Exploring Modifications and Identification of Neurolenin as a Potential Antifilarial Drug Candidate for Lymphatic Filariasis

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Abstract

Lymphatic filariasis (LF), a parasitic illness, is a globally neglected tropical disease that is known to keep 1.4 billion people at risk of infection, mostly in Southeast Asia and Africa (CDC, 2013). LF is a mosquito born disease that is caused by the human parasites: *Wuchereria bancrofti*, *Brugia malayi*, and *Brugia timori*. This study focuses on testing sesquiterpene lactones, called neurolenins, which are secondary plant metabolites from *Neurolaena lobata*, a medicinal herb, native to Guatemala and Central America. The Williams and Shea Laboratories together plan to modify neurolenin to improve its potential as a drug candidate that exhibits anti-filarial activity against LF parasites.

Current drug treatments for LF do not target adult parasites residing in human lymph nodes and vessels, and possible issues of genetic resistance necessitate exploration of drug candidates that target all life stages of the parasite including L3, adults, and microfilaria (mf). Neurolenin B, a sesquiterpene lactone from *N. lobata*, is biologically active *in vitro* against L3, adult, and mf *B. pahangi* and *B. malayi* nematodes. In this study, the Shea Laboratory converted Neurolenin D, another sesquiterpene lactone found *in N. lobata*, to Neurolenin B via acetylation and esterification methods that were analyzed for their efficacy in killing filarial parasites in culture. Using the acetylation technique, synthesized recrystallized Neurolenin B (modified from Neurolenin D) was biologically active with significant killing against adult female, adult male, L3, and mf *B. malayi* and *B. pahangi* parasites. The Neurolenin B product synthesized using an esterification procedure (modified from Neurolenin D), also called isovaleric acid
Neurolenin D ester, was also biologically active with significant killing against L3 *B. pahangi* parasites. The ability for Neurolenin B to kill adult worms makes it a novel drug for LF because current drugs only target mf. Future investigation will continue with Ames testing to screen various forms of neurolenin for potential mutagenicity. Future work with the Shea Laboratory will also examine improvements in solubility for isovaleric Neurolenin D ester for further testing. Both acetylated and esterified Neurolenin B products will be tested against additional parasite cultures to validate the ability of these compounds to efficiently kill filarial nematodes. We also plan to use rodent models to compare the efficacy of the products *in vivo* in collaboration with Glaxo Smith Kline.
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**Introduction:**

**Lymphatic Filariasis**

Lymphatic filariasis (LF), a globally neglected tropical disease, is a mosquito-borne parasitic illness that is known to affect over 120 million people in 81 countries, according to the Centers for Disease Control and Prevention (CDC 2015, Zeldenryk et al., 2011). About 1.4 billion people are at risk of infection (GAELF 2013; CDC 2013). As a Neglected Tropical Disease, LF fits the description of a bacterial or parasitic disease that is marginalized to the poorest and least accessible communities in underdeveloped regions of the world (CDC 2015). LF is the second leading cause of long-term disability worldwide, and is caused by the parasitic nematodes: *Wuchereria bancrofti, Brugia malayi,* and *Brugia timori* (CDC 2015).

Phenotypic appearance of LF is commonly observed as elephantiasis, which is caused by adult parasites causing damage to the lymphatic vessels of humans, the primary host of LF. As a major cause of acute and chronic morbidity affecting males and females at all ages, LF shows clinical signs of enlarged lymph nodes and vessels that commonly causes the enlargement of sexual organs, limbs and feet (Ottesen et al., 1997). LF can also manifest as severe lymphoedema and hydrocele (Cano et al., 2014). LF symptoms are extremely debilitating and lead to permanent disfigurement. This disability often creates a social stigma and long-term socioeconomic impacts, leaving patients unable to work and even unable to access medication (Zeldenryk et al., 2011).

**Transmission and Life Cycle**
According to the World Health Organization, the most prevalent nematode, *W. bancrofti* is most commonly found in the *Culex* (urban areas), *Anopheles* (rural Africa), and *Aedes* mosquito vectors (Pacific islands) (WHO 2015). *B. malayi* and other species in the *Brugia* genus are mostly transmitted by the *Mansonia* mosquito vector, and are specific to Southeast and South Asia. Of the 120 million people infected, one third of the people with LF reside in India, one third reside in Africa, and one third reside in broader South Asia, the Pacific, and the Americas (Anil and Talluri 2015).

The mode of transmission of LF is stimulated by the life cycle of the aforementioned parasites, which reside in mosquito and human hosts (Figure 1). When an infected mosquito takes a blood meal from an uninfected person, it deposits adolescent (L3) nematodes onto the skin around the puncture wound (CDC 2013). Nematodes enter the human host bloodstream only when the mosquito bites. Once in the body, nematodes mature through a physiological molting process where, once adults, they enter the lymph nodes to replicate and produce microfilariae (mf) offspring. The mf are later circulated through the bloodstream and taken up by a subsequent mosquito blood meal (CDC 2013). The mf begin their maturation in the gut and flight muscles of their host mosquito, molting from L1 to L2 to L3 life stages. Once reaching the L3 stage, the nematodes are ready for deposition into the skin of a human host through the proboscis of the mosquito (CDC 2013). It takes about 7-9 days for the L3 adolescents to molt to the L4 stage that mature into adults and reside in the human host lymphatic system. Before maturing into adults, L3/L4 adolescents secrete a
variety of proteases and enzymes that allow for entry into the bloodstream and lymphatic tissue where adults can reside in lymphatic vessels for 4-8 years. Adult parasites in the human host then reproduce and create mf (L1) that migrate to the lymphatic vessels via the bloodstream. It is also important to note that these parasites have a nocturnal periodicity where a large number of parasites exist in the peripheral human blood at night, simultaneous with the peak hours of their mosquito vectors. This may have large implications for vector control, drug ingestion times, and transmission control (CDC 2013, Klei et al., 2002).

**Figure 1.** Life cycle of *Wuchereria bancrofti* (CDC, 2013).

**Human Health and Socioeconomic Impact**
The clinical course of LF disease is often as follows: asymptomatic microfilariae, where patients are generally asymptomatic but may develop chronic inflammatory granulomas depending on the density of mf. These chronic symptoms could also be accompanied with splenic destruction and chyluria (milky urine). The second phase includes adenolymphangitis (ADL) and episodic fever attacks, and painful inflammation of lymph nodes, and testes and spermatic cords in males. This stage is the most common and is often the most recurrent. Secondary infections from ADL include bacterial infection in lesions of affected areas that contribute to extreme pain (Wayangankar et al., 2015).

The third and final stage of the disease results in irreversible lymphedema. Adult parasites are the main contributors of these symptoms as they reside in the lymph nodes and release pro-inflammatory cytokines that contribute to skin exfoliation, genital swelling, and pain. The third stage is the most debilitating and often leads to elephantiasis that afflicts 15 million people worldwide (WHO, 2016). The lymphatic system is the main regulator for fluid balance between blood and tissue and is physically blocked by the damage caused by adult worms. Lymphatic vessel walls thicken and dilate, causing an imbalance in the lymphatic tissue and vessels, preventing the proper function of the system. (CDC 2013; Wayangankar et al., 2015; Zeldenryk et al., 2011).

