Characterization of the effects of flubendazole, a benzimidazole anthelmintic, on filarial nematodes

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"Progress is made by trial and failure; the failures are generally a hundred times more numerous than the successes; yet they are usually left unchronicled."

- *William Ramsay*

"But science and everyday life cannot and should not be separated. Science, for me, gives a partial explanation for life. In so far as it goes, it is based on fact, experience and experiment."

- *Rosalind Franklin*

"How did I escape? With difficulty. How did I plan this moment? With pleasure."

- *Alexandre Dumas*

Abstract

Onchocerciasis and lymphatic filariasis are debilitating diseases which are major causes of long-term disability and impede socioeconomic development in endemic countries. Control of these parasitic diseases have relied on mass drug administration (MDA) of either ivermectin (IVM) or diethylcarbamazine (DEC) in combination with albendazole. These drugs act predominantly as microfilaricides, necessitating yearly dosing in MDA campaigns until death of the adult worm. The risk of severe complications in onchocerciasis patients administered DEC precludes its use within Africa. Limiting these programmes to a single drug IVM, which is contraindicated in regions co-endemic for infections with the eye worm *Loa loa*, jeopardizes our ability to reach elimination goals for onchocerciasis. An urgent need for a safe and effective macrofilaricidal drug is obvious.

Flubendazole (FLBZ), a benzimidazole anthelmintic, is an appealing candidate macrofilaricide. FLBZ has demonstrated profound and potent macrofilaricidal effects in a number of experimental filarial rodent models and one human trial. Unfortunately, FLBZ was deemed unsatisfactory for use in MDA campaigns due to its markedly limited oral bioavailability in formulations marketed for use in the control of human infections with gastrointestinal nematodes. However, great strides have been made in recent years to develop a formulation which provides markedly improved bioavailability following oral administration. The development of FLBZ as a macrofilaricide would be greatly enhanced by a deeper understanding of its filarial effects.

The work presented in this thesis focuses on the consequence of exposure to FLBZ on reproduction and survival of filarial nematodes. Previous studies have outlined the tremendous adulticidal capacity of the drug when administered parenterally. Here, we further contributed to knowledge in this field by providing a more detailed description of changes in the adult worm following exposure to pharmacologically relevant concentrations of FLBZ.

First, we show that short-term *in vitro* exposure does in fact result in damage to tissues required for reproduction and survival of adult *Brugia malayi* at pharmacologically relevant concentrations. Using a histological approach we demonstrate damage to the hypodermis, intestine and developing embryos of adult female worms. Interestingly, we observed no drug-induced damage to stretched *in utero* microfilariae (mf).

Then, we further substantiate that the damage to developing embryos remains after short-term *in vitro* exposure to FLBZ followed by wash-out and transplant of the treated parasite into naïve jirds for long-term residence in a host. Embryogram analysis of recovered female worms indicates that in addition to the late morula exhibiting the greatest damage of all the developing stages, there also appears to be a halting of development at this stage. The release of mf from female worms was found to be impaired; however, released mf were not damaged and *in utero* mf were again found to be comparable to the control. This raises the possibility that FLBZ may be safe to use in *L. loa* endemic regions where rapid killing of mf is of concern. The impairment to the intestine and hypodermis that was observed previously appears to be of less consequence over the long-term.

Next, we implemented a transcriptomic approach to confirm the gross-morphological effects observed histologically and provide a molecular explanation. A great number of downregulated genes were found to be involved generally in developmental processes. This included genes involved in meiosis and mitosis, as well as genes involved in embryo elongation. Impairment in elongation occurring at the morula stage would account for the apparent halting of development observed in the long-term experiments. We also observed changes in genes encoding cuticle components. The role of these genes in embryonic and adult cuticle will be discussed.

Finally, we present the microfilarial effects of FLBZ. By assessing the motility of mf exposed to FLBZ, we determined that it is not directly microfilaricidal in *B. malayi* nor *L. loa,* further supporting FLBZ as a safe macrofilaricide. Lastly, mosquito infectivity assays indicated that treatment of *B. malayi* mf

impaired their ability to cross the mosquito midgut. Ultimately, while some mf did cross the midgut, FLBZ completely abolished their capacity to develop to the infective larval stage, effectively impeding transmission.

The findings presented in this PhD thesis provide invaluable data on the filarial effects of FLBZ, and bring attention to previously unexplored mechanisms by which FLBZ impairs the cycle of transmission. These results contribute to a better understanding of FLBZs action and facilitate its advancement as a macrofilaricide for use in control programmes.

Résumé

L'onchocercose et la filariose lymphatique sont des maladies débilitantes qui causent des invalidités à long-terme et qui entravent le dévelopement socio-économique dans les pays endémiques. Dans le passé, le contrôle de ces morbidités comptait sur des programmes d'administration de masse de médicaments (AMM) avec l'utilisation de l'ivermectine (IVM) ou la diéthylcarbamazine (DEC), en combinaison avec l'albendazole. Ces médicaments agissent principalement comme microfilaricides, et nécessitent des doses annuelles provenant de programmes d'AMM jusqu'à la mort du vers adulte dans l'hôte. Le risque de complications sévères chez les patients souffrant de l'onchocercose qui sont donnés un traitement de DEC empêche l'utilisation de celui-ci en Afrique. La limitation existe chez les programmes d'AMM à cause de la possibilité d'utiliser qu'un seul medicament, l'IVM, qui est contraindiqué dans les régions co-endémiques pour les infections par le vers oculaire *Loa loa*, et ceci compromet les objectifs d'élimination contre l'onchocercose. Il est évident qu'il il existe un besoin urgent pour un médicament sûr et efficace.

Le médicament flubendazole (FLBZ), un benzimidazole anthelmenthique, est un candidat macrofilaricide attirant. Ce médicament a démontré un effect macrofilaricidal profond et fort dans un nombre de modèles rongeurs et dans un essai chez les humains. Malheureusement, le FLBZ a été jugé insatisfaisant pour utilisation dans des programmes AMM à cause de sa biodisponibilité orale limitée dans les formulations disponibles pour contrôler les infections par les nématodes gastrointestinaux chez les humains. Par contre, il eut récement des grands succès avec des nouvelles formulations qui améliorent la biodisponibilité orale du médicament. Le dévelopement du FLBZ comme macrofilaricide serait grandement optimisé par une compréhension enrichie de ses effects filariaux.

Le travail présenté dans cette thèse est concentré sur l'effect de l'exposition du FLBZ sur la reproduction et la survie des nématodes filariaux. Des études antécedentes ont souligné la capacité adulticidale de la drogue administrée par voie parentérale. Ici, nous avons contribué aux connaissances

dans ce domaine en fournissant une description plus détaillée des changements subis par les vers adultes à la suite d'une exposition de FLBZ à des concentrations pharmacologiques pertinentes.

Premièrement, nous démontrons que l'exposition *in vitro* à des concentrations pharmacologiques pertinentes à court-terme résulte en des dommages des tissues requis pour la reproduction et la survie des adultes de *Brugia malayi*. En utilisant une approche histologique, nous démontrons, chez les vers femelles, le dommage à l'hypoderme, l'intestin et les embryons en dévelopement. Il est intéressant de noter que nous n'avons pas observé d'effect causé par le médicament chez les microfilaires (mf) étirés *in utero*.

Ensuite, nous justifions que le dommage chez les embryons en dévelopement est présent suite à une exposition de FLBZ *in vitro* à court-terme, suivi par l'élimination du médicament par lavage complet et la transplantation du parasite traité dans des gerbilles naïves pour une résidence à long-terme dans un hôte. Une analyse embryogramme des vers femelles indique que, en addition du morula avancé qui démontre le plus grand dommage dans les stades de dévelopement, il paraît en plus que le dévelopement arrête à ce point. La libération des mf par les vers femelles a été altérée; par contre, il n'étaient pas endommagés, et les mf *in utero* ont été trouvés comparables au contrôle. Ceci relève la possibilité que le FLBZ peut être sain pour l'utilisation dans les régions endémiques pour *L.loa,* où le décès rapide des mf est une preoccupation importante. La détérioration de l'intestin et de l'hypoderme, qui a été observée auparavant, semble être une conséquence moins importante à long-terme; les implications seront discutées en détail.

En plus, nous avons implémenté une approche transcriptomique pour confirmer les effets bruts morphologiques observés par histologie, et nous avons fournis une éxplication moléculaire. Un nombre importants de gènes sous-exprimés ont été trouvés impliqués dans le processus de dévelopement général. Ceci inclut des gènes ayant des rôles dans la mitose et la méiose, en plus des gènes impliqués dans l'élongation de l'embryon. La cessation de l'élongation qui se produit au stade

morula serait responsable pour l'arrêt apparent du dévelopment observé lors des experiments à long-terme. Nous avons aussi observé des changements dans les gènes qui codent les composants de la cuticule. Le rôle de ces gènes pour la cuticle embryonique et adulte sera discuté. D'ailleurs, nous présentons les effects microfilariaux du FLBZ. En évaluant la motilité des mf exposés au médicament, nous avons déterminé que le FLBZ n'est pas directement microfilaricidal ni pour *B. malayi*, ni pour *L. loa*, qui souligne la sécurité de FLBZ comme microfilaricide.

Finalement, des tests d'infectivité des moustiques ont indiqué que le traitement des mf de *B.malayi* perturbe leur abileté de traverser l'appareil digestif du moustique. En fin de compte, meme si quelques mf ont réussi à traverser l'appareil digestif du moustique, la FLBZ a complètement aboli leur capacité de développer jusqu'au stade larvaire infectif, ce qui entrave leur transmission.

Les découvertes présentées dans cette thèse fournissent des données inestimables sur les effets filariaux du médicament flubendazole (FLBZ), et donnent de l'attention aux mécanismes par lesquels le FLBZ interrompt le cycle de transmission. Ces résultats contribuent à la comprehension amméliorée du fonctionnement du FLBZ, et ainsi facilitent son avancement comme macrofilaricide pour l'utilisation dans des programmes de contrôle.

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The pursuit of my doctoral studies has been one of the most challenging and rewarding experiences of my life. It has been an inspiring, demanding, stimulating, and enriching journey, one that has given me the opportunity to grow not only as a scientist, but on a personal level as well. Certainly, I would not have succeeded in my studies without the support, guidance, and friendship of several people I would like to acknowledge.

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Phil Campeau, thank you for your kindness, your encouragement, your patience and for helping me through the tough times. The past couple of years have been considerably enriched with you as my cheerleader, counsellor, voice-of-reason and JJ partner.

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STATEMENT OF ORIGINALITY

The following aspects presented in this thesis are considered original contributions to knowledge:

CHAPTER 2. MANUSCRIPT I

Maeghan O'Neill, James F. Geary, Dalen W. Agnew, Charles, D. Mackenzie and Timothy G. Geary
In vitro* Flubendazole-induced damage to vital tissues in adult females of the filarial nematode *Brugia malayi

International Journal for Parasitology: Drugs and Drug Resistance (2015). 5(3): 135-140. doi:10.1016/j.ijpddr.2015.06.002.

In this manuscript we investigated the morphological effects of exposure to pharmacologically-relevant concentrations of FLBZ on the model filarial nematode *B. malayi*. This study is the first to implement a scoring system for benzimidazole-induced tissue damage which allowed us to quantify drug-induced damage for the first time. Using a histological method, we identified the tissue types that exhibited the greatest damage and correlated the duration of exposure with histological damage. At all concentrations tested, tissue damage was most prominent in the hypodermis, intestine and developing embryos, even at the shortest duration of exposure (24 hours). These findings provide preliminary information concerning the concentration-time profile of exposure to FLBZ required to elicit detrimental effects as measured by changes in morphology.

CHAPTER 3. MANUSCRIPT II

Maeghan O'Neill, Abdelmoneim Mansour, Utami Dicosty, James F. Geary, Michael Dzimianski, Scott McCall, John McCall, Charles D. Mackenzie, Timothy G. Geary
An *in vitro/in vivo* model to analyze the effects of flubendazole exposure on adult female *Brugia malayi*

Accepted with minor revisions to *PLoS NTDs*

This manuscript reports the long-term effects of exposure to FLBZ in culture on *B. malayi* building on the results of manuscript I. We implemented a mixed *in vitro/in vivo* approach in which adult worms

were incubated in FLBZ *in vitro* over short durations (≤ 24 hours) prior to drug wash-out and transplantation of the parasites to naïve jirds for long-term maintenance *in vivo*. Sacrificing animals with subsequent parasitological examination at various time points following transplantation allowed us to identify damage that was apparently irreversible. Again implementing a histological approach we identified effects on reproduction that were irreversible at 8 weeks post-exposure. This was confirmed by observing a decrease in microfilarial release and damage to intrauterine developing stages. Interestingly, we saw that worms were able to recover from damages to the hypodermis and intestine when maintained *in vivo* over longer durations. This has not been descried in the literature and suggests that worms may have a more sophisticated capacity for healing than anticipated.

CHAPTER 4. MANUSCRIPT III

Maeghan O'Neill, Cristina Ballesteros, Lucienne Tritten, Erica Burkman, Weam I. Zaky, Jianguo Xia, Andrew Moorhead, Steven A. Williams, Timothy G. Geary

Profiling the macrofilaricidal effects of flubendazole on adult female *Brugia malayi* using RNAseq

In preparation for submission to *IJP-DDR*

In this manuscript we describe the transcriptomic changes in *B. malayi* adult female worms in response to FLBZ exposure *in vitro*. We saw downregulation of genes related to reproduction and cuticular synthesis. Changes in expression of genes related to reproduction confirm prior evidence that FLBZ limits embryogenesis and gives the first molecular genetic explanation for this impairment. This study was the first to suggest weakening of the adult cuticle as a mechanism of damage resulting from FLBZ exposure. We have not proven the stage-specific roles (adult vs. embryo) of the collagen genes that were observed to be down-regulated, but these findings provide initial insight into a novel mechanism of action for FLBZ. Finally, dysregulation of the expression of one gene found to overlap all treatment groups was identified as a possible marker of damage. Further experimentation would substantiate this

gene as a FLBZ-specific marker to surmount the difficulties with current methods of assessing drug-induced damage.

CHAPTER 5. MANUSCRIPT IV

Maeghan O'Neill, Jelil A. Njouendou, Michael Dzimianski, Erica Burkman, Patrick C. Ndongmo, Arnaud J. Kenge-Ouafo, Samuel Wanji, Andrew Moorhead, Charles D. Mackenzie, Timothy G. Geary

Microfilaral sensitivity to Flubendazole *in vitro*

In preparation for submission to *Parasites and Vectors*

This manuscript is the first to assess the direct effects of FLBZ on microfilariae. First, this study examined the potential of FLBZ to rapidly kill mf of *L. loa,* a phenomenon that is proposed to result in severe complications in co-infected onchocerciasis patients treated with ivermectin. Assessment of viability, measured by motility, indicated that FLBZ does not rapidly kill *L. loa* mf *in vitro*; a similar lack of toxicity was observed for mf of the model filariid *B. malayi*. Although much work needs to be done to confirm that these effects can be extended to mf present in human patients, these results are an important indication of the safety of FLBZ in *L. loa* endemic regions. Given that the macrofilaricidal activity of FLBZ occurs weeks to months following administration and unaffected circulating mf are available for ingestion by a transmissible host, we measured the effect of FLBZ on transmission of exposed mf. FLBZ exposure impaired the ability of mf to cross the mosquito midgut, regardless of duration of exposure; however, at lower concentrations a proportion of mf crossed the midgut. Although some mf crossed the midgut, FLBZ exposure completely abolished the ability of treated mf to develop to infective L_3s, irrespective of duration of exposure or concentration. The results of this study reveal an important approach in which FLBZ can be implemented to completely block filarial transmission.

CONTRIBUTION OF AUTHORS

The design, execution and analysis of the experiments presented in this thesis were carried out by the author under the supervision of Dr. Timothy G. Geary.

In the first manuscript (Chapter 2), all *in vitro* culture work was completed by the author at the Filariasis Research Reagent Resource Center (University of Georgia). James F. Geary, Dalen W. Agnew and Dr. Charles D. Mackenzie contributed to the development of the quantitative damage scoring system. In addition to the author, James F. Geary, and Dr. Charles D. Mackenzie scored all histological slides.

In the second manuscript (Chapter 3), the *in vitro* culture, transplantation and parasitological assessment was conducted at TRS Labs Inc. (Athens, GA). John McCall and Scott McCall contributed to the experimental design. Assistance in parasite acquisition, *in vitro culture*, necropsy and parasitological assessment was provided by Dr. Abdelmoneim Mansour, and Utami Dicosty. Drs. Abdelmoneim Mansour and Michael Dzimianski conducted the transplant surgery. In addition to the author, James F. Geary, and Dr. Charles D. Mackenzie scored all histological slides.

In manuscript three (Chapter 4), Cristina Ballesteros, Lucienne Tritten, and Weam I. Zaky contributed to the experimental design and analysis of bioinformatics data. Parasite material was supplied by Erica Burkman and Dr. Andrew Moorhead at the Filariasis Research Reagent Resource Center (University of Georgia). The author worked closely with Cristina Ballesteros and Dr. Lucienne Tritten to conduct parasite culture and RNA preparation. Weam I. Zaky completed sample preparation and conducted

RNA sequencing on said samples in the lab of Dr. Steven A. Williams. The author completed the bioinformatics analysis with technical assistance provided by Dr. Jianguo Xia.

The work conducted by the author for manuscript four (Chapter 5) was split between the Research Foundation for Tropical Disease and Environment at the University of Buea in Cameroon and the Filariasis Research Reagent Resource Center (University of Georgia). Dr.. Charles D. Mackenzie contributed to the experimental design of work conducted in Cameroon. Parasite material in Cameroon was acquired by Patrick C. Ndongmo and Arnaud J. Kenge-Ouafo. Jelil A. Njouendou provided assistance for *in vitro* culture in the lab of Dr. Samuel Wanji. Dr. Michael Dzimianski contributed to the experimental design of work conducted in Georgia in the lab of Dr. Andrew Moorhead. Erica Burkman and Dr. Michael Dzimianski assisted in all aspect of mf acquisition, culture and mosquito feeding. All dissections and data analysis was completed by the author.

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LIST OF ABREVIATIONS

ABZ	**Al**Benda**Z**ole
ANOVA	**AN**alysis **O**f **VA**rience
APOC	**A**frican **P**rogramme for **O**nchocerciasis **C**ontrol
ASD	**A**morphous **S**olid **D**ispersion
AUP	**A**nimal **U**se **P**rotocol
BAM	**B**inary **A**lignment/**M**ap
BH	**B**enjamini **H**ochberg
BZ	**B**enzimidazole
CEUAR	**C**ommittee on the **E**thical **U**se of **A**nimals in **R**esearch
CD	**C**yclo**D**extran
DNA	**D**eoxy**R**ibonucleic **A**cid
cDNA	**c**omplementary **D**eoxy**R**ibonucleic **A**cid
dsDNA	**d**ouble **s**tranded **D**eoxy**R**ibonucleic **A**cid
CO_2	**C**arbon **D**ioxide
DE	**D**ifferentially **E**xpressed
DEC	**D**i**E**thyl**C**arbamazine
DEGs	**D**ifferentially **E**xpressed **G**enes
DMEM	**D**ulbecco's **M**odified **E**agle's **M**edium
DMSO	**D**i**M**ethyl **S**ulf**O**xide
EDTA	**E**thylene**D**iamine**T**etraacetic **A**cid
EST	**E**xpressed **S**equence **T**ags
FBS	**F**etal **B**ovine **S**erum
FC	**F**old **C**hange
FDR	**F**alse **D**iscovery **R**ate
FGP	**F**ilarial **G**enome **P**roject
FLBZ	**FL**u**B**enda**Z**ole
FLBZ-H	**H**ydrolyzed **FL**u**B**enda**Z**ole
FLBZ-R	**R**educed **FL**u**B**enda**Z**ole
FR3	**F**ilariasis **R**esearch **R**eagent **R**esource center
GI	**G**astro**I**ntestinal
GDP	**G**uanosine **D**i**P**hosphate
GPELF	**G**lobal **P**rogram to **E**liminate **L**ymphatic **F**ilariasis

GO	**G**ene **O**ntology
GTP	**G**uanosine **T**ri**P**hosphate
h,hr	**h**our
hpe	**h**our **p**ost **e**xposure
IVM	**IV**er**M**ectin
IACUC	**I**nstitutional **A**nimal **C**are and **U**se **C**ommittee
kDA	**k**ilo**DA**lton
L_2	Second stage **L**arvae
L_3	Third stage **L**arvae
LF	**L**ymphatic **F**ilariasis
Mb	**M**ega**b**ase
MBZ	**M**e**B**enda**Z**ole
MDA	**M**ass **D**rug **A**dministration
mf	**m**icro**f**ilaria
OCP	**O**nchocerciasis **C**ontrol **P**rogram
OEPA	**O**nchocerciasis **E**limintation **P**rogram in the **A**mericas
PBS	**P**hosphate **B**uffered **S**aline
Pk	**P**harmaco**k**inetics
QC	**Q**uality **C**ontrol
RAPLOA	**R**apid **A**ssessment **P**rocedure for **LOA**sis
REFOTDE	**R**esearch **FO**undation for **T**ropical **D**isease and **E**nvironment
RNA	**R**ibo**N**ucleic **A**cid
mRNA	**m**essenger **R**ibo**N**ucleic **A**cid
RNAi	**R**ibo**N**ucleic **A**cid **i**nterference
RNAseq	**R**ibo**N**ucleic **A**cid **s**equencing
RPMI	**R**oswell **P**ark **M**emorial **I**nstitute medium
SAEs	**S**evere **A**dverse **E**vents
SAM	**S**equence **A**lignement/**M**ap
TEAs	**T**rans-**E**pidermal **A**ttachments
TMM	**T**rimmed **M**ean of **M**-values
TUNEL	**T**erminal deoxynucleotidyl transferase (TdT) d**U**TP **N**ick-**E**nd **L**abeling
U	**U**nits
UGA	**U**niversity of **G**eorgia, **A**thens
WebGestalt	**WEB**-based **G**ene **S**et **A**na**L**ysis **T**oolkit

Introduction

RATIONALE

Infections with filarial parasites can lead to debilitating diseases which are major causes of long term disability and an impediment to socioeconomic development in endemic countries (1, 2). Despite the magnitude of the problem, there has been ardent optimism about the control of onchocerciasis and eradication of LF with the World Health Organization (WHO) targeting it for elimination (2). However, the target date for elimination continues to be postponed and has recently been extended 10 additional years to 2030 (3).

Current control programmes depend on one of two drugs; ivermectin (IVM) or diethylcarbamazine (DEC), both of which have inherent limitations that are detrimental to elimination efforts (4-6).

DEC is contraindicated in onchocerciasis patients as treatment can promote the ocular pathologies associated with *Onchocerca volvulus* infection(1). Given that upwards of 99% of all onchocerciasis cases are now found in Africa (1), we are left with a single drug for use in mass drug administration (MDA) programmes in Africa; IVM (1). Importantly, IVM is contraindicated in areas co-endemic for *Loa loa,* as IVM treatment has been linked to severe neuropathies and even death in some loiasis patients who have very high microfilarial loads (7). Moreover, suboptimal responses to IVM treatment have been reported in onchocerciasis patients (8). It is also important to note that these drugs are primarily microfilaricidal when used for mass administration, requiring yearly dosing until the death of the adult worms. Therefore, it is critical to continue to search not only for a drug that is safe to use, but also one which has macrofilaricidal efficacy.

Flubendazole (FLBZ), a benzimidazole (BZ) anthelmintic, is an appealing candidate for use in control programmes for onchocerciasis and lymphatic filariasis (LF). When first developed for control of

infections due to gastrointestinal nematodes of livestock animals, FLBZ was found to be highly potent and efficacious (9). What is most appealing is that high macrofilaricidal efficacy is attained in a number of experimental rodent models of filariases, while having little effect on circulating mf (10-12). FLBZ was previously deemed unsatisfactory for use in humans in MDA campaigns due to its limited bioavailability when administered orally. However, a new formulation that afforded high bioavailability following oral administration could render FLBZ an effective treatment for filariasis. With renewed interest into reformulation of FLBZ (7, 13, 14), development of an exposure-efficacy profile *in vitro* can assist in the definition of target pharmacokinetic profiles for dose selection in advanced development.

CENTRAL HYPOTHESIS

The central hypothesis of this project is that exposure to FLBZ at pharmacologically relevant concentrations results in irreversible tissue damage leading to lethality in adult worms while eliciting little damage to mf.

OBJECTIVES

The overall aim of this project was to provide a comprehensive assessment of the *in vitro* and *in vivo* effects of FLBZ on filarial nematodes. The specific objectives for this thesis were:

CHAPTER 2. MANUSCRIPT I. *In vitro* MORPHOLOGICAL DAMAGE

- Describe the time- and concentration-dependant morphological changes in nematode tissue following FLBZ exposure *in vitro*
- Develop a scoring system for quantification of drug-induced tissue damage

CHAPTER 3. MANUSCRIPT II. LONG-TERM DAMAGE

- Examine the long-term concentration-dependant effects of exposure to FLBZ *in vitro* in *B. malayi* survival and viability after *in vivo* maintenance in the peritoneal cavity of jirds

CHAPTER 4. MANUSCRIPT III. TRANSCRIPTOMIC ASSESSMENT

- Confirm morphological damages observed in the previous two chapters using a deep-sequencing approach
- Identify a molecular marker for FLBZ-induced damage

CHAPTER 5. MANUSCRIPT IV. FLBZ EFFECTS ON MICROILARIAE

- Assess the *in vitro* effect of FLBZ on mf of *L. loa* and confirm these effects using the traditional model of filariasis; *B. malayi*
- Determine if FLBZ impairs transmission of mf by impairing their development to the infective L_3 stage in a mosquito host

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Chapter 1. Literature Review

1.1 FILARIASIS

Infections with parasitic nematodes of the family Onchocercidae can result in major health problems and are an impediment to socioeconomic development in endemic countries (1). The pathogenic species of greatest importance are those which cause lymphatic filariasis (*Wuchereria bancrofti* and *Brugia malayi*) and onchocerciasis (*Onchocerca volvulus*). An additional filariae of significance is *Loa loa,* the causative agent of loiasis, which becomes an important consideration for mass drug administration (MDA) programmes. Together, infecting more than 150 million people, these parasitic diseases are among the leading causes of morbidity worldwide (2).

1.1.1 Filarial life cycle

Filarial worms are heteroxenous, alternating between a vertebrate host and an insect vector (Figure 1.1). The threadlike adult worms (macrofilariae) reside in the lymphatic system (LF) or subcutaneous tissue (onchocerciasis and loiasis) of the vertebrate host. Following insemination by the male, ovoviviparous females release upwards of 10 000 microfilariae (mf) daily into lymphatic circulation or the surrounding skin (1). Microfilariae of *W. bancrofti, B. malayi,* and *L. loa* are enclosed in a chitin –containing sheath, while *O. volvulus* mf are unsheathed. From the lymph, mf migrate to the peripheral blood circulation where they become available for ingestion by an insect vector during a blood meal. Mf of *W. bancrofiti* and *Brugia* are transmitted by mosquitoes and have nocturnal periodicity; the number of mf in the blood peaks at midnight, coinciding with the time of peak

feeding by the mosquito vector (3). *O. volvulus* mf are transmitted by black flies and, unlike LF, does not exhibit periodicity (1). *L. loa* mf exhibit diurnal periodicity; during the day mf are found in the blood, but while not circulating, they can be found in the lungs (4) from which mf can invade peripheral blood to be taken up by tabanid flies (5). Within the insect, mf exsheath and penetrate the midgut within 24 hours before migrating to the thoracic muscles, or fat body in the case of *L. loa*. After approximately 14 days, the parasites will have developed into the infective L_3 larval stage. The infective stage is deposited into the skin of another host during a subsequent blood meal. The L_3 invades subcutaneous tissue where it remains, or migrates to the lymphatics, and matures into an adult. Adult worms can live in the host for 7-10 years and mf can live up to 1 year in the host (6).

