the 35 DPT group, the mean recovery and mean mortality percentages were 37.5 and 92.5 respectively with a very small percentage (6.7%) of worms being highly active. Noticeable difference was not observed between the two doses of ABZ-250 mg/kg BW and 150 mg/ kg BW.

No considerable mortality was noticed on 7, 10 and 14 DPT and the majority of the worms were highly active (MS-3). It is likely that due to lack of adequate exposure time to ABZ these worms did not exhibit any changes in motility. On 21 DPT, we were able to isolate susceptible worms which were moderately active (MS-2) with majority of worms being highly active (MS-3). On 28 DPT, we isolated worms which survived treatment (MS-3) while the majority of the worms were already dead or near death (MS-0 or 1). On day 35, 92.5% of the worms were dead, broken or encysted with a very small percentage being highly motile. Therefore, to adequately observe effects of ABZ and still avoid a possible return to baseline levels of gene expression due to decreased levels of the drug, we decided on optimum time points of 21 and 28 DPT for our present study.

Gerbils administered the higher dose (250 mg/kg BW) exhibited some post-treatment pain, possibly due to excess volume of DMSO which is a tissue irritant. Therefore, all treatments used the lower dose (150 mg/kg BW), which was well tolerated by the gerbils.

Experimental Design

Eight days post worm implantation, gerbils were randomly divided into 2 groups- treated and control. Treated gerbils were administered ABZ (150mg/kg BW) by subcutaneous injection. The volume of ABZ solution administered to gerbils weighing between 70 and 100 g was 0.53 –

0.75 ml and contained the required dose of 10.5-15mg/70-100 g animal. These animals were euthanized on either day 21 or 28 post-treatment (DPT). Control animals were euthanized on day 21 or day 28. All the worms contained within the peritoneal cavity were recovered and counted.

Selection of worms for RNA extraction

The viability of adult female worms was evaluated based on the level of worm motility as described earlier in this study. We isolated two sets of worm populations from the treated and untreated groups based on their motility scores to study the effect of ABZ on worms and to see the changes in the two worm populations at genetic level. Two sets of worms were isolated- 1) highly active (motility score-3) that survived ABZ treatment for 28 days and 2) moderately active (motility score-2) that survived the treatment for 21 days, but that demonstrated a reduced motility, suggesting these worms were on their way to succumbing to the drug effect. Similarly, worms were isolated from the untreated group on day 21 and 28, all of which remained highly active. Soon after the worms were isolated and motility scored, they were washed and transferred to a 2ml microfuge tube containing 250µl of 1X PBS. Then they were snap frozen in liquid nitrogen and stored at -80°C for future RNA isolation.

RNA extraction

Extraction of RNA was performed as per a protocol previously used (Laney SJ, 2008). Worms in microfuge tubes were thawed on ice and 750 µl of Trizol LS (1:3 v/v) was added to each tube. A 3mm stainless steel ball was added to each microfuge tube and the tube was vortexed (Mo Bio Laboratories, Inc.) at the highest speed for 30 minutes. Position of these tubes was changed every 10 minutes to ensure all the areas of the tubes were uniformly mixed. A 200 µl of chloroform per 750 µl of Trizol LS was added to each tube. The tubes were vortexed for 15 seconds and allowed to stand at room temperature (RT) for 3 minutes and then transferred to

2ml Phase Lock Gel tubes (Eppendorf). After centrifugation (12000 x g for 15 minutes at 4°C), the top aqueous phase was transferred to a new microfuge tube without touching the interface. Isopropanol (500 µl) was added to the sample and the tubes were kept at RT for 10 minutes after brief vortexing. The tubes were then centrifuged at 4°C for 30 minutes at 12000 x g. Using a flame-drawn Pasteur pipette, the supernatant was removed without disturbing the pellet. The pellet was washed with 1ml 75% cold ethanol and centrifuged at 4°C for 5 minutes at 7500 x g. The supernatant was removed using a flame-drawn Pasteur pipette. After spinning briefly, any remaining supernatant was removed and the pellet was re-suspended in 10-µl Diethyl Pyrocarbonate water. The total RNA concentration was measured using a Nanodrop spectrophotometer (Thermo Scientific, Wilmington, DE) and the samples were stored at - 80°C. These RNA samples will be sent in future to the Microarray Core Facility in Washington School of Medicine, MO for microarray analysis.

