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An investigation into the anthelmintic properties
and synergistic potential of Cry5B

A Thesis submitted in partial satisfaction of the requirements
for the degree Master of Science

in

Biology

by

Sophia Berniece Georghiou

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Professor Emily R. Troemel
Professor Joseph M. Vinetz

2010
The Thesis of Sophia Berniece Georghiou is approved and it is acceptable in quality and form for publication on microfilm and electronically:

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Chair

University of California, San Diego

2010
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ABSTRACT OF THE THESIS

An investigation into the anthelmintic properties and synergistic potential of Cry5B

by

Sophia Berniece Georghiou

Master of Science in Biology
University of California, San Diego, 2010

Professor Raffi V. Aroian, Chair

To examine the anthelmintic properties of Bacillus thuringiensis (Bt) crystal proteins, this thesis investigates the therapeutic effects of Bt crystal protein 5B (Cry5B) as a single dose therapy against a roundworm infection in mice, and considers the synergistic potential of Cry5B in combination with a current anthelmintic. In order to examine the therapeutic effect of crude Cry5B spore crystal lysate (SCL), mice infected with Heligmosomoides bakeri L3 larvae were treated with a single dose of 50 mg/kg Cry5B SCL on day 15 post infection. The compound’s effectiveness was determined by the reduction in fecal egg output and the final worm burden in treated versus placebo-treated control animals. This study finds Cry5B SCL to be highly efficacious in vivo. A
follow-up experiment suggests that the crystals of Bt SCL are solely responsible for this therapeutic effect. This thesis further examines the therapeutic potential of Cry5B and levamisole against *H. bakeri*, using loss of motility as a marker of drug efficacy, and finds that the two compounds demonstrate strong synergy *in vitro* when combined at a 1:1 efficacious dose based upon their Fa30s. Further studies are warranted for an evaluation of any synergism between the two compounds in an *in vivo* system. Finally, the maintenance of *Ancylostoma ceylanicum* is detailed. This thesis confirms Cry5B’s effectiveness as a therapeutic compound for the treatment of gastrointestinal nematode infections and establishes different systems in which to further test its efficacy and synergistic potential.
Introduction

Human intestinal parasitic nematodes are among the most widespread diseases of our time, infecting approximately 2 billion people worldwide (Albonico et al. 2008). The total disease burden of these parasites is estimated to cause upwards of 39.0 million disability adjusted life years (DALYs) lost each year (Bethony et al. 2006; Utzinger and Keiser 2004). The downstream effects of heavy infections include malnutrition, stunted growth, and lowered mental capacity in children. Hookworms, alone, result in the loss of approximately 22.1 million DALYs, and are the leading cause of anemia worldwide (Utzinger and Keiser 2004; Hotez 2008). Parasitic worm infections have been shown to increase susceptibility to other diseases, including AIDS, malaria, and tuberculosis (Alexander and De 2009; Brooker et al. 2007; Elias et al. 2007). Moreover, nematode parasitism traps people in poverty by decreasing both their work productivity and mental capacity (Hotez 2008; Watkins and Pollitt 1997). In a study of children in the Philippines, infection with the whipworm *Trichuris trichiura* at any intensity relative to being uninfected was significantly associated with at least a four-fold higher odds of attaining a low score on tests of verbal fluency (Ezeamama et al. 2005). Tests such as this suggest that even low worm burdens may be enough to see significant mental impairments in infected persons.

Despite the high prevalence and burden of parasitic infections, current treatment options are limited. Only albendazole is extensively used for mass drug administration, as it has adequate efficacy against hookworm and can be delivered as a single, weight-independent dose (Utzinger and Keiser 2008; Crompton et al. 2003). Unfortunately,
albendazole demonstrates poor effectiveness as a single-dose treatment against hookworm and whipworm. In addition, resistance to the benzimidazole family of compounds has already been noted in Ethiopia, Sri Lanka, Vietnam, and Tanzania (Gunawardena et al. 2008; Adugna et al. 2007; Flohr et al. 2007; Albonico et al. 2002; Mazigo et al. 2010). There is a subsequent need for more anthelmintic treatment options, such as drugs with different mechanisms of action to overcome parasites resistant to current compounds, and, in the longer term, a need for synergistic drug combinations.

*Bacillus thuringiensis* (Bt) crystal proteins are the most extensively used, biologically-produced insecticides in the world (Roh et al. 2007). Certain Cry proteins, including Cry5B, have been found to specifically target roundworms (Wei et al. 2003). Specifically, the pore-forming toxin recognizes and binds glycolipid receptors on the intestines of invertebrates (Griffitts et al. 2005). Cry5B is a three-domain crystal protein related by primary sequence and predicted secondary structures to the crystal proteins used to kill insects and many free-living nematodes (Xia et al. 2008). Cry5B has already been shown to have *in vivo* efficacy as an anthelmintic targeting hookworm infections of golden Syrian hamsters (Cappello et al. 2006).

The goal of the first section of this thesis research is to further characterize the anthelmintic properties of Cry5B against the parasitic roundworm *Heligmosomoides bakeri* (formerly *Heligmosomoides polygyrus*). *H. bakeri* is a parasitic nematode that establishes a chronic infection in immunocompetent mice with a high infection rate. For this reason, *H. bakeri* is a widely studied intestinal parasitic nematode, and has been very useful in testing anthelmintics *in vitro* and *in vivo* (Behnke et al. 2009; Boes and Helwig 2000; Fonseca-Salamanca et al. 2003; Monroy and Enriquez 1992; Stepek et al. 2007).
The use of this parasite in characterizing the anthelmintic properties of ivermectin highlights its utility for anthelmintic studies (Omura 2008).

The second section of my thesis employs this same parasite to evaluate the synergistic potential of Cry5B in combination with a current anthelmintic. Drug combinations are considered the best treatment options for many major infectious diseases, including HIV, tuberculosis, and malaria (Harries and Dye 2006; Okell et al. 2008; Portsmouth et al. 2003). These combinations delay the development of resistance of a pathogen, as the pathogen must overcome more than one drug simultaneously, as long as the two drugs differ in mechanism. Interactions between compounds can either be antagonistic, additive, or synergistic. Ideally, compound mixtures show synergy, eliciting a greater therapeutic effect than would be expected from their predicted additive effects. In this case, smaller doses of each drug could be used, with the added advantages of reducing costs and side effects.

In addition to the benzimidazole class of anthelmintics, nicotinic acetylcholine receptor agonists, including pyrantel and levamisole, make up a second class of anthelmintics approved for human helminth treatment. Research utilizing the model organism *Caenorhabitis elegans* has revealed strong synergism between Cry5B and nAChR agonists, but this observation has not been applied to the study of intestinal nematode parasites (Hu et al. 2010). This thesis employs an *in vitro* method for evaluating the synergistic potential of Cry5B combined with the L-type nAchR agonist levamisole against *H. bakeri* (Stepek et al. 2007). The two compounds were then combined for a preliminary *in vivo* test of synergy against *H. bakeri* infections in mice.
Relative reductions in worm burdens and fecal egg counts were used as markers for synergy.

Finally, I present my work in maintaining the hookworm *Ancylostoma ceylanicum* as a model organism in our laboratory. *A. ceylanicum* is a hookworm capable of infecting humans, dogs, and hamsters (Bethony et al. 2006). The parasitic nematode is of the same genus as *Ancylostoma duodenale*, the human hookworm that, with *Necator americanus*, comprise the two dominant human roundworm parasites. As *A. ceylanicum* and *A. duodenale* share the same genus, we hope that any therapy observed in laboratory testing would be indicative of what to expect in clinical trials and field testing of crystal proteins and crystal protein combinations against this critical human parasite.
Introduction: Works Cited


Chapter 1: *Bacillus thuringiensis* Cry5B protein is highly efficacious as a single-dose therapy against an intestinal roundworm infection in mice

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ABSTRACT

Background

Intestinal parasitic nematode diseases are one of the great diseases of our time. Intestinal roundworm parasites, including hookworms, whipworms, and Ascaris, infect well over 1 billion people and cause significant morbidity, especially in children and pregnant women. To date, there is only one drug, albendazole, with adequate efficacy against these parasites to be used in mass drug administration, although tribendimidine may emerge as a second. Given the hundreds of millions of people to be treated, the threat of parasite resistance, and the inadequacy of current treatments, new anthelmintics are urgently needed. Bacillus thuringiensis (Bt) crystal (Cry) proteins are the most common used biologically produced insecticides in the world and are considered non-toxic to vertebrates.

Methods/Principle Findings

Here we study the ability of a nematicidal Cry protein, Cry5B, to effect a cure in mice of a chronic roundworm infection caused by the natural intestinal parasite, Heligmosomoides bakeri (formerly polygyrus). We show that Cry5B produced from either of two Bt strains can act as an anthelmintic in vivo when administered as a single dose, achieving a ~98% reduction in parasite egg production and ~70% reduction in worm burdens when delivered per os at ~700 nmoles/kg (90–100 mg/kg). Furthermore, our data, combined with the findings of others, suggest that the relative efficacy of Cry5B is either comparable or superior to current anthelmintics. We also demonstrate that
Cry5B is likely to be degraded quite rapidly in the stomach, suggesting that the actual dose reaching the parasites is very small.

**Conclusions/Significance**

This study indicates that Bt Cry proteins such as Cry5B have excellent anthelmintic properties *in vivo* and that proper formulation of the protein is likely to reveal a superior anthelmintic.
INTRODUCTION

Neglected tropical diseases (NTDs) have a worldwide devastating impact on the lives of billions of people. Helminth infections comprise approximately 85% of the NTD burden [1]. The top three ailments on this list of NTDs are all caused by intestinal nematodes [2]. These infections consist of ascariasis (caused by *Ascaris lumbricoides*), trichuriasis (caused by *Trichuris trichiura* or whipworm), and hookworm disease (caused by *Necator americanus* and *Ancylostoma duodenale*). Approximately 807-1,221 million people are afflicted with ascariasis, 604–795 million with trichuriasis, and 576–740 million with hookworm infections [3]. The widespread and detrimental effects of parasitic worm infections on human growth, nutrition, cognition, school attendance and performance, earnings, and pregnancy have been well documented [2],[3]. These infections also contribute to increased severity/infectivity of HIV/AIDS, malaria, and tuberculosis due to compromised immune responses [3],[4]. Furthermore, parasitic nematode infections confound vaccination efficacy [5],[6]. Despite the high prevalence and destructive nature of these infections, there are few treatment options. Although four anthelmintics (levamisole/pyrantel and mebendazole/albendazole) are approved by the World Health Organization for use in humans, one, albendazole, is generally preferred in a single-dose regimen over the others since it is relatively more effective against hookworms and whipworms [7],[8]. However, resistance to albendazole may already be appearing [9],[10]. Furthermore, the reliance upon one compound for treating hundreds of millions of people will have devastating consequences if widespread resistance ever becomes a reality. Tribendimidine, developed by the Chinese Centers for Disease Control and Prevention, is emerging as a second anthelmintic with efficacy similar to
albendazole, but is a member of the levamisole/pyrantel class to which resistance in human populations has been reported [11],[12],[13]. Furthermore, none of the compounds have been shown to be totally effective against all helminth infections [8]. Consequently, there is an urgent need for efficacious, safe, inexpensive, single-dose anthelmintics with new mechanisms of action.

This search for new anthelmintics has led to examination of *Bacillus thuringiensis* (Bt) crystal (Cry) proteins. These proteins are the most extensively used biologically-produced insecticides in the world [14]. Bt is a soil bacterium that produces crystal inclusions during sporulation. These inclusions contain Cry proteins that are highly toxic to some invertebrates but nontoxic to humans and other vertebrates [15]. The high efficacy against insects, absence of toxicity towards vertebrates, and low production cost of these proteins has led to their widespread use in pesticides and in transgenic crops [14]. So far, three Bt Cry proteins toxic to a broad range of free-living nematodes and the free-living form of at least one intestinal parasitic nematode have been discovered, including: Cry5B, Cry14A, and Cry21A [16]. Cry13A may also have anti-nematode activity [17]. To date, only one of these, Cry5B, has been shown to be therapeutic *in vivo* with activity against intestinal hookworm parasite (*Ancylostoma ceylanicum*) infections in hamsters when delivered daily, *per os*, over the course of three days [18].

These studies suggest that Cry proteins could provide therapy for intestinal nematode infections. However, it remains to be shown that Cry5B can effect a cure against more than *A. ceylanicum* infections in hamsters or that Cry proteins are efficacious as single-dose anthelmintics. *Heligmosomoides bakeri* (formerly known as *Heligmosomoides polygyrus* and *Nematospiroides dubius*) is one of the most widely
studied rodent intestinal parasite nematodes [19],[20]. The nematode has a high infection rate and is the best model for chronic intestinal nematode infections in immunocompetent mice. *H. bakeri* has also played a key role in the history of anthelmintic development via its use in the discovery of ivermectin [21]. In addition, *H. bakeri* infections in mice are a naturally occurring infection, unlike *A. ceylanicum* infections in hamsters. Thus, curative experiments in *H. bakeri* are complementary to those in *A. ceylanicum*, yielding important information as to how Cry proteins may fare against a broad range of natural intestinal parasites *in vivo*. Herein, we report our investigations into single-dose Cry5B therapy against *H. bakeri*. 


