Discovery of a highly synergistic anthelmintic combination that shows mutual hypersusceptibility

Yan Hu[a], Edward G. Platzer[b], Audrey Bellier[a], and Raffi V. Aroian[a,1]

[a]Section of Cell and Developmental Biology, University of California, San Diego, La Jolla, CA 92093-0322; and [b]Department of Nematology, University of California, Riverside, CA 92521-0415

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The soil-transmitted helminths or nematodes (hookworms, whipworms, and Ascaris) are roundworms that infect more than 1 billion of the poorest peoples and are leading causes of morbidity worldwide. Few anthelmintics are available for treatment, and only one is commonly used in mass drug administrations. New anthelmintics are urgently needed, and crystal (Cry) proteins made by *Bacillus thuringiensis* are promising new candidates. Combination drug therapies are considered the ideal treatment for infectious diseases. Surprisingly, little work has been done to define the characteristics of anthelmintic combinations. Here, by means of quantitative assays with wild-type and mutants of the roundworm *Caenorhabditis elegans*, we establish a paradigm for studying anthelmintic combinations using Cry proteins and nicotinic acetylcholine receptor (nAChR) agonists, e.g., tribendimidine and levamisole. We find that nAChR agonists and Cry proteins, like Cry5B and Cry21A, mutually display what is known in the HIV field as hypersusceptibility—when the nematodes become resistant to either class, they become hypersensitive to the other class. Furthermore, we find that when Cry5B and nAChR agonists are combined, their activities are strongly synergistic, producing combination index values as good or better than seen with antitumor, anti-HIV, and insecticide combinations. Our study provides a powerful means by which anthelmintic combination therapies can be examined and demonstrate that the combination of nAChR agonists and Cry proteins has excellent properties and is predicted to give improved cure rates while being recalcitrant to the development of parasite resistance.

Caenorhabditis elegans | crystal proteins | nAChR agonists | soil-transmitted nematodes | helminth

Intestinal roundworms or nematodes are among the most prevalent parasites of humans today, infecting upwards of 2 billion persons (1–3). The main classes of intestinal nematodes include hookworms (*Ancylostoma duodenale*, *Necator americanus*), whipworms (*Trichuris trichiura*), and roundworms (*Ascaris lumbricoides*). The cumulative impacts of these parasites are tremendous, causing anemia and growth retardation in children, low nutritional status, loss of appetite, impairment of cognitive function and mental performance (e.g., lower working memory), damage to school performance and increased absenteeism, reduction in future wage earning capacity, anemia in pregnant women, increased proportions of stillbirths/perinatal deaths/very-low-birthweight babies, increases in infant mortality, increases in maternal mortality, impaired worker productivity and productive capacity, and weak adults (2, 4–7). Infection by these parasites also indirectly results in tremendous disease burden via impairment of the immune system, leading to increased severity of HIV/AIDS (lower CD4 counts, higher viral load), increased susceptibility to malaria, increased probability of having active tuberculosis (TB) and poor response to TB vaccine, and decreased immune response/failure of vaccine against cholera (8–14). Intestinal roundworms are thus one of the great diseases of our time and play a significant role in keeping infected peoples impoverished (15).

That the effects of intestinal nematodes are more hidden than many other diseases, and that they affect the poorest peoples, have led to a lack of research and commitment for these diseases and a virtually complete lack of new drug (anthelmintic) development. Since 1981, only China has taken a new drug, tribendimidine, into human clinical trials (16). Of the handful of anthelmintics in our arsenal, just two—tribendimidine, a nicotinic acetylcholine receptor, or nAChR, agonist (17), and albendazole, a benzimidazole—are adequate for single-dose mass drug administrations (MDAs). Both have excellent activity against *Ascaris*, moderate (but not ideal) activity against hookworms, and poor activity against whipworm and threadworms (18, 19). Only albendazole is used globally for MDAs, as tribendimidine is not yet approved worldwide (20). In addition, both benzimidazoles and nAChR agonists are susceptible to the development of parasite resistance, in both veterinary and human applications (21–25). There is thus an urgent need for new anthelmintics.

Better yet would be the development of anthelmintic combination therapies. Drug combinations are considered the ideal therapy for treatment of important infectious diseases, including HIV, malaria, and TB (26–28) and play a major role in improving therapeutic efficacy and delaying resistance. For example, the drug combinations found in highly active antiretroviral therapy (HAART) have revolutionized treatment of HIV/AIDS and allowed significant improvements in prolonging the duration and improving the quality of life of infected individuals (28). To date, however, there have been no systematic and quantitative studies of anthelmintic combinations.

