

**EVALUATION OF ANION TRANSPORTERS AS POTENTIAL TARGET SITES  
FOR INSECT AND NEMATODE CONTROL: TOXICOLOGICAL,  
ELECTROPHYSIOLOGICAL, AND MOLECULAR STUDIES**

Dhana Raj Boina

**ABSTRACT**

In this study, four anion transporter (AT) blockers, DIDS (4, 4'-diisothiocyanatostilbene-2, 2'-disulfonic acid), 9-AC (anthracene-9-carboxylic acid), NPPB (5-nitro-2-(3-phenylpropylamino) benzoic acid), and IAA-94 (indanyloxy acetic acid) were selected to evaluate ATs as potential target sites for insect and nematode control.

All the AT blockers showed slowly developing toxicity against second-stage larvae of *Meloidogyne incognita* (Kofoid and White 1919) Chitwood 1949 and adults of *Caenorhabditis elegans* Maupas 1900 but not against third-stage larvae of *Heterorhabditis bacteriophora* Poinar 1975 even at 200 ppm. Symptoms of AT blocker toxicity observed in *C. elegans* adults were increased pharyngeal muscle contractions and decreased locomotion. Exposure of *C. elegans* as fourth-stage larvae to double-stranded RNA (dsRNA) of *ceclc-1* and *ceclc-2* (VGCC genes coding for CeCIC-1 and CeCIC-2, respectively) either alone or together for 24 h decreased their expression in F<sub>1</sub> progeny in a time-dependent manner. Reduction in expression of *ceclc-2* alone or together with *ceclc-1* significantly increased pharyngeal contractions and decreased locomotion in significantly higher percentage of F<sub>1</sub> progeny. The above findings suggested AT

blockers nematicidal activity primarily comes from inhibition of CeCIC-2 channels, while inhibition of CeCIC-1 channels may enhance this activity.

All the AT blockers showed slowly developing toxicity against adults of a susceptible strain (Oregon-R) of *Drosophila melanogaster* Meigen 1830, while DIDS, was equally toxic to dieldrin-resistant *rdl* flies. All AT blockers, except 9-AC, at 100  $\mu$ M showed significant excitatory effect on desheathed central nervous system (CNS) of third-instar larvae of *Drosophila*, while DIDS showed a modest excitatory effect on ascending peripheral nerves. Feeding adult flies on 10% sugar solution mixed with 100 ppm of DIDS for 6 h decreased the midgut pH by 2 units approximately.

All the AT blockers inhibited the growth of larvae (in weight), increased the developmental time, and decreased survival when *Ostrinia nubilalis* (Hübner 1796) second-instar larvae were fed for seven days. All the AT blockers decreased the midgut alkalinity and inhibited chloride ion transport from midgut lumen into epithelia in fifth-instar larvae when fed for 3 h on treated diet. Positive correlations observed among growth, midgut alkalinity, and midgut Cl<sup>-</sup> transport in AT blocker-fed larvae suggested that inhibition of chloride/bicarbonate exchangers by AT blockers may have contributed to midgut alkalinity decrease affecting the digestion and resulting in observed lethal and sublethal effects.

## **DEDICATION**

I dedicate my dissertation to my loving father, Late Shri **Bhaskara Rao Boina**, without whose efforts and sacrifices I could not have reached this position in my life. I am indebted to him in my entire life for whatever he has done to me as a father, as a friend, and as a philosopher.

## ACKNOWLEDGMENTS

I take this opportunity to thank each and everyone who helped me unconditionally in finishing my Ph. D. research and dissertation successfully. First of all, I profusely thank Dr. Jeffrey R. Bloomquist, chair of my advisory committee, for his able guidance, support, and constant encouragement through out my Ph.D. study at Virginia Tech without whose advices and assistance completing my dissertation would have not been possible. I sincerely thank all the committee members for serving on my advisory committee and I really enjoyed your company. First of all, I would like to thank Dr. Jonathan D. Eisenback for providing me the necessary research material. I am also thankful to him for his unhesitated and unconditional help whenever they were needed. I also thank Dr. Donald E. Mullins for his help and advices when needed in carrying out my research. I would like to thank Dr. Edwin E. Lewis for providing me with research material and serving on my advisory committee. I also would like to thank Dr. Eric E. Wong for serving on my advisory committee. I thank Drs. Sally L. Paulson and Kevin M. Myles for allowing me to use their lab space for my research.

I thank Caenorhabditis Genome Center (CGC) for providing *Caenorhabditis elegans* wild strain (N2 Bristol) and *Escherichia coli* strain OP50 for my research. I also would like to thank Virginia Tech graduate school for awarding me a David and Lillian Francis Memorial Fellowship during 2006-07 and graduate student assembly for awarding me two mini research grants for my research and a travel grant for attending and presenting my reseach at the national annual meeting of the Entomological Society of America. I thank AGRO division of the American Chemical Society (ACS) for awading me a travel grant for attending and presenting my research at the national annual meeting

of the ACS. I thank Virginia Crop Production Association (VACPA) for awarding me a scholarship. I would also like to thank Sandra Gabbert, Troy D. Anderson, Nicole Plaskon, and Elaine Morazzani for their help during my research.

I have no words to describe the constant encouragement and support given and sacrifices made by my family through out their life for me, especially my parents, Bhaskara Rao Boina, Narayanamma Boina, and brother, Murali Mohan Boina. With utmost happiness, I thank my beloved wife, Suneeta Gorla, without whose mental support, encouragement, cooperation, and understanding nature I could have not completed this dissertation in time.

Last but not the the least, I want to thank Entomology Department administrative staff, Robin Williams, Kathy Shelor, and Sarah Kenly for their administrative assistance through out my Ph.D. study and making my stay in the department a memorable one.

## TABLE OF CONTENTS

ABSTRACT.....	ii
DEDICATION.....	iiiv
ACKNOWLEDGMENTS.....	v
TABLE OF CONTENTS.....	vii
LIST OF TABLES.....	x
LIST OF FIGURES.....	xi
CHAPTER 1.....	1
INTRODUCTION.....	1
LITERATURE REVIEW.....	5
LITERATURE CITED.....	17
CHAPTER 2.....	32
Nematicidal activity of anion transporter blockers against <i>Meloidogyne incognita</i> (Kofoid and White) Chitwood, <i>Caenorhabditis elegans</i> Maupas, and <i>Heterorhabditis bacteriophora</i> Poinar.	
ABSTRACT.....	32
INTRODUCTION.....	34
MATERIALS AND METHODS.....	36
RESULTS.....	40
DISCUSSION.....	43
LITERATURE CITED.....	47
CHAPTER 3.....	61

Reduced expression of two voltage-gated chloride channel genes affects pharyngeal contraction and locomotion in *Caenorhabditis elegans* Maupas.

ABSTRACT .....	61
INTRODUCTION .....	63
MATERIALS AND METHODS.....	65
RESULTS .....	69
DISCUSSION .....	73
LITERATURE CITED .....	76
CHAPTER 4 .....	93

Toxicity and neurophysiological effects of anion transporter blockers against *Drosophila melanogaster* Meigen.

ABSTRACT .....	93
INTRODUCTION .....	95
MATERIALS AND METHODS.....	96
RESULTS .....	99
DISCUSSION .....	101
LITERATURE CITED .....	106
CHAPTER 5 .....	122

Effect of anion transporter blockers on growth, development, and survival of larvae of the European corn borer, *Ostrinia nubilalis* (Hübner).

ABSTRACT .....	122
INTRODUCTION .....	124
MATERIALS AND METHODS.....	126

RESULTS .....	129
DISCUSSION .....	132
LITERATURE CITED .....	135
CHAPTER 6 .....	147
Summary .....	147
LITERATURE CITED .....	152

## LIST OF TABLES

<b>Table 1.1.</b>	Tissue distribution and proposed functions of different voltage-gated chloride channels in <i>Caenorhabditis elegans</i> .....	29
<b>Table 2.1.</b>	Activity of anion transporter blockers against adults of <i>Caenorhabditis elegans</i> .....	53
<b>Table 2.2.</b>	Activity of anion transporter blockers against second-stage larvae of <i>Meloidogyne incognita</i> .....	55
<b>Table 2.3.</b>	Activity of anion transporter blockers against <i>Meloidogyne incognita</i> eggs.....	56
<b>Table 3.1.</b>	Concentrations of double-stranded RNA used for targeting <i>ceclc-1</i> and <i>ceclc-2</i> in <i>Caenorhabditis elegans</i> RNA interference experiments.....	80
<b>Table 4.1.</b>	Effect of anion transporter blockers against susceptible <i>Drosophila melanogaster</i> adults (Oregon-R).....	110
<b>Table 4.2.</b>	Effect of DIDS against dieldrin-resistant <i>Drosophila melanogaster</i> adults ( <i>rdl</i> ).....	112
<b>Table 5.1.</b>	Effect of anion transporter blockers on <i>Ostrinia nubilalis</i> larval growth.....	140
<b>Table 5.2.</b>	Effect of anion transporter blockers on <i>Ostrinia nubilalis</i> larval midgut alkalinity and chloride ion transport.....	141

## LIST OF FIGURES

<b>Fig. 1.1.</b>	Chemical structures of anion transporter blockers and other stilbene compounds discussed in the text.....	31
<b>Fig. 2.1.</b>	Mortality of <i>Caenorhabditis elegans</i> adults exposed to 40 ppm of anion transporter blockers (solution pH adjusted to 7 with HEPES).....	57
<b>Fig. 2.2.</b>	Mortality of <i>Heterorhabditis bacteriophora</i> third-stage larvae exposed to anion transporter blockers and chlorpyrifos at 200 ppm.....	58
<b>Fig. 2.3.</b>	Spontaneous contraction rates of <i>Caenorhabditis elegans</i> adult pharyngeal muscle after 1 and 6 h exposures to anion transporter blockers at 50 ppm.....	59
<b>Fig. 2.4.</b>	Effect of anion transporter blockers at 50 ppm on locomotory ability of <i>Caenorhabditis elegans</i> adults.....	60
<b>Fig. 3.1a.</b>	Spontaneous pharyngeal muscle contractions rates of F <sub>1</sub> progeny of <i>Caenorhabditis elegans</i> treated as fourth-stage larvae with long double-stranded RNA of <i>ceclc-1</i> and/or <i>ceclc-2</i> for 24 h.....	81
<b>Fig. 3.1b.</b>	Spontaneous pharyngeal muscle contraction rates of F <sub>1</sub> progeny of <i>Caenorhabditis elegans</i> treated as fourth-stage larvae with short double-stranded RNA of <i>ceclc-1</i> and/or <i>ceclc-2</i> for 24 h.....	82

<b>Fig. 3.1c.</b>	Concentration-dependent effect on spontaneous pharyngeal muscle contraction rates of F <sub>1</sub> progeny of <i>Caenorhabditis elegans</i> treated as fourth-stage larvae with long double-stranded RNA of <i>ceclc-2</i> for 24 h.....	83
<b>Fig. 3.2a.</b>	Percent F <sub>1</sub> progeny with decreased locomotion after fourth-stage larvae of <i>Caenorhabditis elegans</i> were treated with long double-stranded RNA of <i>ceclc-1</i> and/or <i>ceclc-2</i> for 24 h.....	84
<b>Fig. 3.2b.</b>	Percent F <sub>1</sub> progeny with decreased locomotion after fourth-stage larvae of <i>Caenorhabditis elegans</i> were treated with short double-stranded RNA of <i>ceclc-1</i> and/or <i>ceclc-2</i> for 24 h.....	85
<b>Fig. 3.2c.</b>	Concentration-dependent effect on percent F <sub>1</sub> progeny with decreased locomotion after <i>Caenorhabditis elegans</i> treated as fourth-stage larvae with long double-stranded RNA of <i>ceclc-2</i> for 24 h.....	86
<b>Fig. 3.3.</b>	Expression level (mRNA transcript) of <i>ceclc-1</i> and/or <i>ceclc-2</i> in 48 (a and c) and 72 h (b and d) old F <sub>1</sub> progeny of <i>Caenorhabditis elegans</i> treated as fourth-stage larvae with long (a and b) or short (c and d) double-stranded RNA of <i>ceclc-1</i> and/or <i>ceclc-2</i> for 24 h.....	87
<b>Fig. 3.4.</b>	Expression level (mRNA transcript) of <i>ceclc-2</i> in 48 (a) and 72 h (b) old F <sub>1</sub> progeny of <i>Caenorhabditis elegans</i>	

