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Nitazoxanide: Nematicidal Mode of Action and Drug Combination Studies

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Abstract

Intestinal nematodes or roundworms (aka soil-transmitted helminths or STHs) cause great disease. They infect upwards of two billion people, leading to high morbidity and a range of health problems, especially in infected children and pregnant women. Development of resistance to the two main classes of drugs used to treat intestinal nematode infections of humans has been reported. To fight STH infections, we need new and more effective drugs and ways to improve the efficacy of the old drugs. One promising alternative drug is nitazoxanide (NTZ). NTZ, approved for treating human protozoan infections, was serendipitously shown to have therapeutic activity against STHs. However, its mechanism of action against nematodes is not known. Using the laboratory nematode *Caenorhabditis elegans*, we show that NTZ acts on the nematodes through *avr-14*, an alpha-type subunit of a glutamate-gated chloride ion channel known for its role in ivermectin susceptibility. In addition, a forward genetic screen to select *C. elegans* mutants resistant to NTZ resulted in isolation of two NTZ resistant mutants that are not in *avr-14*, suggesting that additional mechanisms are involved in resistance to NTZ. We found that NTZ combines synergistically with other classes of anthelmintic drugs, *i.e.* albendazole and pyrantel, making it a good candidate for further studies on its use in drug combination therapy of STH infections. Given NTZ acts against a wide range of nematode parasites, our findings also validate *avr-14* as an excellent target for pan-STH therapy.

1. Introduction

Intestinal parasitic roundworms or nematodes, also known as soil transmitted helminths (STHs), infect upwards of two billion people in the world, including 807–1,121 million people infected by *Ascaris*, 604–795 million by whipworms, and 576–740 million by hookworms [1–3]. The nematode infections lead to high morbidity and a range of health problems, especially in the 400 million infected children. These children are malnourished, show physical and cognitive growth retardation, have reduced energy and strength which leads to school absenteeism, eventually resulting in lower socioeconomic achievements and perpetuating poverty [1, 3–5].

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The two classes of drugs approved by World Health Organization for treatment of STH are benzimidazoles (albendazole and mebendazole) and nicotinic acetylcholine receptor (nAChR) agonists (pyrantel and levamisole), but only the benzimidazoles are commonly used for mass drug administration to treat STH [6]. Interestingly, all the drugs that are used for the treatment of STH infections were developed for veterinary use [7, 8]. Reports of worms developing resistance to or low efficacy of benzimidazoles are surfacing [9–11]. Thus new drugs are necessary. However, the global research funding for discovering new drug(s) to cure STH infections (hookworms, *Ascaris*, whipworms, *Strongyloides*) is small (http://www.policycures.org/downloads/g-finder_2011.pdf). As a result of this poor funding of worm drug research, in last 30 years, only one new anthelmintic drug, tribendimidine in China, has reached clinical trials for humans [12]. This scenario demands discovery of new drugs and/or new ways to improve the efficacy and discourage the evolution of resistance for the drugs that are currently in use.

Nitazoxanide (N-(5-nitrothiazol-2-gammal) salicylamide) (hereafter NTZ), a thiazolide, was discovered in 1984 for its cestocidal effects by Jean François Rossignol at the Pasteur Institute [13]. Later on, it was found to be an effective drug against many protozoan infections like *Cryptosporidia*, *Giardia*, *Entamoeba* [14–16], bacterial infections like *Helicobacter* and *Clostridium* [17–20], and antiviral effects against Hepatitis B and C virus and rotaviral diarrhea [21–24]. This drug is considered safe and is the mainstay for the treatment of *Cryptosporidia* infections in immuno-compromised individuals and patients with Acquired Immune Deficiency Syndrome (AIDS) [25–28]. Serendipitously, NTZ was found to be effective against nematode parasites [29–32] which encouraged several investigations on NTZ's therapeutic effects on different species of parasitic worms. Reports suggest that NTZ is effective in treating a number of parasitic nematodes, *e.g.*, the hookworm *Ancylostoma duodenale* [29, 33], the large roundworm *Ascaris lumbricoides* [29, 31, 32, 34, 35], the whipworm *Trichuris trichiura* [29, 31–34] and *Strongyloides stercoralis* [29, 33]. Successful NTZ treatment regimens for nematode parasites typically involve six doses over two days [33]. However, one trial suggested NTZ does not work well against whipworms at a single-dose [36]. Recent data suggest that NTZ has an all-or-nothing effect and needs to reach high levels to be efficacious against STHs [37], which may explain the value of multiple dosing. In sum, all these reports warrant further investigations of NTZ.

NTZ is proposed to be a noncompetitive inhibitor of the pyruvate:ferredoxin/flavodoxin oxidoreductases (PFORs) of *Trichomonas vaginalis*, *Entamoeba histolytica*, *Giardia intestinalis*, *Clostridium difficile*, *Clostridium perfringens*, *H. pylori*, *and Campylobacter jejuni* and is weakly active against the pyruvate dehydrogenase of *Escherichia coli* [38]. In addition, other mechanisms of action of NTZ in protozoa are inhibition of protein disulphide isomerases (PDI2 and PDI4) [39], altered expression of genes involved in stress response such as heat-shock proteins [40], and interaction with a novel *Giardia lamblia* nitroreductase, GlNR1 [41, 42]. In viruses, NTZ is known to inhibit replication of hepatitis B and C viruses [24] and targets viral hemagglutinin at post translational level in influenza virus [43]. It is also proposed to have an anti-vacuolating toxic activity on *H. pylori* [44].

In contrast, nothing is known about NTZ's mode of action on the nematodes, which is a limitation for its clinical use to cure parasitic roundworms. Finding the mode of action of a drug is important for three important reasons- 1) this knowledge can be used to enhance the efficacy of the drug by modifying the drug molecule, by improving its interaction with its drug target, and/or by discovering analogs with improved potency and efficacy; 2) this information can be used to help select other drugs, with independent mechanisms of action, which might be best combined with it; and 3) this knowledge can be used to understand the development of resistance to that drug and thus lead to ways to detect and delay the evolution of resistance.

In this study, we use *C. elegans* to determine the mode of action of NTZ on nematodes. *C. elegans* is a powerful model to study mechanisms of actions of anthelmintic drugs [8], and our group successfully used it to discover the mechanism of action of the anthelmintic drug tribendimidine [45]. In addition, we study the anti-nematode effects of combining NTZ with representatives from each of the two main classes of approved anti-STH drugs, albendazole (a benzimidazole) and pyrantel (an nAChR agonist) in order to ascertain the potential value of NTZ combination therapy.