According to the Global Programme to Eliminate Lymphatic Filariasis (GPELF), morbidity management, one of its two main initiatives, remains less widespread and successful. Only 26 of the 81 endemic countries even have morbidity management programs and the programs lack a focus on supporting
patients in actively participating in their communities with daily activities both independently and collaboratively. Most management programs only focus on physical impairment and do not address emerging issues such as social stigma and cultural norms. Because LF affects mostly impoverished communities, most patients delay seeking assistance, and free drug availability do not mitigate other costs like travel and time lost from work (Zeldenryk et al., 2011). Many families consider LF patients to be a shameful social burden; LF patients are often hidden from communities. Men who incur genital damage are severely handicapped and have limitations in participating in physical labor, which is a significant challenge since many patients live in communities that are largely agriculture with intensive manual labor. Women pay a high price for social stigmatization because women with lymphedema are restricted from marriage, which is essential for monetary and physical security in many cultures in endemic regions (GAELF 2013).

The WHO indicates that LF causes an estimated annual loss of $1 billion and inhibits up to 88% of total economic activity in endemic countries. GAELF and the WHO also estimate that LF is responsible for the loss of 4.4 million disability adjusted life years (DALYs) in men and over 1.3 million DALYs in women. The DALY index measures the loss of a healthy productive life due to a disability and is a metric for the measurement of the gap between current health status and an ideal health situation where a population is free of disability and disease. Most developing countries have high DALY numbers where large populations persist in the cyclical nature of poverty and disease, particularly exacerbated by neglected tropical diseases like LF (CDC 2013; GAELF 2013).
Current Efforts to Combat Lymphatic Filariasis

The Global Programme to Eliminate Lymphatic Filariasis, launched in 2002, was the first extensive action taken in hopes of eradicating LF by 2020. The two main initiatives, as stated earlier, were to provide community-wide mass drug administration (MDA) via Ministries of Health programmes and pharmaceutical companies like GalxoSmithKline and Merck & Co., and to alleviate the suffering of those with chronic LF symptoms through morbidity management (Zeldenryk et al., 2011, WHO 2016).

One of the current drugs used to treat LF is Diethylcarbamazine (DEC). First discovered in 1948, DEC critically reduces LF transmission, but does not remove all mf, or adult nematodes from a single dose of 6mg of drug/kg of body weight (Taylor, Hoerauf, and Bockarie 2010; Bockarie and Deb 2010). A one-day treatment has been shown to be as effective as the regular 12-day treatment, giving DEC an economic edge in populations that may be able to afford only one trip to the clinic (CDC 2013). Used for more than 50 years, DEC has shown to impact the nematode arachidonic acid signalling pathway in vivo. The drug aims to target the mf in an infected patient to prevent transmission so that mosquitoes cannot transmit the disease to others when taking a human bloodmeal (GAELF 2013; CDC 2013; Taylor, Hoerauf, and Bockarie 2010). DEC is responsive to the density of mf in the blood and incurs side effects when a lower population of mf is present. Common side effects include dizziness, nausea, and muscle pain (CDC 2013). Ivermectin, derived from avermectin, is another broad-spectrum anti-filarial drug that has been popularly used in the treatment for LF since the 1970s.
to today (Crump and Omura 2011). Ivermectin’s mechanism for action is the activation of glutamate-activated chloride channels, causing paralysis of mf (Bockarie and Deb 2010; Arena et al. 1995). Like DEC, ivermectin is only known to have moderate effects on adult worms with quarterly administration, an unreasonable number of dosages for those that cannot afford multiple trips to a health center, which is a majority of the total LF population (Bockarie and Deb 2010).

The third most common drug treatment used is Albendazole, another broad spectrum drug that is used in conjunction with DEC and ivermectin (Taylor, Hoerauf, and Bockarie 2010). Developed in 1975, Albendazole causes degenerative alterations in the LF parasite by inhibiting microtubule polymerization and assembly during the parasite’s life cycle. Albendazole helps to decrease glycogen stores in mf stage parasites, and is also effective with a single dose at 400 mg with a half-life of 8-12 hours (DrugBank 2014).

**Complications with Current Treatments**

The World Health Organization and CDC have made major efforts in Mass Drug Administration to combat LF, but emerging genetic resistance of the parasites to anti-filarial medication has become a concern in efforts for effective treatment. Although there is no formal evidence of resistance to drugs currently used for LF, the recent resistance to ivermectin when used to combat onchocerciasis has raised concerns (Molyneux et al., 2003). As stated earlier, management of chronic symptoms has been largely underdeveloped, and MDA is only undertaken annually and worldwide coverage has yet to be met (GAELF
MDA relies heavily on community health workers to educate patients about the disease and to get compliance by asking patients to taking the necessary medication. MDA is only effective if there is at least 80% coverage within a 5-year period, something that has shown to be variable since the inception of the program (Plaisier et al., 2000). Compliance concerns not only create an issue for those affected by LF, but they are also possibly related to drug resistance development. In areas where compliance is low, community treatment exists at low, sublethal levels and can induce accelerated development of resistance. Drug regimens for LF often include a combination of two current drugs, making resistance less likely when compared to diseases only treated with one drug (Molyneux et al., 2003). However, given the time period (4-8 years) that the adult worms reside in the human host, high compliance of about 80% is necessary for 5 years, a very lengthy compliance period (Michael et al., 2004).

Other than compliance and drug resistance mechanisms, various side effects also contribute to the rising concerns for the current treatments of LF. For example, DEC cannot be co-administered in populations that also have onchocerciasis, another parasitic neglected tropical disease. Patients who are inflicted with both diseases and take DEC incur a heightened inflammatory response when both parasites are targeted (Bockarie and Deb 2010). Patients who have both LF and loiasis cannot be treated with ivermectin as encephalopathy and neurological degradation can often coexist as side effects (Bockarie and Deb 2010). It is evident that those affected with LF can also be prone to other NTDs that inevitably will decrease the efficacy of current treatment options for LF.
Ultimately, the biggest “side effect” with the drugs on the market is that they are not effective in killing adult nematodes. Only mf are efficiently killed by DEC, Albendazol, and ivermectin.

However, MDA has increased coverage from 12 countries in 2000 to 59 countries in 2010, giving more than 500 million people treatment. In fact, 17 countries receive 100% of geographic coverage and have completed five or more rounds of MDA programming. More successfully, in 2008, China and the Republic of Korea have declared elimination of LF as a public health problem (WHO 2016).

**Ideal Drug Targets for Lymphatic Filariasis**

Given the current treatments on the market, it is evident that there is a need for new drug candidates for LF. Specifically, a new therapy that targets adult nematodes has become alarmingly necessary. Current treatments only can work to prevent transmission by killing mf parasites, which may not be sufficient to eradicate LF. Adult worms are the producers of mf and reside in lymph nodes and vessels where long-lived adults eventually can cause elephantiasis and lymphedema. Targeting adult nematodes will not only prevent transmission, but will also help patients that already have the disease (Murthy, Joseph, and Murthy 2011). L3 worms can also be considered as potential drug targets as they resemble adolescent worms that molt to adults, and this is the stage that enters the human host. By targeting L3, we can prevent worms from developing into adults that cause the physical manifestations of the disease. Thus, an effective drug model would target LF worms at three important stages: mf, L3, and adult worms. More
importantly, a gradual killing of worms is desired. Sudden death of worms may lead to an acute inflammatory shock and drive the patient into further presentation of clinical symptoms that may become fatal. Other useful drug properties include the need for non-resistant therapies, regimens that do not require multiple doses, and drugs that have minimal side effects. Additionally, a treatment that is also effective against other NTDs, as are current treatments for LF, would result in a nearly perfect drug candidate. As stated earlier, it is common for those with LF to also contract other NTDs caused by worm-like parasites in human hosts. Some of these NTDs include hookworm, whipworm (trichuriasis), and roundworm (ascariasis) (Micromedex 2015). Thus, creating a drug that also targets other diseases caused by nematodes not only helps a patient with LF, but will also help patients with other worm-related NTDs.