	treated as fourth-stage larvae with long double-stranded RNA of <i>ceclc-2</i> for 24 h.....	88
<b>Fig. 3.5a.</b>	Densitometry analysis of mRNA transcript levels of <i>ceclc-1</i> and/or <i>ceclc-2</i> in 48 and 72 h old F <sub>1</sub> progeny of <i>Caenorhabditis elegans</i> treated as fourth-stage larvae with long double-stranded RNA of <i>ceclc-1</i> and/or <i>ceclc-2</i> for 24 h (data from fig. 3.3 [a and b]).....	89
<b>Fig. 3.5b.</b>	Densitometry analysis of mRNA transcript levels of <i>ceclc-1</i> and/or <i>ceclc-2</i> in 48 and 72 h old F <sub>1</sub> progeny of <i>Caenorhabditis elegans</i> treated as fourth-stage larvae with short double-stranded RNA of <i>ceclc-1</i> and/or <i>ceclc-2</i> for 24 h (data from fig. 3.3 [c and d]).....	90
<b>Fig. 3.5c.</b>	Densitometry analysis of mRNA transcript levels of <i>ceclc-2</i> in 48 and 72 h old F <sub>1</sub> progeny of <i>Caenorhabditis elegans</i> treated as fourth-stage larvae with long double-stranded RNA of <i>ceclc-2</i> for 24 h (data from fig. 3.4).....	91
<b>Fig. 3.6.</b>	Relationship between dsRNA concentration and <i>ceclc-2</i> expression level in 48 and 72 h old F <sub>1</sub> progeny of <i>Caenorhabditis elegans</i> treated as fourth-stage larvae with long double-stranded RNA of <i>ceclc-2</i> for 24 h.....	92
<b>Fig. 4.1.</b>	Effect of DIDS on <i>Drosophila</i> adult midgut pH determined by universal pH indicator paper.....	113
<b>Fig. 4.2.</b>	Central nerve firing rate before and after application	

	of DIDS to desheathed central nervous system preparations from third-instar <i>Drosophila</i> larvae.....	114
<b>Fig. 4.3.</b>	Central nerve firing rate before and after application of DIDS to intact central nervous system preparations from third-instar <i>Drosophila</i> larvae.....	115
<b>Fig. 4.4.</b>	Central nerve firing rate after application of dimethyl sulfoxide twice to desheathed central nervous system preparations ( $n = 3$ ) from third-instar <i>Drosophila</i> larvae.....	116
<b>Fig. 4.5.</b>	Quantified neurophysiological activity of anion transporter blockers on desheathed central nervous system of <i>Drosophila</i> third-instar larvae.....	117
<b>Fig. 4.6.</b>	Quantified neurophysiological activity of anion transporter blockers on intact central nervous system of <i>Drosophila</i> third-instar larvae.....	118
<b>Fig. 4.7.</b>	Quantified neurophysiological activity of peripheral sensory nerves from <i>Drosophila</i> third-instar larvae treated with DIDS.....	119
<b>Fig. 4.8.</b>	Central nerve firing rate after application of $\gamma$ -amino butyric acid and DIDS to desheathed central nervous system preparations from third-instar <i>Drosophila</i> larvae.....	120
<b>Fig. 4.9.</b>	Quantified neurophysiological activity from desheathed central nervous system of <i>Drosophila</i> third-instar larvae treated with $\gamma$ -aminobutyric acid and DIDS.....	121

<b>Fig. 5.1a.</b>	Reduction in <i>Ostrinia nubilalis</i> larval size fed with DIDS-amended diets.....	142
<b>Fig. 5.1b.</b>	Reduction in <i>Ostrinia nubilalis</i> larval size fed with DIDS-amended diets.....	142
<b>Fig. 5.2.</b>	Effect of anion transporter blockers on <i>Ostrinia nubilalis</i> larval development (pupation time).....	143
<b>Fig. 5.3.</b>	Effect of anion transporter blockers on <i>Ostrinia nubilalis</i> larval survival.....	144
<b>Fig. 5.4.</b>	Dose-dependent effect of anion transporter blockers on <i>Ostrinia nubilalis</i> larval midgut alkalinity.....	145
<b>Fig. 5.5.</b>	Dose-dependent effect of anion transporter blockers on <i>Ostrinia nubilalis</i> larval midgut chloride ion transport.....	146

# CHAPTER 1

## INTRODUCTION

Methyl bromide (MeBr) was banned by the United States (US) government for agricultural soil fumigation from the year 2005 due to its strong stratospheric ozone depleting properties (Martin 2003, Talavera and Mizukubo 2005). As a consequence, controlling soil dwelling pests, mainly root-knot nematodes (*Meloidogyne* spp.) became a major problem for successful agricultural crop production. World-wide, these nematodes cause annual economic yield loss of approximately 50 billion US dollars (Dautova et al. 2001). Further, implementation of the Food Quality Protection Act of 1996 will probably result in the cancellation of many organophosphate and carbamate insecticides for pest control due to reevaluation of toxicological safety standards (Fields and White 2002). Therefore, discovering and evaluating novel target sites in nematodes with new or existing chemical compounds that are environmentally friendly and economically inexpensive are major objectives of pesticide toxicologists.

Complete genome sequencing of *Caenorhabditis elegans* Maupas 1900, a free-living soil nematode, revealed the presence of six genes coding for at least six voltage-gated chloride channels (VGCCs). These VGCCs are distributed in various tissues of *C. elegans* playing roles in important physiological functions (Schriever et al. 1999, Nehrke et al. 2000). Similarly, the presence of chloride/bicarbonate ( $\text{Cl}^-/\text{HCO}_3^-$ ) exchangers (CBEs) in apical membranes of midgut epithelia and their role in midgut luminal alkalization was documented in insects, such as larvae of tobacco hornworm, *Manduca*

*sexta* (Linnaeus 1763)(Chao et al. 1989) and larvae of the yellow fever mosquito, *Aedes aegypti* (Linnaeus 1762) (Boudko et al. 2001). The VGCCs and CBEs in nematodes and insects could be major target sites for compounds having insecticidal/nematicidal activity.

A natural stilbene compound, 3, 5-dihydroxy-4-isopropyl stilbene (DST) (Fig. 1.1), produced and released by symbiotic bacteria, *Photorhabdus* spp., of entomopathogenic nematodes, *Heterorhabditis* spp., showed significant toxicity against many plant-parasitic nematodes, including *Meloidogyne incognita* (Kofoid and White 1919) Chitwood 1949 (Hu et al. 1995, 1996, 1999, Hu and Webster 2000). A synthetic stilbene compound, 4, 4'-diisothiocyanatostilbene 2, 2'- disulfonic acid (DIDS), (Fig. 1.1) a well known blocker of anion transporters (ATs), such as VGCCs and CBEs (Cabantchik and Greger 1992). DIDS and other AT blockers, such as NPPB ((5-nitro-2-(3-phenylpropylamino) benzoic acid), 9-AC (anthracene-9-carboxylic acid), and IAA-94 (indanyloxy acetic acid), have been extensively used to characterize the nature and function of VGCCs and CBEs in various biological tissues (Cabantchik and Greger 1992, Machaca et al. 1996, Phipps et al. 1996, Ferroni et al. 1997, Tosco et al. 1998, Wang and Telfer 1998, Pusch et al. 2002, Spiegel et al. 2003, Edwards 2006, Kawamata et al. 2006, Uchiyama et al. 2006, Malekova et al. 2007, Pedersen et al. 2005, Tararhuch et al. 2007), but have never been evaluated for their toxicity against insects and nematodes *in vivo*.

Since insects and nematodes possess ATs (VGCCs and CBEs), it was hypothesized (Bloomquist 2003) and subsequently shown (Boina et al. 2007) that DIDS, having a structural resemblance with DST, along with other AT blockers (NPPB, 9-AC, and IAA-94) have toxicity against insects and nematodes similar to that shown by DST

(Hu et al. 1995, 1996, 1999). Furthermore, much information is available about AT blockers interaction *in vitro* with ATs, but little or no information is available about their effect on insects and nematodes *in vivo*.

Therefore, the present study was designed to evaluate ATs as potential target sites for insect and nematode control by determining the toxicity and mode of action of AT blockers belonging to different classes of organic acids including DIDS (a stilbene disulfonic acid), NPPB (an anthranilic acid), 9-AC (an aromatic carboxylic acid), and IAA-94 (an indanyloxy alcanoic acid) against selected insect species [the European corn borer, *Ostrinia nubilalis* (Hübner 1796) and pomace fly, *Drosophila melanogaster* Meigen 1830] and nematodes (plant-parasitic root-knot nematode, *M. incognita*; free-living soil nematode, *C. elegans*; and entomopathogenic nematode, *Heterorhabditis bacteriophora* Poinar 1975) using toxicological, electrophysiological, and molecular techniques.

My research has three major objectives:

- 1) To determine the toxicity of AT blockers in nematodes
  - i) Lethal effects on *C. elegans*, *M. incognita*, and *H. bacteriophora*
  - ii) Effects on locomotion and pharyngeal muscle contraction in *C. elegans*
  - iii) Ovicidal effects on *M. incognita*
- 2) To evaluate post-transcriptional silencing of predicted target VGCC genes for AT blockers in *C. elegans* using RNA interference
  - i) Effect of soaking *C. elegans* in double-stranded RNA (dsRNA) solution on VGCC genes (*ceclc-1* and *ceclc-2*)

- ii) Effect of soaking *C. elegans* in dsRNA solution on locomotion and pharyngeal muscle contractions
- 3) To determine the toxicity and mode of action of AT blockers in insects
  - a) Pomace fly, *D. melanogaster*
    - i) Lethal effects on adults
    - ii) Neurophysiological activity on the central and peripheral nervous systems
    - iii) Effect on adult fly midgut lumen alkalinity
  - b) The European corn borer, *O. nubilalis*
    - i) Lethal and sublethal effects on larvae
    - ii) Effect on larval midgut luminal alkalinity
    - iii) Effect on larval midgut Cl<sup>-</sup> transport

## LITERATURE REVIEW

### **Phase Out of Methyl Bromide**

MeBr is a colorless and odorless (at concentrations used for fumigation) organic chemical fumigant used for pre-plant soil sterilization (Fields and White 2002, Martin 2003). It acts rapidly and has broad spectrum of activity; killing insect, mite, nematode, weed, fungal, and bacterial pests (Fields and White 2002, Martin 2003). MeBr kills insects by damaging the nerve cell membranes and reacts with sulfhydryl groups of proteins (Fields and White 2002). It was estimated that 75-80% of synthetic MeBr produced in 1990 was used for fumigating agricultural soil (Yagi et al. 1993, Fields and White 2002), mainly for controlling nematodes. Of the total MeBr used for fumigating the agricultural soil in the US, 23% was used in the tomato crop alone for controlling root-knot nematodes and other soil pests (Anonymous 2004). Because of its stratospheric ozone depleting properties (Fields and White 2002, Martin 2003, Talavera and Mizukubo 2005), the US government banned MeBr for agricultural soil fumigation in 2005. However, because a suitable replacement has not been found, special permits under Critical Use Exemptions (CUE) have allowed its continued use at reduced levels, where it must be demonstrated that no viable alternative is available to replace MeBr (Martin 2003).

### **Anion Transporters**

#### ***Voltage-gated Chloride Channels; General Physiological Roles***

VGCCs (a.k.a voltage-dependent/sensitive/activated) are a type of chloride channel whose gating mechanism (opening and closing of the channel pore) depends on

membrane voltage. VGCCs are pore forming proteins in the plasma membrane of excitable and non-excitable cells for transport of the most abundant anion,  $\text{Cl}^-$ , in biological systems (Estevez and Jentsch 2002, Jentsch et al. 2002, Nilius and Droogmans 2003). VGCCs are present ubiquitously, ranging from unicellular bacteria to multicellular animals and plants (Yamamoto and Suzuki 1987, Petalcorin et al. 1999, Schriever et al. 1999, Asmild and Willumsen 2000, Nehrke et al. 2000, Fahlke 2001, Rutledge et al. 2001, Franks et al. 2002). These channels are located either on plasma membrane or on intracellular organelles membranes (Schriever et al. 1999).

Regulation of electrogenesis in excitatory cells, such as neuronal and muscle cells, by increased anion conductance for stabilization of the membrane potential (Jentsch et al. 2002, Nilius and Droogmans 2003), transepithelial transport of ions, cell volume regulation, pH regulation in organelles, control of synaptic activity, endocytosis etc., are the major roles played by VGCCs (Fahlke 2001, Jentsch et al. 2002, Bloomquist 2003, Nilius and Droogmans 2003).