Methods

2.1. Strains and media

Caenorhabditis elegans strains were cultured and maintained on Nematode Growth (NG) or Enhanced NG media plates [46] seeded with *Escherichia coli* OP50 as food source using standard techniques [47]. The nematode strains used in this study were- Bristol N2, *dpy-11(e224)*, levamisole resistant mutant *lev-8 (ok1519) X*, benzimidazole resistant mutant *ben-1(e1880) III*, ivermectin resistant single mutants avr-*14(ad1302) I, avr-15(ad1051) V*, and triple mutant *avr-14(ad1302);avr-15(ad1051);glc-1(pk54::Tc1)*.

All the reagents and chemicals used in the study were obtained from Sigma (St. Louis, MO, USA), unless otherwise indicated. The drug NTZ (Lot No. 82-767-007) was a gift from Romark Pharmaceutical Laboratories (Tampa, FL, USA). The drug powder was stored at room temperature in a desiccator and prepared fresh for use in the assays as follows. Twenty milligrams of the drug was dissolved into 40 μl of 100% DMSO in a 1.5 ml micro-centrifuge tube. Then, serial dilutions of drug were made by transferring 20 μl of this solution into a new 1.5 ml micro-centrifuge tube containing 20 μl of 100% DMSO until the desired final dilution was achieved. Lastly, 10 μl solution from each serial dilution tube was transferred to a new set of 1.5 ml tubes, respectively, and 490 μl of special S-medium (sS-medium) [45] was added to each tube to get a 10x drug solution in 1% DMSO-sS-medium. 20 μl of this 10x drug solution was added to each assay well to achieve desired drug concentration at 0.1% final DMSO concentration.

To test ivermectin sensitivity of the NTZ mutants, we used plate based assays [48]. 10 ng/mL and 50 ng/mL ivermectin was added to the NG plates. Forty μl *E. coli* OP50 was spread on top of these plates, and the plates were left at room temperature overnight to make a bacterial lawn. Forty-fifty L1 worms were inoculated to each plate, and the plates were incubated at 20°C. The worms on the plates were imaged after 44 and 60 hours.

2.2. Intoxication assays

We used three established *C. elegans* intoxication assays from our laboratory— lethality, developmental inhibition, and brood size inhibition— to study the dose dependent effect of the drugs on *C. elegans* [45, 46, 49, 50]. All the assays were conducted in a 48 well polystyrene plate (Becton Dickinson, NJ, USA, Cat No. 35-3078), with each well containing sS-media (pH=7.3), *E. coli* OP50 as food source, and specific concentration of the drug. The experiments were independently repeated for a total of three or more times.

In the lethality assay, 15–20 L4 stage worms were added to each well containing 130 μl sS-media, 40 μl OP50 ($O.D._{600}$=3.0), 20 μl of the 10x drug solution (see above) and 5 μl 5-Fluoro-2′-deoxy-uridine (FuDR) (Sigma, St. Louis, MO, cat. no. F0503). FuDR was added to stop nematodes from reproducing during the assay [46]. Reproduction complicates the 6-day assay because the growth of progeny uses resources (*e.g.*, drug), and the multiple generations get mixed together, making it harder to ascertain effects. We qualitatively confirmed that the mutants *avr-14(ad1302);avr-15(ad1051);glc-1(pk54::Tc1)*), *ntz-13*, and

ntz-20 were still resistant to 1000 μg/mL NTZ in the absence of FuDR, although, as predicted, resistance was easier to score with the addition of FuDR. In each independent trial, each condition was carried out in triplicate. The survival of worms was scored at 25°C after 6 days. A worm was scored as dead when it failed to move after being touched by an eyelash pick.

To study the dose dependent effect of the drug on worm development in a developmental inhibition assay, 15–20 L1 staged nematodes were added to each well containing 155 μl sS-media, 20 μl OP50 ($O.D._{600}$=3.0) and 20 μl of 10x drug solution, in triplicate wells. The number of worms reaching gravid adult stage, *i.e.*, having one or more egg in their uterus, was counted at the end of 60 hours at 20°C.

The dose dependent effect of the drug on the brood size of the worms was studied by transferring an individual L4 worm using a eyelash pick to a well containing 140 μl sS-media, 40 μl OP50 ($O.D._{600}$=3.0), 20 μl of 10x drug solution, in quintuplicate wells. The plates were incubated at 25°C for 64 hours, and the brood size at that time point was counted for each well by transferring the contents of the well to a counting dish.

2.3. Genetic screen for NTZ resistant mutants, complementation testing, molecular characterization

Approximately 52,000 synchronized L4 *C. elegans* larvae were mutagenized using 30 mM ethyl methanesulfonate (EMS) method as described [51]. These mutagenized P_0 L4 larvae were transferred to ENG plates seeded with OP50 and incubated at 20°C overnight to get gravid females, which were bleached as per standard protocol [46]. About 128,000 F_1 embryos were isolated and allowed to hatch overnight in M9 solution [51] at 25°C. The resulting F_1 L1 larvae were seeded on ENG-OP50 plates and incubated at 20°C for 72 hours to get F_1 gravid adults. These F_1 adults were bleached as above to obtain F_2 embryos, which hatched overnight in M9 to produce approximately 450,000 F_2 L1 larvae. Of these 450,000 F_2 L1 larvae, ~36,000 (~8% of total F_2 L1s) were directly screened in 1000 μg/mL NTZ by transferring 10–15 F_2 L1s to individual wells containing 120 μl sS media, 40 μl OP50 ($O.D._{600}$=3.0), and 20 μl drug solution (no FuDR) in a 48 well plate. The plates were incubated at 25°C for 7–9 days, and resistant mutant worms were identified as actively swimming and relatively well developed worms as compared to dead/paralyzed/morbid or less developed worms in the control wells with wild type N2 worms. The resistant worms were picked to individual NG-OP50 plates, incubated at 20°C, allowed to reproduce, and retested on 1000 μg/mL NTZ in 48 well plates.