**Plant-Based Drug Therapies**

Plant based medicine is most commonly used today in traditional Chinese practices, Ayurvedic medicine in India and the Middle East, and in many indigenous cultures of the Americas, which date as far back as 60,000 years ago (Pan *et al.*, 2014). Novel anti-filarial drugs with bioactive components from traditional plants enable alternative methods in combating LF (Murthy, Joseph, and Murthy 2011). New drug therapies to combat LF and other NTDs need to be as potent as current drugs, but they also need to be effective at lower doses with minimal side effects. These characteristics can be met with an exploration of plant based drug therapies. Historically, 35,000-70,000 plant species have been screened for possible drug therapies, and those that have ethnopharmacological
purposes have been used in medicine (Veeresham 2012). During the last 30 years in particular, about 50% of the approved drugs are derived from natural products to combat cancer. In the past 16 years alone, 11% of the 252 drugs considered as “essential” according to the WHO were from a flowering plant origin. Plants represent the core structures for numerous drugs on the market, regardless of modern advances towards synthetic chemistry or molecular modeling design. For example, Merck in 1826 released the first semi-synthetic pure aspirin drug that is based on salicin, a natural product isolated from *Salix alba* (Veeresham 2012). This product later led to the isolation of cocaine, codeine, quinine, and other active compounds that are still in use today. Some plants used in historic traditional practices have been shown to have therapeutic effects against ovarian and breast cancer and even malaria. These plant extracts have been synthesized as drugs approved by the FDA (like Paclitaxel from *Taxus brevifolia* for ovarian and breast cancer and Artemisinin from *Artemisia annua* for malaria) (Veeresham 2012).

**Neurolaena Lobata as a Suitable Drug Candidate for Lymphatic Filariasis**

The Steven A. Williams Lab at Smith College (SAW Lab) is using *Centella asiatica, Neurolaena lobata, and Quisqualis indica* as medicinal herbs native to East Asia, Guatemala, and China respectively to develop drugs that exhibit anti-filarial activity on LF parasites. This investigation will focus on *Neurolaena lobata*, as its extracts have shown to have anti-parastic properties in vitro against *Trypanosoma cruzi, T.vaginalis, Leishmania mexicana* and *Plasmodium falciparum* (Berger et al. 1998, 2001; Franssen et al. 1997; François
et al. 1996; Muelas-Serrano et al. 2000). Found in the Asteraceae family, *N. lobata* is a weedy shrub that originates in Central and South America. It is known to have therapeutic properties in treating malaria, diabetes, cancer, and fevers in traditional medicinal practices of Mayan and Mesoamerican communities (Leon-Levy Native Plant Preserve; Walshe-Roussel et al. 2013). The anti-parasitic element of *N. lobata* is of most interest in the SAW Lab as it pertains to LF and was sparked by a study conducted by Fujimaki *et al* (2005) that defined a crude extract *in vitro* of *N. lobata* leaves as the bioactive component of the plant that showed microfilaricidal properties on *Brugia pahangi* nematodes. A series of concentrations of the extract at 500 µg/mL, 250 µg/mL, 100 µg/mL, 50 µg/mL and 10 µg/mL were tested on mostly adult worms, both female and male. The most promising results indicated a halt of motility at 6 hours post-treatment at 500 µg/mL, or at 24 hours post treatment at 250 µg/mL for both sexes of adult worms. The study also revealed that female adult worms were eventually prevented from further microfilarial release based on concentration and time variables, and that adult males were more commonly reduced in their motility (Fujimaki *et al*., 2005). In this study, *Brugia pahangi* was used as the model nematode because they are almost genetically identical to *Brugia malayi*, but only infect cats and other domesticated animals, making it useful for study in a laboratory. The results from this paper show that *N. lobata* clearly does have anti-parasitic effects on *Brugia pahangi* adult nematodes, an underdeveloped drug target that current treatments do not offer impact.
Research by Trotta in the SAW Lab confirmed the studies performed in the Fujimaki experiment using crude *N. lobata* extracts, Neurolenin A and Neurolenin B sesquiterpene lactone isolates, and a Neurolenin A and B mix (1:1) (Trotta 2013). Adult male, adult female and L3 *B. pahangi* were tested at compound concentrations of 0.6µg/mL, 0.5 µg/mL, and 0.4 µg/mL doses for up to 80 hours post-treatment. Trotta’s studies concluded that only Neurolenin B and the Neurolenin mix were able to kill *B. pahangi* at all life stages, while Neurolenin A showed minimal bioactivity (Figure 2,3,4).

RNA seq gene expression analysis was also performed to elucidate how the extracts of the Central American plant disrupt the physiology of the parasite.

![Figure 2](image)

**Figure 2.** Average Percent Mortality for Adult Female *B. pahangi* treated with Neurolenin A and B. Average of two biological replicates. (+) is a positive control treatment of 0.6 µg/mL Levamisol. “A” represents Neurolenin A. “B” represents “Neurolenin B”. The 1:1 mixture of A and B is a dose containing 0.5 µg/mL.
Neurolenin A and 0.5 µg/mL Neurolenin B (Trotta, 2014).

**Figure 3.** Average Percent Mortality for Adult Male *B. pahangi* treated with Neurolenin A and B. Average of two biological replicates. (+) is a positive control treatment of 0.6 µg/mL Levamisole. “A” represents Neurolenin A. “B” represents “Neurolenin B”. The 1:1 mixture of A and B is a dose containing 0.5 µg/mL Neurolenin A and 0.5 µg/mL Neurolenin B (Trotta, 2014).
Figure 4. Average Percent Mortality for L3 *B. pahangi* treated with Neurolenin A and B. Average for two biological replicates. (+) is a positive control treatment of 0.6 µg/mL Levamisole. “A” represents Neurolenin A. “B” represents “Neurolenin B”. The 1:1 mixture of A+B is a dose containing 0.5 µg/mL Neurolenin A and 0.5 µg/mL Neurolenin B (Trotta, 2014).