Statistical analysis

Data was analyzed using SAS V 9.1 (Cary, NC) software and the results were presented as mean and mean percentage using SAS MEANS procedure.

3. Results

Two gerbils died during the study but all the other gerbils tolerated the transplantation surgery and the ABZ drug well (data not shown).

Effect of ABZ on the motility of worms

The results of the effect of ABZ on adult female worm motility are presented in tables 3.3 and 3.4. A single dose of 150mg/kg BW demonstrated macrofilaricidal effects by day 21. The mean percentage recovery of worms were 85.1 and 68.2 and mean mortality percentages were 48.5 and 68.8 respectively for the 2 sets of treated gerbils (21 DPT and 28 DPT). In the 28 DPT

group, 14.6 % of the recovered worms were highly active (MS-3) while other worms in the same group were dead and/or unrecovered (68.8%) or nearing death (17.9% with MS-1). In the 21 DPT group, 16.2 % of the recovered worms were moderately active (MS=2) while 37.4 % of worms were still highly active (MS=3). All but one worm in the control group were highly motile throughout the study.

Worms selected for RNA extraction

Number and the type of worms selected for RNA extractions are shown in table 3.5. Two sets of worms from treated group: moderately active (MS-2) at 21 DPT and highly active (MS-3) at 28 DPT were selected. Similarly, two sets of highly active worms were selected from untreated control group, coinciding with treated worms at 21 DPT and 28 DPT. Biological replicates included sets of worms isolated from different gerbils of the same group. Thus, 42 moderately active worms were selected from 21DPT group and 40 highly active worms were selected from 28 DPT group. From the control group, 33 and 35 worms respectively were selected to match with day 21 and day 28 PT groups. The results of RNA extraction from these selected worms are shown in table 3.6.

Table 3.1 Pilot study results: Summary of the worms used in transplantation procedure and motility score of recovered worms

S. No.	No. of worms transplanted	Necropsied post-treatment day	No. of worms recovered	MS-3 (HA)	MS-2 (MA)	MS-1 (SD)	MS-0 (Dead)	Lethal % (includes dead and un-recovered worms
1	13	7	10	10	0	0	0	23.1
2	15	7	14	13	1	0	0	6.7
3	15	7	12	12	0	0	0	20.0
4	11	10	10	9	1	0	0	9.1
5	11	10	8	8	0	0	0	27.2
6	12	10	12	12	0	0	0	0.0
7	11	14	11	6	4	1	0	18.2
8	10	14	10	6	3	0	1	10.0
9	11	14	11	5	3	2	1	18.2
10	12	21	11	4	3	0	4	41.7
11	12	21	10	2	2	1	5	58.3
12	12	21	10	2	2	0	6	66.7
13	12	28	11	1	0	2	8	75.0
14	13	28	13	0	1	2	10	77.0
15	15	28	14	2	0	0	12	86.7

16	13	35	6	0	0	1	5	92.3
17	13	35	4	1	0	0	3	92.3
18	14	35	5	0	0	1	4	92.9

MS represents motility score; HA= highly active; MA= moderately active and SD= soon-to-die.