Fortunately, an inexpensive and powerful laboratory nematode exists that, in principle, can be used to facilitate the study of anthelmintics—*Caenorhabditis elegans*. *C. elegans* has played a pivotal role in the discovery of the mechanism of action and resistance of virtually all anthelmintics, namely nAChR agonists, benzimidazoles, aldicarb, ivermectin, pore-forming crystal (Cry) proteins made by *Bacillus thuringiensis* (Bt), and amino-acetylnitriles (29–33). Such studies are far more difficult with parasitic nematodes. Despite these fundamental anthelmintic discoveries made with *C. elegans,* and despite the powerful genetic and toxicological tools available, *C. elegans* has been ignored for studying anthelmintic combinations.

Here we use wild-type and mutant *C. elegans* to characterize in detail how two different classes of anthelmintics interact. We find that nematodes resistant to either Bt Cry proteins or nAChR agonists are reciprocally hypersusceptible to the other class relative to wild-type nematodes. Furthermore, when combined, Cry proteins and nAChR agonists have strong synergy that is on a par with or better than some of the best combination therapies against other diseases. Thus, nAChR agonists and Cry proteins constitute a new and potentially powerful combination therapy against intestinal nematode diseases.

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[1]To whom correspondence should be addressed. E-mail: raroian@ucsd.edu.

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Results

nAChR Agonist Resistant Mutants Are Hypersusceptible to Cry Protein Anthelmintics. Our laboratory has pioneered work on natural Bt Cry proteins that kill nematodes. Cry proteins are intensively used in the control of insect pests, including in aerial eradication campaigns, mosquito control programs, transgenic crops, and organic farming (34, 35). We have characterized Cry proteins lethal to free-living, animal-parasitic and plant-parasitic nematodes (29, 36–38). Although deadly to insects and nematodes, Bt Cry proteins are nontoxic to vertebrates (35) and are ideal candidates for a new class of anthelmintic. To date, one Cry protein, Cry5B, has been demonstrated to have anthelmintic activity in vivo against a hookworm parasite infection in hamsters (29).

As part of our development of Cry proteins as anthelmintics, we decided to quantitatively assay how various anthelmintic-resistant nematodes respond to Cry proteins. This information is important for determining the future usefulness of Cry proteins against anthelmintic-resistant nematodes that might appear during MDAs. Although it is known that various anthelmintic classes (e.g., nAChR agonists, benzimidazoles) have different mechanisms of action, how mutants resistant to one class respond to another class has not been quantitated in detail. We reasoned that *C. elegans*, for which mutants resistant to almost all different anthelmintic classes exist, provides a unique and powerful means to study this important question.

We tested how nematode mutants resistant to nAChR agonists and benzimidazoles (the two classes useful for single-dose MDAs against intestinal nematodes) respond to Cry proteins, using three different intoxication assays and the following mutants: *lev-8(ye493)*, *ben-1(e1880)*, and *bre-5(ye17)*. *lev-8* Encodes an nAChR subunit that mutates to levamisole, pyrantel, and tribendimidine resistance; *ye493* is a null allele (17, 32). *ben-1* Encodes a beta-tubulin conserved in parasitic nematodes and mutates to benzimidazole resistance in *C. elegans* and parasitic helminths (32). It is the only benzimidazole resistance gene identified to date. *bre-5* Encodes a protein involved in the biosynthesis of the invertebrate-specific Cry5B receptor; *ye17* is a null allele (30, 39).

We unexpectedly find that nematodes resistant to nAChR agonists are hypersusceptible (HS) to Cry5B. Using a dose-dependent mortality assay (40), we find that nAChR agonist-resistant *lev-8 (ye493)* nematodes are more susceptible than wild-type nematodes to Cry5B (dose range 1.25–5.0 μg/mL) (Fig. 1*A*). Conversely, benzimidazole-resistant *ben-1(e1880)* nematodes are more resistant to Cry5B than wild-type nematodes (doses 10–40 μg/mL). We calculated LC_{50} values (lethal concentration 50% or dose at which half of the nematodes are dead) from each of these curves, and find that the LC_{50} value of *lev-8(ye493)* nematodes on Cry5B is lower (i.e., 50% of *lev-8* animals are killed at a lower dose) than that of wild type, confirming hypersusceptibility (Table S1). Hypersusceptibility to killing by Cry5B was also seen with *unc-50(ye494)* nematodes, a different nAChR agonist-resistant mutant that alters receptor assembly (32) (Fig. 1*B* and Table S1).