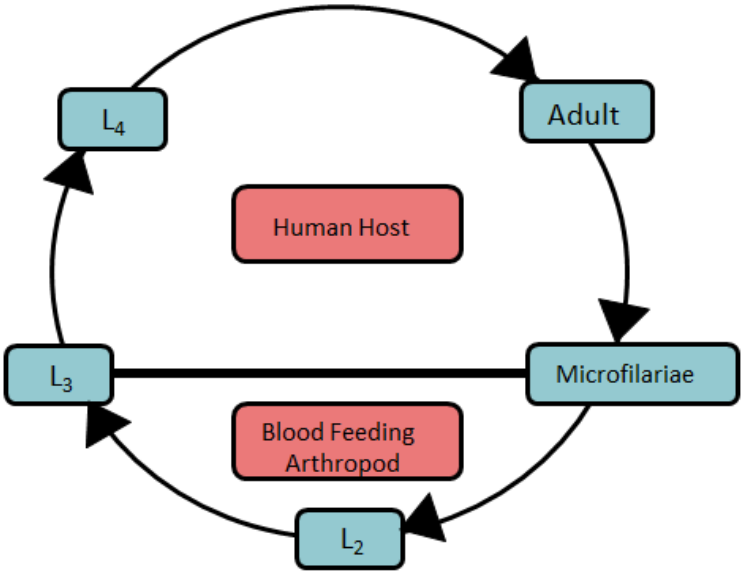

Figure 1.1. General filarial life cycle

1.1.2 Lymphatic Filariasis

Lymphatic filariasis (LF) is a mosquito-borne parasitic disease that is a major cause of long-term disability and an impedes socioeconomic development in more than 80 countries worldwide (Figure 1.2) (7). Of the 1.2 billion people at risk for infection, recent estimates are that 120 million harbour

these long-lived parasites (7). The disease is caused by infection with one of three parasitic nematodes: *Wuchereria bancrofti, Brugia malayi* and *B. timori*. *W. bancrofti* accounts for approximately 90% of all cases and is transmitted throughout the tropics in Asia, Africa and the Americas. *Brugia sp.* are transmitted throughout south-east Asia (1).

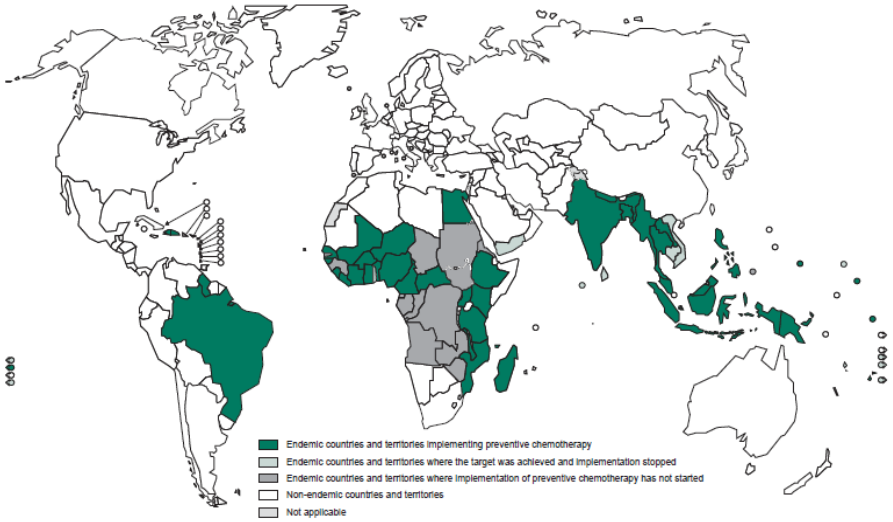

Figure 1.2. Endemicity and distribution of chemotherapeutics for lymphatic filariasis. (8)

Of the 120 million estimated to be infected with lymphatic filarids, approximately one third exhibit clinical manifestations (9). Traditionally, infected individuals have been grouped into four categories: 1. Endemic normal – no mf detected in blood and no clinical signs; 2. Asymptomatic microfilaremia – mf detected, but no clinical signs; or 3. Acute disease – clinical signs, with or without mf; 4. Chronic disease – mostly amicrofilaremic, with or without clinical signs.

While the overwhelming majority of infected people have few observable clinical signs, infection can cause a variety of clinical manifestations. The sign most commonly attributed to LF is lymphedema, and is a permanent sign of chronic infection caused by the adult worm residing in and causing damage to the draining lymph nodes of the affected area. The accumulation of fluid is most commonly seen in the arms and legs, but can also occur in the scrotum of males leading to a

condition termed hydrocele. The progression to elephantiasis in the limbs occurs gradually and is characterized by thickening of the skin and associated fibrosis of underlying tissue (10). These complications limit mobility, access to education, as well as the ability of individuals to obtain and retain employment (2). There is also stigmatization which accompanies the disfiguration associated with advanced pathology (2).

In 2000, the WHO launched the Global Program to Eliminate Lymphatic Filariasis (GPELF) with the goal of eliminating LF as a public health problem by 2020 (11). The program had two goals: (i) interrupt transmission; and (ii) control morbidity. Using a combination of filaricidal drugs and basic health care to treat acute disease and prevent disease progression, the program has led to an estimated 59% reduction of LF incidence in 55 of the 80 endemic countries (12). Continued reduction in parasite burden is expected following expansion of the program into countries where it has yet to be implemented; however, the difficulty in accessing some areas, and the long duration of current treatment regimens indicates a long road to success.

1.1.3 Onchocerciasis

Onchocerciasis, a disease caused by the tissue-dwelling filarial nematode *Onchocerca volvulus*, affects 37 million people worldwide with approximately 123 million people at risk (1). The disease is of greatest concern in sub-Saharan Africa where we find 99% of all cases occurring across 34 countries (Figure 1.3) (13).

Unlike in LF, adult *O. volvulus* reside in parasite-associated nodules in the skin. Adults release mf into the surrounding subcutaneous tissue where disease manifestation typically arises. More than 60% of the population in hyperendemic regions will present with mf in the skin, a tissue in which mf

death provokes a host inflammatory response leading to pathology (1). This is observed as dermatitis,

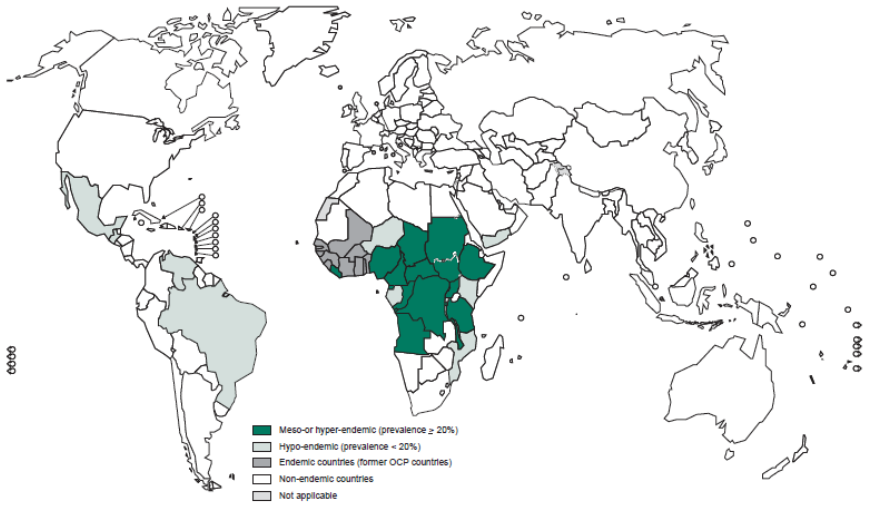

Figure 1.3. Distribution of Onchocerciasis. (8)

intense itching, and, in chronic infections, depigmentation. The most serious pathology occurs when mf migrate to, and die in, tissues of the eye; also known as river blindness, an estimated 500 000 infected individuals are visually impaired, of whom 270 000 are blind (14).

Efforts to control onchocerciasis transmission began in West Africa with the launch of the Onchocerciasis Control Program (OCP) in 11 countries in 1974 (15). This initiative commenced with regular spraying of aerial larvicides for vector management and later expanded to include distribution of oral ivermectin (IVM) in the late 1980s (16). A few years later, the Onchocerciasis Elimination Programme in the Americas (OEPA) was introduced in 13 foci in Latin America with the aim of eliminating morbidity and interrupting transmission (16). Onchocerciasis control was expanded to the remaining 19 endemic African countries with the establishment of the African Programme for Onchocerciasis Control (APOC) in 1995 (16). This program implemented an innovative community-directed treatment mechanism which empowers communities to take on

ownership of delivering IVM to its members. The effectiveness of these control programs is a major achievement in global public health, an achievement which has been highlighted by the awarding of William Campbell and Satoshi Ōmura the Nobel prize in medicine for their discovery of IVM (17). Evidence of interrupted transmission in many endemic regions gives hope for possible elimination (18).

1.1.4 Loiasis

Loiasis is caused by the filarial nematode *Loa loa* and is endemic in rainforest and savannah areas of Central and West Africa (Figure 1.4) (19). Little is known about the epidemiology of *L. loa*. Much of what is known has been derived from clinical reports rather than extensive epidemiological studies, though millions were reported to be infected with prevalences of up to 57% in some villages (20, 21).

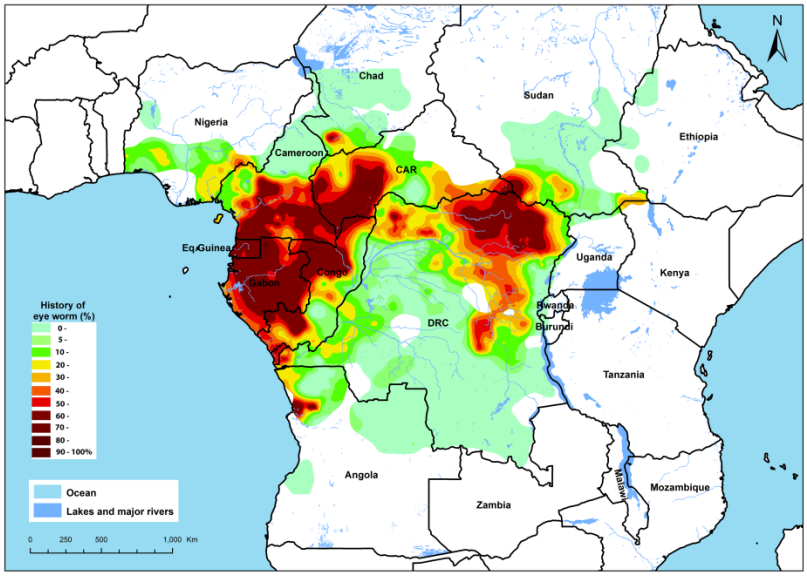

Figure 1.4. Estimated prevalence of Loa loa. (22)

Similar to onchocerciasis, *L. loa* is a tissue dwelling nematode. However, rather than forming nodules, adult *L. loa* actively migrate through subcutaneous tissues (19). The majority of infected people do not show clinical signs; however, when present, symptoms are less serious than those of LF and onchocerciasis. The most common symptom is the Calabar swelling and itching that occur intermittently, which is thought to be caused by antigenic material (4). Commonly called eye worm, *L. loa* adult worms can be seen migrating through the cornea of the eye of its human host. Recently, loiasis has emerged as a public health concern, not due to direct pathology, but because of the negative effects it has on onchocerciasis control programs. Development of severe adverse events (SAEs) in *L. loa* infected individuals included in onchocerciasis MDA programmes revealed a relationship between high microfilarial loads (>30,000 mf/ml) and severe drug-induced neuropathies (23-25). Because of these serious complications, APOC undertook a large scale mapping effort in endemic countries using a questionnaire to assess the history of eye worm termed rapid assessment procedure of loiasis (RAPLOA) (26). The RAPLOA method identified communities which are at high risk for the development of SAEs and is now being adopted by APOC to assess the prevalence of *L. loa* prior to commencing MDA (22, 27).

1.2 Laboratory Models of Filariasis

1.2.1 *Brugia malayi* as a model

Efforts to better understand the biology and mechanism of parasitic infections, as well as investigations into antiparasitic agents, are greatly enhanced by the availability of suitable animal models. Unfortunately, the complex life cycles and host specificities fundamental to the pathological filariae have limited the development of representative animal models.

While animal models do exist for each of the filariae of concern, these models are generally not practical for use in laboratory settings. The unsuitability of these models is largely attributable to ethical considerations and animal housing requirements.

Experimental infections of various non-human primates are the only known models for *W. bancrofti* and *L. loa* . The only models known for *W. bancrofti* are langur monkeys, *Presbytis melalophos* and *Presbytis entellus* (28, 29). *L. loa* readily infects mandrills (*Mandrillus leucophaeus)* (30), baboons (*Papio anubis*) (31), and patas monkeys (*Erythrocubis patas*) (31). The use of non-human primates in research carries several concerns which render these models impractical in most laboratory settings

The natural infecting bovine parasite *Onchocerca ochengi,* a nodule dwelling filaria similar to *O. volvulus*, has been exploited as a model for human onchocerciasis in naturally and experimentally infected cattle in sub-Saharan Africa (32). Low throughput and considerable animal husbandry requirements severely limit the utility of this model. Recent efforts have been made to develop a small animal model of bovine onchocerciasis for use in macrofilaricide screening (33); however, this still requires parasite recovery from parasitized cattle.

The susceptibility of gerbils to *Brugia* spp., discovered in 1970 (34, 35), and the subsequent development of an intraperitoneal infection method (36) were important advances for filarial research. Several laboratory animal models have been shown to be susceptible to *Brugia* spp., including dogs, cats, rats, and mice (37-43); however, the Mongolian gerbil (*Meriones unguiculatus*) has been used preferentially due to the relative ease of husbandry, life cycle maintenance and high percentage of worm recovery.

1.2.2 Genome

As *B. malayi* has been the favoured filaria for laboratory research due to its logistical advantages, it was selected as the representative filarial genome for the Filarial Genome Project (FGP). The WHO-

sponsored FGP was initiated in 1994 with the aim of generating genomic information for the filarial research community (44). Large-scale EST analysis of cDNA libraries laid the groundwork for the whole genome sequence and annotation now in place (44-46).

Like most filarial species, *B. malayi* harbors three genomes: nuclear, mitochondrial and that of a bacterial endosymbiont, *Wolbachia*. The nuclear genome is organized into five chromosome pairs (four automosomes and an XY sex-determination pair), estimated to be 95 Mb (44, 47). Whole-genome shotgun sequencing with nine-fold coverage resulted in approximately 90% assembly into scaffolds (45). It was estimated that 11 500 of the 14 500 – 17 800 *B. malayi* genes are protein encoding (44).

1.2.3 *B. malayi* larval development

The most commonly used chemotherapeutics for filarial infections selectively target larval stages in the blood. Besides this, the importance of impeding the microfilarial stage for limiting transmission has made this stage an area of focus for filarial research.

Relatively little is known about the embryogenesis of the filariae, but it appears to follow a similar sequence to that of *C. elegans*. At the same time that fertilized oocytes complete meiosis I and II, an egg shell is being formed around the new embryo (48). A series of cleavages in the early embryo result in the formation of a solid ball of cells called a morula (48). Continued cell proliferation and movement

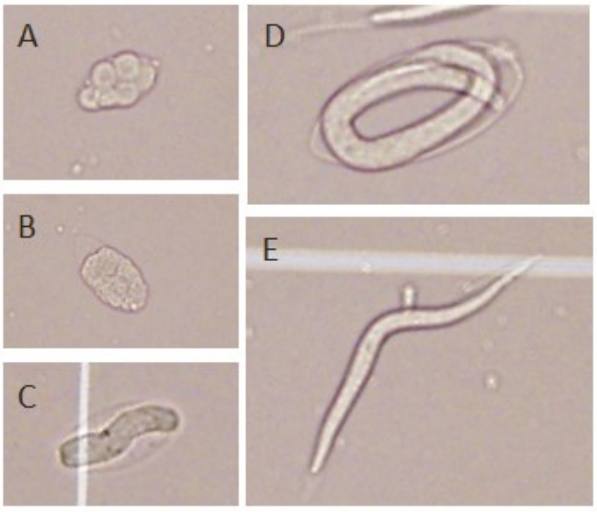

Figure 1.5. Embryonic developmental stages of *Brugia malayi*. A. Early morula, B. Late morula, C. Sausage, D. Pretzel, E. Stretched microfilaria.

occurs as the embryo progresses through three additional stages: a 'sausage' stage, in which the embryo begins to elongate; a 'pretzel' stage, observed as a coiled mf inside the egg shell; and finally a fully stretched mf encased in a sheath (Figure 1.5).

As embryogenesis occurs, developing embryos make their way along the uterus to the vulva to be expelled into the surrounding environment; lymph, in the case of *B. malayi*. In the jird model, mf are expelled into the animal's peritoneal cavity (49).

Sheathed mf migrate from lymph to peripheral blood to be ingested by a mosquito vector during a bloodmeal. In laboratory settings, artificial infection of *Aedes aegypti* mosquitoes is conducted by feeding mosquitoes on membrane feeders filled with blood containing peritoneal-acquired mf. When

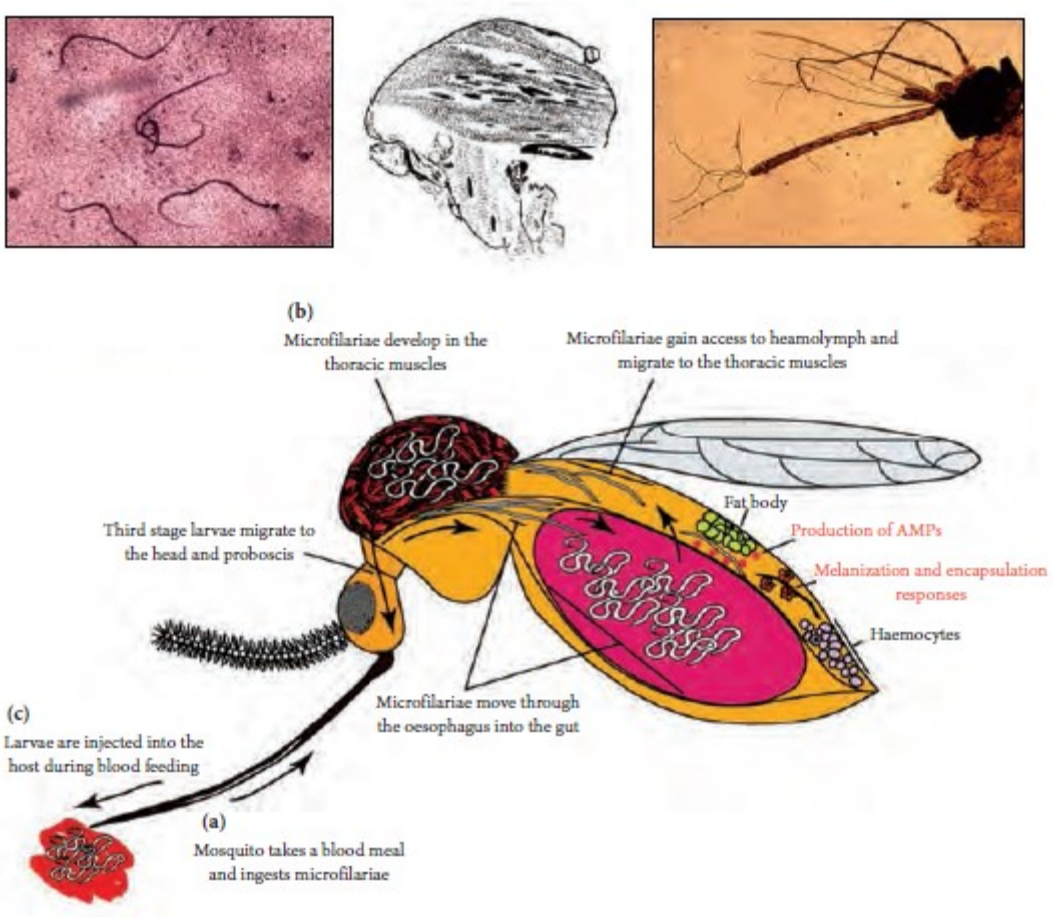

Figure 1.6. Filarial larval development in a mosquito vector. A. Microfilaria in human blood. B. L_2 larvae in thoracic muscle. C. Infective L_3 larvae emerging from proboscis of mosquito. D. Sequence of events occurring during larval development in the mosquito. (50)

female mosquitoes ingest a bloodmeal, the first barrier to infection is the mosquito midgut (Figure 1.6). Invasion of mf into the hemocoel occurs almost immediately and requires the loss of their sheath as well as rupturing of the midgut wall using a cephalic hook (51-53). Once in the hemocoel, exsheathed mf migrate to, and enter into, thoracic muscles where they molt twice, each occurring after 2-3 days (54). Each molt requires the synthesis of a new cuticle to replace the shed one. The infective L_3 larva migrates to the mosquito proboscis, ready to be deposited into a new host with a subsequent bloodmeal (50).

1.3 Chemotherapy

Prior to the establishment and success of filariasis control programs, there were three barriers to disease elimination: i. lack of tools required to interrupt transmission and disease progression; ii. knowledge of parasite dynamics and role in disease; and iii. public awareness (55). In response to this global health crisis, filarial control programs were founded. These programs were largely based on community-wide MDA for at-risk populations.

1.3.1 Current chemotherapeutics

Chemotherapy for onchocerciasis and LF centers primarily on the use of three drugs which have shown to be safe and effective when used appropriately: diethylcarbamazine (DEC), ivermectin (IVM) and albendazole (ABZ). Yearly, single dose-regimens include combinations of DEC (6 mg/kg) or IVM (150-200 μg/kg) with ABZ (400 mg) (55, 56).

Diethylcarbamazine. DEC is the mainstay drug for LF treatment in India and Southeast Asia. The mode of action is not fully understood, but it has been suggested that it involves a synergistic role of the host immune system (57) in the clearance of mf. DEC exhibits some macrofilaricidal effects; however, only a small percentage of the adult worms are killed and the associated adverse

consequences preclude its routine use in higher doses as a macrofilaricide (58). The most concerning aspect of DEC use is the occurrence of profound adverse effects associated with treatment of onchocerciasis. For this reason, DEC is prohibited as a chemotherapeutic in regions where onchocerciasis is co-endemic with LF (59).

Ivermectin. Due to its high efficacy and safety in onchocerciasis treatment, IVM is the pillar chemotherapeutic in Africa for LF where onchocerciasis is co-endemic. IVM is a macrocyclic lactone that results in the clearance of mf for 6-9 months and sterilizes female worms for an equivalent period (60). A synergistic effect of IVM and the host immune system appears to result in mf clearance, whereby hyperpolarization of glutamate-gated chloride channels prevents the excretion/secretion of immune-modulatory molecules from the microfilarial excretory/secretory vesicle (61). While this drug is highly efficacious at clearing mf, it exhibits limited macrofilaricidal effects (1). Microfilarial clearance is important for limiting transmission. However, considering that *O. volvulus* macrofilariae can live up to ten years, continually releasing mf, IVM must be given at least once per year to control transmission and the development of ocular pathology. Although IVM blocks transmission and markedly reduces pathology in onchocerciasis, this requires long-term administration. It is also important to note that it is the macrofilariae which are responsible for pathological effects associated with LF. It is therefore evident that a macrofilaricidal drug is needed to achieve goals for LF and onchocerciasis eradication.

A major limitation of IVM treatment is evident in areas which are co-endemic for *L. loa*. Patients with very high *L. loa* mf loads and occurrence of mf in cerebrospinal fluid have been found to be at risk for development of neurologic severe adverse events (SAEs) following treatment with IVM (23, 62). More than 120 deaths in the last 10 years have been attributed to IVM-related SAEs (63).

Albendazole. ABZ, a benzimidazole (BZ) that interferes with microtubule polymerization in many gastrointestinal (GI) nematodes, is commonly used in combination therapy with DEC or IVM in LF

MDA programmes (64). Studies on the efficacy of combination therapy are limited. ABZ alone was found to be ineffective in reducing microfilaremia (65, 66). A combination of ABZ/IVM was more effective at reducing mf in the blood four months following treatment (65); however, the level of mf was equivalent after twelve months (66). Recent studies implementing triple therapy with IVM/DEC/ABZ are promising. The triple therapy was significantly more effective than the DEC/ABZ combination at reducing microfilaremia (67). Additionally, patients who received triple therapy were mf negative for at least 1 year and, in some cases, 2 years following administration. Patients treated with DEC/ABZ were mf positive at the 1 year timepoint (67). Use of triple therapy has the potential to enhance LF elimination efforts; however, this regimen is only suitable for use outside of sub-Saharan Africa where DEC can be administered safely.

1.3.2 New Drugs

There are important concerns with current chemotherapeutics for filariasis treatment: first, current drugs are strictly microfilaricidal in MDA settings and require long-term administration until death of the adult worm; second, there are safety concerns with both mainstay drugs. It is obvious that a safe and effective macrofilaricidal drug would be of great benefit for current MDA programmes as they transition from control to elimination.

Macrofilaricidal activity is presumably associated with the host immune response and, consequently, host pathology as it reacts to released worm antigen. It is, therefore, ideal to have a drug which can elicit slow killing to avoid such complications.

The only safe and effective macrofilaricides currently available for human use are antibiotics. Antibiotics such as doxycycline (doxy) eliminate the obligate endosymbiont bacteria *Wolbachia,* leading to death of the adult worm (68). It is not yet clear the exact role *Wolbachia* plays in worm physiology, but is it known that it plays an important role in reproduction and adult worm viability (69-71).

Importantly, *L. loa* do not harbor *Wolbachia,* indicating the potential safety of doxy in co-endemic regions. Macrofilaricidal effects of doxy are generally seen following daily administration (100-200 mg) for 4 – 6 weeks (72). Moreover, this schedule has also shown to alleviate pathologic symptoms of LF and onchocerciasis in addition to reducing worm burden (73). This administration schedule is sufficient to lead to effective sterilization of adult females, but only led to partial, rather than complete, killing of adult worms depending on treatment regime (73). Additionally, the duration of therapy impedes the utility of this drug in a field setting where compliance is often a concern.

Emodepside, a cyclooctadepsipeptide drug which acts on potassium channels, has recently been explored as a macrofilaricide due to it's broad nematocidal activity (74). Introduced as a veterinary product, emodepside is available either orally or as a topical treatment for GI nematodes of cats and dogs. High doses resulted in adult worm killing in *Litomosoides sigmodontis*, but not in *B. malayi.* Intrauterine mf were severely damaged, even at low doses (75). The potential utility in *O. volvulus* is yet to be reported, however, the observable effects on mf may preclude its use in *L. loa* co-endemic regions.

Flubendazole (FLBZ) is another promising macrofilaricide and has been shown to be highly effective in a number of animal models of filariasis. This compound is already approved for use in humans (76), an advantage over some macrofilaricidal drugs in the pipeline. A BZ anthelmintic, FLBZ interferes with tubulin polymerization to elicit macrofilaricidal effects. Like other BZs, FLBZ has very low water solubility which limits systemic availability after oral dosing. This requires that FLBZ be administered parenterally to be effective (77-79), a route of administration that is not ideal for MDA programmes. However, the search for alternative pharmaceutical formulations which provide substantial systemic availability of FLBZ following oral administration is underway. Due to recent interest in reformulations, FLBZs activity on filariae is the subject of this thesis and will be described in greater detail.

1.4 Tubulin

Tubulins are small globular proteins, the most abundant being α- and β- tubulins (80). These 55 kDa proteins, consisting of approximately 450 amino acids, assemble to form a heterodimer that binds two molecules of GTP, one of which binds irreversibly to α-tubulin (80). The GTP bound to β-tubulin is exchangeable and undergoes hydrolysis to GDP during the extension of microtubules. Under appropriate conditions, the heterodimers assemble to form a protofilament composed of alternating α- and β- tubulin. A number of these protofilaments come together to form a protein sheet which curls into a tube-shaped protein polymer known as a microtubule (81). A typical eukaryotic microtubule is formed of 13 protofilaments (81); however, the free-living nematode *C. elegans* has cytoplasmic microtubules consisting of only 11 protofilaments (82). Once formed, this structure is not static; it exists in equilibrium with tubulin dimers adding to the plus (+) end and leaving at the minus (-) end. The role of microtubules as key components of cellular function is dependent upon this characteristic dynamic process (81).

Microtubules play a variety of roles at the cellular level: maintenance of cell shape, formation of the mitotic spindle, cellular secretion, nutrient absorption and intracellular transport (81). The ubiquitous and fundamental role of microtubules in cell division is prominent and has been widely studied, yet it is likely the more discrete metabolic functions that predominantly influence homeostasis in multicellular organisms (81). Disequilibrium in cellular homeostasis via impedance of normal microtubule function can be fatal. Microtubules, and consequently the tubulin dimer, are therefore logical drug targets. Three selective sites on either tubulin or microtubules have been identified as pharmacologically relevant: taxol, vinblastine and colchicine binding sites (81, 83). Taxol predominantly binds to a site on microtubules. Unlike the other ligands, taxol stabilizes tubulin to polymerize, independent of tubulin concentration or presence of microtubule associated proteins (83).