Table 3.2 Pilot study results: Worms isolated from the treated used

Group	Total # of gerbils used	Mean # of worms transplanted	Mean % of worms recovered	Mean % of recovered worms with different motility scores (MS)				Mean Mortality % (includes un-recovered and dead worms)
				MS-3	MS-2	MS-1	MS-0	
Treated D-7	3	14.3	83.7	97.2	2.8	0.0	0.0	16.3
Treated D-10	3	11.3	88.2	96.7	3.3	0.0	0.0	11.8
Treated D-14	3	10.6	100.0	53.1	31.3	9.4	6.3	6.3
Treated D-21	3	12.0	86.1	25.8	22.6	3.2	48.4	52.8
Treated D-28	3	13.3	95.0	7.9	2.6	10.5	78.9	80.0
Treated D-35	3	13.3	37.5	6.7	0.0	13.3	80.0	92.5

Table 3.3 Summary of the worms used in transplantation procedure and motility score of recovered worms

S. No.	No. of worms transplanted	ABZ dose (mg/kg BW)	Necropsied post-treatment day	No. of worms recovered	MS-3 (HA)	MS-2 (MA)	MS-1 (SD)	MS-0 (Dead)	Lethal % (includes dead and un-recovered worms)
1	18	0	28	17	17	-	-	-	5.6
2	18	0	28	18	18	-	-	-	0.0
3	17	0	21	17	16	-	1	-	0.0
4	17	0	21	17	17	-	-	-	0.0
5	30	150	21	25	9	4	1	11	53.4
6	30	150	21	27	11	4	3	9 (EC)	40.0
7	30	150	21	26	11	6	1	8 (EC)	30.0
8	30	150	21	27	15	2	2	8	36.7
9	35	150	21	31	11	3	2	15	54.3
10	35	150	21	29	14	4	1	10(EC)	45.7
11	25	150	21	NA	NA	NA	NA	NA	-
12	25	150	21	20	9	2	0	9 (EC)	56.0
13	25	150	21	19	6	4	2	7	52.0
14	25	150	21	22	21	1	0	0	-
15	25	150	21	21	9	5	1	6	40.0
16	24	150	21	22	3	4	5	10	50.0
17	25	150	21	21	7	4	1	9	52.0
18	20	150	21	18	7	2	1	8	55.0
19	25	150	28	18	3	3	1	11+EC	72.0
20	25	150	28	17	5	1	3	8+EC	64.0
21	34	150	28	18	4	6	0	8+EC	70.6
22	34	150	28	27	0	2	11	14	61.8
23	36	150	28	30	5	1	5	19	69.4
24	30	150	28	22	4	3	4	11+BW	63.3
25	30	150	28	16	4	6	6	BW+EC	46.7
26	30	150	28	22	0	4	3	15	76.7
27	30	150	28	19	6	1	1	11+EC	73.3
28	30	150	28	22	1	4	3	14	73.3
29	30	150	28	17	4	1	1	11+EC	80.0
30	25	150	28	9	5	1	3	BW+EC	-
31	34	150	28	10	4	6	0	-	-
32	34	150	28	23	3	2	6	12+BW	67.6
33	36	150	28	24	1	2	5	16+BW	77.8

MS represents motility score; HA= highly active; MA= moderately active and SD= soon-to-die; BW= broken worms, EC= worms encysted.

Table 3.4 Worms isolated from the control and treated gerbils.

Group	Total # of gerbils used	Mean # of worms transplanted	Mean % of worms recovered	Mean % of recovered worms with different motility scores (MS)				Mean Mortality % (includes un-recovered and dead worms)
				MS-3	MS-2	MS-1	MS-0	
Control D-21	2	17.0	100.0	97.1	0.0	2.9	0.0	0.0
Control D-28	2	18.0	97.2	100.0	0.0	0.0	0.0	2.8
Treated D-21	11	27.6	85.1	37.4	16.2	6.8	39.6	48.5
Treated D-28	13	31.1	68.2	14.6	13.2	17.9	54.2	68.8

Table 3.5 Total numbers of worms isolated from the control and treated gerbils based on their motility scores for RNA extraction.