### ***Chloride/Bicarbonate Exchangers in Insects and Their Physiological Role***

CBEs are non-pore forming membrane transport proteins involved in exchange of  $\text{Cl}^-$  for  $\text{HCO}_3^-$  in across membranes in various tissue cells (Sterling and Casey 2002). These exchangers are inhibited on external surface of protein by stilbene disulfonates such as DIDS (Cabantchik and Greger 1992). Insects, especially caterpillars, maintain the metabolically expensive alkaline pH in their midgut lumen by continuously transporting ions or acid/base species across midgut epithelial cells. Various models have been proposed to account for this luminal alkalization, and active pumping of  $\text{H}^+$  into midgut lumen via  $\text{H}^+$ -ATPase coupled with transport of  $\text{H}^+$  in the opposite direction

by  $\text{nH}^+/\text{K}^+$  antiporter is one of them (Azuma et al. 1995). Nevertheless, several studies suggested that transport of one or more base equivalent species such as  $\text{HCO}_3^-$  into the midgut lumen is equally important to account for the highly alkaline pH and to maintain the overall electroneutrality in midgut of *M. sexta* (Dow 1984, 1992, Chao et al. 1989, Moffett and Cummings 1994, Coddington and Chamberlin 1999). Localization of carbonic anhydrase, which catalyzes the formation of  $\text{HCO}_3^-$ , in the midgut epithelia of *M. sexta* larva (Jungreis et al. 1981, Ridgeway and Moffett 1986) supported  $\text{HCO}_3^-$  as a possible base equivalent for exchange of luminal  $\text{Cl}^-$  via CBEs (Chao et al. 1989, Coddington and Chamberlin 1999). Furthermore, Chao et al. (1989) showed that in *M. sexta* larval midgut, transport of  $\text{Cl}^-$  takes place in the lumen to haemolymph direction in two ways: 1) actively from the goblet cavity into goblet cells, possibly through  $\text{Cl}^-$  channels and 2) in exchange for cellular  $\text{HCO}_3^-$  on apical membrane of columnar cells. Boudko et al. (2001) presented evidence for involvement of CBEs in midgut luminal alkalization in *A. aegypti* larvae. Since both *M. sexta* and *A. aegypti* larvae maintain characteristically high alkaline midguts, it appears that CBEs are involved in the midgut luminal alkalization process.

In caterpillars, midgut alkalinity (ranging between 7-12 pH units) plays a major role in protein digestion and absorption as both dietary proteins solubility and availability for digestion, and digestive proteinases activity were highly dependent on midgut alkalinity (Berenbaum 1980, Johnson and Felton 1996, Houseman et al. 1989, Houseman and Chin 1995, Anwar and Saleemuddin 2001).

### ***Voltage-gated Chloride Channels in Nematodes and Their Physiological Roles***

*Caenorhabditis elegans* complete genome sequencing revealed that six genes,

*ceclc-1 to -6*, code for VGCCs and the corresponding proteins are designated as CeCIC-1, -2 (2b), -3 (3b), -4 (4b), -5, and -6 (Schriever et al. 1999, Nehrke et al. 2000). It is believed that CeCIC-1, -2, -3, and -4 channels are located on plasma membrane, while CeCIC-5 and -6 are located on intracellular organelles (Schriever et al. 1999). Conservation of VGCCs in widely divergent animals including nematodes (*C. elegans*) and mutations in VGCC genes causing inheritable diseases in humans, indicate the physiological importance of these channels in biological systems (Estevez and Jentsch 2002, Jentsch et al. 2002).

Tissue localization of six VGCCs in *C. elegans* is presented in Table 1.1. Of the six VGCCs present in *C. elegans*, physiological importance of CeCIC-1 and CeCIC-3 proteins have been documented (Petalcorin et al. 1999, Rutledge et al. 2001). CeCIC-1 channel is involved in maintaining the body morphology either through osmoregulation or body alae (lateral lines formed by the seam cells) formation. Tc-1 transposon-mediated mutation in *ceclc-1* gene of *C. elegans* changed the morphology of worms resulting in significantly wider bodies, especially at fourth-stage larval and young adult stages. Insertion of the deleted gene portion reversed the abnormalities, indicating that functional *ceclc-1* gene protein is necessary for osmotic balance between the extracellular space and the seam cells (Petalcorin et al. 1999). These findings suggest that compounds that target and block CeCIC-1 channel could disrupt its osmoregulatory function, and may lead to increased body diameter. Since nematodes move in a sinusoidal fashion, the number of sinusoidal movement/min and amplitude could decrease in wider bodied worms, leading to decreased locomotion. The localization of CeCIC-2 in nerve cells, pharyngeal-intestinal valve, and the body wall muscles involved in locomotion indicate

an important role in signal transmission and locomotion, but no study so far has reported its physiological role in *C. elegans*.

CeClC-3 channels might mediate the osmoregulatory role of the excretory cells and fluid secretion in the intestinal cells (Nehrke et al. 2000). RNA interference studies targeting the CeClC-3 channel gene showed no role for CeClC-3 in oocyte osmoregulation (Rutledge et al. 2001, Rutledge et al. 2002, Strange 2002), but in the ovulation process by modulating the ovulatory contractions of myoepithelial sheath cells during meiotic maturation (Rutledge et al. 2001). At this point in time, the physiological importance of CeClC-4 channels that are expressed in excretory cells has not been shown scientifically. Furthermore, CeClC-5, and -6 channels being major target sites for nematocidal compounds depends on the ability of such compounds to cross the cell plasma membranes, as the predicted location of these channels is intracellular organelles (Schriever et al. 1999, Nehrke et al. 2000).

In *C. elegans* pharyngeal muscle electrophysiological studies, reducing the extracellular Cl<sup>-</sup> concentration caused a transient depolarization of the membrane potential and produced a burst of action potentials. This finding suggests the resting membrane potential (ranging between -80 and -65 mV) is maintained by Cl<sup>-</sup> conductance through Cl<sup>-</sup> channels (Franks et al. 2002). *Caenorhabditis elegans* pharyngeal muscle action potentials are characterized by a long plateau phase with an average action potential spike duration and amplitude of 0.26 s and 86 mV, respectively (Franks et al. 2002). However, no information is available on VGCCs (possibly, CeClC-2) role in Cl<sup>-</sup> conductance or its contribution in stabilizing the membrane potential in this tissue.

## **RNA Interference Studies in *C. elegans***

Introduction of a double-stranded RNA (dsRNA) in the target organism, of which one strand sequence is complementary to the mRNA of gene of interest, leads to specific degradation of target mRNA transcripts (Fire et al. 1998). This indirect silencing of gene expression, i.e. post-transcriptional gene silencing (PTGS), is known as RNA interference (RNAi).

Once the dsRNA of a specific gene enters the cell, an enzyme called DICER acts upon it and cleaves it into 21 to 23 bp length short fragments. These short-interfering RNAs (siRNAs) then enter into a protein complex known as RNA-induced silencing complex (RISC), where dsRNA is unwound to give single-stranded (ss) siRNA and the activation of RISC. RISC loaded with ss siRNA then searches and recognizes the mRNAs with complementary sequence from mRNA pool and specifically degrades them leading to PTGS (Bhaketia et al. 2005a, Fortunato and Fraser 2005). This phenomenon was first characterized in *C. elegans* by Fire et al. (1998). Subsequently, this technique has been adapted in various organisms ranging from insects to mammalian cells. In *C. elegans*, RNAi can be performed with ease either by 1) injecting dsRNA into the worms, 2) soaking worms in dsRNA, 3) feeding worms on *Escherichia coli* (Migula 1895) Castellani and Chalmers 1919 expressing dsRNA, or 4) transforming the worms with a plasmid constructed to express dsRNA. Further, the gene function or phenotype observed in *C. elegans* by RNAi could be seen or predicted to occur in another organism, particularly plant parasitic nematodes, if *C. elegans* genes have orthologues (same gene) in those organisms. Large-scale functional analysis screenings performed on the whole-genome of *C. elegans* by RNAi revealed the functions for many genes by showing the

resultant phenotype (Kamath et al. 2000, Kamath and Ahringer 2003, Kamath et al. 2003). Accordingly, *C. elegans* has been a model organism for several parasitic nematodes and other organisms in which performing RNAi is difficult for various reasons. Nevertheless, recently many researchers were successful in silencing genes in parasitic nematodes using RNAi (Urwin et al. 2002, Bakhetia et al. 2005b, Chen et al. 2005, Fanelli et al. 2005, Rosso et al. 2005, Geldhof et al. 2006).

In all the studies previously mentioned, researchers were able to determine the function of a gene by RNAi in parasitic nematodes, when the gene sequence was known. However, one can search in the databases for the phenotype of a particular gene obtained from RNAi in *C. elegans* or perform the RNAi in *C. elegans* when the sequence of the gene is not known in parasitic nematodes. Since many aspects of biology between *C. elegans* and parasitic nematodes are similar, it is appropriate to study the function of conserved genes in detail in *C. elegans* until RNAi becomes a robust technique for gene function analysis directly in parasitic nematodes (Britton and Murray 2006).

Search of the Wormbase ([www.wormbase.org](http://www.wormbase.org)), a website for complete information on *C. elegans* RNAi studies revealed that of the six genes (*ceclc-1 to -6* coding for CeCIC-1 to -6) present in *C. elegans*, only *ceclc-3* was characterized functionally by RNAi. This warrants conducting RNAi studies on selected VGCC genes (predicted to have a role in nematode survival) in *C. elegans* (Boina et al. 2007). This approach will also suggest putative VGCC target sites for AT blockers based on phenotypes resulting from RNAi (Bloomquist 2003, Boina et al. 2007). Moreover, RNAi in *C. elegans* can be used to predict the existence of similar mechanisms in *M. incognita*. Rutledge et al. (2001) determined the role of VGCCs in *C. elegans* oocytes by injecting

dsRNAs complementary to VGCC genes *ceclc-1 to -6* into the gonad of *C. elegans* adult and concluded that only dsRNA complementary to *ceclc-3* was able to inhibit the swelling activated currents in oocytes.

## **Anion Transporter Blockers**

### ***A Natural Stilbene, 3, 5-Dihydroxy-4-Isopropyl Stilbene***

Entomopathogenic nematodes belonging to genera, *Heterorhabditis* and *Steinernema* with their symbiotic bacteria, *Photorhabdus* spp. and *Xenorhabdus* spp., respectively, infect the insect hosts and kill them within 24 to 48 h of infection (Hu et al. 1999). The cause of mortality of infected insects could be attributed to the secondary metabolites produced by the symbiotic bacteria present in the gut of entomopathogenic nematodes as well. Of these metabolites, a natural stilbene (DST), was of considerable importance contributing to insecticidal properties (Hu et al. 1996). DST, which is present at about 3 mg/g of entomopathogenic nematode-infected *Galleria mellonella* (Linnaeus 1758) larva, supports this hypothesis (Hu et al. 1999, Hu and Webster 2000). Further, DST at 100 ppm caused 100% mortality in adults of *C. elegans*. A strong antagonistic/inhibitory effect of entomopathogenic nematodes against many soil-living, plant-parasitic nematodes has also been documented (Bird and Bird 1986, Ishibashi and Kondo 1986, Gouge et al. 1994) which may be due to DST release. Cell-free culture filtrates obtained from entomopathogenic nematode symbiotic bacteria, *Photorhabdus* spp. and *Xenorhabdus* spp., were toxic to second-stage larvae of *M. incognita* and inhibited egg hatch at concentrations as low as 12.5 ppm (Hu et al. 1995, 1999). Similar findings on *M. incognita* second-stage larvae and eggs were reported by Grewal et al.

(1999) using cell-free extracts of *Xenorhabdus bovienii* (Akhurst 1983) Akhurst and Boemare 1993, again suggesting that cell-free extracts of entomopathogenic nematode symbiotic bacteria have nematocidal properties.

Interestingly, DST shows no toxicity against entomopathogenic nematodes in which the DST-producing symbiotic bacteria are present. For instance, DST at 100 ppm caused 100% mortality in *C. elegans* adults, but no mortality in *Heterorhabditis megidis* Poinar, Jackson, and Klein 1987 strain 90 was observed at 200 ppm (Hu et al. 1999). These findings suggest that observed resistance to DST in entomopathogenic nematodes could be due to mutation(s) in the target gene that might be protecting them from the toxicity of their own symbiotic bacterial secondary metabolites, such as DST; however, this hypothesis needs to be confirmed with further testing with AT blockers against entomopathogenic nematodes.

### ***Synthetic Stilbene and Other Anion Transporter Blockers***

DIDS, a synthetic stilbene disulfonic acid, is a known blocker of ATs such as VGCCs and CBEs (Steele 1989, Cabantchik and Greger 1992, Boudko et al. 2001). DIDS shares structural similarity with the bacterial stilbene, DST (Fig. 1.1). Therefore, it was hypothesized that any insecticidal or nematocidal activity of DST may come from its action on VGCCs (Bloomquist 2003), although CBEs might also have their function modified, leading to paralysis or death. DIDS and other AT blockers have been widely used for determining the structure and function of Cl<sup>-</sup> channels and CBEs in both vertebrate and invertebrate biological systems (Cabantchik and Greger 1992, Machaca et al. 1996, Phipps et al. 1996, Ferroni et al. 1997, Tosco et al. 1998, Wang and Telfer 1998, Pusch et al. 2002, Spiegel et al. 2003, Edwards 2006, Kawamata et al. 2006, Nagel et al.