METHODS

Animals

Female Swiss Webster white mice were purchased from Harlan Laboratories and were infected at approximately 6 weeks of age at an average weight of 25g. Mice were provided with food and water *ad libitum*. This research was approved by the UCSD Institutional Animal Care and Use Committee (IACUC), protocol number S08140. The maintenance and care of experimental animals complied with the University of California's Animal Care Program's guidelines for the humane use of laboratory animals.

Preparation of Bt strains

Crystal-deficient Bt strains HD1 and 4Q7 were transformed with a plasmid containing the Cry5B gene [23]. *Bacillus thuringiensis* subspecies *kurstaki* HD1-4D8 was ordered through the *Bacillus* Genetic Stock Center. Spore lysates (SLs; HD1 and 4Q7 Cry-deficient strains) and spore-crystal lysates (SCLs; HD1 and 4Q7 transformed with Cry5B plasmid) were prepared using standard methods and then stored at −80° [23]. Bioactivity of SCLs was confirmed against *Caenorhabditis elegans* by a mortality assay over 24 h at 25°C. SLs (Cry-minus) were confirmed to lack toxicity against *C. elegans*.

On the day of use, SL and Cry5B SCL aliquots were thawed and centrifuged at 4,500 rpm for 15 minutes at 4°C and the supernatant was removed. The pellet was then resuspended in distilled water to a final concentration of 2.5 mg/mL, for the HD1 strain, and 2.25 mg/mL, for the 4Q7 strain (protein concentrations were determined by comparing Cry5B band intensities for four different aliquots of SCLs to known amounts of bovine serum albumin on Coomassie-stained 8% SDS polyacrylamide protein gels). The placebo SL
control strains were concentrated to the same extent. The samples were kept on ice until gavage.

**Cry5B curative experiments**

On day 0, mice were infected *per os* with a suspension of 200±10 *H. bakeri* L3 larvae in 0.1 mL of distilled water. Larvae were counted under the microscope, then drawn into a pipette tip and placed into separate glass test tubes until gavage with a blunt-ended syringe. On days 14, 16, 18, and 20 post-infection (P.I.), fecal samples were collected from the mice. Mice were placed individually in empty plastic cages for 1 h each morning, and the fecal pellets were collected into 50 mL centrifuge tubes. The number of eggs present was counted using the modified McMaster technique [22]. Briefly, feces collected from mice were weighed and resuspended in a 1 g:15 mL volume of water. The pellets were allowed to soak overnight before being broken up for 1 h via heavy vortexing. The eggs were counted using a 2-chamber McMaster slide, each chamber holding a 0.6 mL volume of a 1:1 mixture of fecal slurry and saturated sucrose solution. The number of eggs per gram of feces was thus calculated from the following equation: number of eggs counted x (1/0.3 mL slurry) x (15 mL slurry/g feces). For each mouse and each time point, three different egg counts were made and then averaged. Each mouse was treated *per os* on day 15 P.I. with 0.1 mL of relevant treatment (placebo or Cry protein) through a blunt-ended syringe. All mice were killed by exposure to CO₂ on day 20 P.I. and the intestines were removed in their entirety. These were opened longitudinally with a pair of blunt-ended dissecting scissors and then placed into a 50 mL centrifuge tube with 10–20 mL of pre-warmed (37°C) PBS for approximately 1 h to
allow worms to dislodge from the intestine. The solution and intestine were examined under a microscope, using fine tweezers when necessary for further extrication of worms from the intestine, for determination of final worm burden.

**Tribendimidine curative experiments**

Tribendimidine was kindly provided by Dr. Shu-Hua Xiao at the Chinese Centers for Disease Control and Prevention. The drug was suspended in 20 mM citrate buffer pH 7.3 and delivered *per os* on day 15 P.I. in a total volume of 0.1 mL as per Cry5B experiments. For these curative experiments, the mice were infected with on average 150 L3 larvae (six/group except for placebo group, which only had five mice). Placebo control for these experiments was 0.1 mL of buffer only.

**Digestive fluids for Cry5B metabolic fate studies**

Simulated gastric fluid (SGF) was prepared freshly as described in the United States Pharmacopeia and stored at 4°C until use [24]. Cry5B SLC was added to a 1 mL solution of SGF for a final concentration of 2.5 mg/mL and incubated at 37°C [25]. 50 µL aliquots of the digestion stock were removed at each time point as the digestion solution was agitated. Each aliquot was immediately quenched by neutralization with 15 µL of 0.2 M sodium carbonate per 50 µL of SGF [25]. Quenched samples were kept on ice until 2x SDS-PAGE loading buffer was added to each sample. Mixtures were then heated for 5 min in boiling water and stored at −20°C until analysis.

**Statistical analysis**
Data analysis of intestinal worm burdens and fecal egg counts was carried out and plotted using Prism 5 (GraphPad Software Inc., La Jolla, CA, U.S.A.). For worm burdens, average indicates the average worm burdens amongst all the mice in each treatment group. For fecal egg counts, average indicates the egg count per mouse averaged from all mice in the group at a given time point. Fecal egg count data was analyzed via pair-wise comparisons between groups and days through two-way analysis of variance (ANOVA) with repeated measures and Bonferroni post-tests. Results were as follows: $F_{\text{Treatment}} = 70.69$, degrees of freedom (df) = 1, $P<0.0001$; $F_{\text{Time}} = 5.241$, df = 3, $P = 0.003$; $F_{\text{Interaction}} = 11.43$, df = 1,3, $P<0.0001$. Worm burdens for Cry5B treatment versus placebo were compared using Mann-Whitney U test (one-tailed). Values are as follows: $U = 7.5$, $P = 0.0007$ for HD1 Cry5B versus placebo; $U = 2.0$, $P = 0.0012$ for 4Q7 Cry5B versus placebo. Worm burdens for tribendimidine experiment were compared using one-way ANOVA and Tukey's Multiple Comparison Test ($F = 9.387$, df = 3, 19, $P = 0.0005$).
RESULTS

A single dose of Cry5B is able to achieve a significant therapeutic effect against *H. bakeri* parasites *in vivo*

To determine if Cry5B could provide therapy as a single-dose anthelmintic against *H. bakeri*, we treated *H. bakeri*-infected mice with Bt spore-crystal lysates expressing or not expressing (placebo control) Cry5B. When the bacterium Bt sporulates, it produces spores, large crystal protein-containing inclusions, and lysate produced when the mother cell that gives rise to the spore and crystal lyses upon completion of sporulation. Bt spore-crystal lysates (SCLs) from many Bt strains, including Bt *kurstaki* HD1 that targets caterpillars (Lepidoptera), have been extensively tested against mammals (including humans) and found to be non-pathogenic [15],[26],[27]. We transformed a crystal protein-minus HD1 strain with a Cry5B-expressing plasmid. Twenty mice were infected with *H. bakeri* larvae. Fifteen days post-infection (P.I.), we delivered into each mouse *per os* either a single 0.1 mL dose of Cry5B-containing HD1 SCLs (715 nmoles/kg or 100 mg/kg of Cry5B) or, as a placebo control, a single 0.1 mL dose of spore lysates (SLs, crystal-minus) from the parent, untransformed HD1 strain. Beginning the day before treatment (day 14 P.I. or day -1 treatment), and then continuing every other day (day 1, 3, 5 post-treatment), we collected fecal samples from each mouse to measure parasite progeny production (eggs/gram of feces). Five days after treatment (day 20 P.I.), the mice were euthanized and the total number of parasites present in the small intestine tallied.

With regards to progeny production, we found that on the day prior to treatment, the parasites in both groups of mice (placebo treated and Cry5B treated) were producing
statistically indistinguishable amounts of eggs (Figure 1.1, Table 1.1). At days 1, 3, and 5 post-treatment, the placebo group showed no reduction in egg production, consistent with the hypothesis that the parent Bt strain alone has no effect on the parasites. In contrast, a rapid and remarkable reduction in egg production took place in the Cry5B-treated animals, resulting in a 95%, 99%, and 98% reduction on days 1, 3, and 5 post-treatment respectively (Figure 1.1, Table 1.1). With regards to parasite clearance, we found that the single dose of Cry5B achieved a remarkable therapeutic effect, clearing away 67% of the parasites relative to placebo control (Figure 1.2, Table 1.2). Thus, a single dose of Cry5B has strong effects on parasite reproduction and the ability of parasites to maintain an infection.

The reduction in fecal egg count (>97%) was much larger than would be expected from the final mouse worm burden of the SCL-treated animals (67% cleared). There are at least two possible explanations for this—either the treatment was affecting the status of the worms so that any worms left behind were severely compromised in health or the treatment was preferentially eliminating female over male parasites. To distinguish between these possibilities, we made a note of the number of females present in placebo versus Cry5B treated controls during the counting of the worm burdens. In placebo treated mice we found that there were 35.3±5.9 (standard error of the mean, or sem) females while in the Cry5B treated mice there were 7.8±2.3 (sem) females per mouse intestine. Thus, there was a 78% reduction in the number of females present. This drop, although greater than that for males (51% reduction), does not seem sufficient to account for the observed >97% drop in egg production seen. These data suggest that the parasites that remained in the intestine were severely compromised in health.
We also determined if this capacity to clear an infection was dependent upon a particular Bt strain. We performed a similar curative experiment, measuring intestinal worm burdens after treatment, using the Bt strain 4Q7 (derived from Bt *israelensis*, which targets Diptera) either transformed with the Cry5B-expressing plasmid or untransformed. Fourteen mice were infected with *H. bakeri* larvae, and fifteen days P.I. a single dose of 0.1 mL Cry5B-containing 4Q7 SCLs or 0.1 mL 4Q7 SLs (crystal-minus) were delivered *per os*. The dose delivered per mouse was 644 nmoles/kg (90 mg/kg). We found a similar therapeutic effect as above—71% of the parasites were cleared relative to placebo control (Figure 1.2, Table 1.2). We note that in this experiment the total number of parasites present in the small intestine in placebo control animals was greater than in the first experiment. The variability appears to be due to relative infectivity of different batches of L3 parasite larvae. These data demonstrate that, regardless of parent Bt strain and of the initial parasite load, a similar single dose of Cry5B is able to achieve comparable therapeutic effect.

**Cry5B compares highly favorably versus known anthelmintics**

These results are significant when the relative efficacy of Cry5B is compared to other standard anthelmintic treatments. Published reports, employing a treatment timeline against *H. bakeri* parasites that is similar to our own, show that levamisole (10 mg/kg or 49 µmoles/kg delivered on day 12 P.I.) effected a 90% reduction in worm burdens and ivermectin (5 mg/kg or 5.7 µmoles/kg) or pyrantel (50 mg/kg or 84 µmoles/kg) or piperazine (4000 mg/kg or 46 mmoles/kg) delivered on day 18 P.I. effected an 87%, 98%, and 34% reduction in worm burdens respectively ([28],[29]. Another study showed
that 2.9 µmoles/kg of ivermectin delivered on day 10 P.I. effected ~70% reduction in \textit{H. bakeri} worm burdens [30]. We could not find comparable studies with \textit{H. bakeri} and benzimidazoles, although we did find that mebendazole delivered for 7 consecutive days, starting day 9 P.I. at 22 mg/kg/dose or 75 µmoles/kg/dose, achieved an 84% reduction in worm burdens [31]. Benzimidazoles (including albendazole) in general seem to be less active against \textit{H. bakeri} [32]. Therefore, our single dose of ~700 nmoles/kg (which is the highest dose we can currently pipette with SCLs) that achieved ~70% reduction in worm burdens is 70X, 4–8X, 120X and 65,000X lower than the doses of levamisole, ivermectin, pyrantel, and piperazine used in the above studies.

This comparison suggests that the efficacy of Cry proteins relative to known anthelmintics is excellent. To directly compare our results to a known anthelmintic using the same treatment conditions, we performed curative experiments using the newest human anthelmintic and the only one taken to human clinical trials in the past thirty years, tribendimidine. We performed dose-dependent curative assays with tribendimidine against \textit{H. bakeri} infections, finding an estimate dose of ~1 mg/kg or 2.2 µmoles/kg tribendimidine to give a curative effect similar to ~700 nmoles/kg Cry5B (Figure 1.3). Based on this comparison, Cry5B is at least as good as tribendimidine at curing \textit{H. bakeri} infections and in fact appears to be ~2–3 fold superior.

\textbf{Cry5B is rapidly digested in simulated gastric fluids}

These data indicate that Cry5B is an excellent anthelmintic when delivered at a single dose. However, Cry proteins are thought to be digested rapidly in the mammalian
digestive tract, most notably by the acidic stomach [33]. If so, then it is possible that the
dose of Cry protein reaching the parasites might have been very small.

To determine how well Cry5B would survive the mammalian stomach, we
incubated Cry5B HD1-derived SCLs in simulated gastric fluids. We find that Cry5B is
almost completely digested in this environment within four minutes (Figure 1.4). These
data suggest that very little Cry5B is actually reaching the parasites.
DISCUSSION

Our study demonstrates that the Bt Cry protein Cry5B is an excellent anthelmintic in vivo against a natural and chronic intestinal roundworm infection in mice, namely *H. bakeri*. Cry5B is able to achieve significant reductions in parasite egg production (~98%) and intestinal worm burdens (~70%) following a single dose delivered per os at ~700 nmoles/kg. This therapeutic effect, on a mole-by-mole basis, is on par with or superior to those of other anthelmintics commonly used in human therapy. Although this level of efficacy may seem surprising at first glance, upon deeper reflection it is not. Cry proteins, although they only attack the gut cells of invertebrates, are pore-forming toxins (PFTs; [34]). PFTs are the single most common virulence factors made by pathogenic bacteria and are also used by our immune system to combat pathogens [35],[36]. PFTs are potent weapons and the consequences of their attack on the integrity of the plasma membrane are great. In combination with previous data showing that Cry5B is also able to cure *A. ceylanicum* infections in hamsters [18], we have now demonstrated in vivo anthelmintic activity of Cry5B against two very different parasitic nematodes (one a blood feeder, the other not) in two different mammalian hosts. Taken together, along with the fact that Cry5B is active against *Nippostrongylus brasiliensis* larvae, against *Haemonchus contortus* larvae in vitro, against a phylogenetically wide range of free-living nematodes, and against the plant-parasitic nematode *Meloidogyne incognita* [16],[17],[37], our data indicate that Cry5B has very broad anti-nematode activity and that Cry5B has superb potential in human anthelmintic therapy.