S. No.	Group	Total worms isolated	
		Set-1	Set-2 (Biological replicates)
1	Control D-21	17	16
2	Control D-28	18	17
3	Moderately Active (MA) D-21	22	20
4	Highly Active (HA) D-28	21	19

Table 3.6 Total RNA isolated from the selected worms

Sample name	A260	A280	260/280	Concentration (µg/µl)	Volume (µl)	Mass (µg)
C-1	2.569	1.459	1.76	1.027	26	26.70
C-2	2.314	1.213	1.91	0.926	17	15.74
C-3	2.488	1.418	1.75	0.995	26	25.87
C-4	2.890	1.574	1.84	1.156	17	19.65
M-1	1.930	1.137	1.70	0.772	39	30.11
M-2	4.250	2.575	1.65	1.700	30	51.00
H-1	1.259	0.727	1.73	0.580	43	24.94
H-2	1.554	0.867	1.79	0.622	35	21.77

C-1: Highly active worms isolated from control gerbils sacrificed on day-21

C-3: Highly active worms isolated from control gerbils sacrificed on day-28

M-1: Moderately active worms isolated from treated gerbils sacrificed on 21 DPT

H-1: Highly active worms isolated from treated gerbils sacrificed on 28 DPT

C-2, C-4, M-2 and H-2 are biological replicates of C-1, C-3, M-1 and H-1 respectively

4. Discussion:

The concentration and time points at which adult female *Brugia malayi* worms show varying levels of susceptibility to ABZ were determined in a gerbil model. Of all the worms treated with ABZ, two sets of worms were selected; those which were highly active (MS-3) and well tolerated the effects of drug 28 DPT and those which were moderately active (MS-2) exhibiting signs of susceptibility 21 DPT. The mean mortality percentage in these worms

increased (48.5 and 68.8 in 21DPT and 28 DPT respectively) with time in treated group (single dose of ABZ 150mg/kg BW) suggesting that ABZ has a delayed macrofilaricidal effect. Similar delayed macrofilaricidal effects have been seen with flubendazole (BZ group drug) in gerbil model (Denham et al., 1979) and with ABZ in humans (Jayakody et al., 1993) and suggest that these worms were starving to death (Denham et al., 1979). The macrofilaricidal effect of ABZ could have been due to the parasites suffering from metabolic disruption at a number of sites, most of which are involved in energy production(Lacey, 1988a).

Benzimidazoles are only available in oral formulations and are poorly soluble which leads to their decreased absorption through the gastrointestinal tract (Marriner et al., 1986; Murthy et al., 1992; Horton, 2002). This decreased absorption has been shown to be the cause of inconsistent results produced by ABZ either alone or in combination with other anti-filarials (Gaur et al., 2007). Since BZs have been shown to exhibit an increased antifilarial activity through subcutaneous route compared to oral route (Reddy et al., 1983), we administered ABZ dissolved in DMSO subcutaneously in our gerbils. Intraperitoneal administration was avoided to prevent inflammation at the site of worms and also to avoid local gradients of high drug concentration in the peritoneum.

In this study, the viability of the worms was measured in terms of motility. Previously, we had measured the viability of adult female worms by measuring motility, and microfilaria (mf) release and MTT reduction (3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl-tetrazolium bromide) assays. The first two assays yield subjective data where as the latter yields objective data. Despite the fact that MTT assay yields quantitative data on the viability of the parasite, this assay was not employed as MTT treatment yields worms unsuitable for RNA extraction. In addition, in our previous study (unpublished data), a direct correlation between motility and

MTT reduction proved that the worm motility assay can be used alone in gauging macrofilaricidal drug effects. Microfilaria release is also an important parameter in determining female worm viability and worms could still be used in gene expression studies. However, for *in vivo* studies involving differential gene expression in worms, a parameter that quickly determines the viability is a key factor for avoiding non-physiologic changes in gene expression which may occur during in vitro maintenance. Our previous results also showed that at least 24 hours are required to determine viability using mf release assay whereas motility can be assessed in just a few minutes.