Similar striking results were obtained using a developmental inhibition assay. We put first staged larvae from the same mutants on varying concentrations of *Escherichia coli* (*E. coli*)–expressed Cry5B and counted the number of nematodes that were able to develop to adulthood in 60 h at 20 °, a time by which all wild-type nematodes in the absence of toxin develop to adulthood. In this assay, nAChR agonist-resistant *lev-8(ye493)* nematodes are also very HS to Cry5B over a range of doses (1.56–6.25%; Fig. 1*C*). We calculated the IC_{50} values (inhibitory concentration 50% or dose at which half the nematodes fail to develop) for wild-type and *lev-8(ye493)* nematodes, and found that *lev-8* mutants are more than 2-fold more susceptible to Cry5B than wild type (Table S1). *ben-1* Nematodes were not appreciably resistant to Cry5B in this assay (Fig. 1*C*) but appear

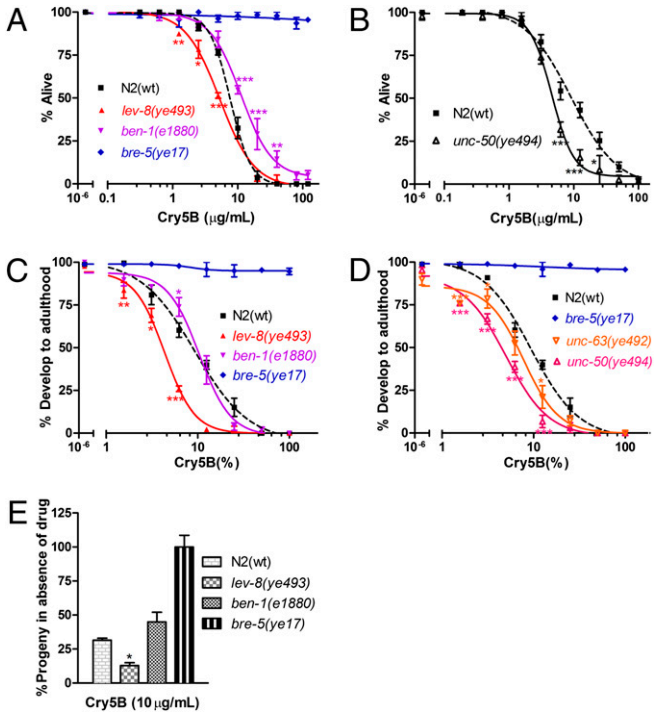

Fig. 1. nAChR agonist–resistant mutants are hypersusceptible to crystal proteins. (*A*) Dose-dependent mortality response of wild-type N2 nematodes and *ben-1*, *lev-8*, and *bre-5* mutant nematodes to purified Cry5B. (*B*) Dose-dependent mortality response of wild-type N2 and *unc-50(ye494)* animals to purified Cry5B. (*C*) Dose-dependent developmental inhibition response of wild-type N2 nematodes and *ben-1*, *lev-8*, and *bre-5* mutant nematodes to *E. coli*–expressing Cry5B. (*D*) Dose-dependent developmental inhibition response of wild-type N2 nematodes and *unc-63(ye492) and unc-50(ye494)* mutant nematodes to *E .coli*–expressing Cry5B. The smaller effect seen with *unc-63* may be due to the possible nonnull nature of this allele. (*E*) Effect of 10 μg/mL purified Cry5B on the 64-h brood sizes of N2 wild-type, *lev-8*, *ben-1*, and *bre-5* mutant nematodes. For *A–D* and in similar figures below, each data point represents mean ± SEM. LC_{50} and IC_{50} values for exposures to Cry5B can be found in Table S1. For *E*, each bar shows 64-h broods from 15 worms; error bars represent SD. For *A–E* and in all similar figures below, $*P < 0.05$ relative to N2; $**P < 0.01$ relative to N2; and $***P < 0.001$ relative to N2. The alleles used here and in all other figures are *ben-1(e1880), lev-8(ye493),* and *bre-5(ye17)*.

HS based on IC_{50} values (Table S1). Using this developmental inhibition assay, we found that two other mutants, *unc-63(ye492)* and *unc-50(ye494)*, resistance to nAChR agonists were also HS to Cry5B (Fig. 1*D* and Table S1).

As a third measure of intoxication, we looked at the response to Cry5B of each of the mutants with respect to nematode fecundity. We found that, normalized to the amount of progeny produced on no-toxin controls, *lev-8(ye493)* adults produce ~60% fewer progeny on Cry5B than wild-type adults (Fig. 1*E*; $P < 0.05$). No significant difference between wild-type and *ben-1(e1880)* nematodes was seen. Thus, based on three different measures of intoxication relevant to anthelmintic control and using up to three different nAChR-resistant mutants, we observed that nematodes resistant to nAChR agonists were HS to Cry5B relative to wild-type nematodes.