Bioactive Components of *Neurolaena Lobata*

The Trotta study from the SAW lab confirms the bioactivity of Neurolenin B as a potential drug candidate for lymphatic filariasis. The isolates Neurolenin A and B from *N. lobata* are two of 11 main sesquiterpene lactones that were tested in the SAW lab in past studies and are considered to have potentially bioactive or cytotoxic/anti-tumor activity (Unger *et al*., 2015; Castillo *et al*., 1982, Manchand and Blout 1978). Sesquiterpene lactones are natural products only in the *Asteraceae* plant family that have a lactone ring and polycyclic 15 carbon systems (Chaturvedi 2011). These sesquiterpene lactones possess the α-methylene-γ-butyrolactone moiety and act by selective alkylation of growth-regulatory immunological macromolecules. This specific moiety of sesquiterpene lactones is
what has been correlated with their bioactivity in past literature. This alkylation process is known as the Michael-type reaction by the $\alpha$-methylene lactone group in particular (Manchand and Blout 1978). The Michael reaction involves a conjugate addition to the alpha, beta unsaturated ketone system that reveals a stable yet reactive nucleophilic enolate (Michael acceptor). Neurolenin A and B in particular are found in *N. lobata* leaves and are of interest because they can be isolated in great quantities and differ by one functional group (Manchand and Blout 1978). The Trotta study and previous studies confirmed that under the specified laboratory conditions, only Neurolenin B was biologically active (Unger *et al.*, 2015; Castillo *et al.*, 1982, Manchand and Blout 1978). Experiments from the Kevin Shea Experimental Teaching 223 Laboratory at Smith College further confirmed these results, but it was found that starting materials were not pure and the overall isolation of Neurolenin B in the Trotta experiment may not have actually been pure Neurolenin B, but rather a mix of various sesquiterpene lactones. The Shea Laboratory, in continuation of the Trotta study, has identified four sesquiterpene lactones that should be the focus of the continued study of anti-parasitic activity as they can be easily altered through the manipulation of one functional group (Figure 5). As the Trotta study indicated, each lactone is
differentially bioactive, and further studies that explore the purification of 11 or more sesquiterpene lactones of *N. lobata* will prove definitive in identifying the most potent and effective drug candidate for lymphatic filariasis.

Figure 5. Four main sesquiterpene lactones from *N. lobata* (Manchand and Blout 1978).

**Purpose of Study**

This project will further investigate the bioactive impact of Neurolenin B (prepared from Neurolenin D) against adult female, adult male and L3 *Brugia pahangi* parasites via *in vitro* drug testing. The ultimate goal of this work is to contribute to the larger effort of discovering a novel potential drug candidate that will kill not only mf, but also adult nematodes, potentially replacing or supplementing current drugs that are being used in MDA for eliminating lymphatic filariasis.

**Materials and Methods**

**Isolation of Neurolenins**

All isolations of Neurolenins were conducted by the Kevin Shea laboratory, Smith College, and used the protocol from Manchand and Blout, 1978, to prepare the extraction of Neurolenin D from *Neurolaena lobata* leaves. Solvents and reagents were purchased from Pharmco-AAPER, Sigma Aldrich,
Fisher Scientific, and Acros Organics and extracts from leaves were prepared with nitrogen wand and magnetic strings. The *Neurolaena lobata* was purchased from Rainforest Remedies or Grenada Market and were crushed and powdered using a food processor. The powder was placed in a thimble in a soxhlet extractor. The powder was extracted with dichloromethane and removed via rotary evaporation. The remaining dark green gum was obtained and run through a column that produced fractions of Neurolenin D that were recrystallized with cold ethyl acetate. There were two main techniques used in this study to convert Neurolenin D to Neurolenin B: acetylation and esterification techniques. The esterification method (Figure 6) was performed using isovaleric acid and the acetylation technique used acetic anhydride (Figure 7). However, the acetic anhydride was later removed before the product was given to the SAW Lab because of its potential toxic effects in the human body.

![Figure 6](image.png)

**Figure 6.** Esterification of Neurolenin D to Neurolenin B with Isovaleric Acid. The resulting product is called isovaleric acid neurolenin D ester (Shea Lab 2015).
Figure 7. Acetylation of Neurolenin D to Neurolenin B. The resulting product is called synthesized recrystallized Neurolenin B (Shea Lab 2015).

Parasite Culture (T.V. Rajan et al. 2003, J. Parasitology 89(4) p 868-870 with modifications made by Susan Haynes, Smith College)

Culture Media (10% FBS)
90 mL Minimum Essential Medium Alpha (no HEPES, just sodium bicarbonate buffer) (Gibco - Life Technologies)
10 mL Fetal Bovine Serum deactivated (Gibco - Life Technologies)
1 mL Pen Strep 5000U (Gibco - Life Technologies)
100 µL Gentamycin (10 mg/mL stock) (Gibco - Life Technologies)
20 µL Ciprofloxacin (10 mg/mL, ophthalmic stock) (TCI America)
20 µL Fortaz (Ceftazidime) (in PBS 10 mg/mL stock) (TCI America)

Total Volume: 101 mL
Prepare under sterile conditions and store at 4°C. Complete medium includes 90mL of the culture medium, 10 mL Fetal Bovine, and 20 µL of fresh Fortaz added on the same day of culture.

Wash Solution
100 mL RPMI 1640 (HEPES Buffered) (Gibco - Life Technologies)
1 mL Pen Strep 5000U
100 µL Gentamycin (10 mg/mL stock)
20 µL Ciprofloxacin (10 mg/mL stock)
100 µL Fungizone (Amphotericin B) (2.5 g/mL stock) (Gibco - Life Technologies)

Total Volume: 100 mL
Prepare under sterile conditions and store at 4°C
Ascorbic Acid: dilute to final concentration of 15 µg/mL of culture medium. Make 1.5 mg/ml (100X) stock and freeze. Only unfreeze stock once and discard stock vial once it is added to the cultures.

*Prepare under sterile conditions and store at -20°C for single use.*

**Nematode Preparation and Procedure:**

**Nematodes:** *B. pahangi* nematodes (L3, adult males and adult females) were obtained from the University of Georgia (Athens, Georgia), College of Veterinary Medicine. Parasites were shipped to Smith College in conical 50 mL tubes in RPMI 1640 solution.

**Preparation:** Wipe down the Tissue Culture room hood (Ford 130) with 70% EtOH. Expose any working surfaces with UV radiation for at least 30 minutes. Let the medium come to room temperature about 20 minutes before use. Before opening the tube of worms, allow the tube to sit upright for 30-60 minutes (at room temperature) thus ensuring the worms have settled to the bottom. Set the incubator CO₂ so the pH is 7.2 - 7.4 at 37°C.

**Washing the Worms:** Pipet the wash solution into (1) 100mm petri dish and (3) 60 mm petri dishes, filling each two thirds full. For L3 worms, use a transfer pipet to aspirate the worms from the bottom of the tube and put into the 100 mm petri dish wash solution. Avoiding any debris, use a p-200 pipet to collect the worms from this wash and move them to the next wash. Do this three times. Pick up as little RPMI 1640 solution as possible when transferring the worms. The goal is to get 40 worms in the p-200 pipet. Pipet tips should be changed between each wash. The number of washes performed depends on the conditions/state the worms arrive in and also their experimental use. They should
be rinsed 3 times, more if necessary. All procedures for L3 worms must be done under a light microscope. For adult females and adult males, follow the same procedures, but instead of using a transfer pipet or p-200 to pick up the worms, use EtOH sterilized forceps for easy transfer. A light microscope is not needed as adult females and adult males can be seen with the naked eye. However, the microscope must be used to view mf produced by adult female worms.

**Plating worms:** Use a 6 well plate (Thermo-Fisher) to deposit the washed worms in complete culture medium. Place 5 ml of culture medium in each well. Add 100 *B. pahangi* L3 worms or up to 5 *B. pahangi* adult females or adult males into each well (Figure 8). Sterilize the fume hood before culturing, and after each washing of the worms.

*Prepare cultures and washes under sterile conditions with gloves and goggles.*

Plate #1: Females
Plate #1: Males

Plate #2: Females
Plate #2: Males
**Figure 8.** Example plate arrangement for adult female and adult male *Brugia pahangi* worms. Each adult well contains 5 worms each in 5 mL of complete medium. Amount in µg/mL refers to the concentration of neurolenin added to each well. This example arrangement shows a replicate experiment.