The retested and validated mutants *ntz-13* and *ntz-20* were out-crossed twice to wild-type by using combination of N2 males and *dpy-11(e224)*, resulting in both *ntz-13*, ntz-20, *ntz-13;dpy-11(e224)* and *ntz-20;dpy-11(e224)* out-crossed lines. For complementation testing with *avr-14(ad1302)*, *avr-14(ad1302)* males were mated with *ntz-13;dpy-11(e224)* and *ntz-20;dpy-11(e224)* hermaphrodites. In addition, *ntz-13* and *ntz-20* males were mated into *ntz-20;dpy-11(e224)* and *ntz-13;dpy-11(e224)* hermaphrodites, respectively. From each mating plate, 10–15 non-dumpy L4s were picked and tested for resistance in 1000 μg/mL NTZ in wells containing 115 μl sS media, 40 μl OP50 ($O.D._{600}$=3.0), 5 μl FuDR, and 20 μl drug solution in a 48 well plate. Dead and alive worms were scored after 6 days at 25°C. To determine the genomic sequence of *avr-14* gene from *ntz-13* and *ntz-20*, *avr-14(ad1302)*, *C. elegans* DNA was isolated using by picking up mutant worms in worm lysis buffer (50 mM KCl, 10 mM Tris pH 8.3, 2.5 mM MgCl2, 0.45% NP-40 (IGEPAL), 0.45% Tween-20, 0.01% gelatin, 0.01% Proteinase K) [52] and PCR amplified using Platinum HiFi DNA polymerase (Invitrogen) from *ntz-13* and *ntz-20* and sequenced using a set of 8 primer pairs listed in Supplementary Table 1.

2.4. Drug combination studies

For the drug combination studies, we selected albendazole and pyrantel to combine with NTZ. Albendazole is currently the best and most widely used drug for mass drug administration of intestinal parasitic worms. We first worked on finding the most appropriate assay to study drug combination for each of the drug by testing albendazole and pyrantel in all the three intoxication assays as described above. The drugs were combined in 1/8x, 1/4x, 1/2x, 1x, 2x, 4x, and 8x ratio of their appropriate IC values [53, 54]. The higher ratios (4x and 8x) were used only when physically not limited by amount of drug. Desired concentration of each of the drug used for combination was prepared as described above to prepare a 20x solution in 1% DMSO-sS media, and 10 μl of this drug solution(s) was added to each well. The final volume of sS media, DMSO, OP50 and number of worms were same as described above for the intoxication assays.

2.5. Statistical analysis

The dose response curves were fitted using GraphPad Prism 5. The lethal concentration 50% (LC_{50}), LC_{90} and inhibitory concentration (IC) values were calculated using GraphPad Prism 5 (GraphPad Software, La Jolla, CA). The drug combination index (CI) values were calculated using CompuSyn software Version 1 (ComboSyn, Inc., NJ, USA, http://www.combosyn.com/). The statistical analyses of data (indicated in the appropriate figure legends) were done using GraphPad Prism 5.

3. Results

3.1. C. elegans N2 is sensitive to NTZ

There is only one published study, with very limited brood size data, documenting the effects of NTZ on *C. elegans* [55]. To quantitatively ascertain the inhibitory effects of NTZ on wild type *C. elegans* wild-type N2 worms, we used three different assays- lethality, developmental inhibition, and brood size inhibition. All of these have been standardized in our lab and successfully used to study effects of different drugs on *C. elegans*, [45, 46, 49, 50].

NTZ is able to kill *C. elegans* at high doses in a dose-dependent, with near complete penetrance at 1000 μg/mL (3.3 mM; Figure 1A–C). The lethal concentration 50% (LC_{50}) value (lethal concentration 50% or the drug concentration at which 50% of the nematode population is dead) at 25°C after 6 days was 521 μg/ml (1.7 mM; Table 1). In the brood size assay, NTZ reduced the reproduction of wild-type *C. elegans* by 53% at 1000 μg/ml (Figure 1D; inhibitory concentration 50% (IC_{50}) value 678.6 μg/ml or 2.2 mM; Table 1). Development of wild-type *C. elegans* from the first larval to adult stage was much more sensitive to NTZ, with an IC_{50} value of 4.5 μg/ml (15 μM; Figure 1E, Table 1).

3.2. Isolation of NTZ resistant mutants

We screened for NTZ resistance using ~36,000 mutagenized F_2 L1s exposed to 1000 μg/ml NTZ at 25°C for 8 days. In total, 59 resistant candidates were rescued from the screen wells, 36 or which either died or failed to produce progeny. The remaining 23 candidates, plus an addition 8 candidates from an earlier pilot screen, were tested against NTZ for reconfirmation, and named *ntz-1* through *ntz-31* (Figure 2A). Six strains showed >30% survival on 1000 μg/mL (3.3 mM) NTZ. We retested these six candidate lines at least three additional times on 1000 μg/mL NTZ and selected the two most reproducibly resistant mutants, namely *ntz-13* and *ntz-20*, for further studies (*e.g.*, *ntz-9* and *ntz-12* showed significantly greater variability in their resistance relative to *ntz-13* and *ntz-20* upon multiple retests). These were out crossed two times to wild-type *C. elegans* N2.

The twice out crossed *ntz-13* and *ntz-20* and their *dpy-11(e224)* versions *ntz-13;dpy-11(e224)* and *ntz-20;dpy-11(e221)* plates were transferred to wells containing 1000 μg/ml NTZ (3.3 mM), incubated at 25°C for 6 days, and scored for viability. The twice out crossed mutant worms were significantly resistant to NTZ with 48% *ntz-13*, 47% *ntz-20*, 39% *ntz-13;dpy-11(e221)* and 37% *ntz-20;dpy-11(e221)* nematodes surviving at the end of 6 days; as compared to 3% and 0.9% for wild-type and *dpy-11* respectively (Figure 2B).

3.3. Ivermectin resistant mutants avr-14;avr-15;glc-1 and avr-14 are resistant to NTZ

We set out to ask if NTZ shares mechanism of action with any other known class of anthelmintics. We tested the dose response of *C. elegans* mutant worms resistant to other classes of drugs on NTZ. Levamisole resistant mutant *lev-8 (ok1519) X*, benzimidazole resistant mutant *ben-1 (e1880) III*, and ivermectin resistant triple mutant *avr-14(ad1302);avr-15(ad1051);glc-1(pk54::Tc1)* were tested on NTZ, and the dose response was compared to N2. We found that the ivermectin resistant triple mutant *avr-14(ad1302);avr-15(ad1051);glc-1(pk54::Tc1)* was significantly (*P<0.0001*) resistant to NTZ with 52% worms surviving at the highest dose of 1000 μg/ml (3.3 mM) as compared to N2 (3.9% survival) at the end of 6 days at 25°C (Figure 3A). Although the drug had a statistically significant effect at 500 μg/mL (1.6 mM) and 1000 μg/mL (3.3 mM; relative to 0 μg/mL), statistically significant resistance with the triple mutant was seen only at 1000 μg/mL (3.3 mM).