As a natural product, it is interesting to compare the efficacy of Cry5B to other natural product anthelmintics. No recently investigated biological treatments against *H.*
*bakeri* demonstrate comparable *in vivo* efficacy using single-dose regimens. Many of these natural compounds, such as the extract of *Embelia schimperi*, nitazoxanide, santonin, and *Myrsine Africana*, showed only small reductions in intestinal worm burden as a single dose, with efficacies of 30%, 21%, 18%, and 10%, respectively [29],[38],[39]. A single dose of 500 mg/kg of *Albizia anthelmintica*, not only revealed low efficacy, with a total worm burden reduction of only 3–23%, but also displayed significant toxicity [28]. Even the macrolactam *N*-methylfluvirucin, delivered at a daily dose of 50 mg/kg over 3 days, effected only a 42% reduction in total worm burden [40]. While other compounds were more efficient, they required extremely high doses and/or multiple-day dosing regimens. These included a daily treatment of ethanol extract of *Canthium manni* (Rubiaceae) at 5600 mg/kg, which showed a 75% decrease in fecal egg count and 84% reduction in worm burden with 7 days of treatment [31]. A 600 mg/kg treatment with extract of stem bark of *Sacoglottis gabonensis* was extremely effective, but exceedingly toxic, with mice showing signs such as depression, drowsiness, unsteady gait and paralysis of the hind limbs, dyspnoea, coma and death apparent within 1–2 min following intraperitoneal injection [41]. Perhaps the most promising of other natural treatments is papaya latex. A single-dose administration of papaya latex at 8 g/kg achieved an efficacy of 84.5%, with fecal egg count reductions of 93.3% [42]. Mice treated daily over 7 days with 133 nmoles of papaya latex showed a decrease in fecal egg count of 87–97% and a 92% reduction of worm burden [43]. In general, few of the natural compounds tested above proved to be practical treatments due to dosing and toxicity issues.

It is clear that Cry5B has great promise as an effective, safe, and much-needed addition to anthelmintic therapy. The vertebrate and human safety profiles of Cry
proteins are outstanding—Cry proteins as insecticides are used around the world on a large-scale in organic farming, in aerial spray campaigns, and in vector (mosquito, black fly) control programs and have even been approved for expression in transgenic foods such as corn, potatoes, and rice [14],[44]. Although Cry5B has not been studied in this regard, it is a member of the same family of three-domain Cry proteins expressed in transgenic crops and used in all these spray programs and thus is predicted to have the same safety profile. Indeed, extensive research from our laboratory has confirmed that the receptor Cry5B needs to bind to in order to intoxicate nematodes is an invertebrate-specific glycan (carbohydrate) [45].

It is interesting to note that, although Cry5B has comparable if not superior activity against *H. bakeri* on a mole-by-mole basis with other anthelmintics, it is likely that only tiny amounts of the protein being delivered *per os* in our experiments are reaching the parasites. In four minutes, virtually all Cry5B is degraded in simulated gastric fluids. These experiments suggest that a simple enteric coating to protect Cry proteins against the stomach while releasing it in the small or large intestines might greatly increase the efficacy of Cry proteins. These data thus emphasize the importance of formulation in the next stage in the evolution of Cry protein anthelmintic development and suggest that such a formulation has the potential to reveal an anthelmintic with therapeutic properties comparable or superior to those currently in use.
ACKNOWLEDGEMENTS

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### Table 1.1: Fecal egg counts in Cry5B HD1 experiment

<table>
<thead>
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<th></th>
<th>Day number relative to treatment</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>−1</td>
</tr>
<tr>
<td>Placebo</td>
<td></td>
</tr>
<tr>
<td></td>
<td>3455±457</td>
</tr>
<tr>
<td>Cry5B (715 nM/kg)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>2698±282</td>
</tr>
<tr>
<td>Reduction relative to placebo</td>
<td>N/A</td>
</tr>
</tbody>
</table>

Numbers shown are eggs/gram of feces/mouse averaged for all 10 mice in each group ± standard error of the mean (sem).
Table 1.2: Worm burdens in Cry5B treatment experiments.

<table>
<thead>
<tr>
<th>Group (no. of mice)</th>
<th>Intestinal worm burden (mean ± sem)</th>
<th>% Reduction relative to placebo</th>
</tr>
</thead>
<tbody>
<tr>
<td>HD1 placebo (10)</td>
<td>59.1±11.1</td>
<td>NA</td>
</tr>
<tr>
<td>HD1 Cry5B (10)</td>
<td>19.4±3.6</td>
<td>67.2</td>
</tr>
<tr>
<td>4Q7 placebo (7)</td>
<td>169.1±14.0</td>
<td>NA</td>
</tr>
<tr>
<td>4Q7 Cry5B (7)</td>
<td>48.1±15.3</td>
<td>71.5</td>
</tr>
</tbody>
</table>

Placebo: crystal-deficient HD1 or 4Q7 strain; Cry5B: the corresponding strain transformed with a Cry5B-expressing plasmid. Cry5B dosage: 715 nm/kg (HD1) or 644 nm/kg (4Q7).

sem = standard error of the mean.

NA = not applicable.
Table 1.3: Worm burdens in tribendimidine treatment experiment.

<table>
<thead>
<tr>
<th>Group (n = number of mice)</th>
<th>Intestinal worm burden (mean ± sem)</th>
<th>% Reduction relative to placebo</th>
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</thead>
<tbody>
<tr>
<td>Placebo (n = 5)</td>
<td>83.8±8.7</td>
<td>N/A</td>
</tr>
<tr>
<td>0.125 mg/kg trib (n = 6)</td>
<td>53.2±9.3</td>
<td>36.5</td>
</tr>
<tr>
<td>0.5 mg/kg trib (n = 6)</td>
<td>33.0±10.7</td>
<td>60.6</td>
</tr>
<tr>
<td>2 mg/kg trib (n = 6)</td>
<td>13.4±9.1</td>
<td>84.0</td>
</tr>
</tbody>
</table>

*sem = standard error of the mean.
N/A = not applicable.
trib = tribendimidine.*
Figure 1.1: Effects of Cry5B on egg production in *Heligmosomoides bakeri* infected mice.

Shown are the average eggs/gram of feces/mouse for both placebo (n = 10) and Cry5B HD1-(n = 10) treated groups the day before treatment (−1) and then every other day thereafter until the animals were euthanized on day 5 post-treatment.
Figure 1.2: Effects of Cry5B on intestinal worm burdens in Heligmosomoides bakeri infected mice.

A. Shown are the intestinal worm burdens in placebo (HD1 SL) and Cry5B (HD1 SCL) treated mice (n = 10 each group). Infected mice were given a single dose of 715 nmoles/kg (100 mg/kg) Cry5B on day 15 P.I. and intestinal worm burdens assessed on day 20 P.I. The worm burdens in each mouse are indicated with a separate symbol. Long horizontal bars represent mean worm burdens; smaller bars indicate SEM (standard error of the mean).

B. Shown are the intestinal worm burdens in placebo (4Q7 SL) and Cry5B (4Q7 SCL) treated mice (n = 7 per group). Mice were given a single dose of 644 nmoles/kg (90 mg/kg) Cry5B on day 15 P.I. and intestinal worm burdens assessed on day 20 P.I. ** P<0.01, *** P<0.001.
Figure 1.3: Effects of tribendimidine on *Heligmosomoides bakeri* infections in mice.

Shown are the intestinal worm burdens in placebo (20 mM citrate buffer pH 7.3) and for various doses of tribendimidine. Data are plotted as in Figure 1. Infected mice (n = 6/group except n = 5 placebo) were given a single dose of tribendimidine on day 15 P.I. and intestinal worm burdens assessed on day 20 P.I. **P<0.01, ***P<0.001 relative to placebo control. Conversion to molar amounts is as follows: 0.125, 0.5, and 2 mg/kg are equivalent to 0.28, 1.1, and 4.4 µmoles/kg respectively.
**Figure 1.4:** Behavior of Cry5B in simulated gastric fluids.

Left lane, markers. Next three lanes: Cry5B loading control 10, 1, and 0.1 µg Cry5B in HD1 SCLs. Next two lanes: Cry5B (10 µg/lane) SCLs in water incubated for 0 or 2 h at 37°. No degradation is seen. Next seven lanes: Cry5B (10 µg/lane) SCLs incubated in simulated gastric fluid (SGF) for the time indicated (in minutes). Cry5B has nearly disappeared after four minutes. Right two lanes: simulated gastric fluids (no Cry5B) incubated for 0 and 2 h at 37° to demonstrate where pepsin runs on the gel. 8% SDS polyacrylamide gel stained with Coomassie blue.
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Chapter 2: An evaluation of the *in vitro* synergistic potential of Cry5B in combination with the nicotinic acetylcholine receptor agonist levamisole
INTRODUCTION

The increasing prevalence and threat of drug-resistant pathogens has led pharmaceutical research to focus on combination drug therapy. Drug combinations are considered optimal therapy for the treatment of many prevalent infectious diseases, including HIV, malaria, and tuberculosis (Harries et al. 2006; Okell et al. 2008; Portsmouth et al. 2003). Combinations of drugs of different mechanisms of action can help to delay the development of resistant pathogens. In addition to addressing the issue of resistance, interactions between drug mechanisms may prove beneficial. Drugs can act synergistically, antagonistically, or additively in combination. Synergistic interactions are desirable as reduced dosages of drugs can be used, with the added advantages of reducing costs and side effects.

The search for synergistic combinations is important for antiviral and antibiotic combination therapy, and could be applied more widely to various treatments, including those for roundworms. The two classes of approved anthelmintics are the benzimidazoles (mebendazole and albendazole) and the L-subtype nicotinic acetylcholine receptor (nAChR) agonists (levamisole and pyrantel). As examined previously, our laboratory is pioneering work on Bacillus thuringiensis crystal proteins as novel anthelmintics, which would establish a third class of treatment options. Ideally, two anthelmintics with different mechanisms of action will show synergy when combined, eliciting a greater response than would be expected if the drugs’ effects were additive. Because crystal proteins have never been utilized as an anthelmintic option, they are ideal for these studies since the pathogens have yet to be exposed to this class of therapeutic agents.
Prior research completed in our laboratory has demonstrated that Cry5B is highly synergistic in combination with the nicotinic acetylcholine receptor agonists levamisole and tribendimidine against the non-parasitic, model laboratory nematode *Caenorhabditis elegans* (Hu et al. 2010). This thesis applies the same technique used to study synergy in *C. elegans* to the mouse parasite *Heligmosomoides bakeri*. Although an *in vitro* application of *H. bakeri* to study anthelmintics has been previously established, this is the first application of this system to quantitate synergistic interactions between drugs (Stepek et al. 2007).

In these assays, loss of motility is used as a parameter for drug effect over time (Stepek et al. 2007). However, instead of scoring change in motility on a graded scale, as published, worms were scored as either “motile” or “immotile,” allowing for quantification of change in motility and determination of the percent of motile parasites at a given drug concentration. In this way, results could be reported similarly to *C. elegans* data, using loss of motility in place of lethality. Inhibitory concentration (IC) values, or the concentration of drug at which a certain percentage of motility was inhibited, could be determined for each drug alone and used as a basis for establishing a 1:1 effective ratio of the treatments in combination. This 1:1 effective ratio, or the dosages at which each drug displayed the same effectiveness (i.e. equal IC values), was then evaluated for effectiveness *in vitro*. Results could then be used, as previously described, to calculate combination index values a la Chou and Talalay (Hu et al. 2010). Herein, I report my findings for evaluating the effects of the L-type nicotinic acetylcholine receptor agonist, levamisole, in combination with Cry5B *in vitro* against *H. bakeri*. 
METHODS

Animals

Female Swiss Webster white mice were purchased from Harlan Laboratories and were infected at approximately 6 weeks of age at an average weight of 25g. Mice were provided with food and water ad libitum. This research was approved by the UCSD Institutional Animal Care and Use Committee (IACUC), protocol number S08140. The maintenance and care of experimental animals complied with the University of California's Animal Care Program's guidelines for the humane use of laboratory animals.

Preparation of Bt strains and proteins

Cry5B was purified from HD1 expressing the Cry5B gene via a sucrose gradient (Cappello et al. 2006). Cry5B was precipitated following purification, suspended in aliquots of double-distilled water, frozen in liquid N₂ and stored at −80°C until use. Bioactivity of the purified Cry5B was confirmed against Caenorhabditis elegans by a mortality assay over 6 days at 25°C. Two batches of purified protein were used for these studies, with LC50 values of 6.127 and 6.000 µg/mL. On the day of use, purified protein aliquots were thawed and centrifuged at 4,500 rpm for 15 minutes at 4°C and the supernatant was removed. The pellet was then resuspended in distilled water to the desired final concentration (protein concentrations were determined by comparing Cry5B band intensities of purified proteins to known amounts of bovine serum albumin on Coomassie-stained, 8% SDS polyacrylamide protein gels). All samples were kept on ice until well addition.
**Levamisole**

Levamisole hydrochloride was purchased as a powder from Acros Organics (cat. no. 187870100). The drug was dissolved in deionized water and freshly prepared prior to each use.