2001, Qiu et al. 2003, Uchiyama et al. 2006, Malekova et al. 2007, Pedersen et al. 2005, Tararhuch et al. 2007). These compounds interact with Cl<sup>-</sup> channels either covalently (irreversibly) or non-covalently (reversibly) (Cabantchik and Greger 1992) changing the conformation of channels, leading to their block and decreased Cl<sup>-</sup> currents.

Other chemical classes of organic acids block a variety of ATs. DIDS and NPPB (Fig. 1.1) at 100 μM inhibited the inwardly-rectifying Cl<sup>-</sup> current of a VGCC natively expressed in spermatid of *C. elegans* (Machaca et al. 1996). Similarly, NPPB at 100 μM inhibited outwardly rectifying Cl<sup>-</sup> current of CeClC-3, a VGCC natively expressed in the oocytes of *C. elegans* (Rutledge et al. 2001). Further, NPPB at > 300 μM completely blocked the voltage-activated Cl<sup>-</sup> conductance of toad skin Cl<sup>-</sup> channel (Nagel et al. 2001). 9-AC, a highly hydrophobic aromatic compound (Fig. 1.1), reduced resting Cl<sup>-</sup> conductance in mammalian skeletal muscle, mostly by blocking VGCC (ClC-1) (Pusch et al. 2002). 9-AC blocks the channel from the intracellular side and is voltage-dependent, while extracellular application of 9-AC at 1000 μM decreased the steady-state current reversibly by 50% in rat type-1 neocortical cultured astrocytes (Ferroni et al. 1997). 9-AC at 30 μM blocked the Cl<sup>-</sup> channels (ClC-1) in rat soleus muscle leading to recovery of muscle excitability and force at 11 mM K<sup>+</sup> and normal pH, similar to that occurs in K<sup>+</sup>-depressed muscles induced by muscle acidification (Pedersen et al. 2005). IAA-94 at 100 μM and DIDS at 200 μM inhibited the amplitude and shortened the duration of excitatory junction potentials in smooth muscle cells of spiral mediolar artery in guinea pigs (Yingzi et al. 2006). In another study, DIDS (500 μM), NPPB (250 μM), and IAA-94 (250 μM) inhibited Cl<sup>-</sup> current significantly compared to control in human T lymphocytes by blocking Cl<sup>-</sup> channels (Phipps et al. 1996).

DIDS fed at 100  $\mu\text{M}$  reduced a highly alkaline midgut luminal pH to neutral within 1 h in *A. aegypti* larvae due to a blocking action on CBEs of midgut epithelial cells (Boudko et al. 2001). Further, DIDS at 100  $\mu\text{M}$  was shown to block the exchange of  $^{36}\text{Cl}^-$  into the embryo cell culture of *M. sexta* (English and Cantley, 1984). Thus, DIDS and other AT blockers have the potential to block  $\text{Cl}^-$  conductance through VGCCs and CBEs and this property of AT blockers could be exploited to evaluate the potential of these target sites for insect and nematode control by determining the toxicity and mode of action of AT blockers *in vivo*.

## **Economic Importance of Insect and Nematode Species Used in This Study**

### ***The European Corn Borer, Ostrinia nubilalis***

The European corn borer is the most damaging and well studied pest of corn in the US and Canada. Though it is a major pest of corn grown for grain, seed, and silage (Quinton et al. 2005), it has a broad host range, infesting more than 200 plant species including many horticultural crops such as bell pepper, tomato, and potato. Larvae are the damaging stage. In corn, young larvae mine the leaf midribs and feed on tassels, leaf sheath tissue, and whorls, while older larvae tunnel the stalk, base of corn ear, ear cob and kernels as well (Mason et al. 1996, Anonymous 2007a). The estimated annual cost for managing this pest and damage caused by this pest to the crops was more than \$1 billion in the U.S. (Mason et al. 1996).

### ***Pomace Fly, Drosophila melanogaster***

*Drosophila melanogaster* is a free-living insect that grows on rotten or over-ripen fruits (Anonymous 2007b). It is used as a model organism in biological research, mainly

in genetics and recently in development biology. It is a small insect with a short life cycle (2-weeks) that is easy to culture and handle.

***Free-living Soil Nematode, Caenorhabditis elegans***

*Caenorhabditis elegans* is a free-living soil nematode (Strange 2006). In wild-type populations, adults are predominantly hermaphrodites with few males (0.1%). The average life cycle is very short, completing in 2-3 weeks under optimal laboratory conditions (Strange 2006). It has egg (14 h), four larval (L1-L4) (35 h), and adult stages (Strange 2006). This nematode is widely used as a model organism in genetics and developmental biology studies.

***Plant-parasitic Root-knot Nematode, Meloidogyne incognita***

Root-knot nematodes belonging to genus, *Meloidogyne* are obligatory, sedentary endoparasites feeding on crop and non-crop plant roots by a specialized complex mechanism (Eisenback 1985). These nematodes are considered to be the most successful parasites of crop plants around the globe, and the host range encompasses plants species across various families (Huang et al. 2003) including more than 2000 plant species (Hussey 1985) causing an annual crop loss value estimated to be \$50 billions throughout the world (Dautova et al. 2001). Among the different species of root-knot nematodes, *M. incognita* is the most important from the economic injury they cause and second-stage larvae are the only free-living stage in its life cycle (Eisenback 1985). These nematodes are mainly controlled by MeBr, a broad spectrum chemical fumigant (Talavera and Mizukubo 2005).

### ***Entomopathogenic Nematode, Heterorhabditis bacteriophora***

*Heterorhabditis bacteriophora* are soil-living cruiser entomopathogenic nematodes.

These nematodes are parasitic to insects and are obligate hosts for symbiotic bacteria, *Photorhabdus luminescens* (Thomas and Poinar 1979) Boemare et al. 1993 (Forst et al. 1997). Third-stage larvae enter the insect host and release symbiotic bacteria into the hemocoel by regurgitation (Ciche and Ensign 2003). These bacteria kill the host within 24 h. Nematodes feed on the bacteria, develop through three life stages, completing 2 to 3 generations in a single host until all the bacteria are consumed and third-stage larvae are released into the soil, en masse. The symbiotic bacteria protect themselves from other harmful organisms and the nematodes by releasing antibiotics and other secondary metabolites (Hu et al 1999, Hu and Webster 2000, Zhou et al. 2002). Therefore, these nematodes are useful in biological control of insects (Gonzalez-Ramirez et al. 2000, Koppenhofer et al. 2004, Aydin and Susurluk 2005, Susurluk 2006, Toledo et al. 2006).

### **LITERATURE CITED**

Anonymous. 2004. USEPA Fact sheet.

<http://www.epa.gov/pesticides/factsheets/chemicals/methyl>

[bromide\\_factsheet.htm](http://www.epa.gov/pesticides/factsheets/chemicals/methyl_bromide_factsheet.htm). Accessed on October 15, 2004.

Anonymous. 2007a. [http://creatures.ifas.ufl.edu/field/e\\_corn\\_borer.htm](http://creatures.ifas.ufl.edu/field/e_corn_borer.htm). Accessed on August 8, 2007.

Anonymous, 2007b. <http://ceolas.org/VL/fly/intro.html>. Accessed on August 10, 2007.

Anwar, A., and M. Saleemuddin. 2001. Regulation of digestive proteolytic activity in the larvae of *Spilosoma oblique* (Lep., Arctiidae). *J Appl Entomol* 125: 577-582.

- Asmild, M., and N. Willumsen. 2000. Chloride channels in the plasma membrane of a foetal *Drosophila* cell line, S2. *Pflug Arch Eur J Physiol* 439: 759-764.
- Aydin, H., and A. Susurluk. 2005. Competitive abilities of entomopathogenic nematodes *Steinernema feltiae* and *Heterorhabditis bacteriophora* in the same host at different temperatures. *Turk J Boil* 29: 35-39.
- Azuma, M., W. R. Harvey, and H. Wiczorek. 1995. Stoichiometry of  $K^+/H^+$  antiport helps to explain extracellular pH 11 in a model epithelium. *FEBS Lett* 36: 153-156.
- Berenbaum, M. 1980. Adaptive significance of midgut pH in larval lepidoptera. *Am Nat* 115: 138-146.
- Bhaketia, M., W. L. Charlton, P. E. Urwin, M. J. McPherson, and H. J. Atkinson. 2005a. RNA interference and plant parasitic nematodes. *Trends Plant Sci* 18: 362-367.
- Bhaketia, M., W. L. Charlton, H. J. Atkinson, and M. J. McPherson. 2005b. RNA interference of dual oxidase in the plant nematode *Meloidogyne incognita*. *Mol Plant-Microbe Interact* 18: 1099-1106.
- Bird, A. F., and J. Bird. 1986. Observations on the use of insect parasitic nematodes as a means of biological control of root-knot nematodes. *Int J Parasitol* 16: 511-516.
- Bloomquist, J. R. 2003. Chloride channels as tools for developing selective insecticides. *Arch Insect Biochem Physiol* 54: 145-156.
- Boina, D., E. E. Lewis, and J. R. Bloomquist. 2007. Nematicidal activity of anion transport blockers against *Meloidogyne incognita*, *Caenorhabditis elegans*, and *Heterorhabditis bacteriophora*. *Pest Manag Sci* (In press).

- Boudko, D. Y., L. L. Moroz, W. R. Harvey, P. J. Linser. 2001. Alkalinization by chloride/bicarbonate pathway in larval mosquito midgut. *Proc Natl Acad Sci USA* 98: 15354-15359.
- Britton, C., and L. Murray. 2006. Using *Caenorhabditis elegans* for functional analysis of genes of parasitic nematodes. *J Int Parasitol* 36: 651-659.
- Cabantchik, Z. I., and R. Greger. 1992. Chemical probes for anion transporters of mammalian cell membranes. *Am J Physiol* 262: C803-C827.
- Chao, A. C., A. R. Koch, and D. F. Moffett. 1989. Active chloride transport in isolated posterior midgut of tobacco hornworm (*Manduca sexta*). *Am J Physiol* 257: R752-R761.
- Chen, Q., S. Rehman, G. Smant, and J. T. Jones. 2005. Functional analysis of pathogenicity proteins of the potato cyst nematode *Globodera rostochiensis* using RNAi. *Mol Plant Microbe Interact* 18: 621-625.
- Ciche, T. A., and J. A. Ensign. 2003. For the insect pathogen *Photorhabdus luminiscens*, which end of a nematode is out? *Appl Environ Microbiol* 69: 1890-1897.
- Coddington, E. J., and M. E. Chamberlin. 1999. Acid/base transport across the midgut of the tobacco hornworm, *Manduca sexta*. *J Insect Physiol* 45: 493-500.
- Dautova, M., M. Rosso, P. Abad, F. J. Gommers, J. Bakker, and G. Smant. 2001. Single pass cDNA sequencing – a powerful tool to analyze gene expression in preparasitic juveniles of the southern root-knot nematode *Meloidogyne incognita*. *Nematology* 3: 129-139.
- Dow, J. A. T. 1984. Extremely high pH in biological systems: a model for carbonate

- transport. *Am J Physiol* 246: R633-R635.
- Dow, J. A. T. 1992. pH gradients in lepidopteran midgut. *J Exp Biol* 172:355-375.
- Edwards, J. C. 2006. The CLIC1 chloride channel is regulated by the cystic fibrosis transmembrane conductance regulator when expressed in *Xenopus* oocytes. *J Membr Biol* 213: 39-49.
- Eisenback, J. D. 1985. Detailed morphology and anatomy of second-stage juveniles, males, and females of the genus *Meloidogyne* (root-knot nematode). Pp. 47-78. In J. N. Sasser and C. C. Carter (Eds.), *An advanced treatise on Meloidogyne* Volume I: biology and control, North Carolina State University Graphics, Raleigh.
- English, L. H., and L. C. Cantley. 1984. Characterization of monovalent ion transport systems in an insect cell line (*Manduca sexta* embryonic cell line CHE). *J Cellular Physiol* 121: 125-132.
- Estevez, R., and T. J. Jentsch. 2002. CLC chloride channels: correlating structure with function. *Curr Opin Struct Biol* 12: 531-539.
- Fahlke, C. 2001. Ion permeation and selectivity in CLC-type chloride channels. *Am J Physiol Renal Physiol* 280: F748-F757.
- Fanelli, E., M. Di Vitro, J. T. Jones, and C. De Giorgi. 2005. Analysis of chitin synthase function in a plant parasitic nematode, *Meloidogyne artiellia*, using RNAi. *Gene* 349: 87-95.
- Ferroni, S., C. Marchini, M. Nobile, and C. Rapisarda. 1997. Characterization of an inwardly rectifying chloride conductance expressed by cultured rat cortical astrocytes. *Glia* 21: 217-227.