Vinblastine has been used as an anti-cancer drug due to its affinity for the tubulin dimer and subsequent inhibition of polymerization (84). There are no reports of this site on nematode tubulin. Colchicine, the classic tubulin-binding drug, was initially developed as a treatment for gout (85). Inhibition of tubulin polymerization occurs when colchicine-bound tubulin associates with the end of a growing microtubule and prevents this subunit from binding a subsequent tubulin molecule (86). Colchicine binds with high affinity to the carboxyl-terminus of β tubulin and induces an irreversible conformational change, due to partial unfolding, which hinders further polymerization (87). The colchicine-binding site, or a site close to it, is the proposed site of relevance for binding of benzimidazoles.

1.5 Benzimidazoles

Because microtubules are highly dynamic structures which are fundamental to cell function and viability, this structure has been a target for drug development to manage organisms varying from fungi (88) to parasitic nematodes (89) and more recently as anticancer treatments (90).

1.5.1 General Description

Benzimidazoles have been long known to be potent anthelmintics for the treatment of gastrointestinal nematodes (91) and have been approved for use in the treatment of roundworm, pinworm, hookworm, lungworm and whipworm infections since the 1970's (92).

BZs are synthesized by the fusion of benzene and imidazole rings to form a general benzimidazole thiazolyl structure from which all BZ anthelmintics are derived (93). Differences arise when modifications are made at various positions around the ring. Modifications of the 2 and 5 positions of the benzimidazole thiazolyl ring generate the most potent anthelmintic BZs, the

methylcarbamates (93). A larger substituent at the 5 position results in more potent anthelmintic activity (94).

1.5.2 Anthelmintic Activity of BZs

Thiabendazole was the first BZ to be identified as having potent anthelmintic effects (95). Since then, approximately 20 more BZs have been commercially developed for use in animals and humans (93, 96). The mode of action is thought to be similar amongst the BZs (89). Early studies of activity focused on antimitotic properties. It was first suggested that the antimitotic activity observed was due to disruption of mitotic spindle formation (97). Hoebeke *et al.* (98) attributed this disruption to interference with microtubule structure and function in mitotic cells following studies on mouse embryonic cells exposed to oncodazole. They concluded that microtubule disappearance, loss of directional subcellular movement, lack of intact spindle and abnormal spatial distribution of chromosomes during mitosis was due to the antitubulin activity of the drug. Using rat brain tubulin, Hoebeke *et al.* (98) substantiated this claim by demonstrating that oncodazole bound rat brain tubulin and disrupted polymerization to microtubules. This was further supported by Friedman and Platzer (99) who demonstrated tubulin binding and polymerization disruption by nine separate BZs, the most potent of which were oxibendazole, parbendazole, mebendazole and fenbendazole. Inhibition of polymerization of bovine brain tubulin was also demonstrated by Laclette *et al.* (100). Assessment of polymerization inhibition in mammalian brain tubulin is achieved by direct spectrophotometric measurement of polymerization. Due to the small quantity of tubulin that can be derived from nematode tissue, polymerization is measured indirectly by assessing inhibition of colchicine binding (101). Friedman and Platzer (102) were the first to assess BZ binding to nematode tubulin by conducting inhibition studies with radiolabelled colchicine. Using crude extracts from *Ascaris suum* eggs, they demonstrated complete inhibition of colchicine binding by

mebendazole and fenbendazole. Mebendazole was also found to completely inhibit colchicine binding to partially purified tubulin from *A. suum* intestinal tissue (103). Subsequent studies validated BZ binding to tubulin via inhibition of colchicine binding by parbendazole, albendazole, mebendazole, fenbendazole, thiabendazole and oxfendazole (104-108). It may be noted that these initial studies of BZ activity on nematodes focused on gastrointestinal nematodes.

1.5.3 BZ Activity in Filarial Nematodes

Antifilarial activity of benzimidazoles became of interest following a report of potent macrofilaricidal efficacy of mebendazole on *B. pahangi* in jirds (109). Since then, using rodent models, high efficacy of flubendazole (77, 78), oxibendazole (110) and mebendazole (78, 111) has been shown. Mebendazole and oxibendazole were macrofilaricidal at doses of 10 – 100 mg/kg administered subcutanteously for five consecutive days (78, 109-111). While both drugs effectively reduced worm burden, they were unable to reduce parasite load to zero. Flubendazole (FLBZ), however, not only reduced worm burden to zero but did so at lower doses (2.5-3 mg/kg; (77, 78)). Notably, FLBZ was macrofilaricidal at single doses in jirds (25 mg/kg) and cats (100 mg/kg; (77)). Interestingly, studies with *B. malayi* and *B. pahangi* have found that the BZs were only effective against macrofilariae and not mf when administered parenterally (77, 109, 110).

It quickly became apparent that high efficacy of BZs in filarial nematodes could only be attained with parenteral injection. This, however, limits their attraction for MDA. Oral administration of BZs is ineffective due to the very low bioavailability associated with their low solubility (112). However, if a formulation were to be developed that increased bioavailability of BZs, perhaps they could be effectively implemented as macrofilaricides for MDA programmes. In beagles infected with *Dirofilaria immitis,* an oral micronized preparation of mebendazole was found to be 100% effective against developing larvae following oral administration at a dose of 40 mg/kg/day for 30 days (113).

A more recent study on cystic echinococcosis in mice found that a hydroxypropyl- β-cyclodextrin formulation of FLBZ enhanced drug availability and led to a significant decrease in weight of *Echinococcus granulosus* tissue cysts (114).

1.5.4 Ultrastucture

Early studies of ultrastructural changes associated with BZ exposure focused on events occurring in parasite intestinal cells. Ultrastructural alterations were observed as early as 6 hr post exposure (hpe) with mebendazole in *A. suum* (115, 116) and *Ascaridia galli* (117). A decrease in glycogen content, loss of apical secretory granules and occurrence of multiple granules near Golgi apparatus were the first observable changes. This was followed by further accumulation of secretory granules surrounding the Golgi, ultimately leading to swelling and complete disruption of microvilli 15-24 hpe (115-117). Corroborating evidence was found in *Toxocara canis (118)*, *Aspicularis tetraptera (119)*, *Heterakis spumosa (120)*, and *Haemonchus contortus (121).*

The suggested role of microtubules in intracellular transport prompted closer investigation of their presence or absence in intestinal cells (115, 120). Borgers *et al.* (115) found that exposure of *A. suum* to mebendazole resulted in loss of apical and central microtubules by 6 hpe. By 12-24 hpe, microtubules could no longer be identified in the cytoplasm of *A. suum* or *H. spumosa* (115, 120). These ultrastructural modifications suggest that loss of microtubules leads to an accumulation of secretory granules in the intestinal cell cytoplasm, two consequences of which were proposed (115). First, this deprives the microvilli of the carbohydrates required to maintain the protective glycocalyx, therefore predisposing the cells to degradation (115). It also leads to deprivation of enzymes required for digestion and absorption, preventing the cell from obtaining vital nutrients (115). Second, it is suggested that secretory granules containing proteolytic and hydrolytic enzymes

destined for secretion may become active within the cell following prolonged storage in the cytoplasm, leading to autolysis (115).

The ultrastructural studies discussed thus far have concentrated on nematodes residing in the gastrointestinal tract. Few studies have investigated the ultrastructural effects of BZs in filarial nematodes. However, these investigations have found similar effects concerning intestinal cell alterations with the exception that there is less extensive tissue damage (122-124). Unfortunately, with the exception of ovarian and embryonic tissue, little information is known about pharmacological effects of BZs in other regions of the nematode body.

1.5.5 Specificity

A number of BZs are recognized for their potent effects on parasitic nematodes while eliciting few or no negative effects on the host. The first experimental indication of specificity arose following the characterization of radiolabelled colchicine binding to *A. suum* embryonic tubulin (102). Mebendazole and fenbendazole had a higher affinity for *A. sum* tubulin, with Ki values 250- and 400-fold greater than for bovine brain tubulin (102). However, a later study reported no difference in mebendazole specificity between *A. suum* intestinal and porcine brain tubulins (103). These conflicting results are difficult to reconcile as they used different methods for acquiring nematode tubulin; the first employed a crude extract, while the second partially purified tubulin via ammonium sulphate precipitation. Both techniques would result in relatively large amounts of contaminating protein to which the radiolabelled ligand and competing BZ could bind non-specifically. Dawson *et al. (106)* took a more direct approach to assessing specificity by purifying mammalian or *A. galli* tubulin with DEAE Sephadex chromatography. Following addition of BZs, the sample was centrifuged to collect polymerized microtubules and protein levels were quantified. Polymerization was also monitored by electron microscopy to visualize microtubule formation. Their results were in

accordance with those of Friedman and Platzer (102), confirming the preferential binding of BZs to nematode over mammalian tubulin. Since then corroborating evidence has also been found in *Nippostrongylus brasiliensis* (125).

1.6 Flubendazole

1.6.1 Anthelmintic Activity

Flubendazole is an appealing prospect for use in MDA for onchocerciasis and lymphatic filariasis. First developed for gastrointestinal nematodes of animals, FLBZ was found to be highly potent and efficacious (126). Since then, FLBZ has been approved for the treatment of human intestinal parasites (127) and has been shown to be highly effective (128, 129).

Zahner and Schares (79) suggested that FLBZ is the most potent of the current BZs in regard to filaricidal activity. In a number of experimental rodent filarial models, FLBZ has high macrofilaricidal efficacy when administered parenterally (63, 77, 78). Macrofilarial clearance of 100% was found in white rats infected with *Breinlia booliati* following subcutaneous delivery of FLBZ (78). Denham *et al.* (77) found that a single subcutaneous injection of FLBZ in jirds infected with *B. pahangi* cleared 97.7% of macrofilariae. FLBZ has been found to not only be effective in rodent models, but there is evidence of antifilarial activity in a trial of humans with onchocerciasis. Parental dosing of FLBZ was found to be highly effective at killing macrofilariae of *O. volvulus* (130).
As found for other BZs, FLBZ treatment of the gastrointestinal nematodes *T. canis* and *A. suum* resulted in complete ablation of the hypodermis as well as a loss of microtubules, followed by complete disruption of intestinal cells (118). Ultrastructural effects of FLBZ on filarial nematodes were slightly different from those of gastrointestinal nematodes. The earliest alteration observed was the disappearance of microtubules from intestinal cells, which reflects what occurs with gastrointestinal nematodes. However, there was no further disintegration of the intestinal cells (131).

Microtubule disappearance from the hypodermis and alterations to oogonia and embryonic cells were also observed (131).

It is important to note that the studies described have found FLBZ to be a potent macrofilaricide, while eliciting little effect on mf (77-79). When FLBZ is administered subcutaneously for 5 consecutive days to mice infected with *L. carnii* (*carinii* now *sigmodontis*), *B. pahangi* or *Acanthocheilonema viteae,* microfilaremia decreased to 0% within 15 - 20 days (79). Microfilaremia also dropped in *B. malayi* infections, though less rapidly. Since the survival time of mf in rodent hosts is unknown, it is not clear if these effects are due to direct toxicity of the drug to mf or reflect the endogenous clearance of circulating mf coupled with cessation of production of mf by treated females. The minimum curative dose for *B. malayi* infection as measured 42 days following administration was 12.5 mg/kg following 5 consecutive daily doses. A minimum curative single dose could not be determined (79). Similar delayed microfilaricidal effects were found in *O. volvulus* infected individuals treated with FLBZ (130). Dermal microfilaremia did not decrease for as long as three months post treatment. The SAEs observed in some individuals infected with *L. loa* is attributed to the death of mf (132). Considering that 124 deaths over the past 10 years have been associated with these SAEs (63), it would be of great benefit to have a drug for MDA that has little to no effect on mf.

1.6.2 Embryotoxicity

Generally, BZs have been known to elicit embryotoxic effects in animal models. Increased incidences of fetal resorption, embryonic death, growth retardation and developmental abnormalities such as encephalocele, hydrocephaly, fused ribs, and fused vertebrae have been reported following BZ treatment of pregnant female animals (133-139). Therefore, this class of drugs is contraindicated in pregnant women. It must be noted, however, that inadvertent exposure of pregnant women to albendazole during MDA has not resulted in reports of negative effects on the fetus (140).

Flubendazole has been shown to elicit embryotoxic effects. In rats, high doses of FLBZ were embryocidal (160 mg/kg). At lower doses (40 – 160 mg/kg), malformations observed included encephalocele, ectrodactyly, shortened backbone and spina bifida occulta (141). FLBZ and ABZ show similar *in vitro* potency in rat embryo toxicity assays (142). Abnormal tissue formation and retardation of growth was observed at concentrations ≥ 0.5 μg/mL. However, no effects were observed at 0.25 μg/mL. The two major metabolites, reduced FLBZ and hydrolyzed FLBZ, were both less toxic than FLBZ (142). Futher *in vivo* studies using an amorphous solid dispersion preparation administered to rats found embryolethal effects at 6.32 mg/kg/day (C_{max} 0.801 μg/mL). Teratogenic effects were observed at just 3.46 mg/kg/day corresponding to a C_{max} of 0.539 μg/mL

1.6.3 Formulations

Several formulations have been explored to enhance the solubility and, thus, bioavailability of FLBZ. The first formulation to be investigated used a cyclodextran (CD) delivery system. CDs are cyclic oligosaccharides that contain a hydrophobic inside surface and hydrophilic outer face (143). This structure provides a unique mechanism of non-covalently retaining hydrophobic molecules. Oral administration of FLBZ-CD formulations drastically increased the plasma levels over that of an aqueous suspension in mice (25x) (114) and rats (16.7x) (144). Oral administration in rats also saw increased plasma levels over the subcutaneous administration of the same formulation (1.55x) (144). A similar, but less dramatic effect was observed in sheep (145) and pigs (146).

The most promising of the more recent formulations is an Amorphous Solid Dispersion of FLBZ (ASD-FLBZ) which has shown encouraging results in murine models of filariasis (147). In rats, a single oral dose of ASD-FLBZ at 2 mg/kg resulted in C_{maz} of 0.389 μg/mL and an AUC of 2.19 μg.h/mL compared to an extremely high dose of 20 mg/kg where we see a C_{maz} of 1.260 μg/mL and an AUC of 13.2 μg.h/ml (142). Using this dosing regimen, FLBZ remained in systemic circulation for

an extended time after administration (8 – 24 hours) and was only minimally metabolized to its two main metabolites; reduced flubendazole (FLBZ-R) and hydrolyzed flubendazole (FLBZ-H) (142).

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Chapter 2. Manuscript I

In vitro flubendazole-induced damage to vital tissues in adult females of the filarial nematode *Brugia malayi*

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2.1 Abstract

The use of a microfilaricidal drug for the control of onchocerciasis and lymphatic filariasis necessitates prolonged yearly dosing. Prospects for elimination or eradication of these diseases would be enhanced by availability of a macrofilaricidal drug. Flubendazole (FLBZ), a benzimidazole anthelmintic, is an appealing candidate macrofilaricide. FLBZ has demonstrated profound and potent macrofilaricidal effects in a number of experimental filarial rodent models and one human trial. Unfortunately, FLBZ was deemed unsatisfactory for use in mass drug administration (MDA) campaigns due to its markedly limited oral bioavailability. However, a new formulation that provided sufficient bioavailability following oral administration could render FLBZ an effective treatment for onchocerciasis and LF. This study characterized the effects of FLBZ and its reduced metabolite (FLBZ-R) on filarial nematodes *in vitro* to determine the exposure profile which results in demonstrable damage. Adult female *Brugia malayi* were exposed to varying concentrations of FLBZ or FLBZ-R (100 nM - 10 μM) for up to five days, after which worms were fixed for histology. Morphological damage following exposure to FLBZ was observed prominently in the hypodermis and developing embryos at concentrations as low as 100 nM following 24 hours exposure. The results indicate that damage to tissues required for reproduction and survival can be achieved at pharmacologically relevant concentrations.

2.2 Introduction

The debilitating diseases onchocerciasis and lymphatic filariasis (LF) are major causes of long term disability and impede socioeconomic development in endemic countries (1, 2). Despite the magnitude of the problem, there is optimism about prospects for the elimination of onchocerciasis (3, 4) and eradication of LF, with the World Health Organization targeting it for elimination by 2020 (2). To increase the likelihood that this goal will be achieved, it is important to address the challenges inherent to current chemotherapeutic strategies used in these programs. The drugs employed in mass drug administration programmes are principally microfilaricidal agents, and also limit reproduction; this strategy reduces transmission and the development of pathology in onchocerciasis but necessitates annual or twice-yearly dosing for many years. An effective and safe macrofilaricide would clearly shorten the time required to reach program goals. In addition, a macrofilaricide would have the benefit of reducing pathology in LF, in which the characteristic sequelae of elephantiasis and hydrocele are initiated by adult worms residing in lymphatic vessels.

Flubendazole (FLBZ), a benzimidazole (BZ) anthelmintic, is an appealing prospective macrofilaricide for use in onchocerciasis and LF. First developed for gastrointestinal (GI) nematodes of animals, FLBZ was found to be potent and efficacious for this indication (5). Subsequently, FLBZ was approved for the treatment of human intestinal parasites (6), an indication for which it is also highly effective (7, 8). What is most appealing in the current context is the very high macrofilaricidal efficacy attained in experimental filarial rodent models (9-11) and in a human trial in onchocerciasis (12).

Early *in vitro* studies of BZ anthelmintic effects focused on GI nematodes. Ultrastructural observations of *Ascaris suum* 6 hr following exposure to mebendazole (13, 14) showed a loss of microtubule structures in intestinal cells. Further exposure resulted in decreased glycogen content, depletion of apical secretory granules, and accumulation of secretory granules near the Golgi,

associated with swelling and disruption of microvilli (13-15). FLBZ-induced damage to reproductive organs of filariae has also been reported (16, 17).

Other investigators reported similar findings after FLBZ exposure in culture of *Toxocara canis* and *A. suum*, including vacuolization of the musculature, female gonadal tissue, intestine, and, to a lesser degree, the hypodermis (18). Swelling of intestinal cell endoplasmic reticulum and complete disruption of intestinal cells occurred. Following FLBZ treatment of infected animals, loss of intestinal microtubules from cells in the GI tract of the filarial nematodes *Brugia malayi* and *Litomosoides sigmodontis* was observed, using transmission electron microscopy, when the parasites were recovered as soon as 6 hr post-dosing (19). Increasingly severe damage to other tissues, including the hypodermis and reproductive tissues, was observed as time after dosing increased.

FLBZ is efficacious in humans infected with *Onchocerca volvulus* (11, 12). Recent efforts have been made to develop a new formulation of FLBZ that would enable oral dosing rather than the parenteral routes used in previous studies (20, 21). Definition of the pharmacokinetic profiles needed for efficacy with an orally-bioavailable formulation would be facilitated by knowledge of the time-concentration exposure profiles at which FLBZ is detrimental to the survival of adult filariae. The present study examines time- and concentration-dependent morphological changes in *B. malayi* adult females caused by exposure to FLBZ *in vitro*.

2.3 Methods

2.3.1 Parasites

Adult female *B. malayi* were isolated from the peritoneal cavity of jirds (*Meriones unguiculatus)* >120 days post-infection as described (22, 23). Briefly, recovered adult worms were washed three times with warm (37°C) RPMI-1640 medium supplemented with 100 U/mL penicillin, 100 ug/mL

streptomycin, and 0.25 ug/mL amphotericin B (Sigma-Aldrich Corp., St. Louis, MO, USA; hereafter referred to as RPMI).

Adult females were exposed to varying concentrations of FLBZ or its reduced metabolite (FLBZ-R) (10 nM, 100 nM, 1 μM, 10 μM; Epichem Pty Ltd, Murdoch, WA, Australia) over a period of 24, 48, 72, 96 or 120 hr, with media changes every 24 hr. FLBZ and FLBZ-R solutions were prepared by dissolving the respective drug in 100% DMSO, and added to RMPI to a final DMSO concentration of 0.1%. Control RPMI contained an equivalent percent of DMSO. Three females were cultured in 1 mL RPMI at 37°C, 5% CO_2 and 95% humidity in each concentration. All worms were fixed for subsequent histological analysis.

Parasite isolation and culture was conducted at the Filariasis Research Reagent Resource Center in Athens, GA, USA.

2.3.2 Assessment of Parasite Motility

Parasite motility was assessed visually under light microscopy. Motility was scored as either: immotile, with no motion during the observation period; slightly motile, where only twitching of the head and/or tail was observed; moderately motile, with slow sinusoidal movements; or highly motile and comparable to the drug-free control. Each sample was observed for at least one minute for scoring of motility

2.3.3 Histological Preparation

B. malayi were fixed in glutaraldehyde (5% in 0.1 M sodium cacodylate buffer, pH 7.2; five worms in 1 mL) for a minimum of 48 hours in preparation for histological processing. Worms from each treatment were combined into groups and coiled prior to embedding in Histogel (FisherScientific), which allowed visualization of various anatomical regions in multiple worms on a single slide. Dehydration, clearing, and vacuum infiltration with paraffin were completed using a

Sakura VIP tissue processor. Parasites were then embedded in paraffin with a ThermoFisher HistoCentre III embedding station. A Reichert Jung 2030 rotary microtome was used to cut 4-5 micron sections, which were dried at 56°C for 2 – 24 hr. Slides were stained with haematoxylin and eosin prior to examination under light microscopy (60 and 100x magnification).

2.3.4 Assessment of worm damage

Sections were assessed independently by three parasitologists familiar with filarial nematode morphology, including one board certified pathologist/parasitologist (CDM); a second board certified pathologist was also consulted in planning and developing the system (DWA). Worms from two independent experiments were examined for damage to the following tissues: body wall, including cuticle, hypodermis and longitudinal muscle; intestine; and reproductive tract, including the uterine wall and embryonic stages (classified as early [ovary, oocytes, early morulae, late morulae] or late [sausage, pretzel, microfilariae]); and pseudo-coelomic space. To aid the comparative analysis of drug-derived effects, tissues were classified into four categories of damage: no damage (0), minor (1), moderate (2), severe (3). This damage score was determined by assessing tissues for nuclear and cytoplasmic distortions, cellular size and shape, membrane integrity, accumulation of debris, and distortion of overall anatomical integrity (Figure 2.1).

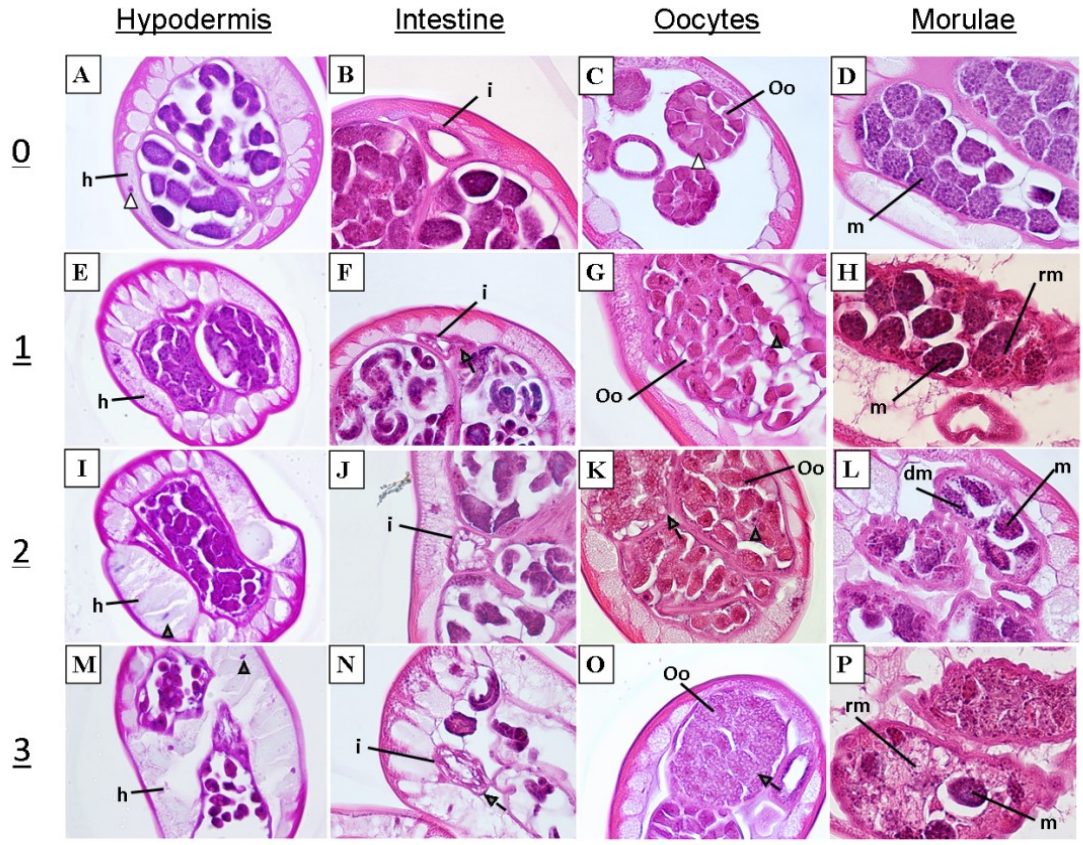

Figure 2.1. Scoring system for tissue damage observed following incubation in FLBZ (E - P) as compared to control worms (A - D).
Damage was scored as mild (1), moderate (2), severe (3), or no damage (0).
HYPODERMIS (h): damage was determined by observing the level of vacuolization (grey arrow) and degree of swelling in the hypodermis. Loss of integrity to the hypodermal wall was considered to be severe. Shrunken and densely staining nuclei (grey arrowhead) as compared to controls (open arrowhead) also elicited a higher damage score.
INTESTINE (i): Intestinal damage was largely determined by the number, size and shape of vacuoles in the tissue. Disruption of the intestinal borders was considered to be severe damage.
OOCYTES (Oo): Early embryos (including oocytes and morulae) exhibited the most damage of all the embryonic stages. Discrimination of the oocyte cell border, degree of vacuolization and nuclear abnormalities dictate the level of damage.
MORULAE (m): damaged morulae exhibited loss of cellular organization. Disintegrating morulae (dm) were classified with a higher damage score. The presence of morulae remnants (rm) also factored into the score given.

Two methods of analysis were performed. The first method adhered to classical techniques used by histopathologists to determine tissue damage, in which all sections on a slide were surveyed, interpreted and translated into a single damage score for each tissue type. The second method involved scoring damage in each tissue type for each worm section on a slide. These scores were then averaged for all sections on the slide to obtain the damage score.

2.4 Results

2.4.1 Quality of sections

Control worms retained well-preserved tissue structure for up to 72 hr in culture. Noticeable loss of normal condition occurred in control groups after 96 hr, seen as an increase in vacuolization in the intestine and hypodermis. A high degree of variability in this latter morphological change was observed among worms in the same treatment group, as well as along the length of an individual worm. Two independent experiments were conducted in an attempt to reduce this variability. Multiple transverse sections were assessed to enable estimation of the proportion of the specimen which was damaged. Samples from the 96 and 120 hr incubations were excluded from further analyses to eliminate the influence of this loss of condition, presumably resulting from the effects of culture, as a variable.

Sections that displayed effects which were clearly consequences of histological processing and not due to drug exposure, such as scored sections, abnormally broken tissue structures, were excluded from analysis.

Limited damage was observed following exposure to 10 nM flubendazole; therefore, this concentration was not included in further experiments or in those with reduced flubendazole.

Control worms were exposed to 0.1% DMSO for 24, 48, or 72 hr; no samples were taken for analysis immediately after removal from the jird.

2.4.2 Assessment of damage

The overall damage score for all tissues at each time and concentration using the survey method and the individual section method are shown in Table 2.1 A, 2.2 A and Table 2.1 B, 2.2 B respectively.

The individual section method returned higher damage scores than the survey method and detected damage that was not scored with the survey method. However, the overall conclusions reached by either method were the same. While the survey method is less labor intensive, it may miss some of the minor changes resulting from drug exposure. A shortcoming of the individual section method centers on processing of the slides. It is assumed that the sections on each slide are representative of damage occurring along the length of the worm, and that a representative number of sections from each worm in the group is assessed.