**in vitro experiments**

For each assay, 2-8 mice were infected *per os* with a suspension of about 200 *H. bakeri* L3 larvae in 0.1 mL of distilled water. Mice were gavaged with a blunt-ended syringe. 15-20 days post infection, mice were killed by exposure to CO$_2$ and the small intestines were removed in their entirety. Intestines were opened longitudinally with a pair of blunt-ended dissecting scissors, and then placed into a 50 mL centrifuge tube with 10–20 mL of pre-warmed (37°C) PBS, pH 7.4, for approximately 1 hour to allow worms to dislodge from the intestine. Each intestine was washed five times with 37°C PBS to clean the worms and intestinal debris was removed with the supernatant. Intestines and solutions were kept in a 37°C CO$_2$ incubator when not in use.

RPMI-1640 medium supplemented with 500 U/mL penicillin, 500 µg/mL streptomycin, 0.6 µg/mL amphotericin, and 25mM HEPES was freshly prepared for each assay (Martinez-Grueiro 2002). RPMI media comprised 70% of the final solution. In each assay, 190 µL of supplemented RPMI was aliquoted into each well of two 48 well plates. 10 wells were used per drug concentration, with a total of 7 drug concentrations per assay, in addition to 10 control wells. Plates with RPMI were kept in a 37°C incubator for half an hour prior to adding worms. Two healthy, active female adult
parasites were picked into each well for a final worm count of 20 parasites per given drug concentration.

Treatments were added in 10 µL aliquots to each well (for a 20X-dilution) and worms were immediately scored for motility, yielding the zero-time point. To score motility, each well was quickly examined under a light microscope. If a worm appeared to be immotile, it was lightly tapped with a metal worm pick three times. If this action did not prompt a change in motility, the worm was scored as immotile. Results were represented as the percentage of motile parasites per given drug concentration over time. Assays were scored every 15 minutes over a time span of 2 hours.

**Statistical analysis**

Data analysis of the percentage of motile parasites over time was carried out and plotted using Prism 5 (GraphPad Software Inc., La Jolla, CA, U.S.A.). For motile *H. bakeri* females, percentages indicate the average percent of motile parasites out of 20, as per 3 assays. Results of combination studies (CI values) were processed using the CompuSyn software package (CompuSyn), modified by a manual correction for mutually nonexclusive drugs, which is not incorporated in the CompuSyn program (Hu et al. 2010). This modification is appropriate for these assays, as we believe that the two classes of drugs have totally independent modes of action and, in any event, result in more conservative (i.e., higher) CI values. For the combination studies in which Cry5B and levamisole were to be added at a 1:1 ratio based on IC$_{30}$ values, pilot dose–response experiments were performed with levamisole and Cry5B. Based on this pilot data, 47 µg/mL of Cry5B and 0.27 µg/mL of levamisole were selected as the 1:1 IC$_{30}$ ratio dose
(and 2-fold dilutions up and down for a total of 7 drug concentrations), and three independent synergy experiments were performed (including Cry5B, levamisole, and Cry5B+levamisole dose–response curves). Upon analyses of the completed data, the average IC$_{30}$ of Cry5B and levamisole alone were found to be 52 µg/mL and 0.24 µg/mL respectively.
RESULTS

The development of a quantitative assay to deduce the efficacy of various anthelmintics in vitro against a chronic gastrointestinal parasite is important to determine the direct effects of these different compounds on free-living parasites. Loss of motility was the standard inhibited biological function that was scored to calculate an IC value (Stepek et al. 2007; Stepek et al. 2006). Heligmosomoides bakeri adult females were obtained from the small intestine of mice sacrificed 15-20 days after infection with 200 L3 H. bakeri. To quantitate the effects of the two anthelmintics against a parasitic nematode, the female worms were subjected to increasing doses of Cry proteins and levamisole. For example, H. bakeri females were subjected to the following seven doses of Cry5B and levamisole: 400, 200, 100, 50, 25, 12.5 and 6.25 µg/mL and 4, 2, 1, 0.5, 0.25, 0.125, and 0.0625 µg/mL, respectively (Figures 2.1 and 2.2). The worms were assayed for motility at each concentration every 15 minutes over a 2 hour period at 37°C.

Levamisole was able to elicit a full dose response (0-100% paralysis) during the 2 hours given experimental conditions. Purified Cry5B was also able to elicit a dose response against H. bakeri females during the same 2 hours, but was unable to inhibit parasite motility past a 60% level, even at the highest concentrations. However, results were reproducible between experiments over the range of concentrations tested for each drug. Dose-reduction index (DRI) values, which determine the magnitude of dose reduction allowed for each drug when given in synergistic combination, as compared with the concentration of a single agent that is needed to achieve the same effect level, were calculated for Cry5B in these three assays at 7.47, 7.06, and 7.75. Levamisole DRI values were 6.63, 5.91 and 5.54. Due to an inability to achieve above 50-60% inhibition
of motility with Cry5B, I combined the drugs at a 1:1 ratio of IC$_{30}$ values as opposed to a 1:1 IC$_{50}$ (Hu et al. 2010). A consistent IC$_{30}$ value was thus determined for each compound at the two hour time point, from the averages of three motility assays for each compound alone, to be utilized as a basis for combination in synergy studies. The average IC$_{30}$ values for purified Cry5B and levamisole alone, after combining the data of the three individual assays, were determined to be 47 µg/mL and 0.27 µg/mL, respectively (Figure 2.1 and Figure 2.2).

These values were exploited as a basis for in vitro combination studies, which tested the two compounds at a constant ratio based upon their 30% efficacy values. In these experiments, the drugs were added to the wells together at a 1:1 efficacious ratio based upon individual drug assay results, and the worms were assayed for motility (Figure 2.3). Individual drug assays were completed in parallel with combination assays as values within the same experiment are used computationally for determination of synergy (and not, for example, by using values of each drug individually from historical data).

From the data of these three replicate combination assays we can calculate the degree of synergy using the combination index (CI) algorithm of Chou and Talalay (Chou 2006). Generally, CI values <1 indicate synergy, while values >1 indicate antagonism. The CI algorithm has been used extensively to calculate the degree of synergy between drug combinations in cancer chemotherapy [typical synergistic CI values 0.1–0.8], viral therapy [synergistic CI values 0.5–0.8], and insecticides [synergistic CI values of 0.3–0.9]. Conservatively, CI values <0.7 were chosen as indicative of synergy and values <0.3 as indicative of a strong synergy (Hu et al. 2010).
With this algorithm, we calculated CI values at ED$_{30}$ of the combination (in accordance with findings for the efficacy of Cry5B alone) and found CI values that spanned between 0.305–0.335 for each experiment, indicating strong synergy between Cry5B and levamisole when mixed at equal efficacious doses (Table 2.1).
DISCUSSION

Levamisole and Cry5B show synergy when combined at a 1:1 efficacy against a gastrointestinal parasite \textit{in vitro}. CI values calculated for these experiments were consistent with studies completed in our lab utilizing \textit{C. elegans}. These experiments were carried out with Cry5B and the nAChR agonist levamisole combined in either a fixed ratio based upon mass (e.g., 1 µg/mL Cry5B mixed with 1 µg/mL levamisole) or efficacy, using LC50 values. Comparable CI values of 0.38–0.15 were calculated from experiments utilizing \textit{C. elegans}, indicative of strong synergy.

This data further suggests that Cry5B and the nAChR agonist levamisole mutually potentiate each other, and that inclusion of a small amount of Cry5B might permit significant reductions in the amounts of nAChR agonists to achieve comparable effects when used alone. These results encourage the further characterization of the interaction of crystal protein, pore forming toxins with the nicotinic acetylcholine receptor agonist class of anthelmintics to generate a powerful new combination for treatment of human intestinal helminths. Furthermore, these findings reinforce the utility of using \textit{C. elegans} for studying drug combinations since similar results were obtained between parasitic and nonparasitic roundworm systems.
**Table 2.1:** CI values for *in vitro* drug synergy combination studies.

<table>
<thead>
<tr>
<th>Experiment</th>
<th>CI value</th>
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<tbody>
<tr>
<td>1</td>
<td>0.305</td>
</tr>
<tr>
<td>2</td>
<td>0.335</td>
</tr>
<tr>
<td>3</td>
<td>0.333</td>
</tr>
</tbody>
</table>

CI values shown are after correction using Chou and Talalay’s algorithm for synergy.
Figure 2.1: Motility of *H. bakeri* females over time at different concentrations of Levamisole

Motile *H. bakeri* females were isolated from the intestine of mice infected two weeks prior to each assay. Each data point represents the average number of motile parasites out of three assays. 20 adult female parasites were used per concentration, per assay. Error bars indicate sem (standard error of the mean).
Figure 2.2: Motility of *H. bakeri* females over time at different concentrations of Cry5B

Cry5B was purified via sucrose gradient for use in motility assays. Motile *H. bakeri* females were isolated from the intestine of mice infected two weeks prior to each assay. Each data point represents the average number of motile parasites out of three assays. 20 adult female parasites were used per concentration, per assay. Error bars indicate sem (standard error of the mean).
Figure 2.3: Motility of *H. bakeri* females over time at different concentrations of Levamisole combined at a 1:1 efficacy with purified Cry5B

Levamisole and Cry5B were combined at a 1:1 efficacy based upon Fa30, or 30% effective concentration values based upon the average of past assays (Figure 3.1 and Figure 3.2). These values were 47µg/mL and 0.27µg/mL for Cry5B and levamisole, respectively. 1x represents the drugs in combination at this concentration, with 2x representing double this concentration, 1/2x representing half this concentration, and so on. Motile *H. bakeri* females were isolated from the intestine of mice infected two weeks prior to each assay. Each data point represents the average number of motile parasites out of three assays. 20 adult female parasites were used per concentration, per assay. Error bars indicate sem (standard error of the mean).
REFERENCES


Chapter 3: Preliminary *in vivo* studies for further characterization of Cry5B- contribution of spores and first attempts to evaluate synergy *in vivo*
INTRODUCTION

When the bacterium *Bacillus thuringiensis* sporulates, the cell produces spores, large crystal protein-containing inclusions, and lysate (Aronson 2002). The lysate is produced when the mother cell that gave rise to the spore and crystal lyses upon completion of sporulation. Bt spore-crystal lysates (SCLs) from many Bt strains have been extensively tested against vertebrates (including humans) and found to be non-pathogenic (Betz et al. 2000; Roh et al. 2007). It has been well documented that the crystal (Cry) proteins produced by this bacteria are responsible for the bacteria’s observed insecticidal activity against a range of invertebrates (de Maagd et al. 2001). Our laboratory has focused upon a specific subset of these proteins, including Cry5B, 14A, 21A, and 13A, which demonstrate activity against nematodes. Furthermore, we have shown that treatment of both a gastrointestinal nematode infection in mice, and a hookworm infection in hamsters, with Cry5B can elicit a therapeutic effect (Hu et al. 2010; Cappello et al. 2006). As it is known that Bt spores can synergize with Cry proteins in attacking insects, we hypothesize that spores may contribute to the effect seen in our PLoS NTD publication (Aronson et al. 1982; Tang et al. 1996; Johnson et al. 1997). Herein, this question is addressed by treating a *Heligmosomoides bakeri* infection in mice with purified Cry5B/HD1, Cry5B/HD1 spore crystal lysate, or purified Cry5B/HD1 with HD1 spores. Differences in fecal egg counts following treatment and final worm burdens are compared to evaluate the contribution of Bt spores to the therapeutic benefit seen in the treatment of parasitic nematode infections in mice.

In addition, the third chapter of this thesis examines general findings utilizing the *in vivo* system established for the testing of crystal protein efficacy against
Heligmosomoides bakeri. These preliminary in vivo studies are of two types: combined replicate experiments testing Cry5B efficacy at different dosage levels in spore crystal lysate and purified forms, levamisole dose-response assays, and a pilot in vivo synergy study that evaluates purified Cry5B in combination with the L-type nicotinic acetylcholine receptor agonist, levamisole. Collectively, these studies provide useful information as to predicting outcomes of given treatments in terms of reductions in worm burdens and fecal egg counts while establishing parameters for future studies. Herein, I relay my findings pertaining to the efficacy of different forms and dosages of Cry5B and levamisole, while considering the potential effectiveness of this combination therapy against a gastrointestinal parasite in vivo.
METHODS

Animals

Female Swiss Webster white mice, purchased from Harlan Laboratories, were infected at approximately 6 weeks of age at an average weight of 25g. Mice were provided with food and water *ad libitum*. This research was approved by the UCSD Institutional Animal Care and Use Committee (IACUC), protocol number S08140. The maintenance and care of experimental animals complied with the University of California's Animal Care Program's guidelines for the humane use of laboratory animals.