**Treatment and Observation:** Plating occurs on Day 0, and drug treatment is added on Day 5. Between Days 0-5, worms are observed and death is noted and subtracted from death count treatment as “death due to shipping or plating.” During day 5, 50 µL of ascorbic acid (Vitamin C) is added to each well to ensure the molting and continuation of the life cycle of L3 to L4 *B. pahangi* (Rajan, 2003). Adult worms do not receive Vitamin C (ascorbic acid) treatment as they do not molt, however they must have old medium exchanged with new medium as mf start appearing in female adult cultures at Day 5. Treatment is added at indicated optimal concentrations that differed between L3 and adults. The acetylated Neurolenin D (Neurolenin B) had optimal concentrations for L3 worms at 1.25 µg/mL, 1.00 µg/mL, and 0.800 µg/mL. The esterification of Neurolenin D to Neurolenin B had optimal concentrations at 1.25 µg/mL, 1.00 µg/mL, 0.800 µg/mL, and 0.700 µg/mL. The adult females and adult males were only treated with the acetylation of Neurolenin D to Neurolenin B at 1.00 µg/mL, 2.00 µg/mL, and 3.00 µg/mL. These optimal concentrations were determined by choosing the top 3 or 4 concentrations that killed the most worms during the desired period (50-70 hours post-treatment). Each compound received from the Shea lab was prepared in a solvent, 70%EtOH, at a concentration of 1mg/mL. Death is recorded when worms become completely immobile and appear hairpin like and dark in morphology. At each observation time, worms were
recorded as either dead or alive. Live worms have sinusoidal motion and move extremely fast if healthy. Before death, it is common for worms to move extremely slowly. This behavior may explain results that appear to show a sudden spike in death. This is not due to a “sudden death” but rather a slow, gradual death. Each culture includes a negative control where no treatment is applied and molt rate is observed (only in L3 cultures) to quantify the health of the culture. L3 worms were scored as either “partial molt” or “full molt” when transparent like-cuticles are shed from worms and can be seen floating in the culture medium. A worm with a cuticle still attached to worms is considered a partial molt. The drug antibiotic Levamisole was used as a positive control for adult cultures. Solid levamisole HCl (TCI America) was dissolved in MEM at a concentration of 1µg/mL for use with B. malayi cultures (only B. malayi parasites were available for adult culture during the time of the investigation). The Levamisol was added at 3.00µg/mL, the highest concentration tested on adult female and adult male worms.

**Analysis:** All cultures were observed every 4 hours starting on Day 5 for up to 72 hours or more (until Day 8 or longer). Average percentage mortality, was recorded in each well by taking the total number of dead worms and dividing by the total number of worms in the well (after accounting for death due to plating or shipping of worms). Results were plotted with time in hours post-treatment (hpt) on the X-axis and and percentage death on the Y-axis. Average mortality for each treatment condition was compared to the health of the average mortality of the negative control and statistical significance of the differences between the
experimental condition and the control condition was calculated using a one-tailed paired student T-test at the last time point. The last time check was at 72 hours post-treatment (hpt) for L3 cultures, and was approximately 98 hours post drug treatment for adult cultures. Significant drug bioactivity was defined as a p-value less than 0.05.

Results

**Neurolenin Treatment of *Brugia pahangi* L3: Confirming Previous Work in the SAW Lab**

All extracts from *Neurolaena lobata* during this investigation were prepared from the Shea Laboratory with the help of Katie McGeough and Megan Neubig. Results were tested for significant killing compared to the health of the negative control time at 72 hpt, the last time check (for L3 cultures).

First we aimed to confirm the findings of previous work done by Kristine Trotta in the SAW Lab, Smith College (Figure 9). Her results indicated that a crude extract from dry *N. lobata* leaves was biologically active at the three highest concentrations: 200 µg/mL, 300 µg/mL and 400 µg/mL compared to the control, which received no treatment. For this investigation, the Shea lab helped us to confirm the results by producing a new extract where it was tested in the SAW Lab. Treatment groups molted quickly, at 20 hpt, where the ideal time of death is expected at around 50-70 hpt (Figure 9). The mortality rate is measured as 1=100% death, where all worms in the well were dead at the indicated hour. For this investigation, the Shea lab prepared the new extract using a charcoal filtration technique. Past work in the SAW Lab did not test the charcoal-filtrated product when working with the crude extract. Charcoal filtration is a method of filtering
out impurities in a sample that uses activated carbons and chemical adsorption. Carbon filtering traps an impure sample in a pore structure of a carbon substrate.

**Figure 9.** Mortality rate for L3 *B. pahangi* testing crude extract from *N. lobata* post-Charcoal Filtration, prepared by the Shea Lab, no biological replicates. Time is in hours post-treatment. Control wells have no treatment with the crude extract (0 µg/mL). Experimental wells have concentrations of 200 µg/mL, 300 µg/mL, 400 µg/mL. “K” is in reference to the crude extract. Mortality rates in cultures treated with all three concentrations of *N. lobata* crude extract were significant compared with the control at 72 hours post-treatment (p<.0001).

The components of the crude extract contained a combination of sesquiterpene lactones and other plant components that are unknown. To confirm that a known sesquiterpene lactone was causing the killing, Kristine Trotta...
modified the crude extract method, and isolated Neurolenin B, a known
sesquiterpene lactone from *N. lobata* that had biological activity against *B.
pahangi* and *B malayi*. In this investigation, with collaboration from the Shea lab,
succeeded in isolating Neurolenin B in the same way, but it was later revealed by
the Shea laboratory that this extract also contained Neurolenin D and other
sesquiterpene lactones. Thus, this isolation was in fact not fully purified (Figure
10). However, this product revealed that a plant extract could be used at much
lower concentrations and still cause mortality. There was about 90% death at the
highest concentration of 1.50 µg/mL, and 30-40% death for the lower
concentrations at 72 hours post-treatment.

A titration of ten different concentrations of this extract was used for this
culture to select thee to four concentrations that had the most significant killing.
This titration of concentrations included 1.50 µg/mL, 1.25 µg/mL, 1.00 µg/mL,
0.800 µg/mL, 0.700µg/mL, 0.600 µg/mL, 0.500 µg/mL, 0.400 µg/mL, 0.200
µg/mL, and 0.100 µg/mL. Concentrations at 1.50 µg/mL, 1.25 µg/mL, 1.00
µg/mL, and 0.800 µg/mL showed the highest mortality rate and were used as the
range of optimal concentrations throughout this investigation (Figure 10).
Figure 10. Mortality rate for L3 *B. pahangi* testing a partially purified Neurolenin B, extract (from Kristine Trotta, 2014, but prepared by the Shea Laboratory, 2015) with no biological replicates using a stock solution of 1 mg/mL. Time is in hours post-treatment. The control has 0 µg/mL of the partially purified Neurolenin B, and was tested against wells with concentrations of 1.5 µg/mL, 1.25 µg/mL, 1.00 µg/mL, 0.800 µg/mL. Mortality rates in cultures treated with 1.5 µg/mL partially purified Neurolenin B extract were significant compared with the control at 72 hours post-treatment (p<0.0001).

**Testing Optimal Concentrations**

Because relatively high mortality was seen using the partially purified Neurolenin B, the crude extract post-charcoal filtration was tested using these same concentrations (Figure 11). The crude extract did not show high mortality rates (only 20% at the highest concentration of 1.25 µg/mL), and is not as biologically active as the partially purified Neurolenin B (Figure 10). However,
even with a low mortality rate, the crude extract at 1.25 µg/mL had statistically significant killing at 72hpt compared to the control well with 0 µg/mL of the extract (p<0.0001).