2.4.3 FLBZ-induced damage

Drug-induced damage was observed in reproductive and hypodermal tissues, and in the intestine to a lesser degree (Table 2.1, Figure 2.2). The damage observed in the hypodermis was predominantly tissue swelling and nuclear abnormalities, including shrinking. Moderate damage to the hypodermis was visible after 24 hr in 100 nM FLBZ (Figure 2.2 A and B, Table 2.1). Increased vacuolization and expansion of the hypodermis was observed in treated vs. control parasites. In some cases, complete disruption of the hypodermal membrane was observed (Figure 2.2 B). Damage to intestinal cells was highly variable. In some cases, intestinal cells were highly vacuolated compared to control; however, the intestinal walls remained intact (Figure 2.2 C).

Table 2.1. Tissue damage scores associated with flubendazole exposure assessed by two methods**:** A. Classical histopathological survey method; B. Individual section scoring method. Scores are averages from two independent experiments.

Treatment	Hypodermis			Intestine			Early Embryos			Late Embryos		
	24	48	72	24	48	72	24	48	72	24	48	72
A												
Control	0.2	0.1	0.3	0	0	0.1	0.6	0.6	0.2	0.3	0.4	0.1
100 nM	0.9	0.4	1.1	0.3	0.3	0.8	0.8	1.4	0.4	0.6	0.2	0.4
1 μM	0.3	0.7	0.4	0.2	0.3	0	0.5	1.3	0.5	0	0	0.4
10 μM	0.9	0.6	0.9	0.3	0.2	0.1	0.6	0.6	0.9	0.2	0	0.2
B												
Control	0.5	1.2	0.6	0	0.2	0.3	0.9	1.1	0	0.2	0.2	0.7
100 nM	1.6	1.5	1.6	1.5	0.2	1.8	1.4	1.9	1.3	0.8	0.4	0.6
1 μM	1.6	1.6	2.1	0.4	1.2	0.4	1.3	1.9	1.3	0	1.3	1.1
10 μM	2	1.8	2	1.1	1.8	0.7	1.6	1.6	2.2	1.5	1.3	1.3

Vacuolization of early embryos within female gonads was the first observable damage to reproductive tissues of treated worms (Figure 2.2 D, E). Early developmental stages exhibited more damage than later stages; oocytes and early morulae (2 – 12 cells) were the most severely damaged. Degradation of early embryos was observed following incubation in 100 nM FLBZ, with evident loss of cellular integrity (Figure 2.2 E). There was little to no damage to later developing stages. Sausage forms exhibited damage similar to what was seen in morulae, however, the pretzel stage and stretched microfilariae (Figure 2.2 F) remained unchanged.

FLBZ had no effect on worm motility (Figure 2.3).

2.4.4 Effects of Reduced Flubendazole

Effects of FLBZ-R were similar to those observed in FLBZ treated worms. Damage was observed in the hypodermis following exposure to 1 μM FLBZ-R for 72 hours (Table 2.2). Moderate damage to the intestinal epithelium required longer incubations in FLBZ-R (Table 2.1 &

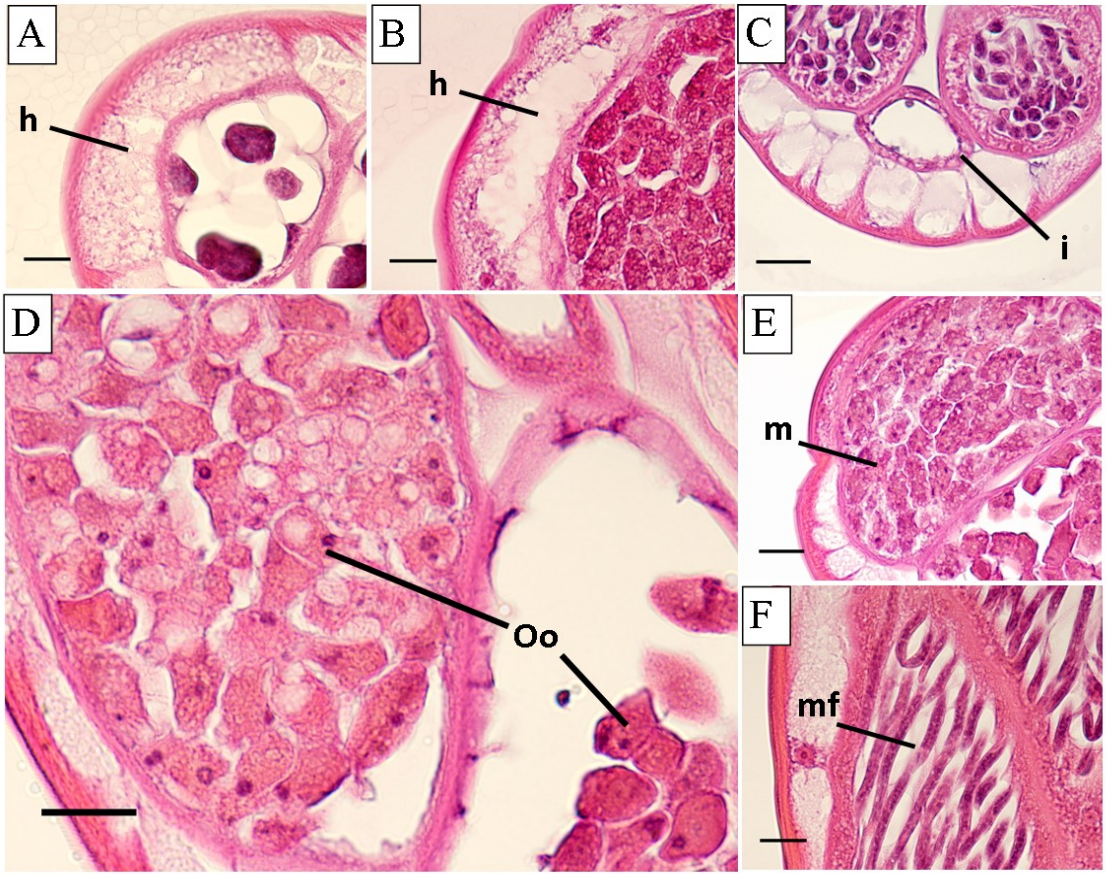

Figure 2.2. Representative damage observed following 24 hour incubation in flubendazole. A and B. hypodermis (h), 100 nM; C. intestine (i), 100 nM; D. oocytes (Oo), 100 nM; E. early morulae (m), 1 μM; F. microfilariae (mf), 10 μM (48 hour). Scale bars are 15 μm.

2.2). Reproductive tissues were damaged following incubation in 100 nM FLBZ-R, similarly to FLBZ. As was found with FLBZ, FLBZ-R-induced damaged presented as increased vacuolization, nuclear abnormalities and disruption of cellular integrity.

FLBZ-R had no effect on worm motility (Figure 2.3).

Table 2.2. Tissue damage scores associated with reduced flubendazole exposure assessed by two methods: A. Classical histopathological survey method; B. Individual section scoring method.

Treatment	Hypodermis			Intestine			Early Embryos			Late Embryos		
	24	48	72	24	48	72	24	48	72	24	48	72
A												
Control	0.6	0.5	0	0	0	0	0.5	0.3	0.2	0.2	0	0.1
100 nM	0.3	0.1	0	0.1	0.4	0	1.4	0.5	0.9	0.7	0.3	0.1
1 μM	0.5	0.5	0.8	0	0	0.6	0.8	0.9	1.1	0.1	0.4	0.3
10 μM	0.6	0.6	0.6	0	0.1	0.3	0.2	0.6	0.9	0.1	0	0.5
B												
Control	0.9	0.8	0.3	0	0.2	0.1	0.7	0.8	0.1	0.1	0.3	0
100 nM	1.1	0.9	0.9	0.4	0.4	0.6	1.7	0.6	1.2	1.4	0.2	0
1 μM	1.4	1	1.8	0.3	0.3	1.3	1.4	0.9	1.6	0.5	0.3	0.2
10 μM	1.2	1.4	1.9	0.2	0.6	0.8	0.8	1.1	1.5	0.2	0.3	0.6

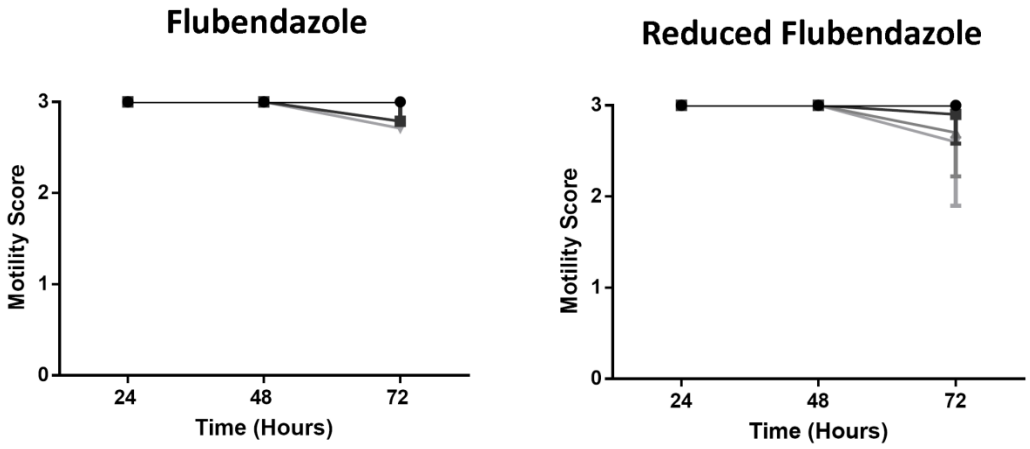

Figure 2.3. Mean motility (±SD) after exposure to flubendazole or reduced flubendazole**;** 10 μM (light grey triangles), 1 μM (grey triangles), 100 nM (grey squares), control (0.1%DMSO; black circles)

2.5. Discussion

In this study, morphological damage following *in vitro* exposure to FLBZ was observed prominently in the female reproductive tissue and hypodermis, and to a lesser extent in the intestine. Previous studies of ultrastructural changes associated with BZ exposure focused on events occurring

in parasite intestinal cells all used electron microscopy, rather than the light microscopic approach used in this study. Ultrastructural alterations were detected as soon as 6 hr of exposure (13-15), including decreased glycogen content, loss of apical secretory granules and occurrence of multiple granules near the Golgi apparatus, ultimately leading to swelling and complete disruption of microvilli (13-15, 18, 24-26). Although these studies described drastic morphological changes elicited by BZs in intestinal cells, they focused on GI nematodes, and the effects may not be the same for filariae. Franz *et al* (1990) found that, while FLBZ exposure resulted in disappearance of microtubules from intestinal cells of *B. malayi* and *L. carinii*, the cells remained intact for up to 6 days post-exposure. It was suggested that this result reflects the limited use of intestinal cells for nutrient uptake by adult filariae. In the closely related parasite *Brugia pahangi*, transcuticular nutrient acquisition occurs in microfilariae and adult worms, yet the adults are also able to acquire nutrients orally (27). Laurence and Simpson (1974) found the pharynx of *B. pahangi* microfilariae to be incompletely developed, suggesting that they acquire nutrients only across the cuticle. The capacity to acquire nutrients transcuticularly suggests that the hypodermis should exhibit more extensive damage than the intestine following FLBZ exposure. In this study, there was observable damage to the hypodermis (Figure 2.1 E, I, M), presenting as increased vacuolization, swelling and nuclear abnormalities. These effects would be expected to inhibit nutrient acquisition not only via physical impairment, but also through limited functioning of hypodermal components. Although highly vacuolated, the intestinal cells, as well as the nuclei, largely remained intact, as reported previously for *B. malayi* and *L. carinii* (19). It has been reported that BZs have little to no effect on the intestinal cells of filarial nematodes (17, 19). In this study, damage to the intestine was highly variable and did not follow obvious concentration- or time-dependent trends.

Our study confirmed prior observations on the effects of BZs on filarial female gonads and developing embryos (16, 17, 19, 28). FLBZ (1 μM) elicited detrimental effects, predominantly vacuolization, in developing embryos of *B. malayi* following 24 hr incubation (Figure 2.2 D, E). BZs

inhibit tubulin polymerization, and given the importance of microtubules for spindle formation and cell division, it is not surprising that pathological alterations were observed in rapidly developing embryos, especially in early stages, more than in the surrounding tissues. Disruption of cell division was also reported in ovaries of *Litomosoides chagasfilhoi* (17), *Dirofilaria immitis* (16), and *O. volvulus* (28).

The type and extent of damage to filarial tissues caused by FLBZ-R is consistent with reports on nematocidal effects of FLBZ-R (29). Given the high degree of first-pass metabolism of BZs, it is promising that a main FLBZ metabolite has intrinsic activity against filariae.

Evaluation of drug-induced effects on tissues based on histochemical analyses is challenging. The present study addressed this issue by conducting multiple independent experiments to assess morphological alterations with multiple observers. This study also aimed to provide a numeric representation of drug induced effects. Previous studies report observed effects but did not indicate the level of damage, range of damage over population tested or portion of the worms that exhibited such damage. This study employed histology and light microscopy to assess damage, which is a cost-effective tool that gives a better indication of tissue level effects to provide a comprehensive assessment of damage than transmission election microscopy, which was the main method used in previous studies.

The limitations imposed by in vitro culture are an additional challenge to evaluating drug-induced damage. The duration of culture of viable microfilariae is relatively short, unless extended slightly through the use of a feeder cell layer(30, 31). In this study we evaluated damage over a period of three days to avoid the use of a feeder cell layer for several reasons. The first was potential uptake of flubendazole by the cells, which could lead to depletion of the drug from the media. Second, mammalian cells can metabolize flubendazole, depleting it from the medium over time. Finally, if the drug is toxic to mammalian cells at the concentrations and durations of exposure employed, it may lead to the release of by-products that could have affected the parasites.

Macrofilarial killing by benzimidazoles generally is seen months after treatment (19, 32), a duration for which *in vitro* culture is not feasible, even with the use of feeder cells. While extended culture would be expected to amplify the effects observed in short term culture, the use of feeder cells could complicate the results.

In conclusion, we found a greater detrimental effect of FLBZ on the hypodermis than the intestine. Given that the hypodermis is suggested to have a greater role in nutrient absorption than the intestine, this suggests that FLBZ may play a role in nutrient deprivation of the worm. Furthermore, the current results support previous findings that BZs have detrimental effects on embryonic development. We found that FLBZ damaged tissues required for development and survival following exposure to ≥100 nM for ≥ 24 hours. This study provides preliminary information concerning the concentration-time profile of exposure to FLBZ required to elicit detrimental effects as measured by changes in morphology.

2.6 Acknowledgements

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CONNECTING STATEMENT I

In the first manuscript, the morphological damage associated with short-term *in vitro* exposure to flubendazole was described using standard histological techniques. Damage was observed in the hypodermis, intestine and developing embryos at all concentrations tested. This effect was also observed with the reduced metabolite (FLBZ-R). In this study we addressed the challenge of utilizing histological technique to assess drug-induced damage by developing a scoring method suitable for quantifying damage. This is the first study to implement a quantification method for assessing the morphological damage of BZs on filarial nematodes.

Importantly, the work presented in the previous chapter demonstrates that FLBZ elicits damage to adult parasite tissues required for survival and reproduction at pharmacologically relevant concentrations. Given that FLBZ requires weeks to months to have macrofilaricidal effects in animal models, it is important to confirm that the damage caused by FLBZ exposure over a few days in culture could lead to lethality if parasites could be maintained over a longer period.

The work described in the following chapter was aimed at determining the long-term effects of FLBZ exposure using an *in vitro*/*in vivo* model. Attempts to maintain parasites in *in vitro* culture for extended periods leads to a rapid reduction in parasite viability, highlighting the inefficiency of this method for testing long-term drug effects. For this reason, we implemented a model in which parasites were exposed to FLBZ *in vitro* for a short period prior to extended maintenance *in vivo*.

Chapter 3. Manuscript II

An *in vitro/in vivo* model to analyze the effects of flubendazole exposure on adult female *Brugia malayi*

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3.1 Abstract

Current control strategies for onchocerciasis and lymphatic filariasis (LF) rely on prolonged yearly or twice-yearly mass administration of microfilaricidal drugs. Prospects for near-term elimination or eradication of these diseases would be improved by availability of a macrofilaricide that is highly effective in a short regimen. Flubendazole (FLBZ), a benzimidazole anthelmintic registered for control of human gastrointestinal nematode infections, is a potential candidate for this role. FLBZ has profound and potent macrofilaricidal effects in many experimental animal models of filariases and in one human trial for onchocerciasis after parental administration. Unfortunately, the marketed formulation of FLBZ provides very limited oral bioavailability and parenteral administration is required for macrofilaricidal efficacy. A new formulation that provided sufficient oral bioavailability could advance FLBZ as an effective treatment for onchocerciasis and LF. Short-term *in vitro* culture experiments in adult filariae have shown that FLBZ damages tissues required for reproduction and survival at pharmacologically relevant concentrations. The current study characterized the long-term effects of FLBZ on adult *Brugia malayi* by maintaining parasites in jirds for up to eight weeks following brief drug exposure (6-24 hr) to pharmacologically relevant concentrations (100 nM - 10 μM) in culture. Morphological damage following exposure to FLBZ was observed prominently in developing embryos and was accompanied by a decrease in microfilarial output at 4 weeks post-exposure. Although FLBZ exposure clearly damaged the parasites, exposed worms recovered and were viable 8 weeks after treatment.

3.2 Author Summary

Onchocerciasis and lymphatic filariasis are debilitating diseases caused by infections with filarial nematodes. The World Health Organization aims to eliminate these infections as public health problems. Despite prolonged control efforts, including chemotherapy through mass drug administration (MDA), transmission and infections persist. Addition of a microfilaricide that is efficacious in a short regimen would enhance prospects for achieving elimination goals. We investigated the long-term effects of the macrofilaricidal drug, flubendazole (FLBZ), on *Brugia malayi*. Adult parasites were exposed in culture to FLBZ at pharmacologically relevant concentrations (100 nM - 10 μM) for up to 24 hr prior to implantation into the abdominal cavity of a jird for long-term maintenance. The greatest drug effect was on embryogenesis; morphological damage was most evident in early developmental stages. There was also a decrease in the release of microfilaria (mf) from the adult. Interestingly, no damage was observed to fully formed mf. Although further studies are required to determine to what extent these findings can be extrapolated to a field setting, an exposure profile which may produce similar effects *in vivo* has been defined.

3.3 Introduction

Infections with filarial parasites that cause lymphatics filariasis (LF) and onchocerciasis can lead to debilitating symptoms and cause great economic losses in endemic countries (1, 2). Control measures have relied on mass drug administration (MDA) of either ivermectin or diethylcarbamazine with albendazole since the Global Programme to Eliminate Lymphatic Filariasis (GPELF), the Onchocerciasis Control Programme (OCP) and the African Programme for Onchocerciasis Control (APOC) were created with the aim of eliminating LF and onchocerciasis as public health problems (3, 4). These drugs appear to act mainly as microfilaricides in an MDA setting that provides yearly dosing for an extended period of time to achieve elimination or local eradication (5). With the recent decline

in individuals reported to be infected with LF and onchocerciasis (6-9), the goal of elimination/control set by the World Health Organization (10) is closer to being achieved. Yet there remain a large number of individuals infected with these parasites. Additionally, MDA programmes for onchocerciasis within Africa are geographically limited due to severe adverse events associated with acute killing of *Loa loa* microfilaria (mf) in individuals bearing high parasitemia following treatment with ivermectin (11). The introduction of a safe macrofilaricidal drug into control programs is predicted to greatly enhance the ability to eliminate these infections in a timely manner.

Flubendazole (FLBZ), a benzimidazole (BZ) anthelmintic, is a candidate macrofilaricide for use in onchocerciasis and LF control programs. Initially introduced for treatment of infections of livestock animals with gastrointestinal (GI) parasitic nematode infections (12), FLBZ was subsequently approved for the same indication in humans (13), for which it is highly efficacious (14, 15). FLBZ has exhibited very high macrofilaricidal efficacy when administered parenterally in experimental filarial models (16-18) and in a human trial in onchocerciasis (19). Unfortunately, available formulations of the drug afford very limited oral bioavailability. Additionally, the formulation used for parenteral dosing in the human onchocerciasis study (19) led to severe injection site reactions, and its development was not pursued.

Recent efforts have been made to re-formulate FLBZ to enable oral dosing (18, 20, 21). Definition of the pharmacokinetic profiles needed for efficacy with an orally-bioavailable formulation would be facilitated by knowledge of the time-concentration exposure profiles at which FLBZ is detrimental to the survival of adult filariae. Previous data show that exposure to pharmacologically relevant concentrations of FLBZ or its bioactive reduced metabolite (R-FLBZ) in culture elicits damage to the hypodermis, developing embryos, and intestine of adult female *B. malayi*, but this damage is not accompanied by apparent changes in motility or viability (22). Developing an exposure-efficacy profile *in vitro* can assist in the definition of target pharmacokinetic profiles for dose selection

in advanced development. We adapted a transplant model for *B. malayi* in an effort to define a concentration of FLBZ that would be lethal after short-term exposures (≤ 1 day). The present study examined long-term concentration-dependent effects of exposure to FLBZ *in vitro* in *B. malayi* survival and viability after recovery from naïve jirds following transplantation.

3.4 Methods

3.4.1 Ethics statement

The transplant surgery was carried out under AUP 15-07 (2) and was approved by the TRS Labs Inc., Institutional Animal Care and Use Committee (IACUC).

3.4.2 Animal care

Male jirds (*Meriones unguiculatus*) approximately 24 -30 weeks of age (55-75 grams) were used as the source for and recipients of parasites in this study. The jirds were multiple housed (3-5/cage) in solid bottom, clear/translucent cages with bedding and wire mesh lids. The study room was maintained on a 12 hour light/dark cycle within a temperature range of 18 - 26 °C and a relative humidity range of 30 to 70%. Jirds were fed ad libitum with an appropriate certified rodent diet and water was provided ad libitum by an automatic watering system and/or water bottles.

3.4.3 Parasites

Adult male and female *B. malayi* were isolated from the peritoneal cavity of jirds >120 days post-infection as described (23, 24). Briefly, recovered adult worms were washed three times with warm (37°C) RPMI-1640 medium supplemented with 100 U/mL penicillin, 100 μg/mL streptomycin, and 0.4% gentamycin (Sigma-Aldrich Corp., St. Louis, MO, USA; hereafter referred to as RPMI).

Adult females were exposed to varying concentrations of FLBZ (10 μM, 1 μM, or 100 nM; Epichem Pty Ltd, Murdoch, WA, Australia) *in vitro* for 6, 12, or 24 hr. FLBZ solutions were prepared by dissolving the respective drug in 100% DMSO, with addition to RMPI to a final DMSO concentration

of 0.1%. Control RPMI contained an equivalent concentration of DMSO. Following exposure, male and female worms (10-15 each) were rinsed with RPMI and then transplanted into the peritoneal cavity of naïve jirds as described (25). The recipient jird was anesthetized with a 1:1:5 cocktail of xylazine:saline:ketamine and the fur was removed from the right ventral abdomen with electric clippers. The skin was wiped with 70% ethanol prior to making a 1 cm incision in the skin and body wall to expose the peritoneal cavity. Worms were aspirated into a Pasteur pipette which was inserted into the incision and the worms expressed into the peritoneal cavity. The incision was closed with Autoclip staples until necropsy 5 days, 4 weeks or 8 weeks later.

3.4.4 Parasite Viability

Recovery: At each end point, jirds were sacrificed and the peritoneal cavity opened for examination for adult worms. Worms were removed from the peritoneal cavity using forceps and placed in RPMI. The carcass was then soaked in warm saline to allow remaining worms to enter the media and the additional worms were placed in the same container of RPMI. The number and sex of adult *B. malayi* were recorded for each animal.

Motility: Parasite motility was assessed visually under light microscopy. Worms were scored as immotile, with no motion during the observation period; slightly motile, where only twitching of the head and/or tail was observed; moderately motile, with slow sinusoidal movements; or highly motile, comparable to parasites obtained from drug-free control jirds. Each sample was observed for at least one minute before scoring. Worms were fixed immediately thereafter as described (22) for subsequent histological analysis.

3.4.5 Effects on Embryogenesis

Microfilarial Release: Microfilariae were obtained from the peritoneal cavity of jirds at the time of necropsy. A glass transfer pipet was used to transfer approximately 10 ml of warm RPMI into the jird peritoneal cavity through an abdominal incision. After massaging the abdomen, the RPMI was

withdrawn and transferred to a conical tube. The number of mf was counted in aliquots of 10 µl in triplicate.

Embryograms: Two to four worms from each jird in each treatment group were placed in a 1.5ml Eppendorf tube with 500 µl PBS, gently crushed with a pestle and vortexed for 5-10 sec. A 10 µl sample was transferred to each chamber of a haemocytometer to count the various embryonic stages following the method of Schultz-Key (26). Embryos were classified into 6 stages: oocyte, early morula, late morula, S-shaped 'sausage' stage, coiled 'pretzel' stage, and stretched mf. A minimum of 100 embryos were counted to assess the proportional representation of each developmental stage.

3.4.6 Histological Preparation

B. malayi were fixed in glutaraldehyde (5% glutaraldehyde in 0.1 M sodium cacodylate buffer, pH 7.2) for a minimum of 48 hr in preparation for histological processing. Worms from each treatment were combined into groups and coiled prior to embedding in Histogel (FisherScientific; Pittsburgh, Pennsylvania, USA), which allowed visualization of various anatomical regions in multiple worms on a single slide. Dehydration, clearing, and vacuum infiltration with paraffin were completed using a Sakura VIP tissue processor. Parasites were then embedded in paraffin with the ThermoFisher HistoCentre III embedding station. A Reichert Jung 2030 rotary microtome was used to cut 4-5 micron sections, which were dried at 56°C for 2 – 24 hr. Slides were stained with haematoxylin and eosin prior to examination under light microscopy.

3.4.7 Assessment of worm damage

Sections were assessed independently by three parasitologists familiar with filarial nematode morphology, including a board-certified pathologist/parasitologist (CDM), as described in (22). Briefly, worms from two independent experiments were examined for damage to the body wall, including cuticle, hypodermis and longitudinal muscle; intestine; and reproductive tract, including the uterine wall and embryonic stages (classified as early [oocytes, early morulae, late morulae] or late

[sausage, pretzel, microfilariae]); and the pseudo-coelomic cavity. For comparative analysis of drug-induced effects, tissues were classified into four categories: no damage (0), minor (1), moderate (2), or severe (3). The damage score was determined by assessing tissues for nuclear and cytoplasmic distortions, cellular size and shape, membrane integrity, accumulation of debris, and distortion of overall anatomical integrity.

Two methods of analysis were performed as previously described (22). The first adhered to classical techniques used by histopathologists to determine tissue damage, in which all sections on a slide were surveyed, interpreted and translated into a single damage score for each tissue type. The second method involved scoring damage in each tissue type for each worm section on a slide. These scores were averaged for all sections on the slide to obtain the damage score.

3.4.8 Statistical Analysis

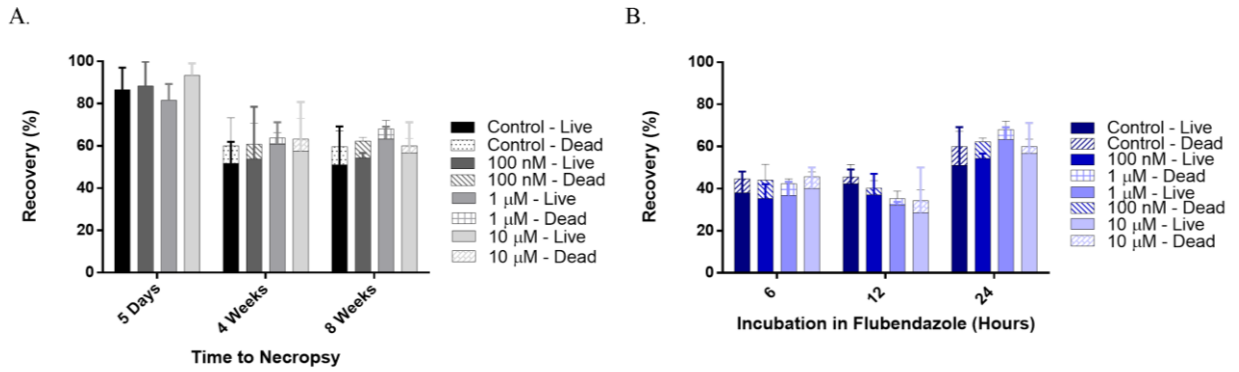
Statistical analyses were performed using IBM® SPSS® Statistics version 24. Percent recovery, microfilarial abundance, and embryogram results were analysed using a two-way ANOVA between treatment groups and time points. Histology and motility scores were analyzed using a Kruskal-Wallis test and Mann-Whitney post hoc with Bonferroni correction. All statistical tests were interpreted at the 5% level of significance.