Preparation of Cry5B samples

Spore lysate (SL; HD1 Cry-deficient strain) and spore-crystal lysate (SCL; HD1 transformed with Cry5B plasmid) were prepared using standard methods and then stored at −80°C (Marroquin et al. 2000). Cry5B was purified from SCL samples by solubilization in 50mM of 1M tripotassium citrate and 1M citric acid monohydrate, combined to pH 3.0, with 0.1M DTT. The solution was rocked at room temperature for five minutes. The resulting mixture was centrifuged at 10,000 rpm for 10 minutes, filtered through a 0.22 µm filter, and precipitated overnight at 4°C with 1 mL of 1M tripotassium citrate. The protein was isolated by centrifuging the solution at 3,500 rpm for 5 minutes and resuspending the pellet in distilled water.

Bioactivity of Cry5B/HD1 SCL was confirmed against *Caenorhabditis elegans* by a mortality assay over 24 hours at 25°C. The LC50 value was 3.099 µg/mL. Bioactivity of purified Cry5B was similarly confirmed against *C. elegans* by a mortality
assay over 6 days at 25°C. The LC50 value was determined to be 4.662 µg/mL. HD1 SL (Cry-minus) was confirmed to lack toxicity against *C. elegans*. On the day of use, purified Cry5B, SL and Cry5B SCL aliquots were thawed and centrifuged at 4,500 rpm for 15 minutes at 4°C and the supernatant was removed.

For the spore study, the SCL pellet was then resuspended in distilled water to a final concentration of 6.25 mg/mL. Purified protein was prepared two ways; one aliquot was resuspended in distilled water to 6.25 mg/mL and a second was resuspended in distilled water to 500 µL of 12.5 mg/mL protein, then diluted to 6.25 mg/mL with 500 µL HDI SL concentrated to double the spore concentration of the Cry5B/HD1 SCL sample for equal spore counts. Spore concentrations were determined by spreading plates of SCL or SL dilutions on LB plates with or without erythromycin, respectively, after killing vegetative cells in the samples by heating them at 65°C for one hour. Upon plating the samples, spore concentrations were found to be 2,000,000 spores/µL for both the HD1 and Cry5B/HD1 samples.

Protein concentrations were determined by comparing Cry5B band intensities for three different aliquots of protein samples to known amounts of bovine serum albumin on Coomassie-stained, 8% SDS polyacrylamide protein gels. All samples were kept on ice until gavage.

**Levamisole**

Levamisole hydrochloride was purchased as a powder from Acros Organics (cat. no. 187870100). The drug was dissolved in deionized water and freshly prepared prior to gavage.
Cry5B/HD1 spore study

On day 0, mice were infected per os with a suspension of 200 ± 10 *H. bakeri* L3 larvae in 0.1 mL of distilled water. Larvae were counted under the microscope, then drawn into a pipette tip and placed into separate glass test tubes until gavage with a blunt-ended syringe. On days 12, 14, 16, 18, and 20 post-infection (P.I.), fecal samples were collected from the mice and fecal egg counts were completed (Hu et al. 2010).

Each mouse was treated per os on day 15 P.I. with 0.1 mL of relevant treatment (25 mg/kg Cry5B SCL, purified protein, or purified protein with HD1 SL) through a blunt-ended syringe. All mice were killed by exposure to CO$_2$ on day 20 P.I. and the intestines were removed in their entirety. These were opened longitudinally with a pair of blunt-ended dissecting scissors and then placed into a 50 mL centrifuge tube with 10–20 mL of pre-warmed (37°C) PBS, pH 7.4, for approximately 1 hour to allow worms to dislodge from the intestine. The solution and intestine were examined under a microscope, using fine tweezers when necessary for further extrication of worms from the intestine, for determination of final worm burden.

**in vivo drug treatment and dose response experiments**

On day 0, mice were infected *per os* with a suspension of 200 ± 10 *H. bakeri* L3 larvae in 0.1 mL of distilled water. Larvae were counted under the microscope, then drawn into a pipette tip and placed into separate glass test tubes until gavage with a blunt-ended syringe. For the Cry5B-treatment experiments, groups of 5 to 9 mice were treated with 10, 25, 30, 50, or 90 mg/kg Cry5B in purified or SCL form. For the levamisole-
treatment experiments, groups of 4 mice were treated with 0.625, 1.25, 2.5, 5, or 10 mg/kg levamisole. Control groups of mice were matched in number to treatment groups. Mice were gavaged with a blunt-ended syringe.

On days 12, 14, 16, 18, and 20 post-infection (P.I.), fecal samples were collected from the mice and fecal egg counts conducted for each sample (Hu et al. 2010). Mice were divided into treatment groups based upon fecal egg counts of day 12 P.I. Each mouse was treated per os on day 15 P.I. with 0.1 mL of relevant treatment (purified Cry5B, levamisole, or water) through a blunt-ended syringe. All mice were killed through CO₂ exposure on day 20 P.I. and intestines removed in their entirety. These were subsequently opened longitudinally utilizing a pair of blunt-ended dissecting scissors and then placed into a 50 mL centrifuge tube with 10–20 mL of pre-warmed (37°C) PBS, pH 7.4, for approximately 1 hour to allow worms to dislodge from the intestine. The solution and intestine were examined under a microscope, using fine tweezers when necessary for further extrication of worms, to determine final worm burden.

**Pilot in vivo synergy experiment**

On day 0, 77 mice were infected per os with a suspension of 200 ± 10 *H. bakeri* L3 larvae in 0.1 mL of distilled water. Larvae were counted under the microscope, drawn into a pipette tip and placed into separate glass test tubes prior to gavage with a blunt-ended syringe. For the levamisole dose curve experiment, 24 mice were infected (with 6 mice used per group) to test levamisole concentrations at 1.25, 1.0, 0.75, and 0.5 mg/kg. For the Cry5B dose curve experiment, 28 mice were infected (with 7 mice used per group) to test purified Cry5B concentrations at 90, 30, 10, and 5 mg/kg.
combination experiment, 18 mice were infected (with 6 mice used per group). The three combination concentrations tested were represented as 1x, 1/2x, and 1/4x, were 15 mg/kg 5B + 0.75 mg/kg levamisole, 7.5 mg/kg 5B + 0.375 mg/kg levamisole, and 3.75 mg/kg 5B + 0.1875 mg/kg levamisole, respectively. A control group of 7 infected mice (treated with water) was also utilized in this experiment. Mice were gavaged with a blunt-ended syringe.

On days 12, 14, 16, 18, and 20 post-infection (P.I.), fecal samples were collected from the mice and fecal egg counts were conducted for each sample (Hu et al. 2010). Each mouse was treated *per os* on day 15 P.I. with 0.1 mL of relevant treatment (purified Cry5B, levamisole, combined treatment, or water) through a blunt-ended syringe. All mice were killed by exposure to CO₂ on day 20 P.I. and the intestines were removed in their entirety. These were opened longitudinally with a pair of blunt-ended dissecting scissors and then placed into a 50 mL centrifuge tube with 10–20 mL of pre-warmed (37°C) PBS for approximately 1 hour to allow worms to dislodge from the intestine. The solution and intestine were examined under a microscope, using fine tweezers when necessary for further extrication of worms from the intestine, to determine final worm burden.

**Cry5B/HD1 spore study statistical analysis**

Data analysis of the intestinal worm burdens and fecal egg counts was carried out and plotted using Prism 5 (GraphPad Software Inc., La Jolla, CA, U.S.A.). For worm burdens, “average” indicates the average worm burdens amongst all the mice in each treatment group. For fecal egg counts, “average” indicates the egg count per mouse
averaged from all mice in the group at a given time point. Fecal egg count data was analyzed via pair-wise comparisons between groups and days through two-way analysis of variance (ANOVA), with repeated measures and Bonferroni post-tests. Results were as follows: $F_{\text{Treatment}} = 53.65$, degrees of freedom (df) = 12, $P<0.0001$; $F_{\text{Time}} = 102.6$, df = 3, $P<0.0001$; $F_{\text{Interaction}} = 13.60$, df = 4, $P<0.0001$. Worm burdens for the different protein treatments were analyzed via pair-wise comparisons between groups and days through a one-way ANOVA and Kruskal-Wallis test, as befitting nonparametric data. Values are as follows: $P = 0.0130$ with a Kruskal-Wallis statistic of 10.78, Difference in rank sum=11.83 for water versus SCL-treatment, 11.17 for water versus purified Cry5B with spores-treatment, and 6.33 for water versus purified Cry5B treatment. Effects of protein treatments on worm burden were analyzed as compared to the water-treated control group via a Mann-Whitney U test (one-tailed). Values are as follows: $U = 7.0$, $P = 0.0461$ for purified Cry5B versus water treatment; $U = 3.0$, $P = 0.0100$ for Cry5B SCL versus water treatment; $U = 0.0$, $P = 0.0025$ for purified Cry5B with HD1 spores versus water treatment.

Statistical analysis of in vivo drug treatment and dose response experiments

Data analysis of all intestinal worm burdens and fecal egg counts was carried out and plotted using Prism 5 (GraphPad Software Inc., La Jolla, CA, U.S.A.). For worm burdens, “average” indicates the average worm burdens amongst all the mice in each treatment group. For fecal egg counts, “average” indicates the egg count per mouse averaged from all mice in the group at a given time point. Fecal egg count data was analyzed via pair-wise comparisons between groups and days through two-way ANOVA.
with repeated measures and Bonferroni post-tests. Worm burden data for multiple treatment groups was analyzed through a one-way ANOVA and Kruskal-Wallis test. Worm burden data for single-treatment effects (purified protein alone, or SCL alone, versus water) was analyzed by Mann-Whitney U-test.

Worm burden statistics were as follows: U = 11.0, P = 0.0013 for the combined 50 mg/kg Cry5B SCL treatment data; U = 12.0, P = 0.0016 for the combined 50 mg/kg purified Cry5B treatment data. Statistics for the other worm burden analyses were: P = 0.0014 with a Kruskal-Wallis statistic of 19.77 for the pilot levamisole dose-response assay, P = 0.0012 with a Kruskal-Wallis statistic of 15.86 for the initial purified Cry5B dose-response assay, and P < 0.0001 with a Kruskal-Wallis statistic of 28.89 for compiled purified Cry5B dose-response results.

Fecal egg count statistics were as follows: for the combined 50 mg/kg purified Cry5B data \( F_{\text{Treatment}} = 68.80 \), degrees of freedom (df) = 1, \( P<0.0001 \); \( F_{\text{Time}} = 2.996 \), df = 4, \( P = 0.0221 \); \( F_{\text{Interaction}} = 7.024 \), df = 4, \( P<0.0001 \); for the combined 50 mg/kg Cry5B SCL treatment data \( F_{\text{Treatment}} = 68.84 \), degrees of freedom (df) = 1, \( P<0.0001 \); \( F_{\text{Time}} = 2.342 \), df = 4, \( P = 0.0600 \); \( F_{\text{Interaction}} = 6.251 \), df = 4, \( P = 0.0002 \). For the pilot levamisole dose-response assay: \( F_{\text{Treatment}} = 4.216 \), degrees of freedom (df) = 5, \( P = 0.0017 \); \( F_{\text{Time}} = 4.855 \), df = 4, \( P = 0.0014 \); \( F_{\text{Interaction}} = 1.091 \), df = 20, \( P = 0.3732 \). For the initial purified Cry5B dose-response assay: \( F_{\text{Treatment}} = 5.696 \), degrees of freedom (df) = 3, \( P = 0.0010 \); \( F_{\text{Time}} = 13.43 \), df = 4, \( P<0.0001 \); \( F_{\text{Interaction}} = 1.581 \), df = 12, \( P = 0.1020 \). For the compiled purified Cry5B dose-response results: \( F_{\text{Treatment}} = 14.34 \), degrees of freedom (df) = 3, \( P<0.0001 \); \( F_{\text{Time}} = 22.83 \), df = 4, \( P<0.0001 \); \( F_{\text{Interaction}} = 2.529 \), df = 12, \( P = 0.0035 \).
Statistical analysis of pilot in vivo synergy experiment

Data analysis of intestinal worm burdens and fecal egg counts was carried out and plotted using Prism 5 (GraphPad Software Inc., La Jolla, CA, U.S.A.). For worm burdens, “average” represents the average worm burdens amongst all the mice in each treatment group. For fecal egg counts, “average” represents the egg count per mouse averaged from all mice in the group at a given time point. Fecal egg count data was analyzed via pair-wise comparisons between groups and days through two-way ANOVA with repeated measures and Bonferroni post-tests. Results were as follows for Cry5B treatment alone: $F_{\text{Treatment}} = 8.09$, degrees of freedom (df) = 4, $P<0.0001$; $F_{\text{Time}} = 15.44$, df = 4, $P<0.0001$; $F_{\text{Interaction}} = 1.33$, df = 16, $P = 0.3042$. For levamisole treatment alone: $F_{\text{Treatment}} = 1.10$, degrees of freedom (df) = 4, $P = 0.3582$; $F_{\text{Time}} = 2.02$, df = 4, $P = 0.0949$; $F_{\text{Interaction}} = 0.31$, df = 16, $P = 0.9946$. For combinations: $F_{\text{Treatment}} = 4.14$, degrees of freedom (df) = 3, $P = 0.0081$; $F_{\text{Time}} = 8.21$, df = 4, $P<0.0001$; $F_{\text{Interaction}} = 1.18$, df = 12, $P = 0.3042$. Worm burdens for treatments versus the water-treated control group were compared using one-way ANOVA and Tukey’s Multiple Comparison Test (one-tailed). Values are as follows: $F = 6.760$, df = 4, $P = 0.0005$ for Cry5B treatments; $F = 5.925$, df = 4, $P = 0.0016$ for levamisole treatments; $F = 4.681$, df = 3, $P = 0.0118$ for combination treatments.
RESULTS

To determine if Cry5B, when combined with spores, was more efficacious against a gastrointestinal parasitic nematode infection, *H. bakeri*-infected mice were treated *per os* with 0.1 mL of either Bt spore-crystal lysate expressing Cry5B, purified Cry5B protein, or purified Cry5B protein with HD1 spores on day fifteen post-infection. All proteins were delivered at a dose of 25 mg/kg. Reductions in fecal egg counts and final worm burdens were compared to a control group treated with distilled water. Six mice were used per treatment group. Beginning three days before treatment (day 12 P.I. or day -3 treatment), and then continuing every other day (day -1, 1, 3, 5 pre- and post-treatment), fecal samples were collected from each mouse to measure parasite progeny production (eggs/gram of feces). Five days after treatment (day 20 P.I.), the mice were euthanized and the total number of parasites present in the small intestine tallied.