Since the ivermectin mutant that we found resistant to NTZ was a triple mutant, we wanted to determine which of those three genes was responsible for the resistance to NTZ. We obtained the mutants avr-*14(ad1302)* and *avr-15(ad1051)* and determined their dose response to NTZ. The single mutant avr-*14(ad1302)* was resistant to NTZ (39.6% alive) similar to the triple mutant *avr-14(ad1302);avr-15(ad1051);glc-1(pk54::Tc1)* (37.7% alive) (Figure 3B) at 1000 μg/mL NTZ (3.3 mM), and both were significantly (*P<0.0001)* resistant relative to N2 wild-type (1.8% alive) (Figure 3B). Thus, mutation in the gene *avr-14(ad1302)* alone is responsible for resistance against NTZ.

We tested whether or not *ntz-13*, *ntz-20*, and *avr-14* might be alleles of each other using standard allelic complementation testing. However, the results showed evidence of semi-dominant effects *for avr-14* and possible semi-dominant effects for *ntz-20* and *ntz-13.* To determine if *ntz-13* and *ntz-20* might be alleles of *avr-14*, we therefore sequenced the entire *avr-14* open reading frame from genomic DNA of each of these mutant lines. For both mutants, we obtained the wild-type *avr-14* sequence. Thus, *ntz-13* and *ntz-20* are not mutations in the coding sequence of *avr-14*.

3.4. NTZ mutants are sensitive to ivermectin

As an ivermectin resistant mutant is resistant to NTZ, we conversely wanted to know if NTZ resistant mutants are resistant to ivermectin. To test this hypothesis, we used a plate based qualitative assay in which ivermectin was added to the plates seeded with *E. coli* OP50 in 0, 10 ng/ml (11 nM) and 50 ng/ml (57 nM) concentrations [56]. Forty L1s of wild-type N2, *ntz-13*, *ntz-20*, avr-*14(ad1302)*, and triple mutant *avr-14(ad1302);avr-15(ad1051);glc-1(pk54::Tc1)* worms were placed on the ivermectin plates, incubated at 20°C and imaged at 44 and 60 hours. Of all the mutants, only the triple mutant showed resistance to ivermectin and grew normally on the plates (Figure 4). That *avr-14* mutant did not grow, but the triple mutant did, is consistent with published results (Figure 4) [48, 56].

3.5. NTZ is synergistic with albendazole and pyrantel

To determine the suitability of NTZ for multi drug combination therapy, we combined NTZ with albendazole (benzimidazole) and pyrantel (nAChR agonist) to ascertain potential synergy between the drug classes. A synergistic effect can increase the efficacy of the drugs, decrease the dosage, and might delay the development of resistance [53]. Combination index (CI) values can be used to define the kind of interaction between drugs as additive, synergistic or antagonistic. CI value of <0.7 indicate synergy [54].

The first task was to find a suitable assay for combination of the selected drugs with NTZ. Albendazole does not kill *C. elegans* in liquid assays and showed a "U-shaped" curve when assaying the inhibition of development (Supplementary Figure 1). Thus, neither lethality nor developmental inhibition was a suitable assay. However, like NTZ, albendazole reduces the 64 hour brood size of *C. elegans* (51% at 1000 μg/mL or 3.3 mM; Supplementary Figure 1). We thus used the brood size assay for combination of albendazole and NTZ. Typically, drugs are combined in the ratios of their IC_{50} values for combination studies using the combination index approach [53, 54]. Since albendazole did not bring down the brood size by half, no IC_{50} value could be calculated and the two drugs were combined in 1:1 ratios of their IC_{25} values (albendazole - 492.3μg/ml or 1.9 mM, NTZ- 422.1 μg/ml or 1.4 mM). The dose response of the individual drugs, the drug combination, and the combination values are given in Figure 5A and Table 2. Albendazole and NTZ combinations at IC_{25} ratios of 1/4, 1/2, 1 and 2x gave extremely low CI values (Table 2), suggesting a highly synergistic effect.

Developmental inhibition assay was used to combine NTZ with pyrantel as pyrantel inhibited nematode development at low concentrations with a dose-dependent response (Supplementary Figure 1). The two drugs were combined in the 1:1 ratios of their IC_{50} values (NTZ-4.5 μg/ml or 15 μM, pyrantel-19.9 μg/ml or 56 μM) NTZ showed strong synergistic interactions with pyrantel at the combination ratios of 1/8x, 1/4x, 1/2x (Figure 5B, Table 2). In summary, NTZ combined synergistically both with albendazole and with pyrantel.

4. Discussion

Here we characterize in detail the activity of NTZ as an anthelmintic against *C. elegans* for the first time. We demonstrate that *C. elegans* viability, reproduction, and development are susceptible to NTZ, and that *C. elegans* mutants resistant to NTZ can be identified. Furthermore, we demonstrate that mutation of the *avr-14* gene, which plays an important role in the response of *C. elegans* to ivermectin, likely leads to NTZ resistance in *C. elegans* (we cannot exclude the remote possibility that both the *avr-14(ad1302);avr-15(ad1051);glc-1(pk54::Tc1)* triple mutant and *avr-14(ad1302)* single mutant contain a secondary background mutation that results in NTZ resistance). DNA sequencing reveals that the resistant alleles obtained in our screen are different than *avr-14* (cloning of the two resistance alleles is reserved for future study). Whereas the *avr-14* ivermectin-resistant mutant is resistant to NTZ, none of the *C. elegans* mutants resistant to NTZ are resistant to ivermectin. We finally demonstrate that both pyrantel and albendazole can be productively combined with NTZ to yield highly synergistic anthelmintic activity.