- Fields, P. G., and N. D. G. White. 2002. Alternatives to methyl bromide treatments for stored-product and quarantine treatments. *Annu Rev Entomol* 47: 331-359.
- Fire, A., S. Xu, M. K. Montgomery, S. A. Kostas, S. E. Driver, and C. C. Mello. 1998. Potent and specific genetic interference by double-stranded RNA in *Caenorhabditis elegans*. *Nature* 391: 806-811.
- Forst, S., B. Dowds, N. Boemare, and E. Stackebrandt. 1997. *Xenorhabdus* and *Photorhabdus* spp.: bugs that kill bugs. *Annu Rev Microbiol* 51: 47-72.
- Fortunato, A., and A. G. Fraser. 2005. Uncover genetic interactions in *Caenorhabditis elegans* by RNA interference. *Biosci Reports* 25: 299-307.
- Franks, C. J., D. Pemberton, I. Vinogradova, A. Cook, R. J. Walker, and L. Holde-Dye. 2002. Ionic basis of the resting membrane potential and action potential in the pharyngeal muscle of *Caenorhabditis elegans*. *J Neurophysiol* 87: 954-961.
- Geldhof, P., L. Murray, A. Couthier, J. S. Gilleard, G. McLauchlan, D. P. Knox, and C. Britton. 2006. Testing the efficacy of RNA interference in *Haemonchus contortus*. *Int J Parasitol* 36: 801-810.
- Gonzalez-Ramirez, M., R. Lezama-Gutierrez, J. Molina-Ochoa, O. Rebolledo-Dominquez, M. Lopez-Edwards, and A. Pescador-Rubio. 2000. Susceptibility of *Mocis latipes* (Lepidoptera: Noctuidae) to *Heterorhabditis bacteriophora* (Rhabditida: Heterorhabditidae). *J. Econ Entomol* 93: 1105-1108.
- Gouge, D. H., A. A. Otto, A. Schirocki, and N. G. M. Hague. 1994. Effects of Steinernematids on the root-knot nematode *Meloidogyne javanica*. *Ann Appl Biol* 124: 134-135.
- Grewal, P. S., E. E. Lewis, and S. Venkatachari. 1999. Allelopathy: A possible

- mechanism of suppression of plant-parasitic nematodes by entomopathogenic nematodes. *Nematology* 1: 735-744.
- Houseman, J. G., and P. S. Chin. 1995. Distribution of digestive proteinases in the Alimentary tract of the European corn borer, *Ostrinia nubilalis* (Lepidoptera: Pyralidae). *Arch Insect Biochem Physiol* 28: 103-111.
- Houseman, J. G., B. J. R. Philogene, and A. E. R. Downe. 1989. Partial characterization of proteinase activity in the larval midgut of the European corn borer, *Ostrinia nubilalis* Hübner (Lepidoptera: Pyralidae). *Can J Zool* 67: 864-868.
- Hu, K., J. Li, and J. M. Webster. 1995. Mortality of plant parasitic nematodes caused by bacterial (*Xenorhabdus* spp. And *Photorhabdus luminescens*) culture media. *J Nematology* 27: 502-503 (Abstract).
- Hu, K., J. Li, and J. M. Webster. 1996. 3, 5-Dihydroxy-4-isopropylstilbene: a selective nematicidal compound from the culture filtrate of *Photorhabdus luminescens*. *Canadian J Plant Pathol* 18: 104 (Abstract).
- Hu, K., J. Li, and J. M. Webster. 1999. Nematicidal metabolites produced by *Photorhabdus luminescens* (Enterobacteriaceae), bacterial symbiont of entomopathogenic nematodes. *Nematology* 1: 457-469.
- Hu, K., and J. M. Webster. 2000. Antibiotic production in relation to bacterial growth and nematode development in *Photorhabdus-Heterorhabditis* infected *Galleria mellonella* larvae. *FEMS Microbiol Lett* 189: 219-223.
- Huang, G., B. Gao, T. Maier, R. Allen, E. L. Davis, T. J. Baum, R. S. Hussey. 2003. A profile of putative parasitism genes expressed in the esophageal gland cells of the root-knot nematode *Meloidogyne incognita*. *Mol Plant Microbe Interact* 16: 376-

381.

- Hussey, R. S. 1985. Host-parasite relationships and associated physiological changes. Pp. 143-153. In J. N. Sasser and C. C. Carter (Eds), An advanced treatise on *Meloidogyne* Volume I: biology and control, North Carolina State University Graphics, Raleigh.
- Ishibashi, N., and E. Kondo. 1986. *Steinernema feltiae* (DD-136) and *S. glaseri*: persistence in soil and bark compost and their influence on native nematodes. *J Nematology* 18: 310-316.
- Jentsch, T. J., V. Stein, F. Weinreich, and A. Zdebik. 2002. Molecular structure and physiological function of chloride channels. *Physiol Rev* 82: 503-568.
- Johnson, K. S., and F. W. Felton. 1996. Potential influence of midgut pH and redox potential on protein utilization in insect herbivores. *Arch Insect Biochem Physiol* 32:85-105.
- Jungreis, A. M., N. D. Barron, and J. W. Johnston. 1981. Comparative properties of tobacco hornworm, *Manduca sexta*, carbonic anhydrases. *Am J Physiol* 241: R92-R99.
- Kamath, R. S., and J. Ahringer. 2003. Genome-wide RNAi screening in *Caenorhabditis elegans*. *Methods* 30: 313-321.
- Kamath, R. S., A. G. Fraser, Y. Dong, G. Poulin, R. Durbin, M. Gotta, A. Kanapin, N. Le Bot, S. Moreno, M. Sohrmann, D. P. Welchman, P. Zipperlen, and J. Ahringer. 2003. Systematic functional analysis of the *Caenorhabditis elegans* genome using RNAi. *Nature* 421: 231-237.
- Kamath, R. S., M. Martinez-Campos, P. Zipperlen, A. G. Fraser, and J. Ahringer. 2000.

- Effectiveness of specific RNA-mediated interference through ingested double-stranded RNA in *Caenorhabditis elegans*. *Genome Biol* 2: 1-10.
- Kawamata, K., H. Hayashi, and Y. Suzuki. 2006. Chloride-dependent bicarbonate secretion in the mouse large intestine. *Biomed Res* 27: 15-21.
- Koppenhofer, A. M., E. M. Fuzy, R. L. Crocker, W. D. Gelernter, and S. Polavarapu. 2004. Pathogenicity of *Heterorhabditis bacteriophora*, *Steinernema glaseri*, and *S. scarabaei* (Rhabditida: Heterorhabditidae, Steinernematidae) against 12 white grub species (Coleoptera: Scarabaeidae). *Biocontrol Sci Technol* 14: 87-92.
- Machaca, K., L. J. Defelice, and S. W. L. Hernault. 1996. A novel chloride channel localizes to *Caenorhabditis elegans* spermatids and chloride channel blockers induce spermatid differentiation. *Dev Biol* 176: 1-16.
- Malekova, L. J. Tomaskova, M. Novakova, P. Stefanik, J. Kopacek, B. Lokatos, S. Pastorekova, O. Krizanova, A. Breier, and K. Ondrias. 2007. Inhibitory effect of DIDS, NPPB, and phloretin on intracellular chloride channels. *Pflugers Arch Eur J Physiol* (In press).
- Martin, F. N. 2003. Development of alternative strategies for management of soil borne pathogens currently controlled with methyl bromide. *Annu Rev Phytopathol* 41: 325-350.
- Mason, C. E., M. E. Rice, D. D. Calvin, J. W. Van Duyn, W. B. Showers, W. D. Hutchison, J. F. Witkowski, R. A. Higgins, D. W. Onstad, and G. P. Dively. 1996. European corn borer ecology and management. North Central Regional Extension Publication 327. Iowa State University, Ames, IA.
- Moffett, D. F., and S. Ann Cummings. 1994. Transepithelial potential and alkalization in

- an *in situ* preparation of tobacco hornworm (*Manduca sexta*) midgut. *J Exp Biol* 194: 341-345.
- Nagel, W., P. Somieski, and U. Katz. 2001. Selective inhibition of Cl<sup>-</sup> conductance in toad skin by blockers of Cl<sup>-</sup> channels and transporters. *Am J Physiol Cell Physiol* 281: C1223-C1232.
- Nehrke, K., T. Begenisich, J. Pilato, and J. E. Melvin. 2000. Model organism: new insights into ion channel and transporter function. *Caenorhabditis elegans* ClC-type chloride channels: novel variants and functional expression. *Am J Physiol Cell Physiol* 279: C2052-C2066.
- Nilius, B., and G. Droogmans. 2003. Amazing chloride channels: an overview. *Acta Physiol Scand* 177: 119-147.
- Pedersen, T. H., F. D. Paoli, and O. B. Nielsen. 2005. Increased excitability of acidified skeletal muscle: role of chloride conductance. *J Gen Physiol* 125: 237-246.
- Petalcorin, M. I. R., T. Oka, M. Koga, K. Ogura, Y. Wada, Y. Ohshima, and M Futai. 1999. Disruption of *clh-1*, a chloride channel gene, results in a wider body of *Caenorhabditis elegans*. *J Mol Biol* 294: 347-355.
- Phipps, D. J., D. R. Branch, and L. C. Schlichter. 1996. Chloride-channel block inhibits T lymphocyte activation and signaling. *Cell Signal* 8: 141-149.
- Pusch, M., A. Accardi, A. Liantonio, P. Guida, S. Traverso, D. C. Camerino, and F. Conti. 2002. Mechanisms of block of muscle type CLC chloride channels (Review). *Mol Memb Biol* 19: 285-292.
- Qiu, W., G. Hu, N. Paolocci, R. C. Ziegelstein, and D. A. Kass. 2003. Differential effects of pulsatile versus steady flow on coronary endothelial membrane potential. *Am J*

- Physiol Heart Circ Physiol* 285: H341-H346.
- Quinton, S. E., G. C. Brown, R. T. Bessin, and D. W. Johnson. 2005. European corn borer (Lepidoptera: Crambidae) effects on qualitative traits of high oil content corn grown in Kentucky. *J Econ Entomol* 98: 395-401.
- Ridgway, R. L., and D. F. Moffett. 1986. Regional differences in the histochemical Localization of carbonic anhydrase in the midgut of tobacco hornworm (*Manduca sexta*). *J Exp Zool* 237: 407-412.
- Rosso, M. N., M. P. Dubrana, N. Cimbolini, S. Jaubert, and P. Abad. 2005. Application of RNA interference to root-knot nematode genes encoding esophageal gland proteins. *Mol Plant Microbe Interact* 18: 615-620.
- Rutledge, E., L. Bianchi, M. Christensen, C. Boehmer, R. Morrison, A. Broslat, A. M. Beld, A. L. George, Jr., D. Greenstein, and K. Strange. 2001. CLH-3, a CIC-2 anion channel ortholog activated during meiotic maturation in *C. elegans* oocytes. *Current Biol* 11: 161-170.
- Rutledge, E., J. Denton, and K. Strange. 2002. Cell cycle- and swelling-induced activation of a *Caenorhabditis elegans* CIC channel is mediated by CeGLC-7 $\alpha/\beta$  phosphatases. *J Cell Biol* 158: 435-444.
- Schriever, A. M., T. Friedrich, M. Pusch, and T. J. Jentsch. 1999. CLC chloride channels in *Caenorhabditis elegans*. *J Biol Chem* 274: 34238-34244.
- Spiegel, S., M. Phillipper, H. Rossmann, B. Riederer, M. Gregor, and U. Seidler. 2003. Independence of apical Cl<sup>-</sup>/HCO<sub>3</sub><sup>-</sup> exchange and anion conductance in duodenal HCO<sub>3</sub><sup>-</sup> secretion. *Am J Physiol Gastrointest Liver Physiol* 285: G887-G897.
- Steele, J. A. 1989. Voltage- and time dependent chloride currents in chick skeletal

- muscle cells grown in tissue culture. *Pflug Arch Eur J Physiol* 414: 265-272.
- Sterling, D., and J. R. Casey. 2002. Bicarbonate transport proteins. *Biochem Cell Biol* 80: 483-497.
- Strange, K. 2002. Of mice and worms: novel insights into ClC-2 anion channel physiology. *News Physiol Sci* 17: 11-16.
- Strange, K. 2006. An overview of *C. elegans* biology. Pp 1-11. In K. Strange (Ed.), *C. elegans methods and applications*. Humana Press, Totowa, New Jersey.
- Susurluk, A. 2006. Effectiveness of entomopathogenic nematodes *Heterorhabditis bacteriophora* and *Steinernema feltiae* against *Tenebrio molitor* (Yellow mealworm) larvae in different soil types at different temperatures. *Turk J Biol* 30: 199-205
- Talavera, M., and T. Mizukubo. 2005. Effects of DL-methionine on hatching and activity of *Meloidogyne incognita* eggs and juveniles. *Pest Manag Sci* 61: 413-416.
- Tararthuch, A. L., R. Fernandez, and G. Malnic. 2007. Cl<sup>-</sup> and regulation of pH by MDCK-C11 cells. *Brazilian J Med Biol Res* 40: 687-696.
- Toledo, J., R. Rojas, and J. E. Ibarra. 2006. Efficiency of *Heterorhabditis bacteriophora* (Nematoda: Heterorhabditidae) on *Anastrepha serpentina* (Diptera: Tephritidae) larvae under laboratory conditions. *Florida Entomol* 89: 524-526.
- Tosco, M., M. N. Orsenigo, G. Gastaldi, and A. Faelli. 1998. pH dependence of Cl<sup>-</sup>/HCO<sub>3</sub><sup>-</sup> exchanger in the rat jejunal enterocyte. *Biochem et Biophysic Acta* 1372: 323-330.
- Uchiyama, H., H. Hayashi, and Y. Suzuki. 2006. Functional characterization of Cl<sup>-</sup>/HCO<sub>3</sub><sup>-</sup> exchange in villous cells of the mouse ileum. *Biomed Res* 27: 265-274.