To see whether the hypersusceptibility applied to Cry proteins other than Cry5B, we tested the response of nAChR-resistant nematodes to Cry21A. Like Cry5B, Cry21A has anti-nematode activity against many free-living nematodes (38) but has only 41% amino acid identity to Cry5B from the N terminus until the end of the toxin domain. Cry21A is distinct from other anthelmintics in that we find that it is more difficult for mutagenized *C. elegans* to evolve resistance to Cry21A than albendazole, ivermectin, levamisole, or

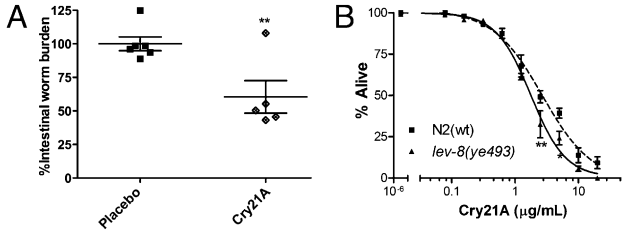

Fig. 2. Effects of Cry21A on *H. bakeri* and on *C. elegans* wild-type and mutant strains. (*A*) In vivo anthelmintic activity of Cry21A against the small intestinal parasite *H. bakeri*. Shown are intestinal roundworm burdens normalized to average in the placebo control (41.7 worms) following treatment of infected mice with placebo (spore lysates, no Cry21A) or Cry21A spore crystal lysate (SCL). Normalized worm burdens in each mouse are shown (n = 6 for placebo; n = 5 for Cry21A; one animal in the Cry21A group died with a tumor before treatment). Long horizontal bar shows mean worm burdens; smaller bars indicate SEM. (*B*) Dose-dependent mortality response of wild-type N2 and nAChR agonist-resistant *lev-8* mutant nematodes to Cry21A SCLs at 20 ° for 3 days. LC_{50} values for exposure to Cry21A can be found in Table S1.

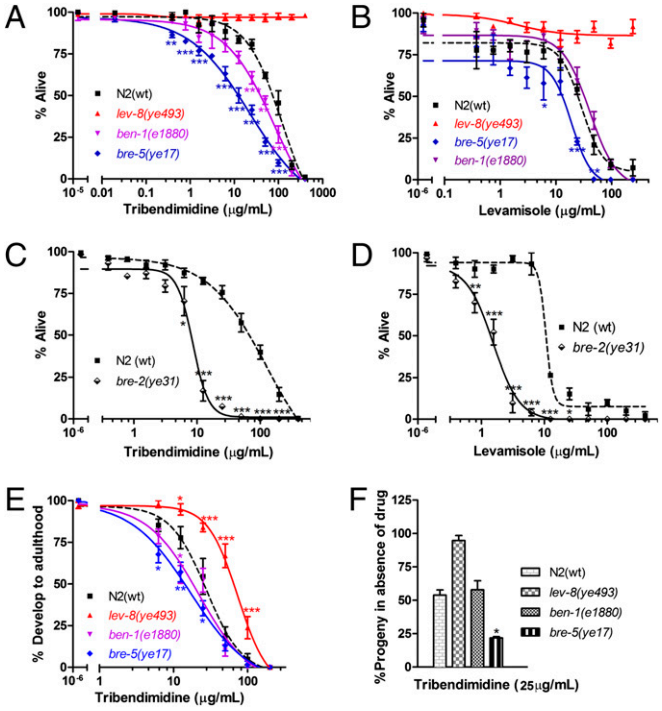

Fig. 3. Cry5B-resistant mutants are hypersusceptible to nAChR agonists. (*A*) Dose-dependent mortality response of wild-type N2 animals and *ben-1, lev-8,* and *bre-5* mutant animals to tribendimidine. (*B*) Dose-dependent mortality response of wild-type N2 nematodes and *ben-1, lev-8,* and *bre-5* mutant nematodes to levamisole. (*C*) Dose-dependent mortality response of wild-type N2 animals and *bre-2(ye31)* mutant animals to tribendimidine. (*D*) Dose-dependent mortality response of wild-type N2 animals and *bre-2(ye31)* mutant animals to levamisole. (*E*) Dose-dependent developmental inhibition response of wild-type N2 nematodes and *ben-1, lev-8,* and *bre-5* mutant nematodes to tribendimidine. (*F*) Effect of 25 μg/mL tribendimidine on 64-h brood sizes of N2 wild-type, *lev-8*, *ben-1*, and *bre-5* nematodes.

Cry5B (Table S2). Cry5B also has excellent properties in this regard relative to albendazole and levamisole.

To date, however, no Cry protein other than Cry5B has been shown to have in vivo anthelmintic activity (29). To see if Cry21A is an anthelmintic, we tested the effects of Cry21A on *Heligmosomoides bakeri* roundworm infections in mice [*H. bakeri* is a natural intestinal parasite of mice and is an important model of roundworm infections in humans (41)]. We infected mice with *H. bakeri* larvae and then delivered per os three doses of Cry21A protein or placebo. Five days later, the mice were euthanized and the number of intestinal adult parasites counted. Cry21A treatment resulted in a 40% reduction in parasite burden relative to placebo (Fig. 2*A*, $P = 0.005$), demonstrating in vivo anthelmintic activity.