C. elegans avr-14 mutant animals are statistically not resistant to 500 μg/mL NTZ, although consistently we do see *avr-14(ad1302)* and *avr-14(ad1302);avr-15(ad1051);glc-1(pk54::Tc1)* mutant animals are more viable than wild-type animals at this dose of NTZ. These data suggest that at low levels of NTZ intoxication, a pathway other than *avr-14* also plays a role in intoxication. Nonetheless at higher levels of NTZ, that result in nearly complete lethality in wild-type, *avr-14* plays a dominant role in NTZ intoxication in *C. elegans*. We also note that some NTZ doses used

are high (*e.g.*, 1000 μg/mL), which might cause off target effects. However, it is likely that the requirement for high dose of NTZ for some assays is due to the notorious nature of the *C. elegans* cuticle, which is thought to be more impermeable than that of parasitic nematodes and which makes it difficult for drugs to get in [57, 58]. Consistent with the relative impermeability of the *C. elegans* cuticle, we have found that *C. elegans* is generally more resistant to anthelmintics than parasitic nematodes are *in vitro*, *e.g.*, albendazole, pyrantel, and NTZ are all more effective against the hookworm *Ancylostoma ceylanicum* and the large roundworm *Ascaris suum in vitro* than against *C. elegans* [37].

avr-14, also known as *gbr-2*, encodes an alpha-type subunit of a glutamate-gated chloride ion channel and has two splice variants [56, 59]. In *C. elegans*, it is expressed in a subset of neurons in the ring ganglia, ventral cord, and some mechanosensory neurons [56]. It is reported that a single mutation in *avr-14* does not confer notable resistance to ivermectin, whereas an *avr-14* double mutant with *avr-15* (a glutamate gated chloride ion channel on muscle cells) and triple mutant with *avr-15* and *glc-1* were significantly highly resistant to ivermectin [56]. In addition, *avr-14* is conserved in parasitic nematodes [56, 60–62] whereas *avr-15* is not [56].

The finding that NTZ acts via *avr-14* is a striking and significant result. Clinically, NTZ dosed over three days has been shown to act against a wide range of soil-transmitted helminths, including large roundworm *Ascaris lumbricoides* (69–95% parasitological cure rate), whipworm *Trichuris trichiura* (76–86% cure rate), the hookworm *Ancylostoma duodenale* (96% cure rate), and the threadworm *Strongyloides stercoralis* (94% cure rate) [33, 35]. Since NTZ works via *avr-14*, then our results, in combination with the clinical results demonstrate that *avr-14* is an excellent target for drug development against a wide range of human STH parasites.

Ivermectin in one model is proposed to kill worms by opening the chloride ion channels very slowly but irreversibly, causing a long-lasting hyper-polarization or depolarization of the neuron or muscle cell and blocking its function [63]. Although *avr-14* is important for ivermectin and NTZ activity against nematodes, our results to date suggest that the two drugs might not share the exact same mechanism of action. Whereas ivermectin paralyzes *C. elegans* at very low concentration, NTZ kills worms only at higher concentrations, although this difference could be due to bioavailability or binding affinities. A single mutation in *avr-14* does not make *C. elegans* resistant to ivermectin, but it does provide sufficient resistance against NTZ. In addition, we found at least two more mutants that were resistant to NTZ but were not resistant to ivermectin. Furthermore, these mutants do not carry a mutation in the *avr-14* coding sequence, indicating that NTZ can kill *C. elegans* worms through additional mechanisms independent of *avr-14*.

Our studies also indicate that NTZ is an excellent candidate for multi-drug combinations. NTZ combines synergistically with two major drugs for human STH therapy- albendazole and pyrantel. Our results are partially in contrast to two other studies [36, 64]. Speich *et al*. 2012 found that combining single-dose albendazole with single-dose NTZ did not provide added benefit against clinical whipworm infections. However, the two treatments (albendazole and NTZ) were given one day apart and thus was not a true combination therapy. In another study on combinations of NTZ with albendazole and pyrantel against two parasitic nematodes *in vitro* and, for NTZ-pyrantel, one parasite *in vivo* [64], NTZ-albendazole combination was found antagonistic; for NTZ-pyrantel opposite results were found, depending upon the parasite. There are, however, some differences between our approaches. For example, our readout is reproduction and development, whereas the previously published *in vitro* studies on the parasite were done using motility. As expected for working with parasites, the numbers of parasitic nematodes analyzed are generally lower

and, as a result, P values are sometimes not significant. Unlike our study with *C. elegans*, single-dose efficacies with the parasites were often not collected in the same experiment as the combination experiment, adding another variable to the analyses. In our case, when we could not achieve IC50 values, we combined at IC25 values. In the case of the parasites, when IC50 could not be reached, estimates of the IC50 values were used. These and other differences (*e.g.*, larger n, relative ease of setting up many conditions simultaneously, access to various measures of intoxication, smaller standard errors and greater reproducibility with *C. elegans*) highlight the utility of *C. elegans* for synergy studies with nematodes ([50] and this study), The caveats are that *C. elegans* is not a parasitic nematode and assays with *C. elegans* lack complexities found i*n vivo*. Nevertheless, *C. elegans* appears to have utility to initially assess the inherent combining properties of multi-drug anthelmintic combinations.

In summary, we have found that NTZ acts in *C. elegans* via the *avr-14* ion channel in a manner likely different than ivermectin. Furthermore, we find that NTZ has potential to be highly synergistic against nematodes in combination with albendazole and pyrantel. Given the wide spectrum of activity of NTZ against of human STH parasites (albeit with multiple doses), our data indicate that development of superior drugs that target *avr-14* has excellent potential to treat a large range of human STH parasites.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Highlights

- We study the anthelmintic nitazoxanide using the nematode *Caenorhabditis elegans*
- *C. elegans* strains resistant to killing by nitazoxanide are identified
- Nitazoxanide kills *C. elegans* through glutamate gated chloride ion channel *avr-14*.
- Additional targets of nitazoxanide are identified as NTZ-13 and NTZ-20
- Nitazoxanide combines synergistically with albendazole and pyrantel.