- Urwin, P. E, C. J. Lilley, and H. J. Atkinson. 2002. Ingestion of double-stranded RNA by parasitic juvenile cyst nematodes leads to RNA interference. *Mol Plant Microbe Interact* 8: 747-752.
- Wang, Y., and W. H. Telfer. 1998. Cyclic-AMP-induced water uptake in a moth ovary: inhibition by bafilomycin and anthracene-9-carboxylic acid. *J Exp Biol* 201: 1627-1635.
- Yagi, K., J. Williams, N.Y. Wang, and R. J. Cicerone. 1993. Agricultural soil fumigation as a source of atmospheric methyl bromide. *Proc Natl Acad Sci USA* 90: 8420-8423.
- Yamamoto, D., and N. Suzuki. 1987. Single-channel recordings of chloride currents in primary cultured *Drosophila* neurons. *Arch Insect Biochem Physiol* 6: 151-158.
- Ying-zi, W., L. Zheng-Jiang, L. Li, F. Ping, S. Jun-Qiang, Z. Lei, M. Ke-Tao, Z. Li, and G. Wei-Jian. 2006. Effects of chloride channel blockers on excitatory junction potentials in smooth muscle cells of cochlear spiral modiolar artery in guinea pigs. *Acta Physiol Sinica* 58: 456-462.
- Zhou, X., H. K. Kaya, K. Heungens, and H. Goodrich-Blair. 2002. Response of ants to a deterrent factor(s) produced by the symbiotic bacteria of entomopathogenic nematodes. *Appl Environ Microbiol* 68: 6202-6209.

Table 1.1. Tissue distribution and proposed functions of different voltage-gated chloride channels in *Caenorhabditis elegans*

VGCC	Tissue distribution	Proposed function
CeCIC-1	Hypodermal cells in head, seam cells on lateral side of body, nerve cells in head ganglia, epithelial D-cells of the vulva, posterior cells of intestine, and cells of spermatheca.	Stabilize membrane potential, (normal nematode morphology).
CeCIC-2 or -2b	Nerve ring, dorsal and ventral nerve cords, tail neurons, vulval muscles, pharyngeal-intestinal valve cells, and body wall muscles involved in locomotion.	Neurotransmission, pharyngeal pumping, locomotion, and chloride secretion/regulation.
CeCIC-3 or -3b	Large H-shaped excretory cell, first four epithelial cells of intestine, muscles controlling defecation, hermaphrodite-specific neurons ( <i>HSN</i> ) innervating	Osmoregulation, fluid secretion in the intestinal cells, and cell volume regulation.

vulval muscles, and oocytes.

CeCIC-4 or 4b	Large H-shaped excretory cell.	Maintenance of osmotic balance and acidification of endocytotic cells
CeCIC-5	Expressed ubiquitously through out the body.	Acidification of endocytotic cells
CeCIC-6	Anterior and posterior portions of intestine, muscle cells of pharynx, vulva, excretory system, and body wall.	Acidification of endocytotic cells

---

Schriver et al. (1999), Nehrke et al. (2000), Bloomquist (2003)

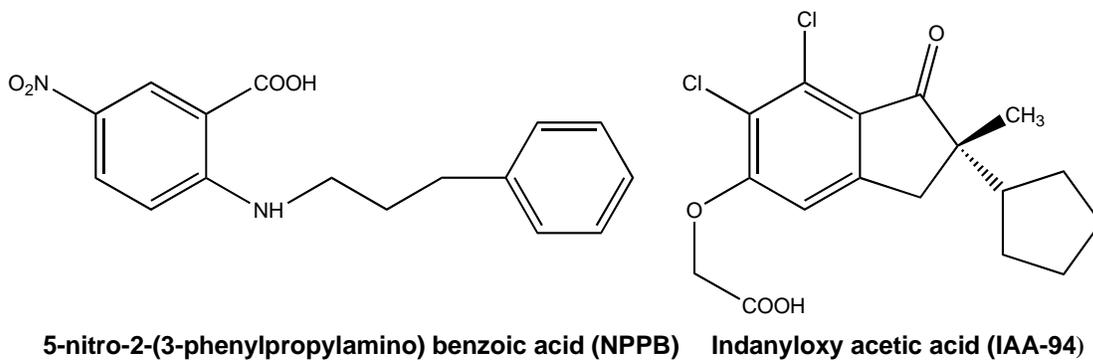
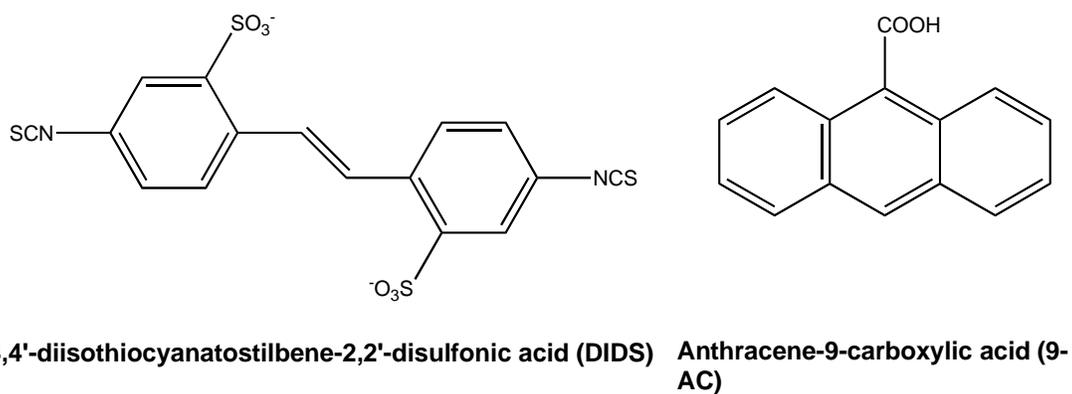
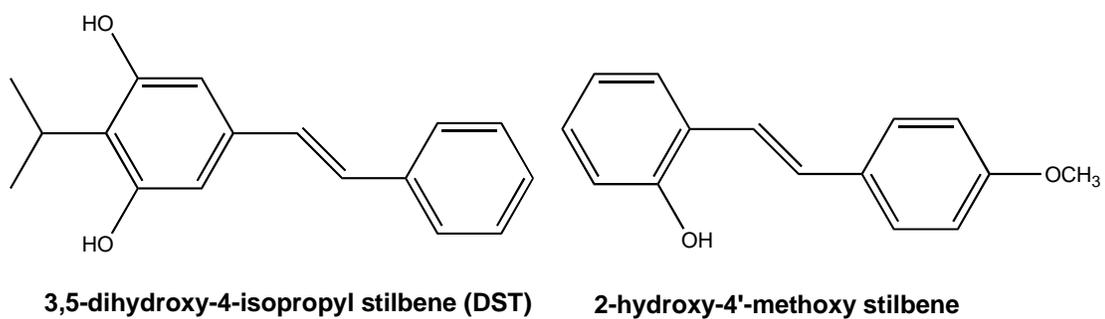


Fig. 1.1. Chemical structures of anion transporter blockers and other stilbene compounds discussed in the text.

## CHAPTER 2

### **Nematicidal Activity of Anion Transporter Blockers Against *Meloidogyne incognita* (Kofoid and White) Chitwood, *Caenorhabditis elegans* Maupas, and *Heterorhabditis bacteriophora* Poinar**

#### **ABSTRACT**

Because methyl bromide was phased out as a soil sterilant, new nematicides are urgently needed. Four different chemical classes of organic acids acting as anion transport (AT) blockers were tested against a free-living nematode, *Caenorhabditis elegans* Maupas 1900: a plant-parasitic nematode, *Meloidogyne incognita* (Kofoid and White 1919) Chitwood 1949): and an entomopathogenic nematode, *Heterorhabditis bacteriophora* Poinar 1975 in toxicity bioassays. The materials tested were DIDS (4, 4'-diisothiocyanatostilbene-2, 2'-disulfonic acid), 9-AC (anthracene-9-carboxylic acid), NPPB (5-nitro-2-(3-phenylpropylamino) benzoic acid), and IAA-94 (indanyloxy acetic acid). All the compounds showed slowly developing nematicidal activity against second-stage larvae of *M. incognita* and adults of *C. elegans*, but not against third-stage larvae of *H. bacteriophora*. Lethal concentration required for 50% mortality of test nematodes (LC<sub>50</sub>) for these compounds were <50 ppm after 48- and 72-h incubation against *C. elegans* and *M. incognita*, respectively, while LC<sub>50</sub> values were <10 ppm for both sensitive species after 168-h incubation. Across both species and time, the LC<sub>50</sub> values generally differed  $\leq 2$ -fold among the four compounds tested in this study. In contrast, none of the compounds (200 ppm) caused more than control mortality to *H.*

*bacteriophora*, even after 168 h of incubation. A 1-h incubation in 50 ppm of AT blocker solution increased significantly (about 20%) the pharyngeal muscle contractions/min in *C. elegans* adults. A 12-h incubation in 50 ppm of DIDS, NPPB, and IAA-94 (but not 9-AC) increased significantly the percentage of nematodes with affected locomotion, while a 24 h incubation affected locomotion in a significantly higher percentage of worms by all AT blockers, including 9-AC. These compounds are potential leads for commercial nematicides. The insensitivity to *H. bacteriophora* is consistent with the natural exposure of this nematode to DST (3,5-dihydroxy-4-isopropyl stilbene), a stilbene produced by its symbiotic bacterium. Based on the known activity of the compounds used in this study, we suggest that anion transporters form the probable target sites for DIDS, 9-AC, NPPB, and IAA-94 in non-entomopathogenic nematodes.

## INTRODUCTION

Among different plant-parasitic nematodes, root-knot nematodes (*Meloidogyne* spp.), are the most important, infesting roots of >2000 plant species resulting in annual crop loss of ~50 billion US dollars throughout the world (Dautova et al. 2001, Huang et al. 2003). Methyl bromide, a soil fumigant used for controlling root-knot nematodes and other soil-borne pathogens, was banned in the US for soil fumigation due to its strong ozone depleting properties (Makhijani and Gurney 1995, Martin 2003). In addition, with the implementation of the Food Quality Protection Act of 1996, many organophosphate and carbamate pesticides are under EPA review for possible phase out (Fields and White 2002), which further shrinks the range of chemical nematicides available for control. Therefore, there is an urgent need for identifying potential target sites in nematodes which can be exploited for their control.

A natural stilbene compound, 3, 5-dihydroxy-4-isopropyl stilbene (DST) (Fig. 1.1), is produced both *in vitro* and *in vivo* by a symbiotic bacterium belonging to the genus, *Photorhabdus* found in entomopathogenic nematodes belonging to the genus, *Heterorhabditis* (Hu et al. 1999, 2000). Concentrations of DST in *Heterorhabditis* spp./*Photorhabdus* spp. complex-infected *Galleria mellonella* (Linnaeus 1758) larvae ranged between 0.7 to 4 mg/g of wet insect tissue (Hu et al. 1999). DST has significant nematicidal activity against some free-living nematodes such as *Caenorhabditis elegans* Maupas 1900 at 100 ppm, but not against the entomopathogenic nematode, *Heterorhabditis megidis* Poinar, Jackson and Klein 1987 strain H90, or the plant-parasitic nematode, *Meloidogyne incognita* (Kofoid and White 1919) Chitwood 1949 (Hu et al. 1995, 1996, 1999). However, DST has shown ovicidal activity against eggs of *M.*

*incognita* (Hu et al. 1999). Although DST has shown nematicidal and ovicidal activity, its target site and mode of action are unknown.