Experimental trials undertaken to determine an effective, but safe dose of ABZ in our gerbils showed no noticeable difference between our selected doses (150 mg/kg BW and 250 mg/kg BW (data not shown)). Our hypothesis is that probably once you get over a certain dose threshold, then time is what is important, so a higher dose does not cause much of a change. The gerbils administered the higher dose (250 mg/kg BW) exhibited some post-treatment pain, possibly due to excess volume of DMSO which is a tissue irritant. Therefore, all the treatments were done at 150 mg/kg BW and all the gerbils tolerated the treatments well.

The time points (day 21 and day 28) chosen in our present study were based on the results of our pilot study experiments (tables 3.1 and 3.2). Forty highly active (MS-3) worms which well tolerated or showed least susceptibility to the effects of ABZ at 28 days post-treatment and 42 moderately active (MS-2) worms showing signs of susceptibility to ABZ at 21 days post treatment were selected for RNA extraction to study the differential gene expression between the two populations. Moderately active worms (MS-2) were preferred over soon-to die (MS-1) worms even though both represented the susceptible group, because, worms in MS-1 group nearing death are expected to have changes in gene expression that are associated with the

death process but unrelated to drug exposure. Worms with MS-2 closely represent the genes responsible for drug susceptibility and comparing MS-3 with MS-2 would give us a better comparison between gene expression levels.

We selected worms at two different time points (MS-3 from day 28 DPT and MS-2 from 21 DPT) in this study. Earlier we had considered choosing highly active and moderately active worms isolated at only one time point post treatment. However, later we found that some worms-moderately active on 28 DPT would have belonged to the highly active group had they been isolated on 21 DPT, confounding the results. Our preliminary data clearly showed that once worms start to show reduced motility they seem to die. So all worms at 21 DPT with reduced motility are almost certainly showing signs of death and are part of the sub-population that is susceptible to the drug. In contrast, worms still highly active by 28 DPT are among the most tolerant of the population to the drug. Therefore, we chose highly active worms on 28 DPT, because they remained highly tolerant to the drug, one week after the moderately active worms were isolated at 21 DPT. MS-3 worms on 28 DPT and MS-2 worms on 21 DPT would represent two distinct populations with significant differences in ABZ susceptibility. Comparing gene expression levels between these two sets of worms may give a clearer picture about genes disregulated due to treatment with ABZ. Furthermore, it should help to elucidate mechanisms involved with the action of ABZ on worms, and at the same time, what allows some worms to survive doses of drugs that kill the majority of worms. Such knowledge may generate important insights into possible mechanisms of drug resistance.

In conclusion, this study demonstrates the macrofilaricidal activity of ABZ when administered subcutaneously to gerbils, and that this activity varies among worms. Understanding the basic information about gene expression in *B. malayi* that survived ABZ

treatment will be a first but critical step in exploring possible molecular mechanisms of BZ resistance. Practically all of our present knowledge on possible mechanisms of BZ resistance is based on candidate gene studies. This candidate gene strategy is not an optimal approach for studying drug resistance since it requires that a single hypothesis be proven correct. Since drug resistance is believed to be a multigenic trait, a candidate gene approach will not identify "novel" genes responsible for resistance. With the availability of microarray technology for *B. malayi*, many genes responsible for resistance can be screened and identified in a single experiment.

Acknowledgements

The authors of this study would like to acknowledge NIAID/NIH Filariasis Research Reagent Repository Center. We would also like to thank Drs.Michael Dzimianski and Prasit Suprakorndej for providing adult female *B. malayi* worms and Mr. Bob Storey for his invaluable technical assistance.