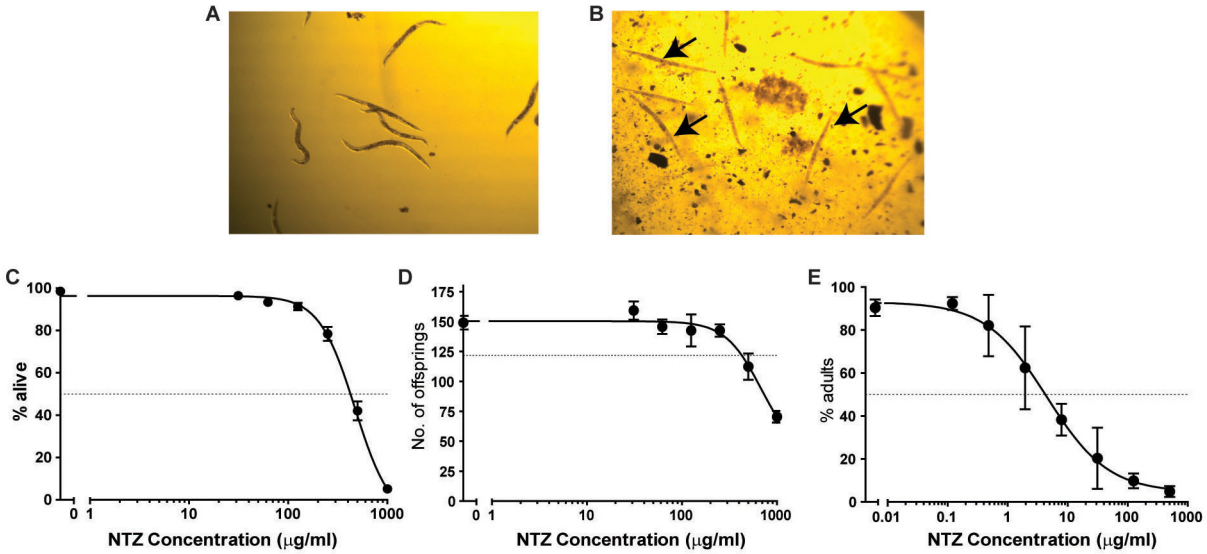

Figure 1.

Effect of NTZ on *C. elegans* N2 wild-type nematodes. **A.** Alive worms in control wells without NTZ, **B.** Dead nematodes, seen as rigid rods indicated by arrows, in 1000 g/ml NTZ after 6 days at 25°C. N2 dose response curves to NTZ in **C.** Mortality assay (n=45–60), **D.** Brood size inhibition assay (n=15) and **E.** Developmental inhibition assay (n=45–60). Error bars represent standard error of the mean, assays repeated at least three independent times.

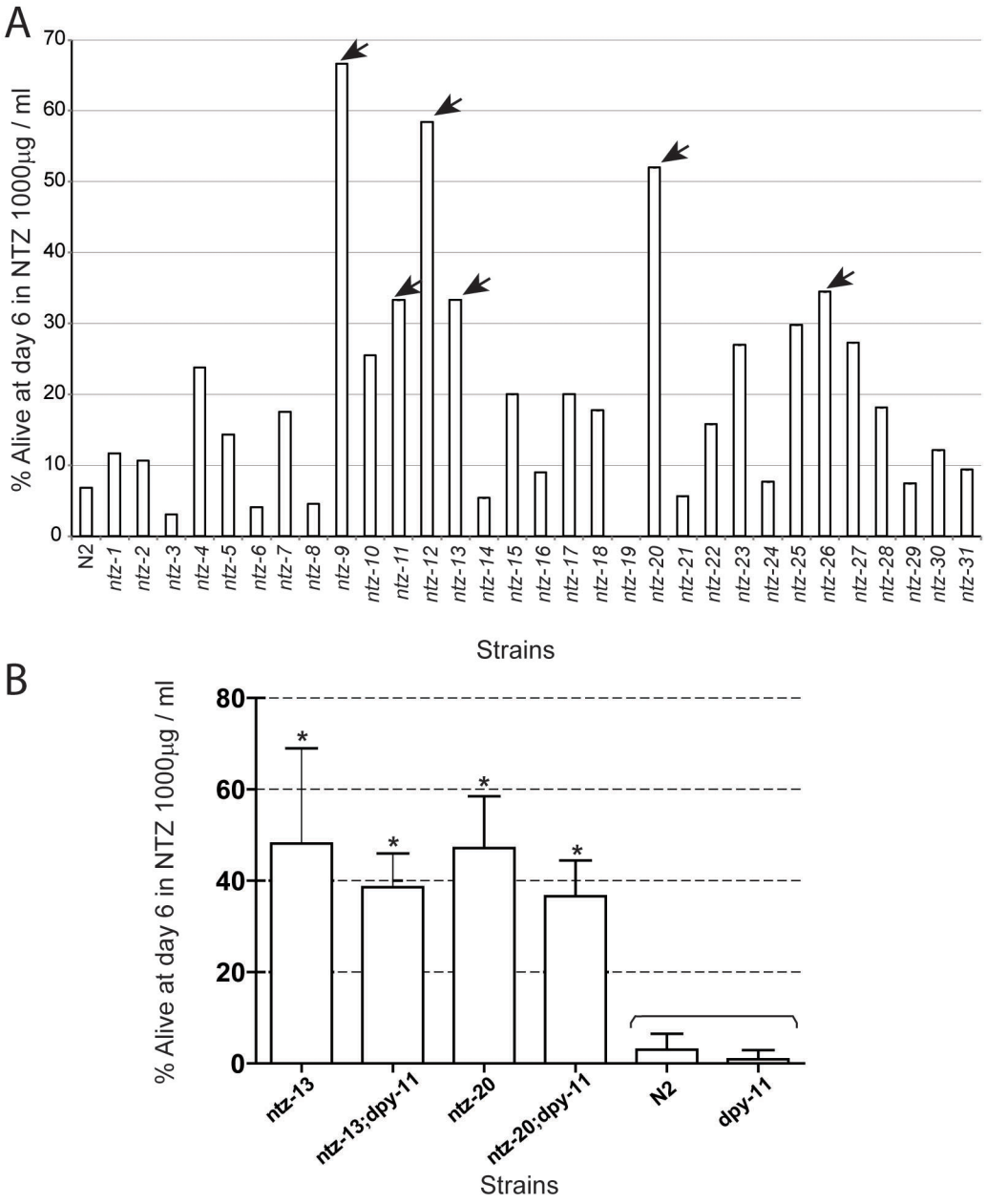

Figure 2.