3.5 Results

3.5.1 Effect of FLBZ on adult worm viability

Recovery. Flubendazole did not affect the recovery of adult worms following 24 hr exposure prior to transplant (Figure 3.1 A). Percent recovery was > 80% in all cases at day five and ranged from 40 – 60 % by four weeks, with no difference between the control and treated groups. Of the adult worms recovered, the proportion of live worms did not differ significantly with exposure (Figure 3.1 A and B). After eight weeks, recovery rates were slightly lower in groups exposed to FLBZ for shorter

durations (6 and 12 hr); however, this was not significantly different from the 24 hr exposure (Figure



3.1 B) or from control groups unexposed to the drug.

Figure 3.1 Effect of FLBZ on recovery of exposed and transplanted adult B. malayi male and female worms from the peritoneal cavity of naïve jirds. A. Five day, four week, or eight week maintenance in jirds after transplantation following 24 hr in culture. Data is a combination of two experiments. In experiment one 20 worms (10 male and ten female) were transplanted into each of three jirds for all treatment groups and maintained for 5 days or 4 weeks. Thirty worms (15 male and 15 female) were transplanted into each of three jirds per treatment group in experiment two and maintained for 4 weeks or 8 weeks. The 4 week data from each experiment did not differ significantly, therefore, they were combined for presentation purposes. B. Worm recovery eight weeks after transplantation following 6, 12, or 24 hr exposure to FLBZ *in vitro*. Thirty worms (15 male and 15 female) were transplanted into each of three jirds per treatment group, following the appropriate duration of *in vitro* exposure to FLBZ. Bars indicate the mean recovery of worms, both male and female, from each jird in a treatment group. Lines represent the standard deviation.

Motility. Motility was unaffected by FLBZ exposure. All recovered worms were highly motile five days following transplantation (Figure 3.2 A). Motility was variable in worms recovered four and eight weeks post-transplantation, but motility impairment and drug exposure were not correlated (Figure

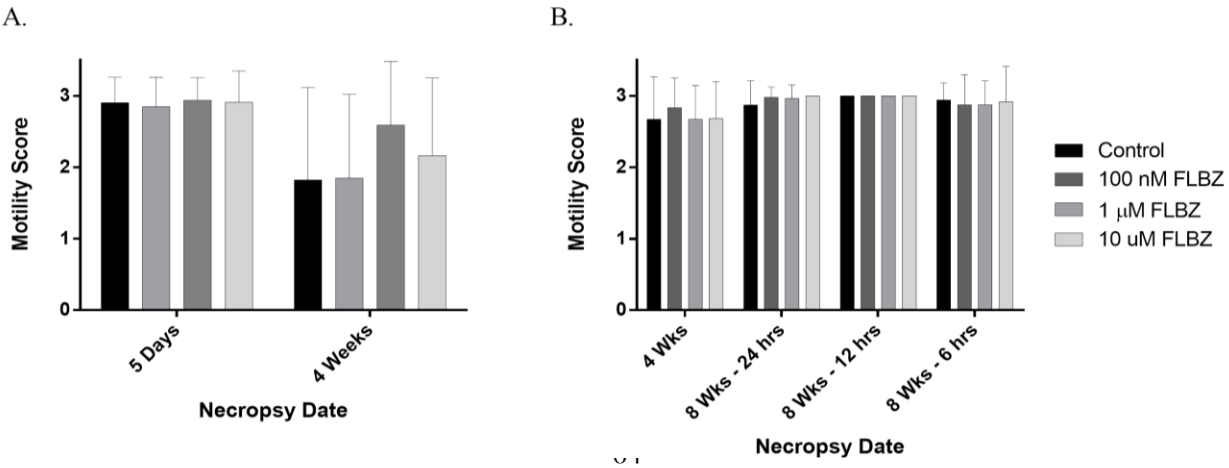


Figure 3.2 Motility of adult worms recovered from the peritoneal cavity of Brugia malayi infected jirds. A. Motility of adults worms from the first experiment where worms were maintained *in vivo* for five days or four weeks following 24 hour *in vitro* exposure to FLBZ. B. Motility of adults worms from the second experiment where worms were maintained *in vivo* for four weeks or eight weeks following 24 hour *in vitro* exposure to FLBZ. We also report motility for eight week *in vivo* maintained worms following 12 and 6 hour *in vitro* exposure.
3.2 A & B). A small proportion of worms were presumed dead due to a lack of motility.

Microfilarial Release. Five days following transplantation into naïve jirds, the number of mf recovered was consistently low and did not differ between control and treated groups (Figure 3.3 A). At four weeks, there was an increase in mf abundance in the control groups, while the number remained low in FLBZ-exposed groups. At eight weeks, there was a significant increase in mf abundance in the control group and slight increases in the treated groups. Incubation time had no effect on mf abundance (Figure 3.3 B). Significantly fewer mf were released from 24 hour treated females following 8 week *in vivo* maintenance (Figure 3.3 B). This did not extend to the 6 and 12 hour exposures as there was a great degree of variability in mf counts from these groups.

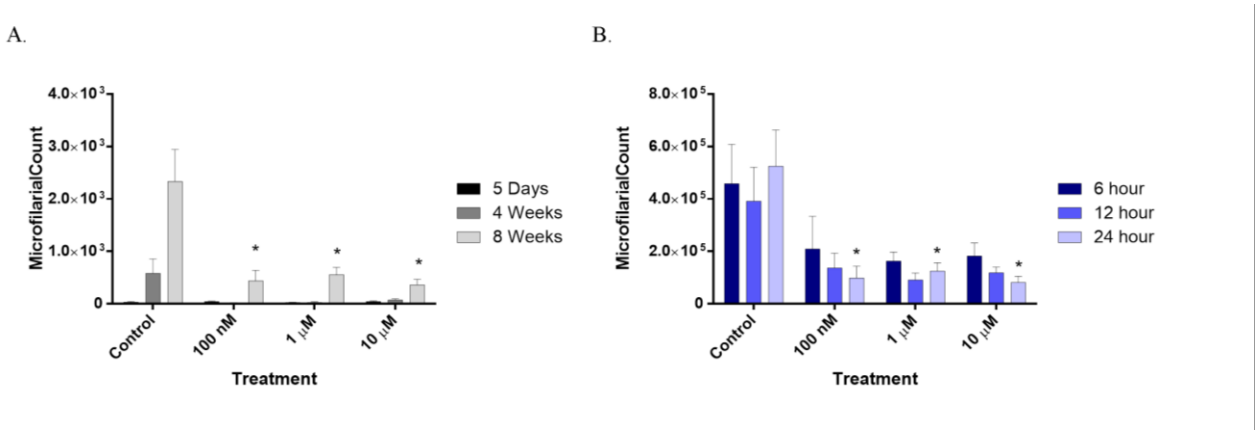


Figure 3.3 Effects of FLBZ on recovery of microfilariae from the peritoneal cavity of jirds. A. Five day, four week, or eight week maintenance after transplantation into naïve jirds following 24 hr culture. Data is a combination of two experiments. Microfilariae were enumerated from the peritoneal wash of each of three jirds which had 10 female worm transplanted in experiment one (5 day and 4 week maintenance) and 15 females in experiment two (4 and 8 week maintenance). Data were corrected for number of females transplanted. The 4 week data from each experiment did not differ significantly, therefore, they were combined for presentation purposes. B. Recovery eight weeks post-transplantation following 6, 12, or 24 hr exposure to FLBZ *in vitro*. Microfilariae were enumerated from peritoneal washes of each of three jirds which had 15 females worms transplanted, following the appropriate duration of *in vitro* exposure to FLBZ. Bars indicate the mean mf counts from each jird in a treatment group. Lines represent the standard deviation

3.5.2 Embryograms

Embryograms of treated worms differed substantially from those of controls (Figure 3.4 A). Late developing stages (sausage, pretzel and stretched mf) were markedly reduced, while early developing morula were significantly increased in treated worms relative to the controls. Treated worms not only contained large numbers of degenerating embryos (Figure 3.4 B) they were also found to contain fewer embryos overall (Figure 3.5).

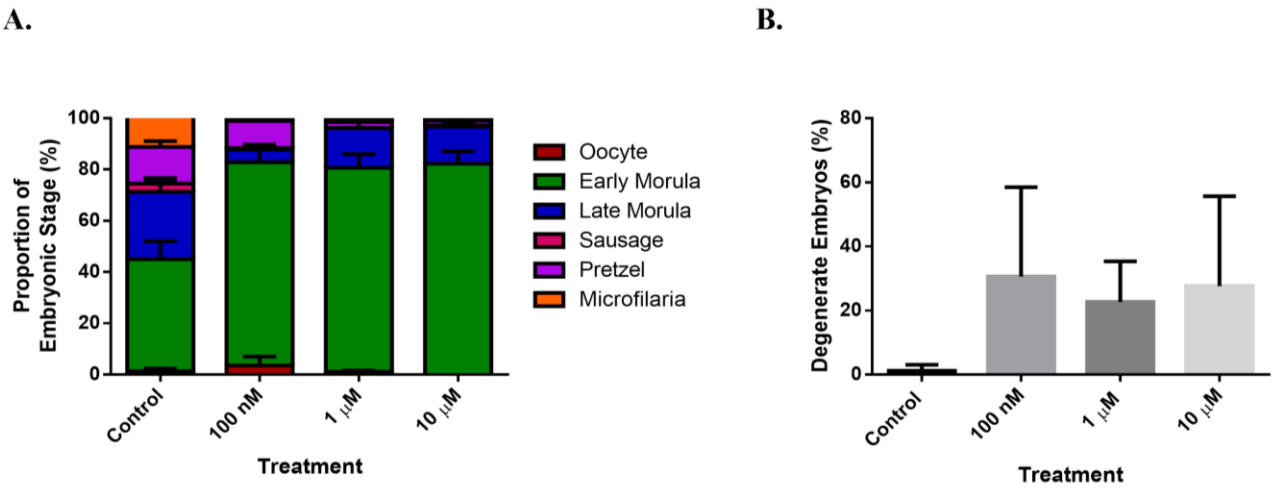


Figure 3.4 Effects of FLBZ on B. malayi embryogenesis. A. Embryograms for untreated and FLBZ-treated (24 hr exposure) females recovered four weeks post-transplantation. Two to four worms per treatment group were used for embryogram analysis. Data shown are % of oocytes, early and late morulae, pretzels and stretched mf in worm homogenates. A significantly higher proportion of early developing embryos was observed in treated groups ($P < 0.001$). B. Proportion of degenerating intrauterine stages.

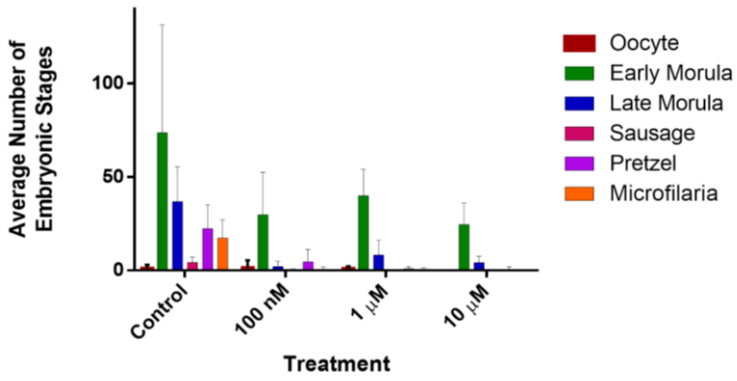


Figure 3.5 Average total number of embryonic developmental stages in in adult females recovered from the peritoneal cavity of Brugia malayi infected jirds four weeks post-transplantation. Numbers are averages of counts from homogenates of a minimum of six female worms per treatment.

3.5.6 Effects of FLBZ on adult worm viability: histology

Tissue damage observed in worms transplanted into naïve jirds differed from that observed in vitro [22]. Damage to the intestine observed *in vitro* was not observed following transplantation (Table 3.1). Minor damage to the hypodermis was observed five days following transplantation in both the treated and control groups; however, this damage appeared to resolve by four weeks. While the damage score in hypodermis of treated groups at four weeks returned a statistically significant p-value, the rather low damage score indicates this is unlikely to be biologically relevant. Damage to the developing embryos was the most prominent effect. While extensive damage to embryos was observed in all treatments at five days, this damage appeared to resolve in the control group by four weeks. Damage to embryos of treated worms, however, was pronounced and did not resolve at four weeks. At this time point damage to treated embryos was significantly higher than the controls. Morula stage embryos were the most extensively damaged (Figure 3.6 D, E) while mf were unaffected (Figure 3.6 F).

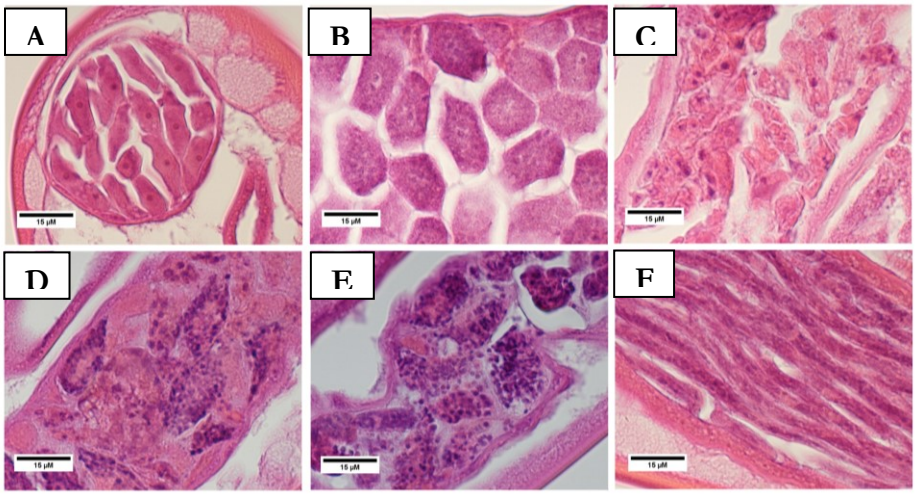


Figure 3.6. Long-term effects of FLBZ on developing embryos. Exposure period was 24 hr in culture prior to re-implantation. Time to recovery after transplant was 4 weeks. A. Oocytes, DMSO control; B. Morulae, DMSO control; C. Oocytes, 100 nM; D. Early Morulae, 1 μM; E. Late Morulae, 100 nM; F. Microfilariae, 10 μM.

Table 3.1 Tissue damage score following recovery of FLBZ-exposed *B. malayi* females from naïve jirds. A, B. Worms incubated for 24 hr prior to transplantation and recovered from jirds for 5 days, 4 weeks or 8 weeks after. Data is a combination of two experiments. In experiment one worms transplanted into each of three jirds for all treatment groups were maintained for 5 days or 4 weeks. Worms transplanted into each of three jirds per treatment group in experiment two were maintained for 4 weeks or 8 weeks. The 4 week data were combined for presentation purposes. A minimum of 25 worms were assessed histologically C, D. Worms incubated for 6, 12, or 24 hr prior to recovery at 8 weeks. Worms were transplanted into each of three jirds per treatment group, following the appropriate duration of *in vitro* exposure to FLBZ. The number of worms assessed histologically ranged from 11 to 39. Damage scored as minor (1), moderate (2), severe (3), or no damage (0) by two methods: A, C. Individual section scoring method; B, D. Classical histopathological survey method.

A

	Hypodermis			Intestine			Early Embryo			Late Embryo		
	5 Day	4 Week	8 Week	5 Day	4 Week	8 Week	5 Day	4 Week	8 Week	5 Day	4 Week	8 Week
Control	1.2	0.2	0	0.7	0.2	0.1	2	0.8	0	1.4	0.3	0.1
100 nM	1.1	0.6*	0.1	0.3	0.3	0.2	2	1.7*	0.6	1.2	0.9*	0.6
1 uM	1.5	0.6*	0.1	0.4	0.4	0	2.3	1.9*	0.9	1.6	1.2*	0.6
10 uM	1.3	0.6*	0	0.2	0.2	0	1.8	1.9*	0.5	2	0.9*	0.2

B

	Hypodermis			Intestine			Early Embryo			Late Embryo		
	5 Day	4 Week	8 Week	5 Day	4 Week	8 Week	5 Day	4 Week	8 Week	5 Day	4 Week	8 Week
Control	0.4	0	0.7	0	0	0	1.9	0.8	1	1.2	0.6	0
100 nM	0.7	0.2	0.3	0.2	0.2	0	1.7	1.7	1	1.4	1.9	0
1 uM	0.5	0	0	0	0	0	1.4	1.4	1	1.3	1.8	0
10 uM	0.4	0.2	0	0	0	0	1	1.8	1.6	2	1.8	0

C

	Hypodermis			Intestine			Early Embryo			Late Embryo		
	6 hr	12 hr	24 hr	6 hr	12 hr	24 hr	6 hr	12 hr	24 hr	6 hr	12 hr	24 hr
Control	0	0	0	0.1	0	0.1	0.9	1.3	0	0.4	0.2	0.1
100 nM	0.1	0.1	0.1	0.2	0.1	0.2	0.6	0.6	0.6	0.1	0.2	0.6
1 uM	0.1	0	0.1	0.2	0.1	0	0.4	0.5	0.9	0.4	0	0.6
10 uM	0	0.1	0	0.1	0.1	0	0.4	0.7	0.5	0.3	0.1	0.2

D

	Hypodermis			Intestine			Early Embryo			Late Embryo		
	6 hr	12 hr	24 hr	6 hr	12 hr	24 hr	6 hr	12 hr	24 hr	6 hr	12 hr	24 hr
Control	0	0	0.7	0.7	0	0	0.9	1.2	1	0	0	0
100 nM	0	0	0.3	0.3	0.3	0	1.2	1	1	0	0	0
1 uM	0	0.5	0	1	0	0	1.7	0.5	1	0	0	0
10 uM	0	0	0	0.7	0	0	1	1.5	1.6	0	0	0

*p-value<0.05

3.6 Discussion

An important step in the development of an anthelmintic is identifying the exposure profile (concentration and duration of exposure) which leads to death or irreversible damage in culture. These data can be used to predict efficacious pharmacokinetic (PK) patterns, leading to more efficient selection of doses for clinical trials in diseases, such as the human filariases, that have long end points, based on PK data rather than efficacy. This is particularly true for FLBZ, which has little apparent acute toxicity for adult filariids in culture (18, 22), but is highly efficacious when administered to infected animals in parenteral regimens that afford prolonged exposure to low blood levels (18). Replicating this exposure pattern necessitates long-term parasite maintenance in culture, which has been difficult to achieve without the presence of feeder cells that may compromise the integrity of added drugs.

Efforts to reformulate FLBZ to provide an orally bioavailable macrofilaricide necessitate replication of the efficacy achieved in a "long, low" exposure profile in a "high, short" paradigm. To determine if short exposures (~1 day) to high but pharmacologically relevant concentrations of FLBZ (e.g., plasma concentrations that can be realistically achieved in an oral dosing regimen) can cause lethal damage to adult filariids, we implemented an *in vivo* protocol in which the long-term effects of short-term exposure to FLBZ on *B. malayi* can be determined by transplanting treated worms into naïve jirds following drug exposure. The choice of FLBZ concentrations and durations of exposure was based on pharmacokinetic studies in rats, mice and pigs(20, 27-29). In rats, a cyclodextrin oral formulation provided a maximum plasma level of approximately 7 μM and remained at a concentration of >1 μM for 6 hr (20). Dosing pigs with a cyclodextrin oral formulation provided FLBZ in the plasma for longer durations, similar to that of the 12 hour time point in this study, albeit at lower concentrations (27). The concentrations chosen have also been shown to elicit detrimental effects on adult female *B. malayi* during *in vitro* incubations, although viability was not apparently compromised (22).

Several important conclusions can be drawn from this study. First, adult filariids have the capacity to recover from damage. This is demonstrated by the observation that control worms recovered 5 days after transplantation showed clear signs of tissue damage; however, damage was not evident in control worms recovered 4 or 8 weeks post-transplantation, suggesting that the process of removal from the initial jird host and maintenance in culture for 24 hr is traumatic, but that the organisms can recover.

Second, exposure to FLBZ for 6-24 hr is deleterious to adult filariids; female *B. malayi* exposed to the drug in culture and recovered 4 weeks after transplant exhibited considerable tissue damage, especially to reproductive tissues. These worms were unable to produce mf.

Third, adult *B. malayi* are resilient to drug-induced damage; worms recovered 8 weeks after transplantation had generally resumed production of mf and had resolved the damage observed at 4 weeks after transplantation. This degree of recovery was unanticipated and suggests the possibility of a more sophisticated and robust healing response to injury in these organisms than we had anticipated.

Worm recovery from control groups was comparable to recovery rates obtained in earlier studies (30, 31). FLBZ exposure had no effect on recovery of adult worms. This result suggests that 24 hr incubation in up to 10 μM FLBZ does not cause irreversible damage that leads to worm death within eight weeks following exposure.

How FLBZ eliminates adult filariae following parenteral dosing *in vivo* remains unknown. Damage from prolonged, low-concentration exposure to FLBZ following parenteral administration results in slow killing in weeks to months [16-19]; efficacy may require an immune response as is thought to be the case for microfilaricidal agents (23, 32, 33). It remains a goal of reformulation efforts to recapitulate the high efficacy of parenteral FLBZ with an oral regimen. The current results suggest that adult female *B. malayi* can recover from a short exposure to FLBZ, but leave unanswered the question of whether oral regimens compatible with field use of a macrofilaricide (up to 7 consecutive days) can cause lethality.

It is evident that the process of transplantation is stressful to the worms, as we observed damage to control worms recovered 5 days post-transplantation; however, they are able to recover from this injury (Table 3.1). While control worms recovered from the transplantation process, FLBZ-exposed worms were less able to do so (Table 3.1), especially in reproductive tissues, suggesting that they are indeed compromised by the drug.

FLBZ damages the hypodermis, intestine and developing embryos in adult female *B. malayi* exposed to the drug in culture (22). In the present study, drug damage to the intestine or hypodermis was resolved after longer residence times in the host. In an early study which injected FLBZ parenterally, no alterations in intestinal cells were reported in recovered worms beyond a decrease in microtubules until the experiment ended at day six (34). It was suggested this was due to the limited role of the intestine in nutrient acquisition by filariid parasites; unfortunately, effects on the hypodermis, which also plays a role in nutrient acquisition, were not reported. That this earlier work confirmed the lack of damage observed in the intestine and hypodermis in the present study does not mean that effects on these tissues can play no role in the macrofilaricidal activity of FLBZ. *In vivo* exposure to a high dose of oral FLBZ, with high macrofilaricidal efficacy, exhibited extensive damage to the hypodermis (unpublished data, CDM) consistent with damage observed *in vitro* (22). However, it cannot be ignored that exclusion of the host at the time of anthelmintic exposure is a limitation to *in vitro* culture systems as it overlooks an important component: the host response. Since we exposed parasites to FLBZ prior to transplantation into a naïve jird, an important factor may be missing in the development of drug-induced tissue damage.

Consistent among FLBZ studies is the damage caused to developing embryos (22, 34, 35). In this study, the embryonic stage which displayed the greatest damage was the morula (Table 3.1, Figure 3.6). Disruption to the integrity of morulae is prominent in treated worms, which exhibited an apparent loss of cellular adhesion and dispersion of these cells (Figure 3.6 D & E). Similar results were observed in

O. gibsoni infected cattle administered five daily doses of mebendazole (MBZ); two weeks following treatment, degenerating morula was the most notable effect (36). That study also report the presence of various developmental stages in the same uterine section, consistent with our results, which showed that early morulae are found alongside degenerating morulae (Figure 3.6 D). Four weeks following treatment, degenerate morulae were mixed with normal mf. Mf exhibited normal appearance for as long as eight weeks post exposure and were found mixed with oocytes and embryonic debris (36). Degeneration of morulae correlated with an increase in released egg antigen that is not observed with exposure to the microfilaricidal drugs IVM and DEC (36).

Embryograms indicate that morulae were also the most abundant stage, which increased with treatment and coincided with a decrease in the proportion of later developmental stages (sausage, pretzel, mf; Figure 3.4). Exposure of filariae to anti-*Wolbachia* antibiotics resulted in a similar phenomenon, whereby the proportion of later developmental stages decreased with increasing drug exposure (37). No previous studies have documented changes in filarial embryogram profiles associated with BZ exposure. However, histological analysis of nodules from human onchocerciasis patients treated intramuscularly with FLBZ revealed that females contained only oocytes and small numbers of mf two month following treatment. The Forsyth (36) and Dominguez-Vazquez (19) studies both administered drug over a period of five days. While we see similar trends in exposed embryos, the data suggest that multiple doses, rather than single exposures as in this study, may be required for high efficacy.

That there was no damage to stretched intrauterine mf at any time point is not surprising, as this observation is consistent with other studies (16, 22, 30, 38). In view of the fact that mf are in an arrested developmental state, there is likely to be a reduced requirement for microtubule-dependant processes with which FLBZ may interfere. However, there was a concurrent impairment of mf release, measured as reduced abundance of mf recovered from the peritoneal cavity (Figure 3.3). At eight

weeks, there was a drastic increase in mf abundance in the control group compared to the treatment groups. There was a slight rebound in mf numbers in treated groups between 4 and 8 weeks after transplantation. Diminution in released mf could result from uterine blockage due to degenerating embryos or an inability of females to release mf. Alternatively, if *B. malayi* embryos follow the same developmental cycle as *Onchocerca volvulus* (26), it may be that embryos present at treatment were damaged and newly developed oocytes were unable to mature to mf. The potential for embryos to recover, or for the females to resume normal embryo production, is yet to be determined. Nevertheless, the limited effects observed on mf is encouraging as it supports the potential safety of FLBZ for use in *L. loa* endemic regions. MDA campaigns for onchocerciasis in Africa are limited due to the activity of ivermectin against *L. loa* mf and association with severe adverse events (11). An ideal drug for onchocerciasis would have little effect on mf in macrofilaricidal regimens.

The present findings demonstrate that FLBZ elicits detrimental effects on developing embryos following long-term maintenance in the peritoneal cavity of jirds. Damage was most evident in early developmental stages and resulted in a decreased output of stretched mf, which is presumed to limit transmission. Conversely, FLBZ did not have direct microfilaricidal effects, which had implications for the utility of FLBZ in areas co-endemic for *L. loa*. This is an important observation, as current MDA programmes are restricted by the SAEs resulting from rapid mf killing by existing drugs. If FLBZ is shown to have a similar lack of microfilaricidal effect on *L. loa,* it could further substantiate its utility as a macrofilaricide in *Loa* endemic regions. It will, therefore, be critical to determine the effects of FLBZ on *L. loa* mf.