We then performed dose-dependent mortality assays with Cry21A and *lev-8* mutant nematodes. As with Cry5B, we found that *lev-8* nematodes were HS to Cry21A (range 2.5–5 μg/mL) and were also HS to Cry21A based on LC_{50} values (Fig. 2*B* and Table S1). Thus, nAChR agonist-resistant mutants are likely to be HS to pore-forming Cry proteins in general.

Cry5B-Resistant Mutants Are Reciprocally Hypersusceptible to nAChR Agonists. Because nematodes resistant to nAChR agonists are HS to Cry proteins, we asked what would happen to nematodes resistant to Cry proteins when exposed to nAChR agonists. We therefore quantified the effects of nAChR agonists, such as tribendimidine, levamisole, and pyrantel, on the same set of anthelmintic-resistant nematodes as above. We observed that *bre-5(ye17)* Cry5B-resistant nematodes were more sensitive than wild-type nematodes to tribendimidine (dose range 0.39–100 μg/mL; Fig. 3*A*), levamisole (dose range 24–48 μg/mL; Fig. 3*B*), and pyrantel (Fig. S1*A*). Based on LC_{50} values, *bre-5(ye17)* nematodes relative to wild-type nematodes are ~5-fold more HS to tribendimidine and ~2-fold more HS to levamisole (Table S1). To see how broadly this result applies to other Cry5B-resistant mutants, we also tested the Cry5B-resistant mutant, *bre-2(ye31)*. These nematodes are also HS to tribendimidine, levamisole, and pyrantel (Fig. 3 *C* and *D* and Fig. S1*B*). Based on LC_{50} values, *bre-2* nematodes are ~8-fold more sensitive to both tribendimidine and levamisole (Table S1). Results with *ben-1(e1880)* nematodes are different for different nAChR agonists. *ben-1* Nematodes are HS to tribendimidine (albeit to a far lesser degree than that of Cry5B-resistant nematodes to tribendimidine; Fig. 3*A* and Table S1) but are resistant to levamisole based on LC_{50} (Table S1).

As above, we tested whether this hypersusceptibility extended to other measures of intoxication of clinical relevance tested above, namely developmental and reproductive inhibition. We found that the development of Cry5B-resistant *bre-5(ye-17)* larvae were HS, relative to wild-type nematodes, to the nAChR agonist tribendimidine in the dose range of ~5–20 μg/mL and based on LC_{50} values (Fig. 3*E* and Table S1). Cry5B-resistant nematodes were also HS, relative to wild-type animals, to the sterilizing effects of tribendimidine (Fig. 3*F; P* < 0.05). Benzimidazole-resistant *ben-1(e1880)* nematodes, albeit less than *bre-5 (ye17)* animals, showed some HS to tribendimidine in the developmental assay (Fig. 3*E* and Table S1), but not in the reproductive assay (Fig. 3*F*).

Cry5B and nAChR Agonists Are Strongly Synergistic. The above data demonstrate that development of nematodes resistant to nAChR agonists actually improves Cry protein nematicidal activity, and that development of nematodes resistant to Cry proteins improves nAChR agonist nematicidal activity. These data, however, do not measure how each class of drug might modulate the activity of the other if the two were combined. That is to say, when combined, are the effects of the two classes antagonistic, additive, or synergistic? A synergistic effect is one that is more than additive; an antagonistic effect is one that is less than additive (i.e., the drugs inhibit each other). Synergistic interactions are sought after because they can result in increased efficacy, decreased dosage, and reduced side toxicity, and can perhaps minimize development of resistance when used clinically (42). Antagonistic effects are generaly avoided.

To quantitate the effects of the two anthelmintics on each other, we subjected wild-type *C. elegans* nematodes to increasing doses of Cry proteins and nAChR agonists individually and in combination

at a constant mass (μg/mL) ratio (Fig. 4). For example, wild-type *C. elegans* were subjected to various doses of Cry5B, tribendimidine, and a 1:1 mass ratio (e.g., 1 μg/mL Cry5B + 1 μg/mL tribendimidine) mixture of the two. The worms were assayed for viability under each of these three conditions, which are plotted in Fig. 4*A*.

