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Anthelmintic effect of herbicidal dinitroanilines on the nematode model *Caenorhabditis elegans*

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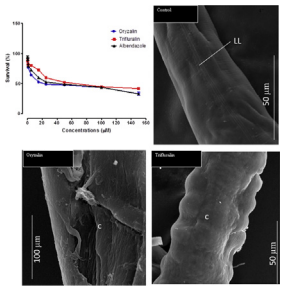
HIGHLIGHTS

- Dinitroanilines affected *Caenorhabditis elegans* adults and inhibited the development of eggs and larvae.
- Dinitroanilines induced several alterations in the ultrastructure at all *C. elegans* life stages.
- Dinitroanilines are promising lead molecules for the development of new compounds for the treatment of helminthiasis.

GRAPHICAL ABSTRACT



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ABSTRACT

Dinitroanilines are known herbicides that impair the polymerization of microtubules. This study investigated the effects of oryzalin and trifluralin on the viability, morphology, and ultrastructure of different life stages of *Caenorhabditis elegans*. Both drugs reduced the survival of the adult population in 50% after three days of treatment with concentrations of approximately 30 μM and 57 μM, respectively. The development of new adults was monitored for seven days and treatment with both drugs also showed a decrease in the adult population. 25 μM Oryzalin or trifluralin inhibited the hatching of eggs by nearly 100%. Both drugs showed remarkable larvicidal activity at 25 μM against the larvae at first and second stages (L1–L2) and at third and fourth stages (L3–L4) after 24 h. Treatment with dinitroanilines led to incomplete egg embryo development. The oryzalin and trifluralin treatments caused the detachment of cuticular layers of adults and larvae and the formation of a large number of intracellular membrane whirls and lipid bodies in the hypodermal cells and non-contractile muscles of adults. Both drugs also provoked the bagging process, which generated lesions in the uterus of the adults. In addition, trifluralin caused the detachment of certain areas of the cuticle adjacent to the hypodermis in a large number of nematodes. Our results suggest that dinitroanilines are a potentially new alternative for anthelmintic chemotherapy.

1. Introduction

The control of helminth infections in animals and plants has been investigated by researchers for decades, yet the anthelmintic

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arsenal against nematodes is limited and is comprised primarily of four chemical classes: the benzimidazoles, imidazothiazoles, tetrahydropyrimidines and macrocyclic lactones (McKellar and Jackson, 2004, Köhler, 2001). On the other hand, praziquantel, a pyrazino quinoline derivative, is the main anthelmintic used against the human parasite *Schistossoma mansoni* (Chai, 2013).

Dinitroanilines belong to the herbicide family and interfere with plant cells by interrupting mitosis and inducing the formation of multinucleated cells. This family is divided into two subfamilies: the methylanilines, which include the most important member of the group, trifluralin; and the sulfonylanilines, represented in this study by oryzalin. Recent studies have shown that dinitroanilines interfere with the stability of microtubules and their function in protozoa parasites as *Toxoplasma gondii*, *Trypanosoma cruzi*, *Trypanosoma brucei*, *Leishmania* sp. and *Giardia lamblia* (Morrissette et al., 2004; Endeshaw et al., 2010; de Souza, 2013).

Microtubules play an important role in several cellular functions and have been considered an ideal target for anticancer and antiparasitic drugs (Jordan and Kamath, 2007).

The benzimidazoles are a class of broad-spectrum anthelmintics that affect the cytoplasmic microtubules by selective binding to the β-tubulin molecules with a high affinity. It has been suggested that benzimidazoles act by inhibiting the microtubule-mediated transport of secretory vesicles in the helminths absorptive tissues, with a consequent release of digestive enzymes and tissue damage (Köhler, 2001). The mode of action of dinitroanilines is similar to that of benzimidazoles; however, the dinitroanilines bind selectively to the α-tubulin subunit, thus inhibiting microtubule polymerization (Lyons-Abbott et al., 2010; Morrissette et al., 2004).

Today, resistance to benzimidazoles is a serious problem in helminthiasis control. Experimental reports have identified single amino acid mutations associated with a loss of affinity of the benzimidazole for the β-tubulin as the major cause of the drug resistance (Alvarez et al., 2001). In this study, we investigated the anthelmintic activity of two dinitroanilines, oryzalin and trifluralin, and their effects on the general morphology and ultrastructure of different stages of *Caenorhabditis elegans*, which is used as a model nematode.