3.7 Acknowledgements

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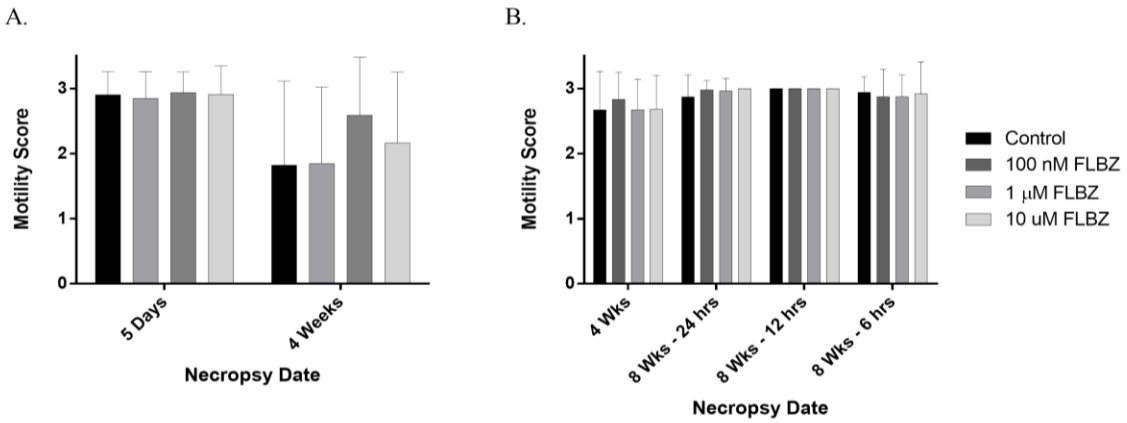
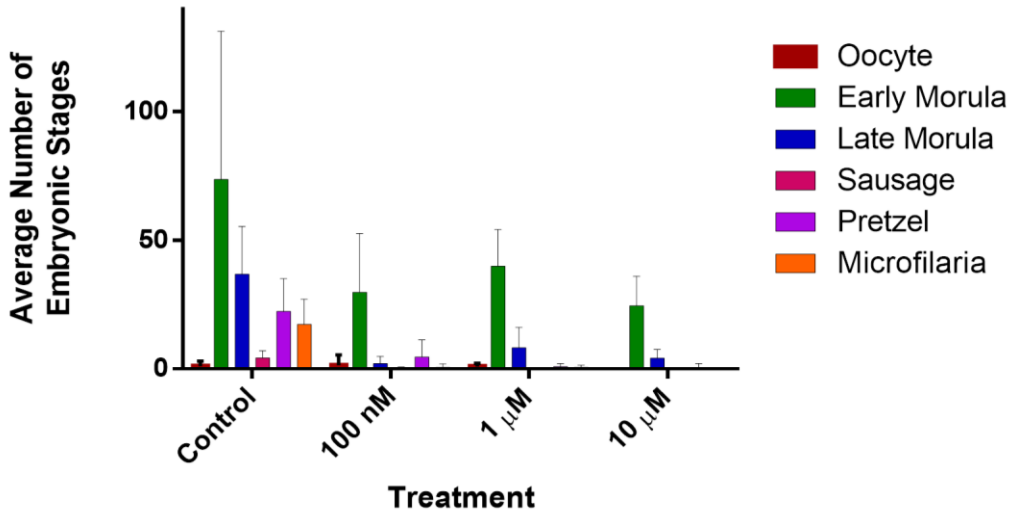


Supplementary Figure 4.1. Motility of adult worms recovered from the peritoneal cavity of *Brugia malayi* infected jirds. A. Motility of adults worms from the first experiment where worms were maintained *in vivo* for five days or four weeks following 24 hour *in vitro* exposure to FLBZ. B. Motility of adults worms from the second experiment where worms were maintained *in vivo* for four weeks or eight weeks following 24 hour *in vitro* exposure to FLBZ. We also report motility for eight week *in vivo* maintained worms following 12 and 6 hour *in vitro* exposure.



Supplementary Figure 4.2. Average total number of embryonic developmental stages in in adult females recovered from the peritoneal cavity of *Brugia malayi* infected jirds four weeks post-transplanation. Numbers are averages of counts from homogenates of a minimum of six female worms per treatment.

CONNECTING STATEMENT II

In the second manuscript, the long-term effects of FLBZ exposure were assessed. Unsuccessful attempts at long-term *in vitro* culture compelled us to consider a different approach. We implemented an *in vitro*/*in vivo* model in which worms were incubated in FLBZ *in vitro* for a short duration before transplantation into a naïve jird for long-term maintenance. This approach permitted us to control the concentration and duration of exposure while still being able to observe long-term effects. The results of this study confirm the damage to the reproductive tissue reported in manuscript I. FLBZ exposure led to a cessation of embryonic development at the morula stage and caused extensive damage to *in utero* developing embryos. Even though an impairment of mf release was observed, *in utero* mf were normal and indistinguishable from mf in the controls, suggesting that reduced mf release is due either to an effect on the adult worm's ability to expel mf, or to an embryostatic effect, rather than direct toxicity to mf. Unexpectedly, damage to parasite tissues observed after 5-day maintenance *in vivo* had resolved by 4 and 8 weeks, suggesting that these parasites can recover from drug-induced damage to a degree that was not anticipated. Evidently the process of transplantation is stressful, presenting as damage even in controls at 5 days, but the ability to recover from this stress provides us with a good model to observe true drug-induced damages.

It is apparent from both manuscript I and II that identifying drug-induced damages through traditional histopathological methods is challenging. Experimental design in the first two manuscripts aimed to address this challenge by employing a quantitative approach to describe pathological chances, as well as conducting multiple experiments with multiple observers and confirming embryonic changes using several methods. Nonetheless, addressing the question using an alternative approach would be advantageous and lend credence to the results obtained thus far.

The scope of the subsequent worked aimed at implementing a transcriptomic approach to better define the consequences of FLBZ exposure. The principle of this objective is partially to confirm results from previous experiments, but also to identify a gene, or set of genes, which could be used as a FLBZ-specific marker of damage. The benefit of utilizing a deep sequencing approach is its high sensitivity and the abundance of information that can be acquired from relatively small sample sizes. The following chapter presents the transcriptomic changes in *B. malayi* following short-term FLBZ exposure.

Chapter 4. Manuscript III

Profiling the macrofilaricidal effects of flubendazole on adult female *Brugia malayi* using RNAseq

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In preparation for submission to IJP-DDR[3]

4.1 Abstract

Background: The use of microfilaricidal drugs for the control of onchocerciasis and lymphatic filariasis necessitates prolonged yearly dosing. Prospects for elimination or eradication of these diseases would be enhanced by availability of a macrofilaricidal drug. Flubendazole (FLBZ), a benzimidazole anthelmintic, is an appealing candidate macrofilaricide. FLBZ has demonstrated profound and potent macrofilaricidal effects in a number of experimental filarial rodent models and one human trial. Unfortunately, FLBZ was deemed unsatisfactory for use in mass drug administration (MDA) campaigns due to its markedly limited oral bioavailability. However, a new formulation that provided sufficient bioavailability following oral administration could render FLBZ an effective treatment for onchocerciasis and LF. Identification of drug-derived effects is important in ascertaining a dosage regimen which is predicted to be lethal to the parasite *in situ*. Evaluation of drug-induced damage to tissues by histological analysis is challenging. In previous studies, exposure to FLBZ induced damage to tissues required for reproduction and survival at pharmacologically relevant concentrations. However, more precise and quantitative indices of drug effects are needed.

Methods/Results: This study assessed drug effects by using a transcriptomic approach to confirm effects observed histologically and to identify genes which were differentially expressed in FLBZ-treated adult female *Brugia malayi*. Comparative analysis across concentration (1 μM and 5 μM) and duration (48 and 120 hr) provided an overview of the processes which are affected by FLBZ exposure. Genes with dysregulated expression were consistent with the reproductive effects observed via histology in previous studies with FLBZ. This study revealed transcriptional changes in genes involved in embryo and larval development. Additionally, significant downregulation was observed in genes encoding components of the cuticle, which may reflect changes in developing embryos or the adult worm cuticle or both.

Conclusion: These data support the hypothesis that FLBZ acts predominantly on rapidly dividing cells, and provides a basis for selecting molecular markers of drug-induced damage which may be of use in predicting efficacious FLBZ regimens.

4.2 Background

Infections with filarial parasites that cause lymphatic filariasis (LF) and onchocerciasis can lead to debilitating symptoms and cause great economic loss in endemic countries (1, 2). Control measures have relied on mass drug administration (MDA) of either ivermectin or diethylcarbamazine in combination with albendazole (ABZ) with the aim of eliminating LF and onchocerciasis as public health problems (3, 4). These drugs act mainly as microfilaricides in an MDA setting, which necessitates yearly dosing for an extended period of time to achieve elimination or local eradication (5). Additionally, MDA programmes for onchocerciasis within Africa are geographically limited due to severe complications associated with acute killing of *Loa loa* microfilaria (mf) in individuals bearing high parasitemia following treatment with ivermectin (6). The introduction of a safe macrofilaricidal drug into control programs is predicted to greatly enhance the elimination of these infections in a timely manner.

Flubendazole (FLBZ), a benzimidazole (BZ) anthelmintic, is a candidate macrofilaricide for use in onchocerciasis and LF control programs. Initially introduced for treatment of livestock for the control of gastrointestinal (GI) parasitic nematode infections (7), FLBZ was subsequently approved for the same indication in humans (8), for which it is highly efficacious (9, 10). FLBZ has exhibited very high macrofilaricidal efficacy when administered parenterally in experimental filariasis models (11-13) and in a human trial of onchocerciasis (14). Although available formulations of the drug afford very limited oral bioavailability, recent efforts have been made to re-formulate FLBZ to enable oral dosing (13, 15, 16).

Early *in vitro* studies of BZ effects focused on GI nematodes. Ultrastructural observations of *Ascaris suum* 6 hr following exposure to mebendazole (17, 18) revealed a loss of microtubule structures in intestinal cells. Further exposure resulted in decreased glycogen content, accumulation of secretory granules near the Golgi and swelling and disruption of microvilli (17-19). Studies on the exposure of *Toxocara canis* and *A. suum* to FLBZ reported vacuolization of muscle, gonadal tissue, intestine and hypodermis (20). FLBZ-induced damage to reproductive organs of filariae has also been reported (21, 22).

Following FLBZ treatment of infected animals, loss of intestinal microtubules from the GI tract of the filarial nematodes *Brugia malayi* and *Litomosoides sigmodontis* was observed when the parasites were recovered as soon as 6 hr post-dosing (23). Increasingly severe damage to other tissues, including the hypodermis and reproductive system, was observed as time after dosing increased.

Definition of the pharmacokinetic profiles needed for efficacy with an orally-bioavailable formulation would be facilitated by knowledge of the time-concentration exposure profiles at which FLBZ is detrimental to the survival of adult filariae. Previous data show that exposure to pharmacologically relevant concentrations of FLBZ elicits damage to the hypodermis, developing embryos, and intestine of adult female *B. malayi*, but this damage is not accompanied by apparent changes in motility or viability (24). These changes were observed via histology, a method which is challenging for evaluating drug-induced damage. Confirmation of histological damage using a transcriptomic approach can assist in defining target pharmacokinetic profiles for dose selection in advanced development. Determination of FLBZ-specific changes in gene expression would aide in defining a molecular marker that predicts drug-induced damage.

The present study examines time- and concentration-dependent transcriptomic changes induced in female *B. malayi* by exposure to FLBZ *in vitro*.

4.3 Methods

4.3.1 Parasites

Adult female *B. malayi* were isolated from the peritoneal cavity of jirds (*Meriones unguiculatus*) >90 days post-infection as described (25). Parasites were supplied by the Filariasis Research Reagent Repository Center (FR3) at the University of Georgia (UGA), Athens, GA. All animal protocols were reviewed and approved by the UGA Institutional Animal Care and Use Committee, and complied with U.S. Department of Agriculture's regulations (USDA Assurance No. A3437-01).

4.3.2 Experimental Design

At UGA, adult female worms were pooled from three individual jirds and randomly distributed among 21 treatment groups (Table 4.1), ensuring each treatment included three technical replicates with 10 worms per replicate. Worms were washed in RPMI-1640 (BioWhittaker® Classic Cell Culture Media, VWR, Mississauga, ON) supplemented with 1% v/v gentamycin (gentamycin solution, 10 mg/ml Sigma Aldrich Inc., St. Louis, MO, USA), prior to shipping on heat pads overnight to McGill in 15 mL of the same solution. Upon arrival, three groups of ten worms were washed and flash-frozen in liquid N_2 in preparation for RNA extraction. The remaining worms were allocated to individual culture plate wells containing 6 mL RPMI-1640 (Sigma-Aldrich Corp., St. Louis, MO, USA) supplemented with 10% v/v heat-inactivated fetal bovine serum (Sigma-Aldrich Corp., St. Louis, MO, USA), 5% penicillin/streptomycin (Sigma–Aldrich Corp., St. Louis, MO, USA) and 2% v/v gentamycin (Gibco, Thermo Fisher Scientific Inc., Grand Island, NY, USA) with or without drug. FLBZ (Epichem, Murdoch, WA, Australia) was prepared in 100% DMSO and diluted in media to a final concentration of 0.1% DMSO; control media also contained 0.1% DMSO. Worms were incubated for 2 or 5 days at 37°C and 5% CO_2, with daily media changes by replacing 3 mL of appropriate media.

Table 4.1. Study design. Worms were allocated to one of three treatment groups. At each time point, three groups of 10 worms were washed, flash-frozen and used for RNA extraction

Treatment Group	Time to RNA Isolation		
	Upon Arrival	2 Days	5 Days
Control (0.1% DMSO)	3 x 10 worms	3 x 10 worms	3 x 10 worms
1 μM Flubendazole		3 x 10 worms	3 x 10 worms
5 μM Flubendazole		3 x 10 worms	3 x 10 worms

4.3.3 RNA Extraction

Total RNA was prepared using a previously described protocol (26) which combines organic extraction using Trizol LS reagent (Ambion, Life Technologies, Burlington, ON) and phase lock gel tubes (5 PRIME, Gaithersburg, MD). RNA was purified and concentrated using columns (RNeasy MinElute Cleanup Kit, Qiagen, Valencia, CA) and treated with DNase (Ambion DNA-free Kit, Life Technologies, Burlington, ON). Samples were shipped on dry ice to the NIH-FR3 (Molecular Division) at Smith College (Northampton, MA) for cDNA library preparation and Illumina sequencing.

4.3.4 cDNA library preparation and RNA Sequencing

RNA concentration and purity were measured using the Qubit RNA BR Assay Kit (Life Technologies, Q10210, Burlington, ON) on an Agilent 2100 Bioanalyzer (Santa Clara, CA). mRNA was obtained by Poly (A) magnetic isolation (NEBNext Poly (A) mRNA Magnetic Isolation Module, NEB, Ipswich, MA). The enriched mRNA served as template for cDNA library preparation with the NEBNext® Ultra RNA Library Prep Kit Illumina (NEB, E7530) and NEBNext Multiplex Oligos for Illumina (Index Primer 1-12) (NEB, E7600) following the manufacturer's instructions. Assessment of quality, DNA concentration and product size of the cDNA was performed for each library using a Qubit® 2.0 Fluorometer (Life Technologies, Q32866), Qubit® dsDNA BR assay kit (Life Technologies, Q32850),

High Sensitivity DNA Analysis Kit (Agilent, 5067-4626) and Agilent 2100 Bioanalyzer. cDNA libraries were sequenced on an Illumina MiSeq Platform employing a 150 base pair paired-end NGS setting.

4.3.5 Data Analysis

RNA Sequencing Analysis

The Mason-Galaxy platform (http://galaxy.iu.edu), a user-friendly web-based computational workflow system, was used to execute the RNAseq analysis. FastQ Groomer (v 1.0.4) was used to convert files to FastQ Sanger format and quality assessed using FastQC (v 0.52). Quality assessment was based on %GC content, Illumina adaptor contamination, and average base quality and content. The GC plot is expected to have a normal distribution at the projected GC, multiple peaks was indicative of contamination. Based on Fast QC statistics on base sequence content, sequences were trimmed from the 5' and 3' ends using FastQ Quality Trimmer (v 1.0.0). Trim Galore (v 0.2.8.1) was used to remove sequences reported as being contaminated with adaptors.

Sequence alignment and transcript quantification

Sequence reads were aligned to the *B. malayi* reference genome (ftp://ftp.wormbase.org/pub/wormbase/species/b_malayi/sequence/genomic/b_malayi.PRJNA10729.WS243.genomic.fa.gz) using TopHat2 (v 0.6), a spliced read mapper built on the short read aligner Bowtie (27). The resulting BAM files were used to obtain RNA sequencing metrics (Table 2) using SAM/BAM Alignment Summary Metrics (v 1.56.0). Aligned reads were enumerated using the HTSeq-count package (v 0.6.1) on the Galaxeast-Galaxy platform (http://www.galaxeast.fr/) (28) using the mode parameter set to 'union' which counts reads overlapping more than one gene model as ambiguous.

Differential gene expression analysis

Differential gene expression was analyzed in edgeR (v 3.10.5) (29) using the web interface NetworkAnalyst (http://networkanalyst.ca) (30, 31). The trimmed mean of M-values (TMM)

normalization method was used to correct for library size and reduce RNA compositional effect (32). Using the Bayes method based on weighted conditional maximum likelihood, tagwise dispersion parameters were estimated for each gene to facilitate between-gene comparisons (33). Hypothesis testing was completed using the exact test for the negative binomial distribution and significance was set as an experiment-wide false discovery rate (FDR) <0.15 (using the Benjamini-Hochberg method (34)).

Bioinformatic analysis

The Wormbase gene name was used to retrieve the protein coding sequence (http://www.wormbase.org/) (35) and the Uniprot accession number (http://www.uniprot.org/). Gene Ontology (GO) terms were obtained from Wormbase and nematode.net (v 4.0; http://nematode.net/NN3_frontpage.cgi) (36, 37). Statistically over-represented GO terms ($p < 0.05$) in gene lists of given treatment groups were identified using WebGestalt (WEB-based Gene SeT AnaLysis Toolkit) (38) using the UniProt accession number for *Caenorhabditis elegans* orthologues (a minimal E-value of $1*10^{-20}$) of *B. malayi* genes. RNAi phenotypes associated with the *C. elegans* orthologs were retrieved from www.wormbase.org.

Venny (v 2.0.2; http://bioinfogp.cnb.csic.es/tools/venny/) was used to create a Venn diagram of overlapping significantly DEGs in all treatment groups.

4.4 Results

4.4.1 Transcriptomic quantification

The average number of paired-end reads generated from polyA-tailed mRNA ranged from 1.7 – 2.8 million reads (Table 4.2). Approximately 80% of the sequenced reads mapped to the reference genome after removal of low quality alignments; this accounts for around 9000 transcripts. Sequencing depth

varied slightly between the treatment groups; however, it is important to note that there is no obvious trend in the number of transcripts between treated and control groups.

Table 4.2. RNA sequencing summary. Picard alignment summary tool was used to summarize the sequencing and mapping of sequences to the *Brugia malayi* transcriptome.

Treatment Group		Average Total # of Reads	Aligned Reads (%)	High Quality Alignments (%)	# of Transcripts
48 hr	Control	2805561	99.9	82.7	9329
	FLBZ 1 μM	1724686	99.9	82.8	8864
	FLBZ 5 μM	2065240	99.9	82.3	9076
120 hr	Control	2462188	99.9	81.8	9054
	FLBZ 1 μM	2087268	99.9	81.1	8827
	FLBZ 5 μM	2771021	99.9	81.8	9126

4.4.2 FLBZ-dependent changes in the transcriptome

Transcriptomic differences between treatment groups were assessed by conducting pairwise comparisons between control and drug-treated groups at each time point. Genes were considered significantly differentially expressed if the FDR of the exact test in EdgeR was <0.15. Pairwise comparisons revealed that the number of DEGs ranged from 94 to 159 (Table 4.3), which accounts for less than 1% of the estimated 14500-17800 protein coding genes in the *B. malayi* genome (39). In general, more genes were downregulated than upregulated. The largest number of DEGs was found after 120 hr exposure to both concentrations of FLBZ. 62.6% - 78.6% of DEGs had a known *C. elegans* orthologue.

Table 4.3. Summary of differential gene expression analysis.

Time point	Treatment Group	Upregulated genes	Downregulated genes	Total # of DEGs	Genes with *C. elegans* orthologue
48 hours	FLBZ 1 μM	18	80	98	77
	FLBZ 5 μM	62	32	94	72
120 hours	FLBZ 1 μM	24	135	159	115
	FLBZ 5 μM	19	104	123	77

Exposure to 1 μM FLBZ resulted in 257 genes that were differentially expressed at both time points, of which only seven overlapped. These genes were glycosyl hydrolase family protein (Bm4567), clec-1 (Bm3563) PAN domain containing protein (Bm6023), unc-22 (Bm7502), peptidase family M1 containing protein (Bm5654), a putative cuticle collagen (Bm9021) and snf-11 (Bm5517). Exposure to 5 μM FLBZ led to 217 DEGs over both time points with only 5 overlapping genes, including an uncharacterized protein (Bm 982), clec-1 (Bm3563), a sugar transporter (Bm5053), oxidoreductase (Bm2014) and snf-11 (Bm5517).

At 48 hr, 8 genes overlapped between the two concentrations: an uncharacterized protein (Bm8094), two ground-like domain containing proteins (Bm 3090,Bm14305), clec-1 (Bm3563), snpn-1 (Bm1903), membrane-anchored cell surface protein (Bm8956), peptidase family M1 containing protein (Bm5654) and snf-11 (Bm5517).

The 120 hr time point saw the greatest number of DEGs as well as the greatest number which were shared between the FLBZ concentrations. 82 of the 282 genes overlapped, including genes involved in signaling (2), metabolism (3), transcription (4), transport (7), development (10), collagen or cuticle related (15) or other/unknown function (41).

Only two genes were differentially expressed in all treatment groups (Figure 4.1). Bm5517, a sodium-dependent neurotransmitter symporter family protein (snf-11), was upregulated in all treatment groups. An orthologue of clec-1 in *C. elegans* (Bm3563) was also differentially expressed in all treatments. Interestingly, Bm3563 was downregulated in all treatment groups except the 5 μM FLBZ, 120 hr group (Figure 4.1).

4.4.3 Ontology of differentially expressed genes associated with FLBZ exposure

To determine the major processes affected by FLBZ exposure, GO terms were mined from Wormbase. Because the *B. malayi* genome is not fully annotated, and annotation relies to some degree

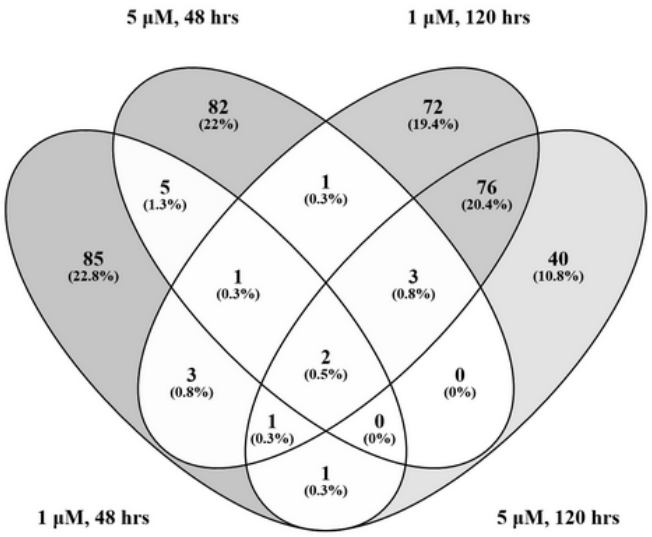

Figure 4.1 DE genes which overlap among the different treatment groups. Venn diagram was created in Venny 2.0.2

on sequence similarity to evolutionarily related species, including *C. elegans*, many of the mined GO terms are associated with the *C. elegans* orthologues. At all time points and concentrations of FLBZ, the most common GO term for up-regulated genes was development (Figure 4.2 A). Development was also one of the more abundant GO terms for down- regulated genes; however, this changed at the 120 hr time point, at which a dramatic increase in the proportion of GO terms relating to the cuticle/collagen was evident (Figure 2B).

Genes assigned the GO term 'structural constituent of cuticle' were generally down-regulated by FLBZ exposure (Table S4.1). The only cuticle gene to be up-regulated was Bm5273, a orthologue of *cut*-3 in *C. elegans*, which is required for alae development in larvae (40). Because the cuticle is initially synthesized in late embryogenesis and during each molt (41), it is not unexpected to find that the majority of the *C. elegans* orthologues are highly expressed in these developmental stages and for which mutations result in impairment of the cuticle (Table S4.1). However, 5 of the *C. elegans* homologues of these 25 genes were most highly expressed in adults (Bm9729, Bm8024, Bm2854, Bm9021, Bm7608),

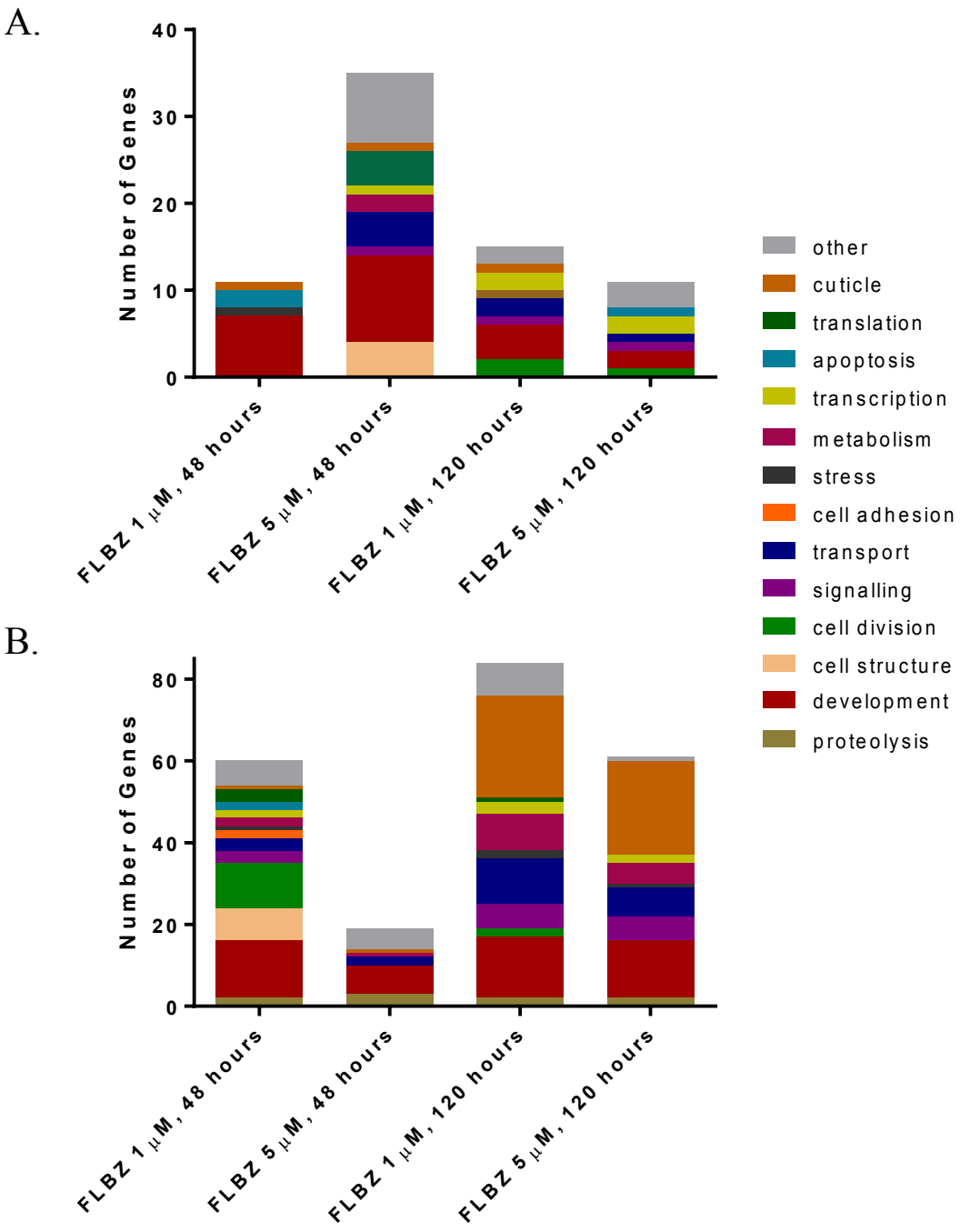

Figure 4.2 Gene ontology of DE genes associated with FLBZ exposure. Up-regulated (A) and down-regulated (B) genes were manually assigned an ontology using information available in Wormbase.
GO term enrichment analysis was conducted with the online interface WEBGESTALT using *C. elegans* orthologues curated from UniProt. Significantly enriched GO terms varied among the treatment groups. The majority of GO terms for DE genes across treatment groups were related to development and cell division. In the 120 hr treatment groups, only two GO terms were enriched at both concentrations: 'structural constituent of cuticle' and 'structural molecule activity.' Interestingly, 'structural molecule activity' was the only GO term enriched in all treatment groups.

Table 4.4 GO term enrichment. Top biological processes and molecular functions associated with *Caenorhabditis elegans* orthologues of *Brugia malayi* genes curated using information available on UniProt databases. Statistically enriched GO terms are reported as p-value.