Forward genetic screen for identification of NTZ resistant worm mutants. **A.** A total of 31 NTZ resistant mutants were identified in the screens and retested on NTZ. Six mutants showing high resistance to NTZ (indicated by arrows) were selected for rigorous retesting for NTZ resistance. **B.** Twice out-crossed *ntz-13* and *ntz-20* mutant worms and their *dpy-11(e221)* marked versions showed high resistance to NTZ. Ten to 15 L4 nematodes of the indicated genotype were plated in three independent wells for each trial. Error bars represent standard error of the mean for three independent trials. For each mutant strain, $P<0.0001$, 1-Way ANOVA, Dunnett's multiple comparison post test compared to respective control.

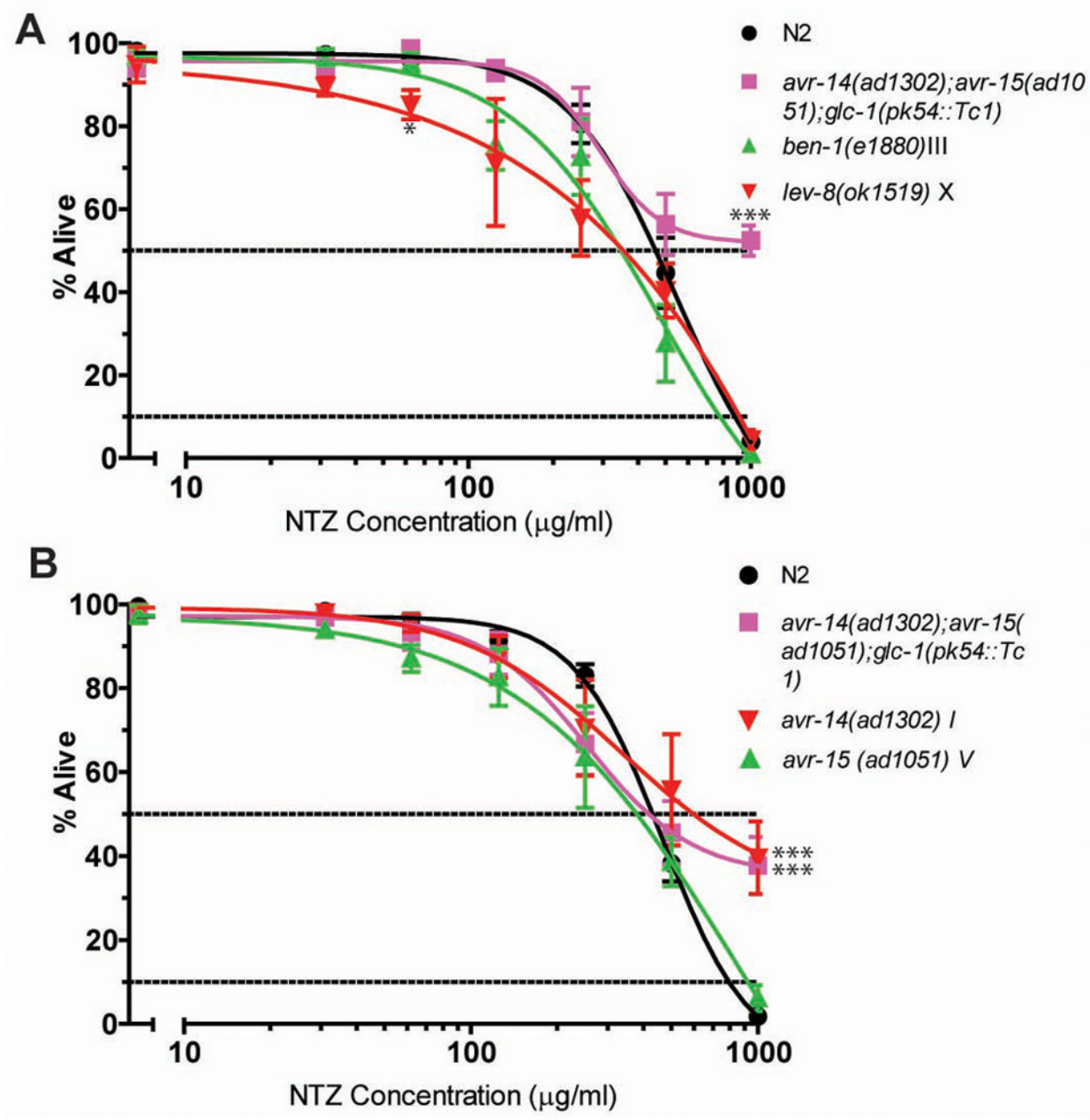

Figure 3.

Dose response curves of *C. elegans* mutants resistant to other classes of drugs to NTZ in a six day lethality assay. **A.** An Ivermectin resistant triple mutant *avr-14(ad1302);avr-15(ad1051);glc-1(pk54::Tc1)* was resistant to NTZ; mutants resistant to benzimidazoles and nAChR agonists were not resistant to NTZ. The levamisole resistant mutant *lev-8 (ok1519)* is significantly hypersensitive to NTZ at the 62.5 g/ml dosage (*P<0.05*), but not at any other dosage. **B.** Dose response of ivermectin single mutants against NTZ revealed that resistance of the triple mutant to NTZ can be accounted for by the *avr-14(ad1302)* mutation alone. * *P<0.05*, *** *P<0.0001*, 1-way ANOVA, Dunnett's Multiple Comparison post test, comparing each mutant to wild-type at any given dose.

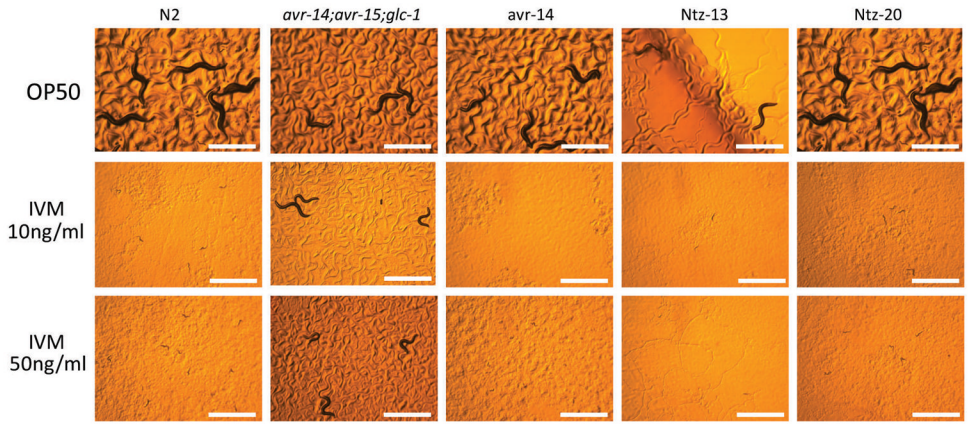

Figure 4.