2. Materials and methods

2.1. Maintenance of C. elegans

Wild-type *C. elegans* Bristol strain N2, obtained from the Caenorhabditis Genetics Center, was grown at 22 °C on NGM (nematode growth medium: 2.5 g casein peptone, 3 g NaCl, 17 g agar, 0.5% cholesterol, 1 mM $CaCl_2$, 1 mM $MgSO_4$, and 25 mM KH_2PO_4/K_2HPO_4 per liter of water) seeded with *Escherichia coli* strain OP50 as described by http://www.sciencedirect.com/science/article/pii/S0014489413001409Brenner (1974).

2.2. Drugs

Oryzalin, trifluralin and albendazole were purchased from Sigma Chemical Company (St. Louis, MO, USA). For the experiments, they were dissolved in dimethyl sulfoxide (DMSO) from Merck. DMSO in the medium never exceeded 0.1% (v/v) and had no effect on the survival of nematodes.

2.3. Anthelmintic activity of dinitroanilines

2.3.1. Assays using adults

The Bristol N2 wild-type *C. elegans* strain was synchronized by incubating the culture with lysing solution (5 M NaOH and 1% hypochlorite), and collecting just the eggs. After three days, the nematodes originated had the same age. The nematodes were maintained as described in Section 2.1. They were collected by washing the bottom of the plates with M9 buffer (3 g KH_2PO_4, 6 g Na_2HPO_4, 5 g NaCl, and 0.25 g $MgSO_4 \cdot 7H_2O$ per liter of water) and centrifuged at 800*g* for 5 min. The supernatant was removed, the helminths were washed in the same buffer, and thirty nematodes in S medium [(S basal 5.85 g NaCl, 1 g K_2 HPO_4, 6 g KH_2PO_4, 1 ml cholesterol (5 mg/ml in ethanol), H_2O to 1 L) 10 ml potassium citrate 1 M pH 6, 10 ml trace metals solution, 3 ml $CaCl_2$1 M, 3 ml $MgSO_4$ 1 M per liter of water] supplemented with *E. coli* were added per well in a 96-well plate and incubated at 22 °C. Different concentrations (1–150 μM) of oryzalin or trifluralin were added just once in the beginning of treatment. The development and survival of the nematodes were observed after three or seven days of incubation.

2.3.1.1. Survival assays. In the survival assays, 30 synchronized adults were treated with 1, 5, 15, 25, 50, 100 and 150 μM of oryzalin, trifluralin or albendazole (used only for comparison) for three days. A group without treatment was used as a negative control in all experiments. After the incubation time, the drugs were removed, the nematodes were washed with M9 buffer and the survival was examined.

The evaluation of the survival of nematodes was performed considering, the motility and the paralysis of the pharyngeal bulb associated with the loss of motility with the occurrence of straight or curved bodies. The survival of adults was counted by optical microscopy excluding the larvae stages, which hatched during the assay. The size and the presence of reproductive organs were used as criteria to follow the original adult population. In the BOD incubator at 22 °C new generations of adults were not observed. The number of living adults was compared to the initial number to calculate the percentage, considering the 30 adults as 100%. Triplicates of six independent experiments were performed.

2.3.1.2. Adult growth assays. The development of the adult population was monitored during seven days, period for the development of multiple generations. 30 synchronized adults were incubated with different concentrations (1, 5, 15, 25, 50, 100 and 150 μM) of dinitroanilines and albendazole and observed after 30 min, one day, three days or seven days. A group without treatment was used as a negative control in all experiments. Only the motile adult worms were considered alive. Triplicates of six independent experiments were performed.

2.3.2. Egg hatch assay

The adults were collected from the NGM plates, centrifuged at 800 × *g* for 5 min, resuspended in lysing solution (5 M NaOH and 1% hypochlorite) and incubated for 5 min in order to synchronize the culture and keep just the eggs.

The eggs were collected and centrifuged at 1000 × *g* for 10 min, suspended in M9 buffer, and counted. Approximately 30 eggs per well in a 96-well plate were incubated for 15 h at 20 °C in S medium supplemented with *E. coli* and the concentrations 1, 25 and 150 μM of oryzalin or trifluralin. At the end of the incubation, the percentage of hatched and unhatched eggs and L1 larvae were determined for each of the different drug concentrations. Triplicates of six independent experiments were performed.

2.3.3. Larval development assay

Eggs were obtained as described in Section 2.3.2 and placed in microtubes containing S medium and *E. coli* with gentle stirring for 15 h at 20 °C. Pool of larvae containing first and the second stages larvae (L1 and L2) (20 larvae in 50 μl) were collected and incubated for 24 h at 20 °C in 96-well plates containing S medium