GO Term	GO Id	48 hours		120 hours	
		FLBZ 1 μM	FLBZ 5 μM	FLBZ 1 μM	FLBZ 5 μM
Biological Process					
anatomical structure development	GO:0048856	6.46E-06			
multicellular organismal development	GO:0007275	2.94E-05			
cell cycle process	GO:0022402	7.46E-05			
M phase	GO:0000279	9.28E-05			
mitotic chromosome condensation	GO:0007076	9.91E-05			
cell cycle	GO:0007049	0.0001			
cell cycle phase	GO:0022403	1.00E-04			
body morphogenesis	GO:0010171	0.0001			0.084
developmental process	GO:0032502	2.00E-04			
anatomical structure morphogenesis	GO:0009653	3.00E-04			
larval development	GO:0002164	0.0012	0.01		
locomotion	GO:0040011	0.0018			0.0033
tissue development	GO:0009888	0.0046			0.0087
regulation of growth rate	GO:0040009				0.0085
molting cycle	GO:0042303				0.0067
positive regulation of growth rate	GO:0040010				0.0084
molting cycle, collagen and cuticulin-based cuticle	GO:0018996				0.0067
positive regulation of growth	GO:0045927				0.0031
regulation of growth	GO:0040008				0.0047
collagen and cuticulin-based cuticle development	GO:0040002				0.0087
Molecular Function					
ATP binding	GO:0005524	8.23E-07			
adenyl ribonucleotide binding	GO:0032559	8.38E-07			
adenyl nucleotide binding	GO:0030554	8.38E-07			
motor activity	GO:0003774	6.03E-06			
purine ribonucleoside triphosphate binding	GO:0035639	1.15E-05			
purine ribonucleoside binding	GO:0032550	1.17E-05			
purine ribonucleotide binding	GO:0032555	1.18E-05			
purine nucleotide binding	GO:0017076	1.18E-05			
nucleoside binding	GO:0001882	1.24E-05			
ribonucleoside binding	GO:0032549	1.24E-05			
structural molecule activity	GO:0005198	1.51E-02	3.95E-05	2.31E-07	1.03E-08
structural constituent of cuticle	GO:0042302			8.37E-09	2.84E-09

one of which (Bm2854, col-19) is an adult-specific marker for modification and assembly of the cuticle in *C. elegans* (42).

Not surprisingly, there was similarity between genes assigned the GO term 'structural constituent of cuticle' and those with 'structural molecule activity.' Fifteen of the 29 genes with structural molecule activity were collagens (Table S4.2). Of the five cytoskeletal components assigned this GO term, two were tubulins, including an α-tubulin (Bm9228, *C. elegans* mec-12; Bm10379, *C. elegans* tba-5) and a β-tubulin (Bm4733, *C. elegans* tbb-1). In addition, 6 DE genes are structural components of the ribosome.

4.5 Discussion

In this deep sequencing-based study, we investigated transcriptomic changes in adult female *B. malayi* occurring in response to FLBZ exposure, to investigate the pathophysiologic significance of this drug and to identify FLBZ-specific markers of damage. While RNAseq is highly informative in detecting alternative splice events, mapping transcription start sites, and strand specific measurements (43), the present study implemented this tool in a broader context, capitalizing on its precision in measuring transcript levels (44). This study also used *C. elegans* orthologues of *B. malayi* genes to better understand the functional significance of DE genes. As the *B. malayi* genome is not completely annotated, many genes remain hypothetical. Although homology is observed for a gene or protein, it may have evolved different functions in the two species. Thus, while the comparative approach may not be perfect, it provides a valuable method of inferring biological function.

Our results indicate that differential gene expression resulting from FLBZ exposure shows strong statistical bias towards GO categories related to structural molecule activity, cuticle, embryogenesis and larval development (Table 4.4). Analysis of significantly enriched GO terms identified those with 'structural molecule activity' as the only GO term to be enriched across both FLBZ concentrations at both time points. This was not completely unexpected as FLBZ, a benzimidazole, is known to inhibit

the polymerization of tubulin, an important structural molecule. In fact, three of the genes with 29 structural molecule activity were tubulins, including two α-tubulins (Bm 10379, Bm9228) and one β-tubulin (Bm4733). Interestingly, two of these (Bm10379 and Bm4733) are functionally redundant in *C, elegans*. TBA-5 (Bm10379 orthologue) localizes to the distal segments of sensory cilia, but is not an essential gene, as deletions yield wild-type phenotypes (45, 46). The benzimidazole sensitive *ben-1* (Bm4733 orthologue) is also redundant in *C. elegans*; animals lacking *ben-1* exhibit a wild-type phenotype (47), indicating that *ben-1* is not essential for growth or movement. Both genes were up-regulated at various time points, but not consistently across treatment groups (Table S4.2). It is surprising that FLBZ would elicit an up-regulation of functionally redundant tubulin genes, given that destabilizing drugs typically decrease tubulin synthesis; however, kinetic studies suggest that tubulin monomers may auto-regulate transcription (48). It may be the case that the increasing monomer pool, which would be expected to negatively regulate tubulin synthesis, fails to do so, leading instead to tubulin synthesis.

The α-tubulin *mec-12* (Bm9228) was upregulated in both FLBZ concentrations at 120 hr (Table S4.2). In contrast to the other tubulins, *mec-12* is highly specific for touch receptor neurons in *C. elegans* and is required for their proper function (49). Mutants in this gene lack the 15 protofilament microtubules characteristic of mechanosensory neurons [48].

Differential expression of tubulin genes provides proof-of-concept of the mechanism of action of FLBZ. It is also interesting to note that the expression of other tubulin-related genes is also altered. The downregulated gene *Bma-hcp-6* ($\log_2$FC of -1.43) plays an important role during mitosis. The *C. elegans* orthologue *hcp-6* attached microtubules to the kinetochore during mitosis and prevents chromosome twisting (50). Another downregulated tubulin-related gene was the heavy chain of dynein (*Bm-dhc-1*; $\log_2$FC of -1.14). Dynein is a molecular motor involved in microtubule-based movement and plays a critical role in several aspects of early embryogenesis, including positioning the microtubule

organizing center during cell division (51). RNAi of *dhc-1* in the one-cell stage of *C. elegans* embryos resulted in failure of pronuclear migration and chromosome separation (52). The dynein intermediate accessory chain (*Bm-dyci-1*), which is also essential in early embryogenesis (53) but has also been shown to function in dendrites and contributes to the formation of cilia (54) in *C. elegans*, was up-regulated in this study ($\log_2$FC = 2). The importance of *dyci-1* in the function of dendrites may account for its dissimilar pattern of regulation compared to the heavy chain.

The nematode cuticle is a collagen-rich extracellular matrix which covers underlying epithelial cells and is required for normal body morphology, movement, and interactions with the external environment (55). The cuticle is synthesized five times during development to the adult stage and is first synthesized during late embryogenesis (56).

In the current study, 26 DE genes were annotated as cuticle components (Table S4.1). Five of these overlapped with a set of DE genes detected during culture of *B. malayi*, indicating that these genes may be related to changes associated with *in vitro* culture, as opposed to a drug-induced effect (26). Six additional genes overlapped with a study assessing differential gene expression of ivermectin-treated *B. malayi* (Ballesteros *et al,* in preparation); however, in the ivermectin study, these genes were up-regulated, while they were down-regulated by exposure to FLBZ (Table S4.1), suggesting they may still be unique to FLBZ treatment. Indeed, all the cuticle-related DE genes in the current study were down-regulated (Table S4.1). Previous studies have shown that short term exposure to FLBZ leads to extensive damage to *B. malayi* hypodermal tissue (24). Given that components of the nematode cuticle are synthesized and delivered to the surface through the hypodermis (55), it is not unexpected that hypodermal tissue damage would result in a dampening of cuticule synthesis.

Working with gravid female worms introduces an additional layer of complexity when assessing the consequence of changes in expression of cuticle-related genes. Differential expression of these genes could stem from a general effect on embryogenesis, through which fewer embryos develop to the

stage at which the cuticle is first synthesized. Alternatively (or simultaneously), FLBZ may act on the hypodermis of the adult worm, impairing normal turnover of cuticular components. "Structural constituent of cuticle" (GO: 0042302) is overrepresented among genes with both embryonic and somatic tissue expression (Table S1). *C. elegans* orthologues of the *B. malayi* cuticle genes reveal that the majority exhibit highest expression in embryos and larval stages (Figure S4.1). Genes with highest expression in embryos and larval stages have RNAi phenotypes such as larval lethal, molt defective and dumpy embryos, suggesting that FLBZ-induced cuticular changes are primarily relevant in the context of embryonic development. Concomitantly, the *C. elegans* orthologues of several *B. malayi* DE genes exhibit high expression either in all stages (*col-107, col-182, dpy-31, T19B10.2*) or in adults (*col-97, col-130, col-19, col-89*, Figure S1).

A few of the DE genes deserve more comment. RNAi knockdown of T19B10.2 (Bm5834 orthologue) reduces the life span of *C. elegans* (57). Knock-down of this gene also impairs the ability of the nematode to resist hypertonic stress (58). The zinc-metalloprotease *dpy-31* is required for normal cuticle formation and proper body morphology and is essential to embryonic development (59). *dpy-31* is primarily expressed in hypodermal cells, but is also found in head neurons in adults and in rectal and vulval epithelial cells (59). The not well characterized *col-130* is one of the two genes whose expression is predominantly confined to adult worms (Figure S4.1). The other gene is *col-19*, an adult-specific marker for collagen assembly (42). Expressed exclusively in the adult cuticle, RNAi led to structural defects in the cuticle, including disrupted cuticular ridges. The downregulation of these genes suggests that FLBZ is eliciting negative consequence on the adult hypodermis and cuticle, if only indirectly. While no studies demonstrate cuticular damage by FLBZ, a scanning electron microscopy study of *Wuchereria bancrofti* exposed to either DEC or DEC+ABZ found that ABZ exposure results in cuticular damage (60). Lateral swelling, as well as spike-like and large leaf-like projections were seen DEC+ABZ exposed adult females but not in those exposed to DEC only (60). Using a TUNNEL-based assay,

ABZ was found to damage the adult cuticle in the bovine filariid *Setaria cervi* (61). This study also found extensive damage to the hypodermis. Hypodermal damage is a common theme among the benzimidazoles, and FLBZ is no exception (20, 22, 24, 62-65).

We know relatively little about the normal rate of turnover of cuticular components in adult filariae. Early studies reported that adult surfaces appeared to be quite stable with limited protein shedding (66, 67) but later work suggested that small amounts of surface proteins are released, albeit slowly (68-70). Presumably, this signifies a slow, but present, turnover of cuticular components. Given that filariae are long-lived parasites, some rate of turnover of cuticle proteins is expected to occur, and the inhibition of this process by FLBZ could lead to slow death of the worm.

Another possible explanation for the DE of apparently adult-expressed collagen genes is that components incorporated into the cuticle of developing embryos may originate from the female, as is the case for microfilarial sheath proteins (71, 72). Further experimentation is required to determine the consequences of FLBZ exposure on the integrity of the adult cuticle.

Fertilization in filariae is internal; female worms contain oocytes, sperm, developing embryos, and stretched microfilariae in their uteri. Impairments to embryogenesis and larval development emerged as common themes in our analysis of over-represented functional categories of DE genes. Genes which are critical to embryogenesis, or which are embryonic lethal when deleted, were common among the most notable functional categories; structural molecules, cuticle-related genes, and those involved in mitosis and meiosis.

Differentially expressed genes related to development were associated with, and overlap among, a number of GO terms, including; Cell Cycle (GO: 0007049) Cell Cycle Phase (GO: 0022402), Cell Cycle Process (GO: 0022403), M Phase (GO:0000279), Anatomical Structure Morphogenesis (GO:0009653) and Developmental Process (GO: 0032502).

Effects on embryogenesis can be seen as direct effects on various stages of the cell cycle (GO: 0007049, GO: 0022402, GO: 0022403, GO:0000279). The downregulated tubulin-related *hcp-6* (Bm8795 orthologue, Log_2FC -1.57), in addition to structural molecule activity, also functions in embryogenesis through its role in chromosome condensation and segregation (50). In addition to impairing chromosome segregation in response to RNAi in *C. elegans*, *hcp-6* null mutants exhibit embryonic lethality and larval arrest (50). A number of other genes involved in meiotic chromosome condensation and segregation were down-regulated by FLBZ exposure. *viln-1* (Bm2146 orthologue), a gelsolin-related protein predicted to have actin binding activity, is also involved in chromosome condensation. Villin proteins have gelsolin-like domains which give them actin-severing activity important to actin filament turnover during cell division (73). The dynein heavy chain (Bm589, Log_2FC -1.14) and an orthologue of MIX-1 (Bm13786, Log_2FC -1.57) are also involved in chromosome segregation and were found to be down-regulated by FLBZ exposure.

Chromosome inheritance during meiosis relies on induction of double-stranded DNA (dsDNA) breaks and subsequent repair of these breaks (74). Segregation of chromosomes and repair of dsDNA breaks requires a cohesion protein complex which helps to hold sister chromatids together (74). The loading of this complex in *C. elegans* requires the activity of PQN-85 (Bm6552) (75). RNAi of *pqn-85 in C. elegans* result in 100% embryonic lethality and lagging of chromosomes at anaphase (76). The B. malayi homologue of this gene was down-regulated in the present study (Log_2FC -1.63). We also saw down-regulation of Bm5562 (Log_2FC -1.44), the *C. elegans* orthologue (*rad-50*) of which functions in repair of dsDNA breaks (77).

A common trend among genes with roles in anatomical structure morphogenesis (GO:0009653) and developmental process (GO: 0032502) is involvement in embryonic elongation. Early embryogenesis entails rapid cell proliferation, but little change in the shape of the embryo (78). Approximately mid-way through embryogenesis, the embryo begins to elongate. Rapid and drastic changes occur during

the elongation phase whereby the embryo decreases in circumference three-fold and increases four-fold in length (78, 79). This change in shape is heavily dependent on hypodermal development, as stretching of this tissue is essential during the elongation process (79). In preparation for morphogenesis, epithelial actin filaments and microtubules organize circumferentially (78). NMY-1 (encoded by Bm4244), a non-muscle myosin which was down-regulated in this study (Log_2FC -1.68), forms filamentous structures in proximity to actin. Jointly they form a constriction apparatus in which NMY-1 functions as the motor driving actin constriction (80). Microtubules and the embryonic sheath function together to apply uniform pressure to internal cells as actin filaments contract circumferentially (78). The rate of constriction is regulated by *sma-1*,(Bm14776 orthologue in *C. elegans*) which was also found to be down-regulated in this study (log_2FC -1.62). *sma-1* stabilizes actin fibers during elongation by linking them to the embryonic hypodermis (81). Mutations in *sma-1* slow elongation as actin filaments dissociate from the membrane (82). Pressure distributed evenly across the worm creates an internal hydrostatic pressure that has been suggested to drive elongation (83).

Studies have shown that muscle contractions are also necessary for elongation (84). At the 1.75-fold stage, underlying muscle begins to contract (79). Contraction is transmitted through the hypodermis to the external surface mechanically through trans-epidermal attachments (TEAs) (79). It is unknown if the contractions promote morphogenesis or if the attachment structure plays a role in cell shape. In the present study, two genes related to this process were down-regulated by exposure to FLBZ. *Bma-myo-3* (Bm5021, log_2FC -1.53) encodes a myosin heavy chain necessary for initiating the assembly of thick filaments (85). Loss-of-function mutations in *C. elegans myo-3* prevent elongation (85), highlighting the importance of muscle interaction in morphogenesis. The *C. elegans* orthologue *vab-10* (Bm7639, Log_2FC -1.36) encodes a spectraplakin cross-linker of actin and microtubules (86). It is required for the interaction between TEAs and the circumferential actin bundles (79) and is suggested to protect cells from tension that builds up in the epidermis (87).

Down-regulation of genes involved in early embryo morphogenesis may explain the apparent halt of embryogenesis associated with FLBZ exposure (88). Short-term *in vitro* exposure to FLBZ followed by long-term maintenance *in vivo* resulted in an increase in late morula stage embryos, the stage preceding the onset of elongation. The great degree of damage seen in this transitionary stage *in vitro* (24) is also presumably, in part, due to the downregulation of elongation promoting genes.

Other DE genes involved in anatomical morphogenesis are generally related to late embryogenesis. Down-regulation was seen in genes involved in development of the gut/pharynx (Bm3576, Bm5753), reproductive system (Bm5753, Bm8739, Bm7169, Bm3563, Bm3633), hypodermis (Bm3576), and nervous system (Bm3576, Bm1770).

It should be noted that virtually all FLBZ-affected genes involved in development were down-regulated; only one gene was up-regulated, accounting for approximately 2% of DE genes in this functional category. Considering that FLBZ destabilizes microtubules, which are integral to developmental processes, it is not surprising that these processes would be impeded. Conversely, there is an alternative explanation which may be occurring simultaneously. The evolutionary life-history theory predicts that there is a trade-off between reproduction, growth and survival depending on the availability of resources (89). It is conceivable that the insult of drug exposure, or resulting damage to tissues involved in nutrient acquisition (24), could stimulate down-regulation of genes not required for immediate survival, such as those involved in reproduction.

The difficulty in assessing drug-induced damage motivated us to search for FLBZ specific markers of damage. Comparison of DE genes in each treatment group uncovered two genes which were common in all treatment groups; Bm3563 and Bm5517 (Table 4.1). To determine the suitability of these genes as markers, their expression was compared to results reported in a study outlining the transcriptomic changes associated with *in vitro* culture of *B. malayi* (26), as well as a study that identified DE genes associated with ivermectin exposure (Ballesteros et al, in prep). Bm5517, a sodium-dependent GABA

transporter (90) which was up-regulated in all groups in the present study, was also up-regulated in both of the previous transcriptomic studies, rendering it an unsuitable marker. Bm3563 was not differentially expressed following exposure to ivermectin (Ballesteros et al, in prep), but was up-regulated in the culture effect study (Ballesteros et al, 2016). However, FLBZ exposure caused down-regulation of this gene across all treatment groups. The large difference in expression profiles of this gene between the two studies lends support to the use of this gene as a marker of FLBZ-induced damage.

Bm3563 is an orthologue of *C. elegans clec-1*, which encodes a C-type lectin. Very little is known about *clec-1*; however, >120 C-type lectin domain containing proteins are predicted in the genome of *C. elegans,* some of which have characterized physiological functions (91, 92). Parasitic nematodes exploit lectin receptors to evade the host immune response by secreting C-type lectins (93, 94). However, RNAi studies of *clec-1* in *C. elegans* suggest that it is involved in body morphogenesis, larval development and growth (95), events which are commonly impaired by FLBZ. Further studies are needed to explore the utility of this gene as a marker of damage.

Conclusion

RNAseq is a valuable resource as it facilitates a better understanding of molecular interactions in complex biological systems. We implemented a transcriptomic approach to identify genes and overarching processes were being affected in *B. malayi* when challenged with FLBZ. Analysis of GO terms highlights the influence FLBZ on filarial embryonic development, consistent with previous findings (24, 88). Significantly enriched GO terms commonly had RNAi phenotypes of embryo/larval lethality or impairments to overall morphogenesis for *C. elegans* homologues. We also noted changes in cuticular components, for which all significantly DE genes were down-regulated. The tissues in which these changes occur are yet unknown; the contribution from the adult cuticle vs. developing embryos is the subject of future work. One DE gene overlapped all treatment groups and emerged as a possible

marker of FLBZ-induced damage, Bm3563. Although more work is necessary to confirm the utility of this gene as a marker, it would be highly beneficial to pharmacodynamic experiments if a dependable, FLBZ-specific marker of drug lethality was available.

4.6 Acknowledgements

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Supplementary Table 4.1. Differentially expressed genes assigned the GO term 'structural constituent of cuticle' (GO:0042302). Fold Change (log_2) is reported for 120 hour exposure to flubendazole. Data on developmental stage which exhibits the highest expression in *C. elegans* was mined from wormbase. E – embryos, L – larvae, A - Adults

B.malayi Gene	*C.elegans* orthologue	Fold Change (log_2)		Stage with highest expression	RNAi phenotype
		1 μM FLBZ	5 μM FLBZ		
Bm6587	Y46G5A.29	-5.88			
Bm4954	K09E2.1	-5.31			
Bm4334	cutl-20	-4.69		E	
Bm8605	col-104	-4.04	-3.88	L	
Bm11095	col-107	-3.97		All	dumpy, locmotion variant
Bm4904	col-182	-3.77	-4.19	All	sterile, exploding through vulva, larval arrest, lethal
Bm9729	col-97	-3.49	-3.36	L_4, A	dumpy, morphology and locomotion variant
Bm9092	col-48	-3.20	-2.59	L	lethal, molt defect, body vacuole
Bm3465	cut-1/3	-3.11	-2.69	E, D	larval lethal, molt defect
Bm2336	ptr-1	-3.09		E, L	dumpy, dauer cuticle varient
Bm5827	T19A5.3	-3.04		E	larval letal, molt defect
Bm3273	cut-1/3	-2.72	-4.51	E, D	protruding vulva
Bm7894		-2.39	-3.58		
Bm2786	col-14	-2.38	-2.79	L	protruding vulva
Bm8024	col-130	-1.95	-1.88	A	embryonic lethal
Bm5922	cutl-21	-1.90		E	
Bm5432	dpy-31	-1.56		All	embryonic lethal
Bm2605		-1.53			
Bm2854	col-19	-1.52	-1.22	A	breaks in alae, multiple alae
Bm9021	col-89	-1.50	-1.24	L, A	dumpy
Bm5834	T19B10.2	-1.50	-0.96	All	larval lethal, molt defect, lethal, shortened lifespan
Bm11071	col-73	-1.36		L	dumpy
Bm6421	col-109		-1.4	L	egg laying defective
Bm11074	col-165	-1.26	-1.63	E	dumpy, dauer cuticle variant, pharyngeal morphology varient
Bm7608	col-97	-1.18		L_4, A	dumpy, morphology and locomotion variant
Bm5273	cut-3	1.17		E	dumpy, missing alae, pharynx morphology variant

Supplementary Table 4.2. Differentially expressed genes assigned the GO term 'structural molecule activity' (GO:0005198).

B. malayi Gene	*C.elegans* orthologue	Fold Change (log_2)				Functional Role
		48 hours		120 hours		
		1 μM FLBZ	5 μM FLBZ	1 μM FLBZ	5 μM FLBZ	
Bm4116	unc-54	-1.35				myosin
Bm1706	mrpl-9	0.99				mitochondrial ribosomal protein
Bm14055	M28.5		1.12			non-histone ribosomal protein
Bm7366	mrps-18A	1.06				mitochondrial ribosomal protein
Bm8439	col-45	1.74				constituent of cuticle
Bm9238	act-4		1.02			actin
Bm7802	npp-11		-3.49			nucleoporin
Bm14726	rpl-36		1.45			ribosomal protein
Bm5011	mrpl-36		1.47			mitochondrial ribosomal protein
Bm10379	tba-5		1.55			alpha tubulin
Bm10355	col-68		4.15			constituent of cuticle
Bm11071	col-73			-1.36		constituent of cuticle
Bm4904	col-182			-3.77	-4.19	constituent of cuticle
Bm11095	col-107			-3.97		constituent of cuticle
Bm4733	ben-1			0.71		beta tubulin
Bm9729	col-97			-3.49	-3.36	constituent of cuticle
Bm2605	col-89			-1.53	-2.17	constituent of cuticle
Bm8043	col-104			-2.40	-2.58	constituent of cuticle
Bm9092	col-48			-3.19	-2.59	constituent of cuticle
Bm2786	col-14				-2.79	constituent of cuticle
Bm9228	mec-12			0.97	0.962	alpha tubulin
Bm4024	grl-4			-1.98	-2.23	hedgehog-like protein
Bm4605	col-34				-2.57	constituent of cuticle
Bm11125	mrpl-17				-2.88	mitochondrial ribosomal protein
Bm11074	col-165			-1.26	-1.63	constituent of cuticle
Bm6158	F53B6.4			1.21	1.11	locomotion
Bm6421	col-109				-1.35	constituent of cuticle
Bm2854	col-19			-1.52	-1.22	constituent of cuticle
Bm8024	col-130			-1.95	-1.87	constituent of cuticle

CONNECTING STATEMENT III

In the previous manuscript, I used a deep sequencing approach to describe the transcriptional changes in adult female *B. malayi* exposed to FLBZ. Most prominently, genes involved in development and cuticle synthesis were commonly found to be differentially expressed. These results validate the impairments of embryonic development presented in the first two manuscripts and provide a description of molecular events resulting in the gross-morphological changes observed via histology. We found downregulation of cuticle-related genes, some of which are embryo specific, but also some which are only expressed in adults. We suggest there may be an alternate mechanism of action by which FLBZ produces macrofilaricidal effects through weakening the cuticle barrier. Additionally, one gene was identified as a possible FLBZ-specific marker of damage. The availability of such a marker would greatly enhance the assessment of drug-induced damage given the inherent limitations of current methods.

A common trend across the benzimidazole anthelmintics is lack of activity on the microfilarial stage of filarial nematodes. Results of the first two manuscripts reinforce this trend. The following chapter presents a different approach to identifying microfilarial effects of FLBZ. The objective of this work was two-fold; first we aimed to determine if FLBZ had direct microfilaricidal effects on *L. loa*, an effect which is of great concern with the current chemotherapeutic drugs used to treat filariasis. Second, given that we already knew that FLBZ was not microfilaricidal in *B. malayi*, we investigated the capacity for treated mf to infect mosquitoes, which would give us an indication of the transmission limiting potential of FLBZ.

Chapter 5. Manuscript IV

Microfilarial sensitivity to flubendazole *in vitro*

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5.1 Abstract

The benzimidazole anthelmintic flubendazole (FLBZ) is a potent and efficacious macrofilaricide following parenteral administration. Generally, FLBZ elicits little to no effect on circulating microfilariae (mf) in animal models and in one human trial in patients infected with *Onchocerca volvulus*. The severe complications observed following the treatment of patients with high *Loa loa* microfilaraemia with ivermectin (IVM), the only chemotherapeutic approved for onchocerciasis control in Africa, is of great concern in co-endemic areas. This study examined the potential of FLBZ to rapid kill mf of *L. loa,* the phenomenon proposed to underlie the complications. Mf of *L. loa,* acquired from experimentally infected baboons, were exposed to FLBZ, its reduced metabolite, albendazole and IVM *in vitro*. Viability of *L. loa* mf *in vitro*, measured by motility, was not affected by FLBZ. This result was substantiated with mf of the conventional filarial model *Brugia malayi*. Given that the macrofilaricidal activity of FLBZ occurs weeks to months following administration and unaffected circulating mf are available for ingestion by a vector mosquito, we measure the effects of FLBZ exposure on transmission of treated mf. *Aedes aegypti* mosquitoes were fed on blood containing FLBZ-exposed *B. malayi* mf. Mosquitoes were dissected 24 h or 14 days post-feeding to determine the number of mf which permeated the midgut and developed to the infective L_3 stage. At the highest concentration, FLBZ impaired the ability of mf to cross the mosquito midgut, regardless of duration of *in vitro* exposure; however, even lower concentrations reduced the proportion of mf which crossed the midgut. Although some mf crossed the midgut, FLBZ exposure completed abolished the capability of treated mf to develop to infective L_3s, irrespective of duration of exposure or concentration.

5.2 Background

Infections with filarial nematodes of the Onchocercidae family are among the most debilitating of the neglected tropical diseases and are estimated to affect more than 150 million people (1, 2). *Wuchereria bancrofti* and *Onchocerca volvulus,* the causative agents of lymphatic filariasis (LF) and onchocerciasis respectively, are of greatest concern as infection can result in significant debilitation and is a major hindrance to socioeconomic development in endemic regions. *Loa loa* has received less attention. Although generally asymptomatic, loiasis can be rather disconcerting, causing pruritus, calabar swelling and subconjunctival migration of adult worms (3). However, the main concern regarding *L. loa* is the severe adverse events (SAEs) observed following administration of ivermectin (IVM) for the treatment of LF and onchocerciasis in co-endemic regions; high microfilarial loads of *L. loa* (>30,000 mf/ml) are associated with rare cases of IVM-induced SAEs, including encephalopathy and death (4, 5). The death of *L. loa* mf following IVM treatment is thought to be responsible for the SAEs as IVM is known to rapidly decrease circulating mf (6, 7).