**Figure 11.** Mortality rate for L3 *B. pahangi* testing the crude extracts post-charcoal filtration, prepared by the Shea Lab, at three concentrations that were most successful with the partially purified Neurolenin B, no biological replicates. Time is in hours post-treatment. The control has 0 µg/mL treatment of the crude extract, and was tested against wells with concentrations of 1.25 µg/mL, 1.00 µg/mL, and .800 µg/mL. Mortality rates in cultures treated with 1.25 µg/mL crude extract post-charcoal filtration were significant compared with the control at 72 hours post-treatment (p<0.0001).

*Brugia pahangi* L3 in vitro Cultures: Results from Chemical Modifications of *N. lobata* to Neurolenin B

To continue testing neurolenins from *N. lobata*, the investigation moved to testing Neurolenin D and then Neurolenin B (prepared from Neurolenin D) via an
acetylation and esterification technique. It was found that the Neurolenin D
sesquiterpene lactone purified from *N. lobata* by the Shea Laboratory did not
show high mortality rates against L3 *Brugia pahangi* at any tested concentrations
(Figure 12). Less than 20% of the worms at the highest concentration (1.25
µg/mL) died at 72 hpt. The control well here shows higher mortality rate than all
other concentrations due to the poor health of the worms from shipping and
handling.

The mortality rate in the culture testing the synthesis of Neurolenin B from
Neurolenin D via the acetylation technique, at 1.25 µg/mL had statistically
significant killing at 72 hpt compared to a control with 0 µg/mL (Figure 13). This
drug treatment was biologically active against L3 *B. pahangi*. The recrystallized
Neurolenin B via the acetylation technique showed 60% death at 72 hpt, at the
highest concentration tested, 1.25 µg/mL, and was later tested at higher
concentrations in adult *B. malayi*, which also showed statistically significant
killing.
Figure 12. Mortality rate for L3 *B. pahangi* testing Neurolenin D sesquiterpene lactone, using a 1 mg/mL stock solution prepared by the Shea Laboratory, with no biological replicates. Time is in hours post-treatment. The control has 0 µg/mL treatment of Neurolenin D. Mortality rates in cultures treated with 1.25 µg/mL Neurolenin D was significant compared with the control at 72 hours post-treatment (p<0.0005).
Figure 13. Mortality rate for L3 B. pahangi testing a synthesized recrystallized Neurolenin B sesquiterpene lactone via acetylation of Neurolenin D using a 1 mg/mL stock solution, prepared by the Shea Laboratory, no biological replicates. Time is in hours post-treatment. The control has 0 µg/mL treatment of the synthesized recrystallized Neurolenin B and was tested against wells with concentrations of 1.25 µg/mL, 1.00 µg/mL, and 0.800 µg/mL. The mortality rates in cultures treated with 1.25 µg/mL synthesized recrystallized Neurolenin B was significant compared with the control at 72 hours post-treatment (p<0.0001).

The synthesis of Neurolenin B via the esterification technique developed in the Shea Laboratory showed statistically significant killing at the highest concentration of 1.25 µg/mL (Figure 14). The synthesis of Neurolenin B, in this technique, was accomplished by modifying an ester to Neurolenin D. The highest concentration in this culture showed a mortality rate of approximately 65% at 72
hours post treatment. The next portion of the results section, testing adult female and adult male worms, was the focus of this investigation, and is discussed extensively below.

**Figure 14.** Mortality rate for L3 *B. pahangi* tested with isovaleric acid Neurolenin D ester (Neurolenin B) prepared via esterification of Neurolenin D using a 1 mg/mL stock solution, prepared by the Shea Laboratory, no biological replicates. Time is in hours post-treatment. The control has 0 µg/mL treatment of the isovaleric acid Neurolenin D ester, and was tested against wells with concentrations 1.25 µg/mL, 1.00 µg/mL, 0.800 µg/mL, and 0.700 µg/mL. The mortality rate in cultures treated with 1.25 µg/mL isovaleric acid Neurolenin D ester was significant compared with the control at 72 hours post-treatment (p<0.0001).

*Brugia malayi* adult female and adult male: Results from Synthesized Recrystallized Neurolenin B via Acetylation of Neurolenin D
Mortality rates for adult females treated with recrystallized Neurolenin B synthesized from Neurolenin D via the acetylation technique at the highest concentration (3.00 µg/mL) showed statistically significant killing when compared to the control (0 µg/mL) at 72hpt (Figure 15). At the highest concentration, there was 100% mortality at both 72hpt. Similarly, mortality rates for adult males treated with recrystallized Neurolenin B synthesized from Neurolenin D via the acetylation technique at the highest concentrations (300 µg/mL) showed statistically significant killing when compared to the control (0 µg/mL) at 72hpt and 98 hours post-treatment (Figure 16). At the highest concentration, there was 100% mortality at 98hpt. The adult cultures used *B. malayi* as the experimental model and were observed for longer periods of time.
post-treatment when compared to the L3 cultures.

**Figure 15.** Mortality rate for adult female *B. malayi* tested with synthesized recrystallized Neurolenin B via an acetylation technique using a 1mg/mL stock solution, prepared by the Shea Laboratory. This graph shows an average of three biological replicates where time is in hours post-treatment. The negative control has 0 µg/mL treatment of the synthesized recrystallized Neurolenin B, and was tested against wells with concentrations 1.00 µg/mL, 2.00 µg/m, and 3.00 µg/mL. The positive control contains 3.00 µg/mL of Levamisol. The mortality rate in cultures treated with 3.00 µg/mL synthesized recrystallized Neurolenin B were significant compared with the control at 72 hours post-treatment (p<0.0001).
Figure 16. Mortality rate for adult male B. malayi tested with synthesized recrystallized Neurolenin B via an acetylation technique using a 1mg/mL stock solution, prepared by the Shea Laboratory. This graph shows an average of three biological replicates where time is in hours post-treatment. The control has 0 µg/mL treatment of the synthesized recrystallized Neurolenin B, and was tested against wells with concentrations 1.00 µg/mL, 2.00 µg/mL, and 3.00 µg/mL. The positive control has 3.00 µg/mL of Levamisol. The mortality rate in cultures treated with 3.00 µg/mL synthesized recrystallized Neurolenin B were significant compared with the control at 72 hours post-treatment (p<0.0001).

Discussion

In this study, Neurolenin B synthesized from Neurolenin D via an acetylation method and an esterification method was tested on B. pahangi L3 and and B. malayi adult female and adult male parasites as a potential drug candidate to treat LF. The optimal dose range experiments for L3 worms was found to be 1.5 µg/mL or 1.25 µg/mL, whereas for adult males and adult females, the optimal
dose was found to be 3.00 µg/mL. This dose range reflects optimal concentrations of treatment where worms die in a moderately gradual death in the desired range of 50-100 hpt. Adult worms were observed for longer periods of time because this study displayed the first time pure Neurolenin B (synthesized recrystallized Neurolenin B) was tested on adult worms. A bioactive treatment must be potent enough to kill worms gradually within a few days, but not kill the worms so quickly where antigen from the worms is also released quickly. The faster the antigen is released, the more likely it will lead to antigen dumping that would shock the human host immune system. A treatment that kills all worms quickly would be one where total worm death occurs between 10-30 hpt, and where death is not gradual. These *in vitro* studies may not behave the same as *in vivo* studies, but this investigation aims to explore dose-dependent mechanisms in the worms, that must be considered in any future animal experiments.