With regards to progeny production, we found that during the day prior to treatment, the parasites in all groups of mice were producing statistically indistinguishable amounts of eggs (Figure 3.1, Table 3.1). During days 1, 3, and 5 post-treatment, the water-treated group showed no significant reduction in egg production, establishing a baseline against which reduction in fecal egg counts of treatment groups could be measured. Treatment groups all showed a dramatic reduction in egg production, with Cry5B SCL, purified Cry5B, and purified Cry5B combined with HD1 spores resulting in a 94%, 85%, and 90% reduction, respectively, in the day following treatment (Figure 3.1, Table 3.1). With regards to parasite clearance, we found that a single dose of 25 mg/kg Cry5B SCL cleared away 68% of the parasites relative to control (Figure 3.2, Table 3.2). Purified Cry5B and purified Cry5B with HD1 spores were able to elicit
similar reductions in worm burdens, at 43% and 67%, respectively (Figure 3.2, Table 3.3). Thus, a single dose of 25 mg/kg Cry5B has strong effects on parasite reproduction and the ability of parasites to maintain an infection. All reductions in worm burden elicited by Cry5B treatments were significant when compared to the control alone. Reductions in worm burdens were not significant when compared to each other in any spore/protein or protein-treatment group, suggesting that the observable effect could not be explained by the presence of spores in the treatment.

As noted with our prior Cry5B therapy experiment, the reduction in fecal egg counts for each treatment group (>85%) was much larger than would be expected from the final mouse worm burden of all treated animals (43%-67% cleared). This is significant considering the low concentration of protein that was given (25 mg/kg)—suggesting that the treatment was negatively affecting the health of the worms through a causal effect that is not explained by the reduction in worm burden. Furthermore, although fecal egg counts were not all reduced to the same level, there was no significant difference between treatment groups in reduction of fecal egg counts. All treatment groups showed a significant difference in fecal egg count reduction relative to the water-treated control. These data suggest that the health of parasites remaining in the intestine was severely compromised to the same degree, regardless of the presence of spores. Based upon our data, the capacity of Cry proteins to clear an infection and compromise the health of the parasites is independent of the presence of spores in the given treatment. Furthermore, there is no significant difference in the efficacy of Cry5B in SCL or purified form when delivered at 25 mg/kg.
To better characterize *in vivo* studies testing anthelmintic efficacy, worm burden and fecal egg count results from many experiments were considered for analysis. Doses of 50 mg/kg Cry5B in SCL and purified forms were tested twice, with results of the two different experiments considered in combination (Figures 3.3 and 3.4). For the 11-mice treatment groups, 50 mg/kg spore crystal lysate reduced worm burdens by 66.9%, while the same dose of purified Cry5B reduced worm burdens by 62.1% relative to the water-treated control group. Both effects were very significant (p < 0.01) and the reductions were not statistically distinguishable from one another. Fecal egg count data reiterated this finding; the egg production of the water-treated control group was statistically insignificant in comparison to treatment groups up until the days following treatment (p < 0.001). In the day following treatment, SCL reduced fecal egg counts by 94.3% while purified protein reduced counts by 92.9% (Figure 3.5). Purified protein was used for follow-up studies, since Bt cells proved much harder to concentrate to desired dosages than purified protein.

Purified Cry5B was tested at concentrations of 90, 30, and 10 mg/kg prior to the *in vivo* dose curve assay later conducted in tandem with the synergy assay. The different treatment dosages are analyzed here in combination with findings from that synergy study (Figure 3.6). The combined tests comprised 11 mice per group, and it was found that 90, 30, and 10 mg/kg purified Cry5B effectively reduced worm burdens by 58.4%, 57.5%, and 37.5%, respectively, as compared to the experimental control group. Fecal egg counts were decreased by 88.5%, 83.7%, and 79.4%, respectively, as compared to the control on the day following treatment. None of the decreases were significant from one another for the different treatment concentrations.
Levamisole was tested at concentrations of 10, 5, 2.5, 1.25, and 0.625 mg/kg prior to an \textit{in vivo} dose curve assay, which, like the purified Cry5B dose response assay, was conducted in parallel with the \textit{in vivo} synergy assay. Although levamisole treatment was able to reduce worm burdens by 100\%, 99\%, 91\%, 92.5\%, and 34.3\%, respectively, at these concentrations, fecal egg counts were not significantly reduced, as compared to the control group and the other treatment groups, during the course of the experiment (Figure 3.7). The decreases in worm burdens between different treatment concentrations were only significant between 10 mg/kg and 0.625 mg/kg dosages, $p < 0.05$.

An overall evaluation of combined worm burden and fecal egg count data showed that these \textit{in vivo} studies using \textit{H. bakeri} were extremely variable between mice in treatment groups. We also had difficulty in developing a full dose response range for either anthelmintic. One consistent finding, however, was that Cry5B treatment of infection always significantly decreased fecal egg counts far more dramatically than would be expected from reduction in worm burden alone. Additionally, preliminary dose response data, and following synergy study results, reveal that levamisole may not share this same therapeutic effect.

In the preliminary study assessing the synergistic potential of Cry5B and levamisole \textit{in vivo}, we designed an experiment similar to \textit{in vitro} studies, employing dose curves of each drug alone to predict and analyze results in combination. Instead of scoring inhibition of motility, however, reduction in final worm burdens and fecal egg counts were used as indicators of drug effect at different concentrations. For the dose curves, drug concentration ranges were chosen based upon prior knowledge of drug effect against \textit{H. bakeri} for the parameters of our experiment. To study the effect of drug
combinations, small concentrations of each compound were chosen, as we hoped to observe a therapeutic effect much greater than otherwise expected, as defines synergy.

As predicted by pooled in vivo data, final worm burdens of mice treated with either levamisole or purified Cry5B did not show a strong dose response to given treatments. Purified Cry5B resulted in worm burden reductions of 54.4, 60.8, 29.9, and 20.7% at 90, 30, 10, and 5 mg/kg dosages, respectively (Figure 3.8). Only reductions between 30 and 5 mg/kg dosages were significant when compared to one another. One mouse in the 90 mg/kg treatment group had an unusually high worm burden upon sacrifice, which may explain the deviation from a normal dose curve, but as the animal did not show compromised health and only 7 mice were used per treatment group, the data was not excluded from analysis. Levamisole resulted in worm burden reductions of 62.5, 39.2, 52.3, and 41.3% at 1.25, 1.0, 0.75, and 0.5 mg/kg dosages, respectively (Figure 3.9). None of these decreases were found to be significant from each other. As there was no true dose response in the reductions in worm burden of the assays, we could not calculate the degree of synergy of the two compounds in combination with regards to worm burden reductions using the combination index (CI) algorithm of Chou and Talalay (Chou 2006; Figure 3.10).

Analysis of fecal egg count data, however, showed a different synergy profile for the two compounds in combination. Fecal egg counts of the given treatments revealed that levamisole was unable to elicit a significant reduction in egg production relative to the water-treated control group during the entire course of the experiment, just as in the preliminary dose response study. In fact, fecal egg counts actually increased the day following treatment at 1 mg/kg, 0.75 mg/kg, and 0.5 mg/kg (Figure 3.9). 1.25 mg/kg of
levamisole resulted in a 39% reduction in mean fecal output as compared to the control group, but 1, 0.75, and 0.5 mg/kg levamisole showed a 49, 19, and 11% increase in fecal egg counts, respectively. As a nicotinic acetylcholine receptor agonist, levamisole is expected to increase parasite egg output, but the results are surprising when considering the >40% reductions in worm burdens at every concentration of levamisole tested. The in vivo levamisole dose response assay deserves confirmatory testing to corroborate this effect.

In contrast, Cry5B treatment demonstrated dramatic decreases in fecal egg counts at every concentration tested, especially on the day following treatment. This result was consistent between experiments, and at all doses of Cry5B tested. Compared to the control, 90, 30, 10, and 5 mg/kg doses reduced fecal egg counts by 88, 86, 79, and 57%, respectively (Figure 3.8). None of these effects were significant when compared to one another. The results are striking, though, when compared to the corresponding final worm burdens at each concentration, as the decreases are much larger than expected from the reductions in parasite burden alone, at roughly 20-60%.

The resulting reductions in fecal egg counts of the combined treatments were slightly higher than expected, considering the corresponding reductions in worm burdens. A combined treatment of 15 mg/kg Cry5B and 0.75 mg/kg levamisole showed a 71% reduction in fecal egg counts (Figure 3.10). A combined treatment at half that concentration resulted in a 33% reduction in egg production, while treatment with one-quarter of the concentration elicited a 74% decrease in egg counts, as compared to the control group. Acknowledging inconsistencies in fecal egg counts of the dose response
and combination assays, however, we were again unable to analyze drug interactions for synergy using combination index values.
DISCUSSION

The Cry5B/HD1 spore study shows that spores do not significantly contribute to the therapeutic effect of Cry proteins, and confirms previous findings that the Bt Cry protein Cry5B is an excellent anthelmintic \textit{in vivo} against a natural and chronic intestinal roundworm infection in mice. Cry5B is able to achieve significant reductions in parasite egg production and intestinal worm burdens in both purified and SCL forms following a single dose delivered \textit{per os} at only 25 mg/kg. There is no significant difference between the presence and absence of spores in combination with the protein with regards to observed therapeutic effects, as evidenced by lack of statistical change in worm burdens and fecal egg counts.

The preliminary experiments with Cry5B and levamisole provide valuable insights into the therapeutic effects of a pore forming toxin and a nicotinic acetylcholine receptor agonist in a living system. Results suggest that there are remarkably significant differences in pharmacological effects between anthelmintics of different mechanisms. Although Cry5B treatment has yet to render a complete cure in terms of worm burden reduction, it demonstrates a potent and consistent efficacy with regards to reducing fecal egg output. Since the lifecycles of intestinal roundworms depend upon the passage of eggs through host fecal matter, therapies that hamper the ability of parasites to produce eggs or that neutralize female parasites can be extremely effective in preventing disease transmission. Based upon our initial studies with levamisole, nAChR agonists may not be preferred anthelmintics as they may stimulate egg production by parasites that are not eliminated from the gut following treatment. This finding may elucidate broader
considerations with regard to anthelmintic effects, which could be missed by analysis of final worm burdens alone.

Another consideration with regard to limiting the metrics in anthelmintic evaluations is that studies employing immunocompetent hosts demonstrate marked variability, even within treatment groups. Our results are consistently nonparametric for both fecal egg counts and worm burdens. Consequently it is difficult to obtain a full dose response range for given treatments. Based upon this observation, it would be desirable to increase the size of treatment groups for future in vivo studies. In establishing dose curves for any given anthelmintic, it may be more informative to have fewer treatment groups, with more animals in each group, as opposed to more treatment groups.

Although levamisole and Cry5B show synergy when combined at a 1:1 efficacious ratio against a gastrointestinal parasite in vitro, data is not sufficient to confirm the same for the two in vivo. While the CI values of in vitro experiments against a gastrointestinal parasite compare favorably with studies completed with C. elegans, CI values for the in vivo synergy experiment could not be calculated due to discrepancies in dose curve data for both fecal egg counts and worm burdens. Additional studies are necessary to map dose responses in worm burden and fecal egg count reductions following levamisole- and purified Cry5B-treatment of H. bakeri infections.

To further evaluate whether Cry5B and the nAChR agonist levamisole mutually potentiate each other in vivo, it may be helpful to include another parameter for drug effect, such as recovery of immune function. With regard to Cry5B, carrying out the experiment over a longer time frame, or sacrificing the mice later in the study, may determine whether lowered fecal egg counts, indicate that the worms are actually being
eliminated from the host. As previously noted, larger treatment groups should be utilized to mitigate the impact of variations in worm burden and fecal egg count data.

Furthermore, it would be beneficial to examine drug effect against not only *H. bakeri* but also rodent analogs of human parasites, such as whipworm and hookworm, or *Trichuris muris* and *Ancylostoma ceylanicum*. Overall, the promising data presented in this chapter encourages further characterization of the interaction of crystal proteins with the nicotinic acetylcholine receptor agonist class of anthelmintics.
### Table 3.1: Fecal egg counts in spore study

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<th>Day number relative to treatment</th>
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<td>Water</td>
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<tr>
<td>Cry5B SCL</td>
<td>6689±873</td>
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<tr>
<td>Purified Cry5B</td>
<td>4744±372</td>
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<td>Purified Cry5B + spores</td>
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Numbers shown are eggs/gram of feces/mouse averaged for all 6 mice in each group ± standard error of the mean (sem).
Table 3.2: Fecal egg count reductions in spore study relative to control

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</tbody>
</table>

Numbers shown are percent reduction in eggs/gram of feces/mouse averaged for all 6 mice in each group as compared to water-treated control for each day.
**Table 3.3:** Worm burdens in spore study.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Intestinal worm burden (mean ± sem)</th>
<th>% Reduction relative to control</th>
</tr>
</thead>
<tbody>
<tr>
<td>Water</td>
<td>131.3 ± 6.1</td>
<td>N/A</td>
</tr>
<tr>
<td>Cry5B SCL</td>
<td>42.7 ± 8.9</td>
<td>67.5</td>
</tr>
<tr>
<td>Purified Cry5B</td>
<td>74.5 ± 10.6</td>
<td>43.3</td>
</tr>
<tr>
<td>Purified Cry5B + HD1 spores</td>
<td>43.5 ± 6.2</td>
<td>66.9</td>
</tr>
</tbody>
</table>

All proteins were given at 25mg/kg mouse body weight.