A synthetic analog of DST, DIDS (4, 4'-diisothiocyanatostilbene-2, 2'-disulfonic acid) (Fig. 1.1), is an anion transporter (AT) blocker known to block chloride ion (Cl<sup>-</sup>) transport through voltage-gated chloride channels (VGCCs) and chloride/bicarbonate (Cl<sup>-</sup>/HCO<sub>3</sub><sup>-</sup>) exchangers present on plasma membranes of both electrogenic and non-electrogenic cells of biological systems (Cabantchik and Greger 1992, Boudko et al. 2001, Jentsch et al. 2002, Maduke et al. 2000, Nilius and Droogmans 2003). The recent discovery of six VGCCs (CeClC-1 to 6) in *C. elegans* (Schriever et al. 1999, Nehrke et al. 2000, Strange 2002, Denton et al. 2003), DST nematicidal activity against *C. elegans*, and the structural similarity between DST and DIDS (Bloomquist 2003), suggested that the nematicidal properties of DST may be due to the inhibition of VGCCs in nematodes and that DIDS could have the same nematicidal properties as DST (Bloomquist 2003). A wealth of information is available documenting the interaction of DIDS with ATs (reviewed by Cabantchik and Greger 1992). However, no information is available on the activity of DIDS or other AT blockers towards nematodes, *in vivo*. Therefore, four AT blockers belonging to different classes of organic acids (Fig. 1.1), including DIDS (a stilbene disulfonic acid), 9-AC (anthracene-9-carboxylic acid; an aromatic carboxylic acid), NPPB ((5-nitro-2-(3-phenylpropylamino) benzoic acid; an anthranilic acid)), and IAA-94 (indanyloxy acetic acid; an indanyloxy alkanolic acid) were selected for testing in this investigation. Our objectives were: 1) to determine the activity of DIDS, 9-AC, NPPB, and IAA-94 against eggs and second-stage larvae of *M. incognita*, adults of *C. elegans*, and third-stage larvae of *Heterorhabditis bacteriophora* Poinar 1975; 2) to

compare the results obtained in this study with that of DST; and 3) discuss probable target sites of DST and AT blockers for nematode control.

## MATERIALS AND METHODS

### Chemicals

All the tested AT blockers, DIDS, 9-AC, NPPB, and IAA-94 and dimethyl sulfoxide (DMSO) were purchased from Sigma-Aldrich (St Louis, MO). Chlorpyrifos (O,O-diethyl O-(3,5,6-trichloro-2-pyridyl) phosphorothioate with 99.5% purity) was purchased from Chem Service (West Chester, PA).

### Nematode Cultures

*Caenorhabditis elegans* wild strain N2 Bristol was cultured on nematode growth medium (NGM) agar plates seeded with *Escherichia coli* (Migula 1895) Castellani and Chalmers 1919, strain OP-50 (Brenner 1974) (Both *C. elegans* and *E. coli* were kindly provided by Caenorhabditis Genetics Center, Minneapolis, MN [<http://www.cbs.umn.edu/CGC>]). Nematodes (3-5-days old) were collected from the culture plates for bioassays.

*Meloidogyne incognita* cultures were maintained on susceptible tomato (*Lycopersicon esculentum* (Linnaeus.) Mill.) plants (Rutgers variety) grown in a greenhouse. Eggs were collected using the methods of McCarter et al. (2003) and Talavera and Mizukobo (2005). In brief, galled roots of tomato plants infested with *M. incognita* were collected. To free the eggs from the gelatinous egg masses, roots were shaken in 10% bleach containing 6% sodium hypochlorite (wt/wt) for 3 min in a 1000 mL conical flask. The contents of flasks with eggs were poured onto a 325-mesh sieve

placed over a 500-mesh sieve and washed several times with tap water to remove any traces of bleach. Eggs were retained on the 500-mesh sieve. Collected eggs were either used directly for ovicidal assays or placed on a modified Baermann funnel to collect second-stage larvae daily for bioassays.

Third-stage larvae of *H. bacteriophora* emerged from infected *G. mellonella* larvae were collected daily by the White trap method (White 1927) and used directly in bioassays. The nematode population densities (no. nematodes/mL) in distilled water were standardized before running bioassays (Glazer and Lewis 2000).

#### **Nematicidal Activity on *C. elegans*, *M. incognita*, and *H. bacteriophora***

Nematicidal activity of AT blockers was tested against adults, second-stage, and third-stage larvae of *C. elegans*, *M. incognita*, and *H. bacteriophora*, respectively. In a 96-well plate, 20  $\mu$ L of distilled water containing 17 ( $\pm$ 3) suspended nematodes was placed in each well and the volume was made to 300  $\mu$ L with distilled water. Stock solutions of DIDS, 9-AC, NPPB, or IAA-94 prepared in DMSO were added to each well to obtain final concentrations of 2, 5, 10, 20, and 40 ppm. Wells with DMSO alone (final concentration not more than 0.5%) served as controls. *Heterorhabditis bacteriophora* bioassays included an organophosphate compound, chlorpyrifos, as positive control and a higher concentration of AT blockers (200 ppm) was tested as lower concentrations were not effective against *H. bacteriophora* third-stage larvae. Plates were sealed with Parafilm and incubated at room temperature (21°C) in the dark. Mortality counts were taken at 24, 48, 72, 120, and 168 h after incubation. Nematodes that were immobile for 30 s observation under stereo microscope were considered dead and included in mortality counts. Since AT blockers are organic acids and the pH of stock treatment solutions

ranged from 3.4 to 5.1, we wanted to confirm that the resulting mortality of nematodes was not merely due to the acidic nature of these compounds. Therefore, a bioassay, as described above, was conducted using these blockers at 40 ppm against *C. elegans* adults, wherein the pH of blocker solution was adjusted to neutral (pH 7) with 10 mM HEPES and DMSO/HEPES alone served as a control. All the pH-balanced experiments were conducted twice.

### **Ovicidal Activity on *M. incognita***

In a 96-well plate, each well was filled with 280  $\mu$ L of distilled water to which 20  $\mu$ L distilled water containing 135 ( $\pm$ 12) eggs of *M. incognita* was added. Stock solutions and various final concentrations of DIDS, 9-AC, NPPB, or IAA-96 were prepared as described above. Wells with solvent (DMSO) alone served as controls. The plates were sealed with Parafilm and incubated at room temperature (21°C) in the dark. Observations on egg hatching were taken by counting the number of second-stage larvae (mobile + non-mobile) 24, 72, 120, and 168 h after incubation.

### **Effect on Pharyngeal Muscle Contraction and Locomotion**

Tests were conducted using adult *C. elegans* collected from culture Petri plates and incubated with 50 ppm AT blockers in 96 well plates as described above. For pharyngeal muscle contractions, 1 and 6 h after incubation, 20 nematodes were removed from each treatment well and placed on a microscope slide with a drop of *E. coli* bacteria (strain OP50). A cover slip or Parafilm was placed over the nematode/bacteria suspension and the pharyngeal pumping rate (contractions/min) for each nematode was recorded visually for 1 min under a stereomicroscope (Olympus BX40). Effect on locomotion was determined by a radial migration assay (Salcini 2001). After 12 and 24 h

incubation, 12 to 15 nematodes from each treatment were picked and transferred to the center of a 30-mm (inner diameter) ring made of *E. coli* lawn in the center of a NGM agar plate (90-mm diameter). One hour after nematode release, nematodes that reached the bacterial ring were counted and subtracted from the total number of nematodes released, giving the number of nematodes with affected locomotion. Experiments were replicated three times.

### **Statistical Analyses**

Toxicity data for each dosage were pooled and were subjected to probit analysis using the Polo Plus program (LeOra Software 2005) after correcting for control (DMSO) mortality. Lethal concentration required for 50% mortality of test nematodes ( $LC_{50}$ ) along with corresponding 95% confidence limits were calculated for each blocker at each incubation time. For ovicidal activity, the proportion of eggs hatched was calculated as the number of second-stage larvae divided by total number of eggs incubated. From the data, effective concentration required for 50% egg hatch inhibition ( $EC_{50}$ ) was calculated for each blocker by probit analysis using PROC PROBIT program (SAS Institute 2005) after correcting for control egg hatch. Statistically significant differences in  $LC_{50}/EC_{50}$  values among blockers at a given incubation time period within or among species were determined based on non-overlap of 95% confidence intervals. Mean ( $\pm$ SEM;  $n = 20$ ) pharyngeal muscle contraction rates and mean ( $\pm$ SEM;  $n = 3$ ) percent nematodes with locomotion affected were calculated for each AT blocker. Data were subjected to one-way ANOVA using PROC GLM program for statistical significance between treatment means and means were separated using Tukey's studentized range test or Student Newman Keuls test at  $\alpha=0.05$  level of significance (SAS Institute 2005).

## RESULTS

### Nematicidal Activity on *C. elegans*, *M. incognita*, and *H. bacteriophora*

All the tested blockers showed slowly developing nematicidal activity against *C. elegans* and *M. incognita* that increased with time. The control mortality in *C. elegans* and *M. incognita* exposed only to the vehicle DMSO ranged from 0% after 24-h incubation to 10% after 168-h incubation. Activity of DIDS, 9-AC, NPPB, and IAA-94 at 40 ppm, after adjusting the solution to neutral pH with HEPES buffer, was measured against *C. elegans* adults (Fig. 2.1). Percent mortality in control (DMSO/HEPES) ranged from 2% after 24-h incubation to 13% after 168-h incubation (Fig. 2.1), similar to that of DMSO alone. The percent mortality was above 50% in DIDS, NPPB, IAA-94 and was 45% in 9-AC after 48-h incubation in buffered media. Further, 100% mortality of *C. elegans* adults was recorded in all the blocker treatments after 120-h incubation in buffered media (Fig. 2.1).

None of the blockers at the highest concentration tested (40 ppm) caused  $\geq 50\%$  mortality in *C. elegans* after 24-h incubation; therefore,  $LC_{50}$  values could not be determined at this time point. All the blockers achieved  $\geq 50\%$  mortality in *C. elegans* by 48-h incubation, with DIDS the most active compound (Table 2.1), followed by NPPB, 9-AC, and IAA-94 in decreasing order of activity. No statistically significant differences were observed after 48-h incubation between the  $LC_{50}$  values based on overlap of 95% confidence limits (Table 2.1). After 72-h incubation, the activity of all the blockers increased by 2- to 3-fold compared with 48-h incubation. Again, DIDS had the lowest  $LC_{50}$  value and was significantly different from 9-AC and IAA-94. NPPB had the next highest activity, but did not differ statistically from DIDS. Similarly, a near 2-fold

decrease in LC<sub>50</sub> values of blockers against *C. elegans* adults was observed at 120-h incubation compared to that of 72-h incubation. The LC<sub>50</sub> values ranged from 4.5 to 11.6 ppm with DIDS again showing the highest activity, which was statistically significant from that of IAA-94. The LC<sub>50</sub> values of the blockers dropped another 2-fold after 168-h incubation, but no statistically significant differences in activity were observed between them (Table 2.1).

Concentrations of blockers required for 50% mortality of *M. incognita* second-stage larvae were slightly higher than for *C. elegans* adults at all time points (Table 2.2), but the difference had narrowed considerably by 168-h incubation. Mortality in *M. incognita* did not reach 50% at the highest concentration of blockers (40 ppm) tested after 48-h incubation. At 72-h incubation, LC<sub>50</sub> values of blockers ranged from 15.7 to 30.3 ppm with DIDS < NPPB < 9-AC < IAA-94 in that order of activity, but no statistically significant differences were observed between them (Table 2.2). Compared to 72-h incubation, a near 2-fold increase in activity against *M. incognita* second-stage larvae was observed in all the blockers after 120-h incubation, but no statistically significant difference in activity was observed (Table 2.2). Similarly, a near 2-fold increase in activity of these blockers was seen at 168-h incubation compared to 120-h incubation and the LC<sub>50</sub> values were without any significant differences (Table 2.2).

In contrast to *C. elegans* and *M. incognita*, none of the blockers tested was found active against third-stage larvae of *H. bacteriophora* (Fig. 2.2). Mortality was <15% at the highest concentration (200 ppm) of blockers tested after 168-h incubation. In contrast, significantly higher mortality of *H. bacteriophora* was observed with

chlorpyrifos at concentrations <200 ppm (data not shown) and it was 100% at 200 ppm following 24-h incubation (Fig. 2.2).