From these data, we can calculate the degree of synergy using the combination index (CI) algorithm of Chou and Talalay (42). In general, CI values <1 indicate synergy; >1 indicate antagonism. The CI algorithm has been used to calculate the degree of synergy between drug combinations in cancer chemotherapy [typical synergistic CI values 0.1–0.8 (43–46)], viral therapy [synergistic CI values 0.5–0.8 (47, 48)], and insecticides [synergistic CI values of 0.3–0.9 (49, 50)]. To be somewhat conservative, we chose CI values <0.7 as indicative of synergy and <0.3 as indicative of a strong synergy (42, 51).Using this algorithm, we calculated CI values at the ED_{50} (median-effect dose), ED_{75}, ED_{90}, and ED_{95} of the combination and found CI values that ranged between 0.52–0.12, indicating strong synergy between Cry5B and tribendimidine when mixed at equal mass doses (Table 1). Synergy between the two compounds was also indicated using isobolograms at this mixture ratio (Fig. S2).

A similar experiment was also carried out with Cry5B and the nAChR agonist levamisole combined in a fixed ratio based on mass (e.g., 1 μg/mL Cry5B mixed with 1 μg/mL levamisole) as shown in Fig. 4*B*. CI values of 0.38–0.15 were calculated from these data, indicative of strong synergy (Table 1).

To determine whether Cry5B and nAChR agonists are synergistic at other drug combination ratios, we combined Cry5B and tribendimidine at a 1:1 ratio based on their LC_{50} values (and 2-fold dilutions up and down from there; Fig. 4*C*). Again, excellent synergy with CI values ranging from 0.48–0.27 (EC_{50} – EC_{95}) were seen (Table 1). Thus, Cry5B and tribendimidine also synergize when combined at a ratio based on similar efficacies.

We also calculated the dose-reduction index (DRI) for anthelmintics in different combinations at specific effect levels (Table S3). DRI is a measure of how many fold the dose of each drug in a synergistic combination may be reduced relative to the dose of each drug alone while still achieving the same effect. For example, at the ED_{90} for the Cry5B and levamisole combination, Cry5B can be reduced ~6-fold and levamisole ~39-fold relative to what it would take for each of these drugs to achieve the same effect on their own. These data suggest that Cry proteins and nAChR agonists mutually potentiate each other and that, for example, inclusion of a small amount of Cry5B might permit significant reductions in the amounts of nAChR agonists to achieve comparable effects when used alone. To test this conclusion, we "spiked" dose-dependent tribendimidine mortality assays with a small dose of Cry5B (2 μg/mL), which normally permits ~95% viability, and in parallel ran nonspiked assays. The small amount of Cry5B produced a synergistic interaction with tribendimidine (Fig. 4*D*). For example, the same lethality was seen with 20 μg/mL tribendimidine + Cry5B spike than was seen with 200 μg/mL tribendimidine alone.

Table 1. Combination index values of Cry5B and nAChR agonists at different effect dose levels

Combination	Combination index values			
	ED_{50}	ED_{75}	ED_{90}	ED_{95}
Cry5B + tribendimidine (1:1 mass)	0.52	0.30	0.17	0.12
Cry5B + levamisole (1:1 mass)	0.38	0.26	0.19	0.15
Cry5B + tribendimidine (1:1 LC_{50})	0.48	0.38	0.31	0.27

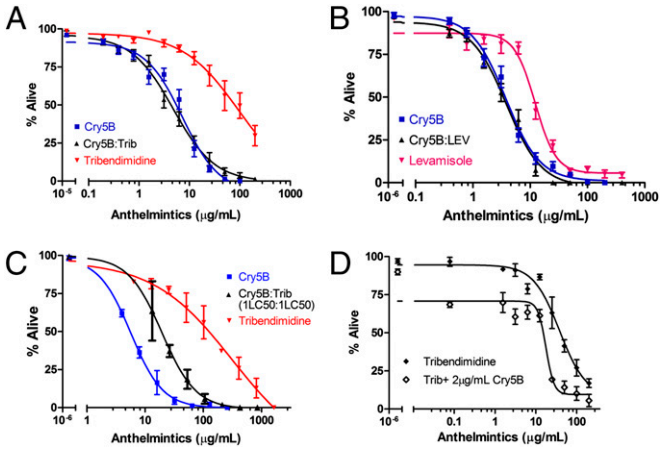

Fig. 4. Cry5B and nAChR agonist combinations are strongly synergistic. (*A*) Dose-dependent mortality response of wild-type N2 animals to purified Cry5B, tribendimidine, and 1:1 ratio of Cry5B:tribendimidine based on mass. The *x* axis is plotted in terms of total dose (e.g., 1 μg/mL = 1 μg/mL of Cry5B, 1 μg/mL of tribendimidine, or combination of 0.5 μg/mL of each). (*B*) Dose-dependent mortality response of wild-type N2 animals to purified Cry5B, levamisole, and 1:1 ratio of Cry5B:levamisole based on mass. (*C*) Dose-dependent mortality response of wild-type N2 animals to purified Cry5B, tribendimidine, and 1:1 ratio of Cry5B:tribendimidine based on LC_{50} values. (*D*) Dose–response curve of wild-type N2 animals to tribendimidine without and with a low dose of Cry5B (2 μg/mL), which normally gives ~5% mortality. LEV, levamisole; Trib, tribendimidine.