Due to the limitations imposed by IVM-associated SAEs in *L. loa* patients, mass drug administration (MDA) programmes have been suspended or limited in areas which are highly endemic for *L. loa* (7, 8). This has led the WHO to propose alternative strategies which primarily focus on interrupting transmission (2). The current recommended strategy involves administration of albendazole twice yearly combined with vector control (9, 10); however, the length of time necessary for this strategy to be effective has not been defined. Filariasis control programmes would be greatly enhanced by a macrofilaricidal drug which elicited no or slow killing of mf, thereby lessening the chances of SAEs. Flubendazole (FLBZ), a benzimidazole (BZ) anthelmintic approved for the treatment of infections due to gastrointestinal nematodes of livestock (11) and humans (12-14), is an appealing candidate to address these concerns.

As a general feature, FLBZ elicits much greater effects on adults and developmental stages of filariae than mf in animal models (15-17) and in a single *O. volvulus* human trial (18). Wider testing in humans was restricted due to problems related to the route of administration; macrofilaricidal effects are only observed with parenteral administration. Injection-based drugs have limited appeal for use in field settings, and in the human trial led to injection site reactions which precluded further development. Fortunately, Johnson & Johnson have undertaken preclinical development of FLBZ (19) to repurpose the drug by developing an oral formulation which provides high bioavailability and efficacy. A highly bioavailable, orally administered macrofilaricide with limited macrofilaricidal activity would be an important contribution to campaigns that aim to eliminate filariasis as public health problems. Considering that FLBZ has limited effects on mf in many animal models, it is important to confirm these observations by directly measuring the effects of FLBZ exposure on *L. loa* mf. This is especially true in light of the fact that orally bioavailable formulations provide much higher systemic exposure than achieved with parenteral administration (20, 21), and the acute effects of exposure to high levels of FLBZ have not been reported.

Another important consideration is that it can take weeks to months for FLBZ to produce macrofilaricidal effects (15-18). In the meantime, circulating mf which are not killed by FLBZ are available to be ingested by the mosquito host, furthering the reinfection cycle. IVM treatment of infected jirds has been shown to suppress the development of *B. malayi* and *B. pahangi* mf within a mosquito host that has fed on treated animals (22), suggesting that drugs which are not directly microfilaricidal may have effects on mf that are only detectable in a model that requires development. Therefore, we conducted studies to determine if high FLBZ concentrations elicited direct microfilaricidal activity and also if FLBZ exposure affected the capacity of mf to develop in a mosquito host.

5.3 Methods

5.3.1 Ethics Statement

Loa loa. Microfilaremic blood was acquired from experimentally infected baboons using animal procedures in accordance with animal care and use protocols at the National Institutes of Health (USA). Ethical approval for use of baboons in this study was obtained from the Ministry of Scientific Research and Innovation of Cameroon. The use of non-human primates for research was approved by the Committee on the Ethical Use of Animals in Research (CEUAR) within the Research Foundation for Tropical Diseases and Environment (REFOTDE), Cameroon.

Brugia malayi. Microfilariae and mosquitoes were supplied by the Filariasis Research Reagent Repository Center at the University of Georgia, Athens, GA. All animal protocols were reviewed and approved by the UGA Institutional Animal Care and Use Committee, and complied with U.S. Department of Agriculture's regulations (USDA Assurance No. A3437-01).

5.3.2 Parasites

Loa loa mf were obtained from experimentally infected baboons (*Mandrillus* sp.). Blood samples were collected in EDTA coated tubes and transported to REFOTDE at the University of Buea on ice. Blood constituents were separated on a Percoll gradient (40%, 50%, 65%) centrifuged at 4000 g x 10 min. Mf are found in the top layers containing platelets and lymphocytes. The top layers were removed, passed through a 5 μm syringe filter to remove cells and placed in a Petri dish filled with Dulbecco's Modified Eagle Medium (DMEM; Wisent) supplemented with penicillin (100 U/ml) and streptomycin (100 μg/ml; Invitrogen) to allow mf to migrate into the media. Mf were then concentrated by centrifuging at 2000 g x 10 min.

Brugia malayi mf were isolated from the peritoneal cavity of jirds (*Meriones unguiculatus)* >120 days post-infection as described (23, 24). Mf for infectivity assays were isolated from peritoneal washes via passage through a 5 μm membrane filer and overnight incubation in DMEM medium supplemented with 100 U/mL penicillin and 100 μg/mL streptomycin (Sigma-Aldrich) to allow the greatest number of mf to migrate into the media.

5.3.3 Culture

Mf were distributed among various treatment groups; FLBZ (10 μM, 1 μM, 100 nM; Epichem Pty Ltd), reduced FLBZ (10 μM, 1 μM, 100 nM; Epichem Pty Ltd), albendazole (10 μM, 1 μM, 100 nM; Sigma-Aldrich), IVM (1 μM; Sigma-Aldrich), or 5% DMSO as a positive control for mf immobilization. All drugs were prepared in 100% DMSO and diluted in media with a final concentration of 0.1% DMSO. Incubation in drug-containing media was conducted for a maximum of 72 hours.

For infectivity assays mf were exposed to FLBZ (10 μM, 1 μM, 100 nM), FLBZ-R (1 μM) or IVM (1 μM) for 2, 6 or 24 hours prior to washing in serum-free media.

5.3.4 Motility

Motility was scored every 24 h using a four-point scoring system; 3 – highly active, 2 – sluggish, 1 – twitching of head/tail, and 0 – immotile. Percent motility was determined using the following formula:

$$\frac{(\#\text{ mf scoring 3 x 1}) + (\#\text{ mf scoring 2 x 0.66}) + (\#\text{ mf scoring 1 x 0.33}) + (\#\text{ mf scoring 0 x0})}{\text{total \# of mf}}$$

Mortality was determined as the percent of mf with a motility score of 0.

5.3.5 Mosquito Feeding

Cultured *B. malayi* mf were concentrated by centrifugation (2000 g x 5 min) and added to heparinized dog blood (FR3, University of Georgia) at 24 – 32 mf / μl. *Aedes aegypti* Black Eye Liverpool strain mosquitoes were allowed to feed on infected blood maintained at 37°C using an artificial membrane

feeding apparatus for 2h. Blood-fed mosquitoes were maintained in the FR3 insectary at the University of Georgia at 27°C and 80% humidity. Mosquitoes were collected from the insectary 24 h and 14 days after feeding for either immediate dissection or fixation in 90% ethanol for later dissection.

5.3.6 Mosquito Dissection

Freshly collected mosquitoes were kept on ice until dissection. Following removal of the wings and legs, mosquitoes were placed on a glass slide with a drop of RPMI. Midguts were carefully excised with fine forceps, taking care not to rupture the blood-engorged midgut. The midguts were cover-slipped and the number of mf counted under 400x magnification.

Dissections of ethanol-fixed mosquitoes were conducted following WHO suggested methods with a few modifications (25). Mosquitoes were washed in descending dilutions of ethanol for 30 min (70%, 55%, 25%) followed by a final 30 min wash in distilled water.

Midguts – Midguts were carefully excised from 24 h fixed mosquitoes and stained whole in Mayer's haemalum for 1 h before washing in distilled water for 15 min. Individual midguts were spread on glass slides and left to dry overnight prior to enumerating mf under 600x magnification

L3 - Fourteen day mosquitoes were stained in Mayer's haemalum at room temperature for seven days. Mosquitoes were washed for 3 days in distilled water and stored in glycerol until dissection. The head, thorax, and abdomen were separated and placed into individual drops of glycerol. Fine needles were used to tease apart tissues to enumerate L_3s under a stereoscopic dissecting microscope at 8x – 35x magnification.

5.3.7 Statistical Analysis

Statistical analyses were performed using a two-way ANOVA in the GraphPad Prism 6 package. All statistical tests were interpreted at the 5% level of significance.

5.4 Results and Discussion

5.4.1 Effects of Flubendazole on mf of *Loa loa*

Limitations of filaricidal drugs used in Africa center on the risk of IVM-induced SAEs in *L. loa* patients. Chemotherapeutic efforts would be greatly enhanced by a macrofilaricide that elicited little or no damage to mf of *L. loa*. Because FLBZ is a highly effective macrofilaricide, it is important to understand its effects on mf at concentrations which might be obtained in plasma with an orally bioavailable formulation if it is to be used in the field.

Our results confirm the limited effect of FLBZ on mf reported in previous studies (15-17, 26, 27). Neither *in utero* fully stretched mf nor circulating mf exhibited detrimental effects associated with FLBZ treatment. In this study, *B. malayi* mf obtained from the jird peritoneal cavity were exposed to FLBZ. Lack of motility was considered indicative of mf death. All drug-treated groups had motility similar to that of the control (Figure 5.1 A), with only slight motility impairment. FLBZ had no observable microfilaricidal activity (Figure 5.1 C).

L. loa mf were less fit under these culture conditions than *B. malayi* (Figure 5.1 B), with the motility of mf in all treatments decreasing over time. Some differences in motility were observed between the treated groups and the control but the greatest difference was observed in the 5% DMSO group which served as a positive control for motility impairment. Importantly, there was no microfilaricidal effect of FLBZ on *L. loa*. Increased mortality of drug-treated mf was only observed at the 72 h time-point and was < 25%. These results are promising, as they suggest that FLBZ is not quickly lethal to *L. loa* mf, which may prevent SAEs in patients with high microfilaraemia. In patients, treatment with IVM leads to a sharp decrease in mf to 40% of the original *L. loa* mf load 24 h after treatment and to 20% of the original load by 72 h (28).

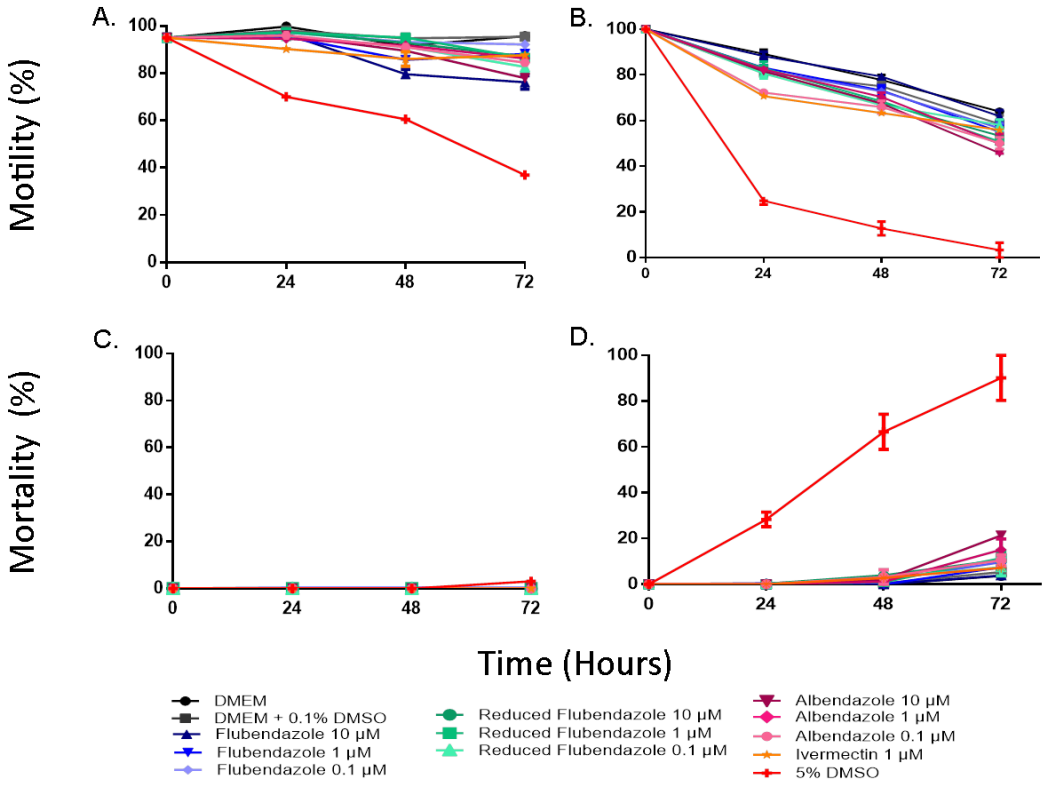

Figure 5.1 Effect of flubendazole on the viability of microfilariae of Loa loa (B and D) and Brugia malayi (A & C). Motility (A & B) was scored on a four point scale percent motility calculated using the formula in 5.3.4. Mortality (C & D) was calculated as the percent of microfilariae which were completely immotile and presumed dead.

Slight motility impairment was observed in drug-treated mf compared to the controls. A dramatic loss of motility was not seen, nor was there a striking microfilaricidal effect of the drugs tested (Figure 5.1 B and D), which may have been expected of a potential SAE-inducing drug. However, this assay only measures direct microfilaricidal activity, and it is known that IVM, for instance, is not lethal to mf in culture (29). Instead it's microfilaricidal effects require, or are greatly enhanced by, a host immune component (30, 31). The limitation of our studies on the effect of FLBZ on *L. loa* mf *in vitro* lies in the absence of host components in the system; we have not demonstrated that exposure of mf *in vivo* to FLBZ at high concentrations may lead to effects similar to those observed with IVM or DEC. However, in human onchocerciasis and animal models for lymphatic filariasis, FLBZ elicits filaricidal

effects over long durations (15, 18). In addition, *O. volvulus* mf counts in skin did not diminish in human onchocerciasis patients given FLBZ parenterally until several months post-treatment. It still must be proven that an oral formulation would not provide sufficiently high exposure to produce a drastically different response.

5.4.2 Effects of flubendazole on infectivity of mf to mosquitoes

FLBZ requires weeks to months to produce observable macrofilaricidal effects (15-18). In the meantime, circulating mf are available to be ingested by the mosquito host, furthering the reinfection cycle. It would be highly beneficial to MDA programmes if a drug which was not directly microfilaricidal, and therefore potentially safe to use in *L. loa* co-endemic areas, was also able to limit parasite transmission. We therefore examined the transmission limiting ability of FLBZ by determining infectivity of treated mf for the mosquito host, including development to the L_3 infective stage.

FLBZ led to significantly fewer mf crossing the mosquito midgut in a concentration-dependant manner following 24 h exposure (Figure 5.2 A). A significantly higher number of mf were retained in the mosquito midgut compared to the control. The 10 μM FLBZ group had the greatest impairment at all time points of exposure (2,6 and 24 h; Figure 5.2 B). The number of mf retained in the midgut following 2 and 6 h exposure to lower concentrations (100 nM, 1 μM) was not different from the control (Figure 5.2 B). Interestingly, FLBZ-R (1 μM) had no effect on midgut penetration. It is important to note that this metabolite produced histological damage to adult *B. malayi* (26) and is known to be bioactive (32).

Although FLBZ exposure inhibited the ability of mf to cross the midgut, a proportion of these mf crossed into the abdominal cavity. The fate of these mf was assessed by determining if mf which had penetrated the midgut developed to the infective L_3 stage.

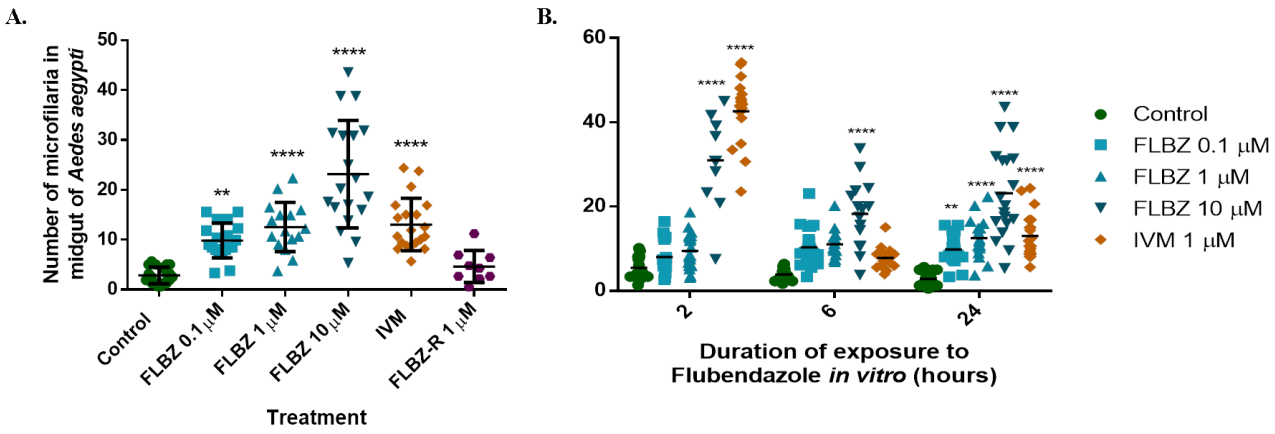

Figure 5.2 Consequence of flubendazole exposure on midgut penetration of Brugia malayi microfilariae to Aedes aegypti mosquitoes. A. Number of microfilariae retained in the mosquito midgut 24 h following feeding on microfilariae which were exposed to flubendazole, reduced flubendazole or ivermectin for 24 h *in vitro*. B. Number of microfilariae retained in the mosquito midgut 24 h following feeding on microfilariae which were exposed to flubendazole or ivermectin for 2, 6, or 24 h *in vitro*.

Infectivity in the controls was 37.5% (Figure 5.3). Strikingly, exposure to FLBZ at all concentrations and for all durations completely abolished the ability of mf to develop to infective L_3s (Figure 5.3).

The midgut wall of the mosquito host acts as a physical barrier to invasion by mf. Invasion into the mosquito hemocoel was once thought to require the mf to first lose their sheath before rupturing the membranes of the midgut wall using a cephalic hook (33). Later studies found mf to lose their sheath as they migrate through the midgut (34, 35).

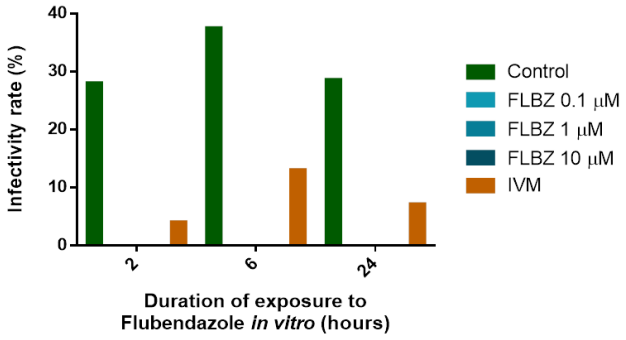

Figure 5.3 Infectivity rate of Aedes aegypti fed on flubendazole treated Brugia malayi microfilariae. Infectivity rate was calculated as the percent of mosquitoes which harbored infective L_3 larvae 14 days following feeding.

Whether the loss of the sheath occurs before or during midgut migration, this step is an obvious requirement for development to later larval stages.

Although the factors leading to exsheathment are unknown, the process is thought to be developmentally regulated (36, 37) and can be stimulated by calcium-dependant proteases as well as other proteases or endopeptidases (22, 38, 39). Given that these factors need to be secreted from the worm to break down the sheath, it is possible that FLBZ blocks the secretion of such factors. BZs (40, 41) including FLBZ (40), inhibit protein secretion from GI nematodes and *B. malayi* mf. It would not be surprising if a similar phenomenon occurs with the filarial nematodes. We did not investigate the presence or absence of sheaths on mf that penetrated the midgut. If, at lower FLBZ concentrations, the mf penetrated the midgut without losing their sheath, this could account for their inability migrate and further develop to the infective stage.

5.5 Conclusions

FLBZ did not show rapid killing of *L. loa* mf *in vitro*. If rapid death of *L. loa* mf is responsible for the severe complications observed in IVM treated patients, it is tempting to speculate on the safety of FLBZ in loiasis areas, as it does not lead to rapid mf death. Further experiments are needed to explore the consequence of FLBZ exposure *in vivo*. While not directly microfilaricidal, FLBZ completely abolished the development of mf to infective L_3s in *Aedes aegypti*. Consequently, FLBZ treatment is likely to block transmission of filariae to another host. However, we do not know if mf can recover from FLBZ exposure, as is the case with adult *B. malayi* (42).

The results of this study suggests that FLBZ could be a safe macrofilaricide to use in regions co-endemic for *O. volvulus* and *L. loa* owing to an absence of rapid microfilaricidal effects, while having the added benefit of blocking further transmission.

5.6 Acknowledgements

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Chapter 6. Summary and Conclusion

6.1 Summary

Neglected tropical diseases (NTDs) are tragically common infections of the poorest people in underdeveloped countries. Predominantly comprised of chronic parasitic infections, NTDs typically result in prolonged periods of disability and perpetuate a vicious cycle of poverty through a continual impairment of productivity (1). In recent years, increased awareness and interest in combatting these diseases has encouraged great expansion in the field of NTD research (2).

Two such diseases are lymphatic filariasis and onchocerciases, caused by infection with filarial nematodes. As with many parasitic infections prominent in developing countries, vaccines are not available and chemotherapeutics are limited by accessibility, cost, and possibility of serious side effects (3). Unfortunately, this restricts clinicians to a single drug which can be used effectively in onchocerciasis control programmes; IVM. Moreover, treatment must be provided yearly for extended periods as the drug is only effective at killing larval stages and not the adult. While IVM clearly affects adult filariae, as shown by extended sterility following a single dose, macrofilaricidal effects are limited at best. As such, it is of paramount importance to develop alternative chemotherapies to be implemented in control strategies if the goal of elimination is to be achieved.

One method of approaching this challenge is to investigate a drug that has been previously approved for use in humans and has shown macrofilaricidal effects in animal models. FLBZ fits this profile and has recently drawn much interest for that reason. Indeed, FLBZ exhibits profound and potent macrofilaricidal effects in multiple animal models (4-9) and in one human trial of onchocerciasis in the early 80s (10) following parenteral administration. Serious injection site reactions in that human trial, possibly due to the choice of delivery vehicle or the highly insoluble nature of FLBZ, precluded its use

as a field safe macrofilaricide. Recent efforts to reformulate FLBZ for oral administration (6, 11) underscores the necessity to develop a better understanding of its macro- and microfilaricidal effects, as well as the identification of a dose regimen at which these effects can be predicted to occur in humans.

The goal of the experiments reported in this thesis was to gain a deeper understanding of the filaricidal effects of FLBZ. The first aspect to be addressed was the identification of morphological changes to tissues following drug exposure and linking the severity of these changes to a dosage range. The histological assessment we conducted allowed us to identify three tissue types that exhibited the greatest damage following short-term *in vitro* culture; the hypodermis, intestine and developing embryos. Damage to developing embryos is consistent with the general mechanism of action of BZs; inhibition of microtubule polymerization has been clearly shown to impair the rapid cell division occurring during embryogenesis (12-15). Together, damage to the hypodermis and intestine signal a hindrance in nutrient acquisition. The functionality of the filarial intestine is a yet controversial subject, as the degree to which the parasite obtains nutrients from ingested host material is yet unknown. Nutrient acquisition in the filarial nematodes is thought to occur predominantly by uptake across the cuticle (16-18). FLBZ-induced accumulation of vacuoles and swelling observed in the hypodermis would certainly impair nutrient acquisition mechanically, in addition to the diminution of intracellular transport expected to occur in the absence of microtubules. BZ-induced impairment of nutrient acquisition has been suggested as the ultimate cause of worm death (19-21), a consequence that would account for the slow killing of adult parasites characteristic of BZs (6, 9). Importantly, these damaging effects were observed at all concentrations tested, indicating that damage to macrofilarial tissues required for reproduction and survival occur at pharmacologically relevant concentrations (22-25).

It was then important to confirm that these effects persisted over longer durations. The next set of experiments conducted corroborated, and further described, the damage to embryos. Embryograms

conducted four weeks post-exposure suggested that FLBZ causes a cessation of embryonic development at the late morula stage, the stage prior to the onset of embryonic elongation. A decrease in mf released from FLBZ-exposed adult females was also observed at four and eight weeks post-exposure. Initially, this would seem to imply a detrimental effect on mf; however, intrauterine mf in treated females were morphologically indistinguishable from those in the untreated controls. This is not surprising, since FLBZ has little effect on circulating mf in animal models (5, 9, 10). The limited effects observed on mf supports the potential safety of FLBZ for use in *L. loa* endemic regions. MDA campaigns for onchocerciasis in Africa are limited due to the activity of IVM against *L. loa* mf and its association with severe adverse events in some patients bearing high loads of *L. loa* mf (26). An ideal drug for onchocerciasis would have little effect on mf in macrofilaricidal regimens.

Unexpectedly, damage to the hypodermis and intestine observed following short-term culture resolved after longer residence in a host. This contradicts the dramatic histological damages observed in the hypodermis following *in vivo* exposure to high doses of oral FLBZ (6). We suggest that, because the parasites were exposed to drug *in vitro* prior to long-term maintenance *in vivo,* exclusion of the host at the time of anthelmintic exposure removes an important variable; the host response. The host response likely plays a larger role at the time of drug exposure than originally expected.

The difficulty in assessing drug-induced damages using traditional histological techniques compelled us to explore alternative methods to characterize drug effects that result in lethality. Using a deep sequencing approach we further confirmed the effects of FLBZ exposure on embryogenesis and helped clarify the molecular underpinnings of the gross-morphological changes we observed previously. The expression of a number of genes involved directly in meiosis and mitosis was down-regulated. We also observed down-regulation of expression of genes involved in embryo elongation, which is another microtubule dependent process. Changes in expression of genes involved in

elongation may at least partially explain the FLBZ-induced cessation of embryogenesis at the morula stage observed in the *in vitro/in vivo* model experiments.

Downregulation of expression of genes encoding cuticle components suggests a weakening of the adult cuticle as a mechanism of damage resultant from FLBZ exposure. This list consists mostly of genes expressed predominantly in embryos and larvae, but it also includes genes that are adult specific. We know relatively little about turnover of cuticle components, but there is some evidence that suggest a slow, but detectable, natural turnover (27-29). Down-regulation of collagen genes have yet to be identified as adult or embryonic in origin in *B. malayi*, but these findings provide initial insight into a potentially novel mechanism of action for FLBZ.

Assessment of drug-induced effects would be greatly enhanced if a quantifiable and drug-specific marker of damage were available. The challenges of assessing damage histologically prompted us to search for a molecular marker of damage. One gene, found to overlap all treatment groups, was identified as a candidate marker. Further experimentation would substantiate this gene as a FLBZ-specific marker to surmount the difficulties with current methods of assessing drug-induced damage.

The final aspect of this thesis was examination of the possible effects of FLBZ exposure on mf. Early work in this project confirmed results of previous studies which indicated that FLBZ exposure had little to no effect on mf. This suggested to us that FLBZ might be a safe, effective macrofilaricide to use in *L. loa* endemic regions. To further investigate this prospect we assessed the viability of *L. loa* mf exposed to FLBZ *in vitro*. By measuring motility, we determined that FLBZ did not have direct microfilaricidal effects on mf of *L. loa* or *B. malayi* , the conventional filarial model. This lends further credence to the possibility of implementing FLBZ as a safe macrofilaricide. Further experiments are required to ensure this trend holds true in human patients, but it this provides important initial observations strengthening an exciting prospect.

The intriguing lack of efficacy of FLBZ on mf led us to question the possibility of delayed, non-lethal drug effects related to disruption of microtubule-related processes. Microfilariae are in a state similar to diapause in which they are not highly metabolically active (30). We investigated the possibility that FLBZ exposure affects mf when they reactivate during the mosquito infection process. High concentrations of FLBZ impaired the capacity of mf to cross the mosquito midget. We suggest that FLBZ inhibits the release of proteins required during the process of midgut penetration. Inhibition of protein secretion by BZ anthelmintics has been reported in GI nematodes (31, 32), including FLBZ (31). Lower concentrations of FLBZ followed a similar trend but were less effective. However, the results on infectivity were rather striking. Irrespective of concentration or duration of exposure, FLBZ exposure completely abolished the development of mf to the L_3 infective stage in mosquitoes. This effect would effectively break the reinfection cycle by preventing transmission.

6.2 Conclusions

The work outlined in this thesis serves to clarify our understanding of the macro- and microfilarial effects of FLBZ, as well as identify an effective concentration range and duration of exposure required to elicit lethal damage, to aid in its development as a macrofilaricide. We provide evidence of damaging effects to tissue required for adult reproduction and survival at pharmacologically relevant FLBZ concentrations. We have also endeavored to provide molecular explanations for gross morphological tissue damages. Furthermore, our findings brought forth compelling evidence for the safety of FLBZ as an appealing candidate for use in *L. loa* endemic regions.

Finally, the knowledge acquired through this project has shed light on the potential role of FLBZ in onchocerciasis and LF control programmes and has contributed to advancing it's preclinical development.

6.3 References

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