All neurolenin products were generated by the Shea Laboratory and given to the SAW Laboratory at a stock concentration of 1mg/mL in 70% EtOH unless otherwise noted. These stocks were diluted to 0.100-3.00 µg/mL for L3 and adult female and male cultures for this study. For L3 cultures, the concentrations selected that showed the highest mortality rates were 1.50 µg/mL, 1.25 µg/mL, 1.00 µg/mL, .800 µg/mL, and .700 µg/mL. For adult cultures, the concentrations selected that showed the highest mortality rates were 1.00 µg/mL, 2.00 µg/mL, and 3.00 µg/mL. The positive control prepared for the adult cultures, Levamisol, was prepared in a 1mg/mL stock solution in MEM at the highest concentration tested in adults, 3.00 µg/mL The significance for the mortality rate at the highest
concentration in each adult and L3 culture was tested against 0 µg/mL in the control well at 72hpt and 98hpt because we were following common pharmaceutical reporting procedures. In these procedures, statistical significance showing proof of the effectiveness of a drug at just one concentration is enough to justify any kind of biological or anti-parasitic property.

The adult and L3 life stages of the parasite were the focus of this study because adult worms are the primary cause for serious clinical symptoms in LF and are not efficiently killed by current drugs. As aforementioned, current treatments only target mf which prevent the early stages of transmission. At the L3 stage, the worms enter the human host from the mosquito vector and by targeting L3 worms, we can prevent the worms from making it to the adult stage. L3 worms are also exemplary experimental parasites because they can grow and molt in vitro without an animal host, giving an indication of worm health. This study used B. pahangi in L3 cultures and B. malayi adult parasites because of worm availability from the University of Georgia and also because the two species are almost identical as experimental laboratory models.

**Testing the crude N. labota Extracts: in vitro L3 Cultures**

The crude extracts at the highest concentration, 400 µg/mL (p<.0001) had a mortality rate that showed statistically significant killing when compared to the control (0 µg/mL) well at 72hpt (Figure 9). The worms were dying too rapidly, at around 20 hpt, which, in this study is considered dangerous as it may lead to immune shock or rapid “antigen dumping” in an animal or human host. The crude extract was tested to determine if there was any kind of biological activity the
plant, before attempting isolation of specific sesquiterpene lactones or neurolenins.

This experiment showed that L3s die when treated with the crude extract at high concentrations. The crude extract was also tested at much lower concentrations determined after purifying the crude extracts and testing partially purified Neurolenin B and purified Neurolenin B via acetylation and esterification methods (Figure 11). These concentrations for desired activity of the synthesized compound were much lower, compared to crude extract, showing activity at 1.25 µg/mL, 1.00 µg/mL, 0.800 µg/mL, and 0.700 µg/mL. At these low concentrations, the crude did not show high mortality rates, or strong biological activity. Therefore in crude extracts, there are sesquiterpene lactones that have efficacy at high doses, but do not have efficacy at much lower doses. Since the purified, non-crude extracts have the ability to kill worms at much lower doses, this means that the purified non-crude extracts must have much higher concentrations of the biologically active form of neurolenin.

**Testing Neurolenin B: in vitro L3 Cultures**

This experiment aimed to first reconfirm the studies in previous SAW Lab experiments. The first study confirmed that Neurolenin B was the most biologically active sesquiterpene lactone from *N. lobata*, as shown in the literature and as shown in the experiments from Kristine Trotta, Smith College (Trotta, 2014). However, the Shea lab, using Kristine Trotta’s method of isolating Neurolenin B, confirmed that the Neurolenin B extract Trotta isolated was not in fact pure Neurolenin B, but was a partially purified extract that still had traces of
other sesquiterpene lactones and other components of *N. lobata*. With this partially purified Neurolenin B product, an optimal range of concentrations was determined that killed the worms slowly and gradually, and within the 50-70 or more hpt time period (Figure 10). The optimal range of concentrations was determined by planning a titration experiment that tested a sequence of concentrations designed to detect a range that killed the worms most effectively (three to four concentrations), in the desired time frame. The range of optimal concentrations lies in this range at 1.5 µg/mL, 1.25 µg/mL, 1.00 µg/mL, and 0.800 µg/mL where there was statically significant killing at 1.25 (p<. 0001) for the partially purified Neurolenin B extract.

In collaboration with the Shea lab, a newly synthesized and purified Neurolenin B product was used to test against the L3 *B. pahangi* worms. A fully purified Neurolenin B was synthesized by acetylating Neurolenin D. This method used acetic anhydride as a reagent, but this was later removed by recrystallizing the purified product. This recrystallized product at 1.25 µg/mL had a mortality rate that was statistically significant when compared to the control (0 at 1.25 µg/mL) at 72hpt. Although there was only a 60% mortality rate at the highest concentration of 1.25 µg/mL by 72hpt (Figure 13), this marks the first time that a fully purified Neurolenin B sesquiterpene lactone was synthesized in a laboratory from the manipulation of Neurolenin D and tested on live parasites. In this particular experiment, death recorded began at approximately 45 hpt due to logistical and time complications, thus preventing the display of preceding data points. It is evident that more purified samples of Neurolenin B may require a
higher dosage range to achieve 90% or more mortality rate at the 50-70 hour post-treatment period for L3 larvae. However, if this culture was monitored for a longer period of time, it is expected that a slow gradual death would be achieved eventually. This culture proves that the acetylated product had effective killing potential it was later tested at slightly higher concentrations on adult female and male worms.

Another technique used to synthesize the Neurolenin B product was an esterification procedure that also required modifications to Neurolenin D. The modifications involved isovaleric acid and placement of an ester group on the Neurolenin D sesquiterpene lactone. The mortality rate of the product with a concentration of 1.25 µg/mL (p<.0001) had statistically significant killing when compared to the control well (0 µg/mL) at 72hpt. The optimal range of high bioactivity and high mortality rates are similar to the previous experiment and are as follows: 1.25 µg/mL, 1.00 µg/mL, 0.800 µg/mL, and 0.700 µg/mL (Figure 14). However, there were solubility problems and the product was not fully dissolved in the solvent when tested against L3 *B. pahangi*. Therefore, the product derived from an esterification technique would likely be much more potent if a new solvent was more effective in dissolving the product. Based on the collective data, it is expected that a dissolved product would kill worms with a 90-100% mortality rate, or higher than the 65% mortality rate achieved in this study on L3 larvae. Future work should test the esterified product on adult worms after finding a safe solvent candidate for the worms that also dissolves the esterified product completely.
Regardless of the method used to synthesize Neurolenin B, the sesquiterpene lactone itself had great efficacy across all cultures in terms of interrupting the molting process. Each culture with a Neurolenin B product had only partial molting, indicating that the L3 to L4 process was targeted. This may lead to insights about the mechanism of action of Neurolenin B and its potentially negative effects on cuticle formation. It may also lead to some conclusions regarding effects on inhibiting the life cycle growth of these parasites as a whole.