Discussion

Here we quantitatively analyze two important aspects of anthelmintic interactions—how nematodes resistant to one anthelmintic class behave on other classes, and how two anthelmintic classes behave in combination with each other. These results are more readily achievable with the use of *C. elegans* than with parasitic nematodes. Each data point on our graph represents ~100–200 nematodes, and each data point is taken under culture conditions in which the nematodes are fully healthy and capable of completing a full life cycle, unlike in vitro assays with parasitic nematodes. Intense quantitative assays such as these are also not practical with in vivo parasite assays, which would require hundreds of vertebrate hosts per graph.

The results are striking—we find that nematodes resistant to nAChR agonists are hypersusceptible (HS) (relative to wild-type nematodes) to two Cry protein anthelmintics and, reciprocally, nematodes resistant to a Cry protein are HS to three nAChR agonists. Hypersusceptibility was seen with three different clinically relevant measures of anthelmintic activity—mortality, developmental inhibition, and sterilization of the nematodes. Why resisting an anthelmintic that acts at the neuromuscular junction should make nematodes more susceptible to pore-forming toxins that attack the intestine and vice versa is unexpected, and suggests large gaps in our understanding of nematode physiology. Although some hypersusceptibility was seen between benzimidazole-resistant nematodes and tribendimidine, the extent was less robust and did not extend to all measures of activity or to levamisole.

Mutual hypersusceptibility has, to our knowledge, not been previously identified with any anthelmintic combinations and has profound implications for anthelmintic chemotherapy. Hypersusceptibility is a key characteristic of one of the most successful antiinfective therapies today, HAART. In the HIV field, hypersusceptibility is often associated with the nucleoside reverse transcriptase inhibitors (NRTIs) and the nonnucleoside reverse transcriptase inhibitors (NNRTI) combinations (52). Evolution of viral resistance to one class results in hypersusceptibility to the second class. HS combinations are associated with improved clinical outcome and virological response (52–54) and with improved resistance outcomes, such as delaying/suppressing resistance,

reducing the risk of cross-resistance, and even reversing an existing resistance phenotype (53–57). The experience with HAART suggests that the combination of L-subtype nAChR agonists such as tribendimidine with Cry proteins should result in significantly improved clinical outcomes while simultaneously working to prevent the emergence of resistance to either drug.

Our HS studies were complemented with measurements of synergy between L-subtype nAChR agonists and Cry5B. To our knowledge, quantitation of the synergy between any anthelmintics has yet to be reported. These data demonstrate that Cry5B and L-subtype nAChR agonists show strong synergy when combined, and greatly exceed that predicted from simple additive effects. Synergy is highly desirable. When the drugs used in the combination therapy are synergistic, the therapeutic efficacy of each component is greatly increased. As a result, much smaller doses of each drug can be used in the combination therapy than would otherwise be required if each drug were used individually. This results in reductions in drug side effects and in cost. With regards to resistance, there is precedent for synergistic interactions precluding the development of resistance, such as in the case of synergy between Cry and the cytolytic proteins found in mosquitocidal Bt strains (58–60)

The implications of our findings are profound. Due to the threat of resistance to individual anthelmintics and the fact that we have no single drug that has high efficacy against all of the parasites, therapeutic agents with novel modes of action and, more importantly, intelligently designed combination therapies are urgently needed for the treatment of these diseases (9, 18, 22). The excellent combination of Cry proteins and L-subtype nAChR agonists therefore potentially represent an evolution in anthelmintic chemotherapy, as this combination displays two different characteristics—hypersusceptibility and synergy. Such a combination is predicted to be highly valuable in MDA treatments for intestinal nematodes in which tens to hundreds of millions of children and pregnant women are targeted for single-dose treatment. In such circumstances, having a powerful combination therapy is likely to maximize therapeutic outcome in a single dose while delaying/preventing resistance that may be inevitable with massive use of single anthelmintics.

In summary, we demonstrate that Cry proteins and nAChR agonist anthelmintics possess a powerful set of characteristics defined by mutual hypersusceptibility and strong synergy. These data define a potentially unique combination therapy for treating intestinal nematode infections that is predicted to be a very potent and recalcitrant to the development of parasite resistance. These studies also demonstrate the unique utility of *C. elegans* in the study of anthelmintic therapy, and open the door to similar characterizations of other anthelmintic combinations and therapies.