*sem = standard error of the mean.*

*N/A = not applicable.*
Figure 3.1: Effects of Cry5B SCL, purified protein, and purified protein with HD1 spores on fecal egg counts of *Heligmosomoides bakeri* infected mice.

Shown are the average eggs/gram of feces/mouse for both control (n = 6) and Cry5B spore crystal lysate, purified protein, and purified protein with HD1 spore-treated groups (n = 6) treated groups three days before treatment (−3) and then every other day thereafter until the animals were euthanized on day 5 post-treatment. Results analyzed with Two-way ANOVA and Bonferroni post-test for each treatment group relative to the water-treated control. ***p<0.001
Figure 3.2: Effects of Cry5B SCL, purified protein, and purified protein with HD1 spores on intestinal worm burdens in *Heligmosomoides bakeri* infected mice.

Shown are the intestinal worm burdens in water, Cry5B/HD1 SCL, purified Cry5B, and purified Cry5B with HD1 spores-treated mice (n=6 mice per group). Infected mice were given a single dose of 178 nmoles/kg (25 mg/kg) Cry5B on day 15 P.I. and intestinal worm burdens assessed on day 20 P.I. The worm burdens in each mouse are indicated with a separate symbol. Long horizontal bars represent mean worm burdens; smaller bars indicate sem (standard error of the mean). Data was analyzed using the nonparametric Mann-Whitney test to compare treatment groups to the water-treated control group. Effects of Cry5B treatment were compared to each other via One-way ANOVA with a Kruskal-Wallis test and differences were not found to be significant. **p<0.01,*p<0.05
Figure 3.3: Effects of Cry5B SCL on intestinal worm burdens in *Heligmosomoides bakeri* infected mice.

Shown are the intestinal worm burdens in water and Cry5B (HD1 SCL) treated mice (n = 11 each group). Infected mice were given a single dose of 357.5nmole/kg (50 mg/kg) Cry5B on day 15 P.I. and intestinal worm burdens assessed on day 20 P.I. The worm burdens in each mouse are indicated with a separate symbol. Long horizontal bars represent mean worm burdens; smaller bars indicate sem (standard error of the mean). **p<0.01
Figure 3.4: Effects of purified Cry5B on intestinal worm burdens in *Heligmosomoides bakeri* infected mice.

Shown are the intestinal worm burdens in water and purified Cry5B treated mice (n = 11 each group). Infected mice were given a single dose of 357.5 nmol/kg (50 mg/kg) Cry5B on day 15 P.I. and intestinal worm burdens assessed on day 20 P.I. The worm burdens in each mouse are indicated with a separate symbol. Long horizontal bars represent mean worm burdens; smaller bars indicate sem (standard error of the mean). Results were analyzed with a Mann-Whitney U test. **p<0.01
Figure 3.5: Effects of 2 different forms of Cry5B on egg production in *Heligmosomoides bakeri* infected mice.

Shown are the average eggs/gram of feces/mouse for both water (n = 11) and Cry5B-(n = 11) treated groups two days before treatment (−3) and then every other day thereafter until the animals were euthanized on day 5 post-treatment. Results were analyzed using Two-way ANOVA with a Bonferroni post-test. ***p<0.001
Figure 3.6: Effects of 3 different dosages of Cry5B on intestinal worm burdens and fecal egg counts in *Heligmosomoides bakeri* infected mice.

A. Shown are the combined intestinal worm burdens in mice from 2 experiments treated with water or 90, 30, or 10 mg/kg purified Cry5B (n = 16 each group). Infected mice were given a single dose of Cry5B on day 15 P.I. and intestinal worm burdens assessed on day 20 P.I. The worm burdens in each mouse are indicated with a separate symbol. Long horizontal bars represent mean worm burdens; smaller bars indicate SEM (standard error of the mean). Results were analyzed via one-way ANOVA with a Kruskal-Wallis test. ***p<0.001, *p<0.05

B. Shown are the fecal egg counts from mice in “A” treated with water or purified Cry5B. Error bars represent standard error of the mean (SEM). Results were analyzed with Two-way ANOVA and a Bonferroni post-test. ***p<0.001, **p<0.01, *p<0.05
Figure 3.7: Effects of 5 different dosages of levamisole on intestinal worm burdens and fecal egg counts in *Heligmosomoides bakeri* infected mice.

A. Shown are the intestinal worm burdens in mice treated with water or 10, 5, 2.5, 1.25 or 0.625mg/kg purified Cry5B (n = 4 each group). Infected mice were given a single dose of levamisole on day 15 P.I. and intestinal worm burdens assessed on day 20 P.I. The worm burdens in each mouse are indicated with a separate symbol. Long horizontal bars represent mean worm burdens; smaller bars indicate sem (standard error of the mean). Results were analyzed via One-way ANOVA with a Kruskal-Wallis test. **p<0.01, *p<0.05

B. Shown are the fecal egg counts from mice in “A” treated with water or levamisole. Error bars represent standard error of the mean (sem). Results were analyzed with Two-way ANOVA and a Bonferroni post-test.
Figure 3.8: Pilot Synergy Study: Effects of purified Cry5B at 4 different dosages on intestinal worm burdens and fecal egg counts in *Heligmosomoides bakeri* infected mice.

A. Shown are the intestinal worm burdens in mice treated with water or 90, 30, 10, or 5mg/kg purified Cry5B (n = 6 each group). Infected mice were given a single dose of purified protein on day 15 P.I. and intestinal worm burdens assessed on day 20 P.I. The worm burdens in each mouse are indicated with a separate symbol. Long horizontal bars represent mean worm burdens; smaller bars indicate sem (standard error of the mean). Results were analyzed via One-way ANOVA with a Tukey post-test. ***p<0.001, **p<0.01

B. Shown are the fecal egg counts from mice in “A” treated with water or purified protein. Mice were divided into treatment groups based upon fecal egg count data from three days prior to treatment (-3). Error bars represent standard error of the mean (sem). Results were analyzed with Two-way ANOVA and a Bonferroni post-test. ***p<0.001, **p<0.01, *p<0.05
Figure 3.9: Pilot Synergy Study: Effects of levamisole at 4 different dosages on intestinal worm burdens and fecal egg counts in *Heligmosomoides bakeri* infected mice.

A. Shown are the intestinal worm burdens in mice treated with water or 1.25, 1, 0.75, or 0.5mg/kg levamisole Cry5B (n = 6 each group except water; n = 7). Infected mice were given a single dose of levamisole on day 15 P.I. and intestinal worm burdens assessed on day 20 P.I. The worm burdens in each mouse are indicated with a separate symbol. Long horizontal bars represent mean worm burdens; smaller bars indicate sem (standard error of the mean). Results were analyzed via One-way ANOVA with a Tukey post-test. **p<0.01, *p<0.05

B. Shown are the average eggs/gram of feces/mouse for both control (n = 7) and levamisole-treated groups (n = 6) in “A” three days before treatment (−3) and then every other day thereafter until the animals were euthanized on day 5 post-treatment. Results analyzed with Two-way ANOVA and Bonferroni post-test.
Figure 3.10: Pilot Synergy Study: Effects of Cry5B/levamisole combination treatment on intestinal worm burdens and fecal egg counts in *Heligmosomoides bakeri* infected mice.

A. Shown are the intestinal worm burdens in drug combination (n= 6) and water/control-treated mice (n= 7). Infected mice were treated on day 15 P.I. and intestinal worm burdens assessed on day 20 P.I. 1x, 1/2x, and 1/4x represent 15mg/kg 5B + 0.75mg/kg levamisole, 7.5mg/kg 5B + 0.375mg/kg levamisole, and 3.75mg/kg 5B + 0.1875mg/kg levamisole, respectively. The worm burdens in each mouse are indicated with a separate symbol. Long horizontal bars represent mean worm burdens; smaller bars indicate sem (standard error of the mean). Percentages represent percent reduction in worm burden as compared to water-treated control. Data was analyzed using One-way ANOVA with a Tukey post-test. *p<0.05

B. Shown are the average eggs/gram of feces/mouse for both control (n = 7) and combination-treated groups (n = 6) three days before treatment (−3) and then every other day thereafter until the animals were euthanized on day 5 post-treatment. 1x, 1/2x, and 1/4x represent 15mg/kg 5B + 0.75mg/kg levamisole, 7.5mg/kg 5B + 0.375mg/kg levamisole, and 3.75mg/kg 5B + 0.1875mg/kg levamisole, respectively. Results analyzed with Two-way ANOVA and Bonferroni post-test. *p<0.05
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Chapter 4: The establishment and maintenance of *Ancylostoma ceylanicum* as a model organism for laboratory studies
INTRODUCTION

*Heligmosomoides bakeri* is an ideal laboratory model for a gastrointestinal parasitic nematode infection as it is easily maintained in a laboratory setting and its utility in anthelmintic testing is well documented (Behnke et al. 2009; Boes and Helwig 2000; Fonseca-Salamanca et al. 2003; Monroy and Enriquez 1992; Stepek et al. 2007). The development of infectious, L3 larvae of *H. bakeri* is easily arrested at 4°C, and the larvae remain highly infective for up to 6 months after culturing. The organism also has a short lifecycle, in which the time from egg to egg can take about 14 days. Furthermore, *H. bakeri* has a high infection rate in an immunocompetent host, with about 75% of larvae establishing in the small intestine of mice following oral gavage. However, nematode phylogeny is extremely complex, and effects of treatments evaluated against just one parasitic roundworm do not necessarily hold true between nematodes. Albendazole, for example, which is currently the most effective anthelmintic recommended for mass drug administration due to its efficacy against hookworm infections, was only able to effect a 48% reduction in *H. bakeri* worm burden when delivered as a 100 mg/kg dose on day 6 post-infection (Sayles and Jacobson 1983).

With these considerations, our laboratory would prefer to focus on model roundworm parasites such as *Ancylostoma ceylanicum* for anthelmintic studies. *A. ceylanicum* is of the same genus as one of the most pernicious of human gastrointestinal nematode infections. *Ancylostoma duodenale* and *Necator americanus* are estimated to infect 600 to 700 million people worldwide, and as blood feeding parasites they are a major cause of anemia and malnutrition (Bethony et al. 2006). Furthermore, resistance to the benzimidazole family of compounds, to which albendazole belongs, has already been
noted in Ethiopia, Sri Lanka, Vietnam and Tanzania (Gunawardena et al. 2008; Adugna et al. 2007; Flohr et al. 2007; Albonico et al. 2002). A laboratory model of these human parasites would clearly be preferred for potential anthelmintic efficacy and synergy studies, as examined in this thesis.

*A. ceylanicum* is a laboratory hookworm model capable of infecting immunocompetent hamsters. Preliminary *in vivo* and *in vitro* studies have shown that crystal proteins such as Cry5B demonstrate therapeutic effects against *A. ceylanicum*, but the results have not been followed-up (Cappello et al. 2006). Although it is desirable to utilize this human hookworm analog for further investigation into the anthelmintic properties and synergistic potential of crystal proteins, the maintenance of the roundworm’s lifecycle presents several challenges. First, the larvae have a low infection rate in hamsters. Only about a third of infectious larvae establish in the small intestine of their host (Cappello et al. 2006). Also, the larvae only live for about one month at room temperature. The lifecycle is difficult to maintain as even heavily infected hamsters have very low fecal egg counts. Fecal collections are arduous to conduct as a large number of hamsters must be employed to obtain enough samples and the samples must be kept moist so that the eggs do not dry out. Additionally, *A. ceylanicum* is a skin-penetrator in addition to being transmitted through a fecal-oral route, and is capable of infecting humans in addition to hamsters, so extra precautions must be taken when attempting to work with infected animals or their fecal matter.

For the above reasons, and due to the lack of funding that is available for human intestinal roundworm studies, very few laboratories utilize *A. ceylanicum* as a model for human parasitic roundworms. In fact, only two other laboratories in the United States
maintain *A. ceylanicum*. Yale University and George Washington University comprise the entire American task force investigating hookworm pathology for innovative anthelmintic and vaccine development. As our laboratory seeks to develop new drugs for treatment of human helminth diseases, it is in our best interest to overcome these challenges and establish *A. ceylanicum* as a laboratory model with which to conduct further studies as to the efficacy of crystal proteins against human gastrointestinal nematodes. In the final chapter of this thesis, I present a description of techniques, garnered from collaboration with researchers at George Washington University, used to maintain *A. ceylanicum* in our own laboratory, as well as a modified protocol for egg isolation, allowing for the establishment of developmental assays to determine anthelmintic effect on different stages of the hookworm lifecycle.
METHODS

Hamsters

Male Golden Syrian hamsters were purchased from Harlan Laboratories and were infected by oral gavage with 150 L3 larvae of *A. ceylanicum*. The hamsters were approximately 4-5 weeks of age. Hamsters were provided with food and water *ad libitum*. Hamsters were housed 4-5 animals per cage and 15-20 animals were used to maintain the parasite lifecycle. This research was approved by the UCSD Institutional Animal Care and Use Committee (IACUC), protocol number S09067. The maintenance and care of these experimental animals complied with the University of California's Animal Care Program's guidelines for the humane use of laboratory animals.