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CHAPTER IV

CONCLUSIONS

Lymphatic filariasis (LF) is an endemic disease caused by vector borne, filarioid nematode parasites *Wuchereria bancrofti*, *Brugia malayi and Brugia timori.* This disease is a major cause of acute and chronic morbidity and incapacitation with devastating public health and socio-economic consequences, associated with conditions of poverty. Albendazole (ABZ) is a commonly used benzimidazole (BZ) anthelmintic, but drug resistance has been widely reported in livestock nematodes. For developing effective strategies to monitor and prevent the development of resistance, a better understanding of the genetic and molecular basis of anthelmintic resistance in parasites is required.

The ultimate goal of our study was to gain basic knowledge of gene expression in *B. malayi* that survive treatment with BZ to gain insights into possible molecular mechanisms of BZ resistance.

In the *in vitro* part of our study, the thiabendazole (TBZ) concentration and time points at which lethal effects are produced in *B. malayi* adult females were determined by measuring worm motility and mf release. Thiabendazole showed increased effects on motility with higher drug concentrations, and the effects increased over time. Statistically significant differences in motility were seen between drug concentrations. Based on our results, worm motility and mf release assays appear to be the best available indicators of worm viability, still leaving worms usable for RNA extraction to study gene expression. Motility assay was preferred as its rapidity in determining the worm viability thereby minimizing the possibility of exposure to environmental contamination that could lead to additional gene expression changes other than

those imposed by the drug. Our work is the first *in vitro* study to determine the viability of adult *B malayi* worms following exposure to TBZ. This study shows that TBZ can have macrofilaricidal effects *in vitro* (similar to the results of a previous *in vivo* study using ABZ in humans) and both worm motility and mf release assays are good indicators of worm viability, but motility assay is preferred.

Through our *in vivo* study, we aimed to determine the concentration and time points at which adult female *B. malayi* worms show varying levels of susceptibility to ABZ in a gerbil model. Of all the worms subcutaneously treated with ABZ, two sets of worms were selected; those which were highly active (MS-3) and well tolerated the effects of drug at 28 DPT and those which were moderately active (MS-2) exhibiting signs of susceptibility at 21 DPT as our preliminary data showed that all MS-2 worms are destined to die. The mean mortality percentage in these worms increased with time in the treated group suggesting that ABZ had a delayed macrofilaricidal effect. In conclusion, this study demonstrates the macrofilaricidal activity of ABZ when administered subcutaneously to gerbils, and that this activity varies among worms.

With the availability of microarray technology for *B. malayi*, many genes responsible for drug action and/or resistance can be screened and identified in a single experiment. RNA extracted from selected worms from our study was submitted to Washington University School of Medicine, Microarray Core Facility (MCF) for microarray analysis. The facility uses a second-generation filarial microarray developed by the filarial microarray consortium. This array contains 65mer oligonucleotides derived from 15,412 *B. malayi* clusters, 1016 *Onchocerca volvulus* clusters, 872 *W. bancrofti* clusters, and 803 genes of *Wolbachia* endobacteria from *B. malayi*. All the procedures, including cDNA synthesis and labeling, hybridization, scanning,

gridding and data analysis, required for microarray analysis of RNA samples will be performed at the MCF. Results of microarray analysis will provide a list of genes, which are differentially expressed. Further detailed analysis of the significantly disregulated genes will be done in the future.

Gaining basic knowledge of gene expression in *B. malayi* and identifying differentially expressed genes in worms that are able to survive levels of ABZ that kill a majority of the worms exposed to the drug and comparing their gene expression levels may give a clearer picture about genes disregulated due to treatment with ABZ. Furthermore, it should help to elucidate mechanisms involved with the action of ABZ on worms, and at the same time, what allows some worms to survive doses of drugs that kill the majority of worms. Such knowledge may generate important insights into possible mechanisms of drug resistance. It is also expected that findings will be applicable to other human filarial nematodes including *W. bancrofti* and *O. volvulus* and parasitic nematodes of livestock where anthelmintic resistance is now reaching critical and alarming levels.