Materials and Methods

***C. elegans* Strains and Reagents.** *C. elegans* strains were cultured using standard techniques including the use of *Escherichia coli* strain OP50 as standard food source (61). Different strains were allowed to grow for different amounts of time at 20 ° from the L_1 to L_4 stage before testing on drugs to reflect slight differences in their growth rates relative to N2 wild type: *unc-63(ye492), lev-8(ye493) unc-50(ye494), bre-2 (ye31),* and *bre-5 (ye17)* were allowed to develop for 45 h. N2 wild-type animals and *ben-1 (e1880)* were used at 44 h. The preparation of Cry5B, tribendimidine, levamisole, pyrantel, Cry21A, and all worm plates and buffers were as described elsewhere (17, 29, 38, 40, 62).

Intoxication Assays. Mortality assays with nAChR agonists and purified Cry5B were as described for 6 days at 25° (17, 40). Assays with Cry21A spore-crystal lysates (SCLs) were carried out in the presence of 15 μg/mL tetracycline to prevent Bt from infecting and at 20° for 3 days (62). The reason for the lower temperature and reduced time with Cry21A assays was to keep the dose–response range similar to that for other drugs, as the Bt spores present in Cry21A SCLs enhance mortality. Each experiment was performed with ∼20 L4 nematodes per well in triplicate wells and then repeated in three independent repeats [∼100–240 nematodes/data point; except for the pyrantel and *bre-5(ye17)* assay, which was carried out in duplicate]. All liquid assays were carried out in special S medium.

Developmental inhibition assays for tribendimidine were as previously described at 20 ° (17). Triplicate wells and three independent assays were carried out as described above. To measure developmental inhibition by Cry5B, 20 L1 nematodes were seeded on ENG-IC plates with various percentages of Cry5B-expressing *E. coli* diluted with non–Cry5B-expressing *E. coli* as described elsewhere (40) and incubated at 20 ° for 60 h. The number of nematodes that did/did not reach gravid adulthood was tallied for each plate. The experiment was independently repeated three times.

To calculate brood sizes, individual L4 worms were placed with an eyelash in a 48-well plate containing 40 μL OP50 (OD_{600} = 3.0) and tribendimidine or purified Cry5B in a total volume of 200 μL (five nematodes/treatment/assay; three independent assays). The plates were incubated for 64 h at 25 °. The progeny were pipetted onto an empty NG agar plate for counting.

Effect of Cry21A on *H. bakeri* Infections in Mice. Swiss Webster female mice (6–8 weeks of age) were orally infected with 150 *H. bakeri* third-stage larvae. Animals were split into two groups, and on days 15, 16, and 17 postinfection were given either 99 nM/kg of Cry21A SCL in 0.1 mL H_2O or 0.1 mL spore lysates of the Bt host strain 4D22. At day 22, infected mice were euthanized and their small intestines removed and dissected. Adult *H. bakeri* present in the small intestine were then counted.

Statistical and Synergy Analyses. LC_{50} values and 95% confidence limits were calculated by combining all data from the three independent experiments and using PROBIT (from XLSTAT add-on to EXCEL, Addinsoft). All other data analyses and plots were carried out with Prism 5 (GraphPad Software). For brood size data, pair-wise comparisons between groups were carried out via one-way ANOVA and Tukey's HSD test. For developmental and mortality data, pair-wise comparisons among groups and doses was carried out via two-way ANOVA and Bonferroni post tests. Cry21A in vivo treatment results were analyzed by Student's *t* test (unpaired, one-tailed). Drug combination curves were plotted using Prism 5. Results of combination studies (CI and DRI values, isobologram plots) were processed using the CompuSyn software package (CompuSyn), with the modification that we manually added on the correction for mutually nonexclusive drugs, which is not incorporated in the CompuSyn program. This modification is appropriate, 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. The formula of Chou and Talalay used for calculating CI values is given in *SI Text*. For the combination studies in which Cry5B and tribendimidine were to be added at a 1:1 ratio based on LC_{50} values, we performed pilot dose–response experiments with tribendimidine and Cry5B just before the actual experiments. Based on these pilot data, we then selected 4 μg of Cry5B and 102 μg of tribendimidine as the 1:1 LC_{50} ratio dose (and 2-fold dilutions up and down) and performed three independent synergy experiments (including Cry5B, tribendimidine, and Cry5B+tribendimidine dose–response curves). In the actual experiments, upon analyses of the completed data, the LC_{50} of Cry5B and tribendimidine alone were found to be 4.6 μg/mL and 78 μg/mL respectively. Thus, the actual ratio present in this experiment was 1 × LC_{50} Cry5B: 1.5 × LC_{50} tribendimidine.

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