**Bio-inactive Neurolenin D: in vitro L3 Cultures**

The study confirmed that Neurolenin D by itself was relatively bio-inactive against L3 *B. pahangi* worms (Figure 12). This demonstrates that not all sesquiterpene lactones in *N. lobata* extract contribute equally to its anti-parasitic activity. It is also important to note that the worms in this culture were in poor health after shipment, particularly the control well, where there was a higher mortality rate compared to other wells with treatment. However, collaboration with the Shea lab has led to some other conclusions about Neurolenin D. The *N. lobata* plants were obtained from Belize, which were found to have high amounts of Neurolenin D. *N. lobata* plants obtained from other locations apparently vary in the amounts of different sesquiterpene lactones based on the genetic variability of *N. lobata* in different geographic locations and climates (Sotes *et al.*, 2015). Because Neurolenin D was available in high amounts in the plants used in this study, it was easy to isolate large quantities of Neurolenin D to synthesize Neurolenin B, the most bioactive sesquiterpene lactone according to experiments cited in the in literature (and also according to our results). Some of these papers
claim that Neurolenin B blocks the NPM/ALK pathway that triggers a signaling cascade, typically leading to cell survival and “immortality” (Unger, 2015).

**Neurolenin B: in vitro Adult Male and Adult Female Cultures**

Both adult male and female cultures had mortality rates that showed statistically significant killing at 3.0 µg/mL (p<0.0001) when testing synthesized recrystallized Neurolenin B (using the modification of Neurolenin D to Neurolenin B via the acetylation method) against *B. malayi* nematodes at 72 hours post-treatment and 98hpt. In adult male cultures, 100% mortality was first recorded at approximately 85hpt with a concentration of 3.00 µg/mL, and for adult female cultures, 100% mortality was first recorded at approximately 70hpt with a concentration of 3.00 µg/mL.

*B. malayi* nematodes were used in the adult cultures due to the lack of *B. pahangi* nematodes available from the suppliers (University of Georgia). For the adult female cultures, an average of three biological replicates showed moderate increased death in the control wells due to worms injured in the process of plating and likely also because of the poor health of the worms due to shipment. During the course of monitoring the worms, mf were also monitored in the female cultures, *in vitro*. Throughout the three adult cultures, the mf all died at 3.00 µg/mL alongside the death of the adult females. However, at 2.00 µg/mL and 1.00 µg/mL, mf slowed in motility, but did not die during the monitoring period. We can assume here, that if this product was used at the lower concentrations (1.0 µg/mL and 2.0 µg/mL) as a suitable drug candidate for LF, it must be used in conjunction with current drugs in the market that kill mf. For the adult worm
cultures, a positive control (Levamisol) and a negative control (no drug treatment) were used. Levamisol is a known treatment for parasitic worm infections for veterinary use that has many adverse side effects like the depletion of white blood cells and complications with the nervous system. It is not approved for human use (Drugs.com). The Levamisol treatment in both cultures achieved 100% mortality at around 30-40hpt.

The adult cultures also were monitored well beyond 70 hpt, unlike the L3 cultures. The wells were monitored for a longer period of time because the wells with concentrations 3.00 µg/mL, for both adult female and adult male worms, died much faster than wells with the other concentrations. The adult cultures were meant to be monitored as closely as possible, or at least until more worms died at the lower concentrations to get an accurate estimate of total adult death in all wells.

Another observation from the adult cultures includes a trend where the adult female worms died at a faster rate than the adult male worms. The adult female worms started showing death at approximately 30hpt, whereas adult males started showing death at approximately 40hpt. This may give some insights about the mechanism of action of the drug treatment.

Adult nematodes are the primary target of this study, as adult worms are not efficiently killed by current drug treatments for LF. Developing a drug that can efficiently kill adults, mf and L3 is a high priority of this project and these results on adult make and female cultures are very encouraging that we are close to achieving that objective.
Conclusions and Future Research

We conclude from this collaboration with the Shea Laboratory that both acetylation and esterification techniques to convert Neurolenin D to Neurolenin B produced active compounds with efficacy against adult, L3 and mf *B. pahangi* and *B. malayi* nematodes. We can conclude from the results described in this study that Neurolenin B is biologically active while Neurolenin D is relatively biologically inactive. Synthesized recrystallized Neurolenin B (from Neurolenin D using the acetylation method) showed statistically significant killing and was biologically active when comparing mortality rates at the highest concentrations (3.00 µg/mL and 1.25 µg/mL) with the control wells (0 µg/mL) against L3, adult female and adult male nematodes, and mf at 72hpt and 98hpt. Synthesized recrystallized Neurolenin B, produced in the Shea Laboratory using the acetylation technique, is the purist form of Neurolenin B we have been able to test so far. Isovaleric acid Neurolenin D ester (which results in Neurolenin B) showed significant killing and was biologically active when comparing mortality rates at the highest concentration, 1.25 µg/mL against L3 nematodes at 72hpt. There were solubility issues discovered in the esterified product, and it is expected that the product is more potent than our measurements indicate, due to these solubility issues. Despite this solubility issue, significant L3 death was demonstrated at ~50hpt. The results from the esterified Neurolenin D product, along with its solubility issues led us to use the acetylated Neurolenin D product on adult nematodes, which showed significant killing and biological activity at 3.00 µg/mL.
We plan to continue conducting biological replicate experiments in adult and L3 worms for both Neurolenin B produced from Neurolenin D by acetylation and esterification techniques to confirm biological activity and significant killing across all life stages of the parasite. It is clear that the required dosage differed for L3 and adult nematodes. It would be helpful to perform RNA-seq expression analysis on adult males and adult females, along with L3, to see how life stage and sex can impact which genes are differentially expressed in response to treatment. By observing gene expression analysis, we can better understand the mechanism of action Neurolenin B has on filarial nematodes.

For future studies, we aim to explore growing techniques of *N. lobata* in a location site at or near Smith College. The Shea Laboratory observed varying concentrations of the different neurolenins based on the origin of the plant. The plants purchased for this study were from Belize, and were observed to have a high concentration of Neurolenin D. We expect that *N. lobata* found in other parts of Central and South America will have genetic variability resulting in different concentrations of the various neurolenins. We hope to grow *N. lobata* at the Smith College Botanical Garden to have a ready and consistent supply of neurolenins. We also plant to use DNA barcoding to confirm the origin of any plant material we obtain for our work.

In addition to demonstrating worm killing, we also want to demonstrate that Neurolenin B is not mutagenic or toxic. The Ames mutagenicity test will be used to determine the mutagenicity of the synthesized Neurolenin B produced by acetylation and esterification. Mutagens have the ability to alter DNA sequences
by deleting or changing nucleotides. The Ames test is used to test the
mutagenicity of different compounds in bacteria. It uses mutant strains of
Salmonella typhimurium bacteria that can detect many different mutagenic agents.
By detecting potential mutagenic agents, we can determine if Neurolenin B is
mutagenic before proceeding to testing in animal models, or even for human
ingestion.

N. lobata has been used historically in traditional and indigenous
medicine, and no signs of mutagenicity or toxicity have been previously reported.
However, testing for potential mutagenicity remains a necessary step when
considering Neurolenin B as a suitable drug candidate for LF. Furthermore, we
hope to observe more L3 and adult cultures testing both Neurolenin B products
that were derived from Neurolenin D via acetylation and esterification techniques
to gather more data, biological replicates, and further confirmation of the results
presented in this study.

Ultimately, we hope to eventually partner with Glaxo Smith Kline, a
pharmaceutical company, to start testing our Neurolenin B products on rodent
hosts infected with B. pahangi to validate Neurolenin B as a suitable drug
candidate for LF.
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