Fecal egg collections

Feces were collected from two cages of the infected hamsters every day starting on day 18 post-infection. Cages of animals were rotated for collections, so that no animal was utilized two nights in a row. For each fecal collection, one cage of hamsters (4-5 animals) was placed in a raised, wire-bottom cage over a plastic collection pan. Six paper towels were used to line the pan bottom, and roughly 300 mL of tap water was added to the pan. The fecal collection chamber was left overnight with enough water in the pan to keep the towels moist overnight (amount varies depending on room humidity and time frame of collection). Feces were isolated with a stainless steel scraper and placed in a 50 mL conical tube, precluding food particles as much as possible. Feces could be kept at 4°C for up to one week before proceeding to culture infectious L3 larvae. Immediate culturing of larvae, however, is recommended to obtain a higher yield.
Conducting fecal egg counts

Fecal egg counts for *A. ceylanicum*-infected animals could be conducted in the same fashion as counts for *H. bakeri*-infected animals (Hu et al. 2010). Fecal egg counts will peak around day 19 post-infection (Cappello et al. 2006). Typically, a good yield fecal egg count is around 1500 eggs per gram of feces. Fecal egg counts can be carried out up to 3 months post-infection.

Culturing of infectious larvae

All steps in the culturing of infectious *A. ceylanicum* L3 larvae should be done at room temperature. Equal parts feces and tap water are mixed together in a plastic tub with a stainless steel scraper for 2 minutes. If fecal pellets are still hard and the mixture doesn’t homogenize, the slurry is allowed to sit for an hour to absorb water before further mixing. Activated charcoal is added at slightly more than the same volume as the total mixture (total volume water and fecal matter) to absorb ammonia. If there is a lot of excess water in the mixture, more charcoal is added. Once the mixture is well blended and holds its shape, the contents are transferred to 150 mm plastic petri dishes until the dishes are three-quarters filled and contents are well distributed. The lids are labeled with the date and species. Dishes are stacked upright in a 28°C incubator. A large pan of water is set in the incubator to add humidity. The cultures do not need to be checked before 7 days in the incubator, except to add more water to the pan of water, if necessary.

Harvesting infectious larvae
All steps in the harvesting of infectious larvae are carried out using tap water at 40°C. 1 L plastic, conical graduates are filled with warm water to about 5 cm below the top, with one graduate per culture dish. 1 layer of cheesecloth is spread over the top of the graduate and secured with a rubber band, so that it dips into the water at the top of the container. Two layers of 11.8” x 11.8” chemwipes are spread on a clean surface and one hookworm culture/petri plate is emptied on top of the sheets. The sheets are then twisted around the top of the culture and placed, twisted side up, in the top of the graduates. The cultures should be covered with water. This arrangement is left overnight to allow larvae time to migrate through the sheets to the bottom of the graduates.

The following day, the chemwipe/charcoal mixtures are disposed of and the supernatants in the graduates are removed by aspiration to 50 mL. At this point, all sediments/cylinder contents are combined together. The remaining slurry is left for 1.5 hours. The supernatant is then removed back to 50 mL. Warm water is added to the graduate and the solution is left another 1.5 hours before aspirating down to 50 mL. The remaining solution is transferred to a 250 mL graduate and allowed to settle for 30 minutes. Finally, larvae are concentrated in 1X BU salt buffer in a 50 mL conical tube. The final volume in the tube should not exceed 20 mL. The lid is kept loose on the tube and a strip of parafilm is used to seal the opening. The tube should be stored horizontally, in the dark, and at room temperature. Larvae can be kept in this way for a little over one month and still retain high infectivity.

**Obtaining hookworm adults from infected animals**
Starting day 19 post-infection (when fecal egg counts of infected animals typically peak), hamsters may be sacrificed by CO₂ exposure in a closed container. The chest cavities of the animals are opened and the small intestines are removed in their entirety. These are opened longitudinally with a pair of blunt-ended dissecting scissors and then placed into a 50 mL centrifuge tube, with 10–20 mL of pre-warmed (37°C) PBS, pH 7.4, for 1 hour to allow worms to dislodge from the intestine. The solution and intestine are examined under a microscope, using fine tweezers when necessary for further extrication of worms from the intestine. At this point, worms can be picked for in vitro assays or tallied following treatment in in vivo assays.

**Isolating eggs for in vitro developmental assays**

Embryonated *A. ceylanicum* eggs are isolated from the feces of hamsters following an overnight fecal collection. Using 6 grams of fresh feces, a general parasitology protocol has been modified for hookworm egg isolation (Mes et al. 2007). All steps are done at room temperature. 6 grams of fresh feces are weighed in a 50 mL centrifuge tube. 25 mL of 13% NaCl solution is added to the sample and thoroughly vortexed to liberate the eggs from the feces. The solution is then poured through a metal tea strainer with pore diameter 1 mm² and the flow-through is collected in a glass beaker. The beaker contents are poured into a second 50 mL tube and centrifuged at 3,400 rpm for 5 minutes at room temperature. The eggs are found at the top of the solution following centrifugation. At this point, the supernatant is poured into another 50 mL centrifuge tube and an equal volume of water is added to the solution. The solution is
mixed by gentle shaking and inversion and then centrifuged for 1 minute at 3,400 rpm.

The dilution allows the nematode eggs to pellet at the bottom of the tube.

The supernatant is removed by aspirator and the pellet is resuspended in 5 mL of a 17% sucrose solution by gentle pipetting. The resulting solution is centrifuged at 3,400 rpm for 5 minutes, leaving the eggs at the top of the flotation solution. The supernatant is then transferred to a 15 mL tube and diluted with an equal volume of tap water. The solution is mixed thoroughly by gentle shaking and inversion before centrifuging at 3,400 rpm for 5 minutes. With the eggs now at the bottom of the tube, the supernatant is removed by aspirator down to 0.5 mL. Yield is confirmed using a simple light microscope and pipetting 20 µL aliquots of suspended eggs onto a plate for visualization.

Eggs can be used to set up in vitro developmental assays at this point.
DISCUSSION

The preceding methods section presents experimentally effective protocols for maintaining *A. ceylanicum* in a laboratory. Although only a few labs in the world utilize human hookworm models, many variations of these techniques exist, as determined through correspondence with laboratories on the east coast of the United States, in the United Kingdom, and in China. Literature searches as to hookworm maintenance also present a multitude of observations into the lifecycle and maintenance of these parasites, but details are often lacking. For example, infections seem to be better maintained in male hamsters, although no studies have been conducted to determine why this is so.

Other key details noted here, that seem to be left out in other protocols detailing hookworm larvae culturing and maintenance, were discovered only after receiving training at George Washington University. One such factor was the level of moisture present when doing a fecal collection or culturing the larvae. Fecal samples should not be allowed to dry out at any point in the process, and, as such, it is of critical importance that cultures be kept in a humidified incubator. Another useful protocol was to house animals in greater numbers, so that larger samples could be obtained during fecal collections to maximize larval yield, especially when fecal egg counts were low. These key details have a major impact on the potential for successfully establishing this model organism in a lab. Giving funding limitations and the consequences of trial and error, the time spent trying to figure out these details independently can be costly.

UCSD is now the third institution in the United States to maintain *Ancylostoma ceylanicum*. Furthermore, we have the necessary tools to set up developmental assays evaluating mono-anthelmintic and anthelmintic combinations against hookworm.
Knowing that crystal proteins are effective against *A. ceylanicum in vitro*, we can evaluate the therapeutic effects of different crystal proteins, anthelmintics, and combinations against hookworm adults as well. With these essential tools and techniques at our disposal, we are better able to investigate the anthelmintic effects and synergistic potential of crystal proteins.
REFERENCES


Conclusion

From this work it may be concluded that the pursuit of nematicidal *Bacillus thuringiensis* crystal proteins as novel anthelmintics is most promising. These agents are simple and inexpensive to generate and can be readily produced on a large scale. As our laboratory has shown, the glycolipid receptor recognized by the proteins are specific to invertebrates, and as such Cry proteins have an excellent safety profile for vertebrates (Griffitts et al. 2005; Betz et al. 2000). As this thesis has demonstrated, Cry5B has a very significant therapeutic effect against a gastrointestinal nematode in the *in vitro* and *in vivo* systems investigated. Furthermore, the spores produced by *B. thuringiensis* when Cry proteins are generated do not significantly contribute to the nematicidal properties of the protein. The fact that Bt Cry proteins have never been promoted for human use further encourages their consideration as novel anthelmintics, since targeted pathogens have not had an opportunity to evolve resistant strains. With regard to anthelmintic combination therapy, results of *in vitro* studies with *H. bakeri* are promising. Further exploration is warranted *in vivo*, especially with larger treatment populations to see statistically significant dose response curves.

Cry5B has excellent *in vivo* efficacy as an anthelmintic, targeting both hookworm infections of golden Syrian hamsters as well as *H. bakeri* infections in Swiss white mice (Cappello et al. 2006; Hu et al. 2010). Cry5B significantly reduced worm burdens when tested in two different acrystalliferous strains of Bt. Cry5B/HD1 SCL, delivered as a single 100 mg/kg dose, reduced worm burdens by 67.2% while Cry5B/4Q7 SCL, delivered as a single 90 mg/kg dose, reduced worm burdens by 71.5%. As investigated,
neither Bt strain nor the presence of spores significantly influenced the observed therapeutic outcome. Furthermore, the extent of purification of Cry5B does not seem to significantly influence its efficacy. In the pilot in vivo spore study, 25 mg/kg Cry5B was able to achieve statistically significant reductions in worm burden at 43% and 68% in purified and spore crystal lysate form, respectively. As seen in the in vivo synergy study, reductions were reproducible between experiments with the purified protein, as a dose of purified Cry5B at 30 mg/kg reduced worm burdens by 61%, and a dose of 5 mg/kg reduced worm burdens by 21%. These two reductions were statistically significant from each other. Fecal egg count results were even more dramatic, as even the lowest concentration of Cry5B tested, at 5 mg/kg, effected more than a 50% decrease in fecal egg output following treatment. The ability to eliminate egg production in gastrointestinal parasitic infections is an important property of any anthelmintic to prevent transmission, and data generated in the preliminary in vivo synergy study suggests that crystal proteins are preferable, in this regard, to an anthelmintic in the nAChR agonist class, which stimulates parasite egg production. Cry5B is clearly a powerful, innovative anthelmintic that merits further testing and formulation.

At this point in the synergy studies it is too early to say whether crystal proteins and nicotinic acetylcholine receptor agonists mutually potentiate the mechanisms of each other, or synergize. In vitro results utilizing H. bakeri, and similar studies with C. elegans, suggest that levamisole and Cry5B are strongly synergistic when combined at a 1:1 efficacy. An analysis of combinations using the Chou and Talalay algorithm for synergy yielded consistent CI values: 0.38–0.15 for C. elegans, and 0.305-0.333 for H. bakeri, indicative of strong synergy. However, an in vivo analysis of the Cry
protein/nAChR agonist combination did not reproduce these findings. Instead of looking at lethality or inhibition of motility over drug concentration, reductions in fecal egg counts and final worm burdens were used as markers for drug effect, but CI values could not be calculated for the data due to deviations in dose curves. As noted previously, larger treatment groups and/or additional markers of drug effect, such as immune function recovery, may show the statistical significance needed for CI calculations. Different evaluations of worm clearance, such as sacrificing the mice at a later time following treatment or noting the distance that worms have traveled down the small intestine, might also allow for a greater evaluation of anthelmintic therapy and reveal significant differences between different doses of treatments.

Further investigation of the interaction between crystal proteins and currently available anthelmintics is necessary to confirm whether nematocidal crystal proteins, such as Cry5B, hold any potential for synergistic combinations. In addition to different markers of drug effect, such as recovery of immune function, other parasitic nematodes may be utilized in these studies to better characterize drug interactions across roundworm phylogeny. An analysis of different anthelmintics in combination with Cry5B, utilizing *H. bakeri* in our established *in vivo* system, would be desirable to test how these combinations interact in a live host and allow for a more focused approach to potential crystal protein synergy.

The establishment of *Ancylostoma ceylanicum* in our laboratory offers yet another system in which to determine crystal protein therapy and synergistic potential. As a relative of the human parasitic roundworm *A. duodenale*, *A. ceylanicum* will hopefully give us some idea of what to expect in regards to treating human parasitic infections with
crystal proteins. *In vitro* and *in vivo* experiments already showcase Cry5B therapy against adult *A. ceylanicum*, and the modification of a protocol allowing isolation of embryonated eggs allows for the implementation of developmental assays to further assess crystal protein therapy against the roundworm at different stages of its lifecycle.

In conclusion, Cry5B is a powerful anthelmintic against at least two parasitic roundworms, and efforts should be made to formulate and protect the protein from digestive break-down in order to preserve its efficacy. Although Cry5B and levamisole display hallmarks of synergy in *in vitro* experiments against two different nematodes, further study is warranted to determine whether crystal proteins synergize with any currently available anthelmintics in an *in vivo* system.
Conclusion: Works Cited