Ivermectin (IVM) susceptibility of the two NTZ resistant mutants identified in forward genetic screens at 44–45 hours. The ivermectin resistant triple mutant *avr-14(ad1302);avr-15(ad1051);glc-1(pk54::Tc1)* and the ivermectin sensitive single mutant *avr-14(ad1302)* are controls. (Scale bar = 1 mm).

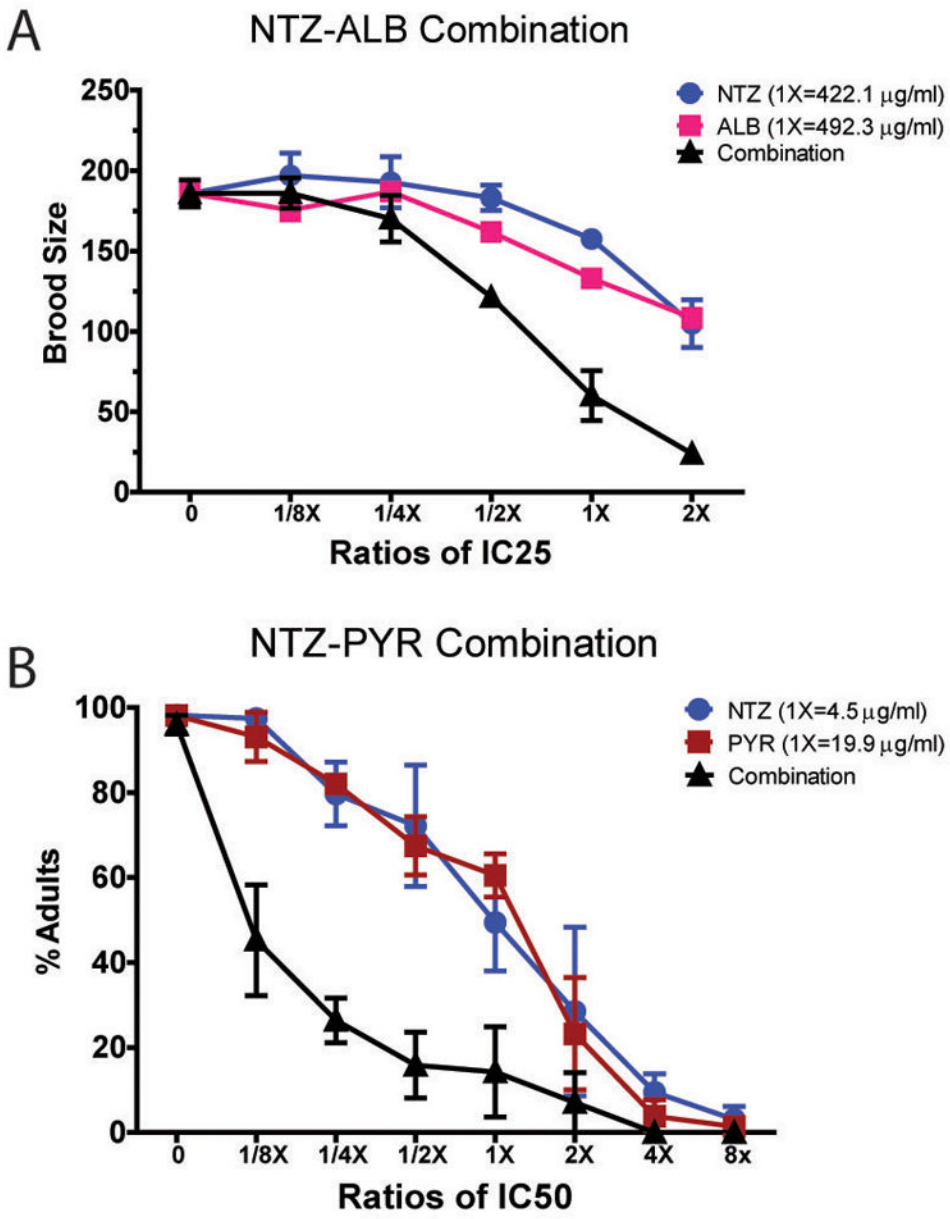

Figure 5.

Combination of NTZ with albendazole (ALB) and pyrantel (PYR). A. NTZ combination with albendazole in a brood size inhibition assay, B. NTZ combination with pyrantel in a developmental inhibition assay. Error bars show standard error.

Table 1

IC_{50} and IC_{90} values of N2 and mutant *C. elegans* N2 worms on NTZ

Strain	Intoxication Assay	IC_{50} (95%confidence Interval) (μg/ml)	IC_{90} (95%confidence Interval) (μg/ml)
N2	Lethality	521.0 (414.2 to 656.1)	946 (599.9–1494)
N2	Brood size	678.6 (158.2 to 2912)	No IC_{90}
N2	Developmental	4.5 (1.5–13.7)	59.97 (4.4–811.2)
ben-1	Lethality	359 (295–438)	835.6 (570 to 1223)
lev-8	Lethality	341 (238–488)	1379 (692.1 to 2747)
avr-14; avr-15; glc-1	Lethality	921 (652–1300)	6518 (2080 to very high)
avr-15	Lethality	332.3 (272.3 to 405.7)	1303 (815.5 to 2083)
avr-14	Lethality	630.2 (438.4 to 905.7)	4509 (1552 to 13102)

Table 2

Combination index values of drug combinations. The synergistic effects are indicated as bold font.

Drug Combination Ratio	Combination Index Value
Nitazoxanide- Albendazole	
(Brood Size, Drugs combined in 1:1 ratio of their IC_{25} values)	
1/8x	855.669
1/4x	0.52520
1/2x	0.00312
1x	5.77E-5
2x	1.70E-6
Nitazoxanide-Pyrantel	
(Developmental Inhibition, Drugs combined in 1:1 ratio of their IC_{50} values)	
1/8x	0.32736
1/4x	0.26595
1/2x	0.26608
1x	0.45328
2x	0.38749
4x	0.08642
8x	0.17285