### **Ovicidal Activity on *M. incognita***

None of the blockers at tested concentrations inhibited 50% egg hatch of *M. incognita* at 24- and 72-h incubation. Inhibition of 50% egg hatch with DIDS and NPPB was obtained at 120-h incubation, but not with 9-AC and IAA-94. At 120-h incubation, the effective concentration required for 50% inhibition of egg hatch by DIDS and NPPB was 29.1 and 46.5 ppm, respectively (Table 2.3). The EC<sub>50</sub> values for 9-AC and IAA-94 were 48.0 and 42.3 ppm, respectively at 168-h incubation, while DIDS and NPPB activity increased only modestly beyond that measured at 120-h incubation (Table 2.3). No significant differences in EC<sub>50</sub> values were recorded between blockers for any exposure time period based on overlap of 95% confidence intervals.

### **Effect on Pharyngeal Muscle Contraction and Locomotion**

Additional effects of AT blockers were determined on locomotion and contraction frequency of pharyngeal muscle. At 50 ppm (this concentration is an LC<sub>50</sub> value between 24- and 48-h exposures), there was a significant increase (about 20%) in pharyngeal muscle contraction frequency across all the compounds ( $P < 0.05$ ) (Fig. 2.3). However, the increase had subsided after 6 h of exposure, and pharyngeal contraction returned to control levels of about 200/min ( $P > 0.05$ ) (Fig. 2.3). AT blockers at 50 ppm showed no significant effect on nematode locomotion compared to control after 6-h incubation (data not shown). After 12-h incubation, the percentage of nematodes with decreased locomotion was significantly higher in DIDS, NPPB, and IAA-94 compared to control. These three AT blockers were statistically not significant from each other, while NPPB

and IAA-94 were statistically not significant from 9-AC (Fig. 2.4). After 24-h incubation, all four AT blockers decreased locomotion compared to control, with NPPB being the most active. NPPB was equivalent with DIDS, DIDS was equivalent with IAA-94, while IAA-94 was significantly superior to 9-AC in affecting the locomotion of nematodes (Fig. 2.4).

## DISCUSSION

In this study, DIDS, NPPB, 9-AC, and IAA-94 demonstrated nematicidal activity against second-stage larvae of the plant-parasitic nematode, *M. incognita*, adults of free-living nematode, *C. elegans*, and ovicidal activity against *M. incognita*. The observed nematicidal activity of blockers against nematodes was not merely due to their acidic nature, as the blockers showed similar activity against *C. elegans* after the pH of the solution was adjusted to neutral with HEPES, compared with blockers without any pH adjustment. Incubation times of 48 and 72 h were required to obtain the same level of activity in *C. elegans* and *M. incognita*, respectively, indicating a slowly developing activity of AT blockers compared to DST. However, the difference in activity of blockers observed initially against *C. elegans* and *M. incognita* was minimized by the time exposure reached 168 h. The faster activity of these blockers against *C. elegans* might be partly attributable to using an actively feeding adult stage of this nematode and a non-feeding second-stage larvae of *M. incognita*, which possibly leads to ingestion of more test compound by *C. elegans*.

Our results with AT blockers are generally in agreement with results of Hu et al. (1999) for DST lethality against nematodes. Similar to our findings, DST, a bacterial

stilbene, has shown nematicidal activity against *C. elegans* fourth-stage larvae and adults with an  $LC_{50}$  of 50 ppm after 24-h incubation (Hu et al. 1999). Unlike the nematicidal activity of AT blockers observed against *M. incognita* in our study, DST was inactive against second-stage larvae of *M. incognita* (Hu et al. 1996, 1999). However, DST at  $\geq 12.5$  ppm did significantly inhibit egg hatching of *M. incognita* in 5 days (Hu et al. 1999). Similar to DST, we found that all AT blockers showed slowly developing ovicidal activity against *M. incognita*, as significant egg hatch inhibition (50% of control) was obtained at  $\geq 120$  h after incubation. The observation that DST was active against *M. incognita* eggs but not second-stage larvae was not explained by Hu et al. (1999).

The blockers tested in our study did not affect the entomopathogenic nematode, *H. bacteriophora*, at 200 ppm, even after 168-h incubation. These results are in accord with DST toxicity to the entomopathogenic nematode, *H. megidis* strain H90, as no mortality of third-stage larvae was observed at 200 ppm after 24-h incubation (Hu et al. 1999). The resistance observed in *H. megidis* strain H90 to DST (Hu et al. 1999) is probably due to the need for protection from DST and like metabolites released by its own symbiotic bacteria, *Photorhabdus* spp. We hypothesize that the same mechanism may confer cross resistance against AT blockers to *H. bacteriophora* in our study.

Our study with AT blockers and the previous findings of Hu et al (1995, 1996, 1999) with DST against free-living, plant-parasitic, and entomopathogenic nematodes suggest that DST and AT blockers may have the same mode of action, as was suggested by Bloomquist (2003). Differences in binding to the target protein, in detoxification in the body, and in transport to the target site could account for the variations observed in activity between DST and AT blockers, or among AT blockers within a species and

between species. In addition, substitutive atoms on the stilbene moiety play a major role in its activity against nematodes (Suga et al. 1993, Suga 1994). However, the reasons for DST inactivity observed against *M. incognita* second-stage larvae in the Hu et al (1999) study remain unknown.

These same pharmacokinetic and pharmacodynamic processes could contribute to the slow onset of toxicity observed for the AT blockers. Possible reasons for the lag in toxicity could include slow penetration/distribution of AT blockers, *in vivo*.

Alternatively, metabolic bioactivation could be involved, but at the present time there is no evidence it occurs. Mortality in control treatments was  $\leq 10\%$ , indicating that aging of the worms does not have any obvious effect on the rate of intoxication by AT blockers. Finally, although the stilbenes used in the present study had a slow action and relatively low potency, a synthetic stilbene compound, 2-hydroxy-4'-methoxy stilbene (Fig. 1.1), caused 95% mortality of pine wood nematodes in 48-h incubation tests at 1 ppm (Suga 1994), confirming that commercial levels of activity are available from this chemistry following appropriate chemical substitution of the stilbene moiety.

The AT blockers, including DIDS, 9-AC, NPPB, and IAA-94, are known for blocking (modifying)  $\text{Cl}^-$  conductance/transport across cell membranes of excitatory and non-excitatory cells via channels such as VGCCs as well as exchangers, such as  $\text{Cl}^-/\text{HCO}_3^-$  exchangers (Cabantchik and Greger 1992, Boudko et al. 2001, Fahlke 2001). These blockers have been widely used for characterizing cell membrane  $\text{Cl}^-$  channels and exchangers pharmacologically in many vertebrates and non-vertebrates, including nematodes (Machaca et al. 1996, Ullrich and Sontheimer 1996, Ferroni et al. 1997, Li et al. 2000, Nagel et al. 2001, Pusch et al. 2002, Qiu et al. 2003, Cavarra et al. 2004).

Therefore, it is likely that these compounds are indeed acting on ATs in nematodes to cause mortality, as predicted by Bloomquist (2003). Although VGCCs are found in nerve cells, the slowly developing activity of these blockers is an indication that they are not acting upon VGCCs of nerve cells, or at least not in a way that rapidly affects function.

*Caenorhabditis elegans* oocyte membranes possess CeClC-3 (*ceclc-3*) and NPPB at 100  $\mu$ M inhibited its Cl<sup>-</sup> current up to 90% (Rutledge et al. 2001). Ovicidal activity of AT blockers observed in the present study against *M. incognita* is an indication of VGCCs presence in *M. incognita* oocytes and blockage of this channel might have resulted in inhibition of egg hatch. The increase in pharyngeal pumping rate we observed for AT blockers is similar to the effects of low chloride saline on *C. elegans* pharyngeal muscle, where a transient depolarization and increased excitability was observed (Franks et al. 2002). Further, decrease in locomotion of AT blocker-treated adults suggests that AT blockers were inhibiting VGCC involved in locomotion as well. It is also possible that the sensory cues associated with finding food (*E. coli*) may have been blocked due to exposure to AT blocker. Further tests on this aspect are needed to confirm this hypothesis. Taken together, these findings suggest that VGCCs or transporters present on pharyngeal muscle and body wall muscles were blocked in exposed nematodes, but perhaps with a longer time course, since the pharyngeal and body wall structures were intact and not exposed by dissection as in electrophysiological studies (Franks et al. 2002).

In conclusion, our studies show that AT blockers are nematicidal to *M. incognita* and *C. elegans*, but not to *H. bacteriophora*, consistent with its insensitivity to a related

stilbene produced by a symbiotic bacterium. ATs in different tissues are probably the target sites for blockers and DST in nematodes. Increased pharyngeal contractions and decreased locomotion observed in AT blocker-treated *C. elegans* adults suggested that CeCIC-2 channels expressed in pharyngeal and body wall muscles and CeCIC-1 channels expressed in seam cells of body wall could have been blocked by AT blockers. Similarly, observed ovicidal activity could be due to the inhibition of orthologue CeCIC-3 channels expressed in oocytes of *M. incognita*. Disruption of physiological processes by VGCC inhibition leads to nematode mortality or egg mortality.

#### LITERATURE CITED

- Bloomquist, J. R. 2003. Chloride channels as tools for developing selective insecticides. *Arch Insect Biochem Physiol* 54: 145-156.
- Boudko, D. Y., L. L. Moroz, W. R. Harvey, and P. J. Linser. 2001. Alkalinization by chloride/bicarbonate pathway in larval mosquito midgut. *Proc Natl Acad Sci USA* 98: 15354-15359.
- Brenner, S. The genetics of *Caenorhabditis elegans*. 1974. *Genetics* 77: 71-94.
- Cabantchik, Z. I., and R. Greger. 1992. Chemical probes for anion transporters of mammalian cell membranes. *Am J Physiol* 262: C803-C827.
- Denton, J., K. Nerke, E. Rutledge, R. Morrison, and K. Strange. 2003. Alternative splicing of N<sup>-</sup> and C<sup>-</sup> terminal of a *C. elegans* CIC channel alters gating and sensitivity to external Cl<sup>-</sup> and H<sup>+</sup>. *J Physiol* 555: 97-114.
- Cavarra, M. S. S. M. del Monaco, and B. A. Kotsias. 2004. Chloride channel blockers activate an endogenous cationic current in oocytes of *Bufo arenarum*. *J Comp*

- Physiol A* 190: 531-537.
- Dautova, M., M. Rosso, P. Abad, F. J. Gommers, J. Bakker, and G. Smant. 2001. Single pass cDNA sequencing – a powerful tool to analyze gene expression in preparasitic juveniles of the southern root-knot nematode *Meloidogyne incognita*. *Nematology* 3: 129-139.
- Fahlke, C. 2001. Ion permeation and selectivity in ClC-type chloride channels. *Am J Physiol Renal Physiol* 280: F748-F757.
- Ferroni, S., C. Marchini, M. Nobile, and C. Rapisarda. 1997. Characterization of an inwardly rectifying chloride conductance expressed by cultured rat cortical astrocytes. *Glia* 21: 217-227.
- Fields, P. G., and N. D. G. White. 2002. Alternatives to methyl bromide treatments for stored-product and quarantine insects. *Annu Rev Entomol* 47: 331-359.
- Franks, C. J., D. Pemberton, I. Vinogradova, A. Cook, R. J. Walker, and L. Holde-Dye. 2002. Ionic basis of the resting membrane potential and action potential in the pharyngeal muscle of *Caenorhabditis elegans*. *J Neurophysiol* 87: 954-961.
- Glazer, I., and E. E. Lewis. 2000. Bioassays of entomopathogenic nematodes. Pp. 229-248. In A. Navon and K. R. S. Ascher (Eds.), *Bioassays of entomopathogenic microbes and nematodes*. CABI publishers, New York.
- Hu, K., J. Li, and J. M. Webster. 1995. Mortality of plant parasitic nematodes caused by bacterial (*Xenorhabdus* spp. And *Photorhabdus luminescens*) culture media. *J Nematology* 27: 502-503 (Abstract).
- Hu, K., J. Li, and J. M. Webster. 1996. 3, 5-Dihydroxy-4-isopropylstilbene: a selective nematicidal compound from the culture filtrate of *Photorhabdus luminescens*.

- Canadian J Plant Pathol* 18: 104 (Abstract).
- Hu, K., J. Li, and J. M. Webster. 1999. Nematicidal metabolites produced by *Photorhabdus luminescens* (Enterobacteriaceae), bacterial symbiont of entomopathogenic nematodes. *Nematology* 1: 457-469.
- Hu, K., and J. M. Webster. 2000. Antibiotic production in relation to bacterial growth and nematode development in *Photorhabdus-Heterorhabditis* infected *Galleria mellonella* larvae. *FEMS Microbiol Lett* 189: 219-223.
- Huang, G., B. Gao, T. Maier, R. Allen, E. L. Davis, T. J. Baum, R. S. Hussey. 2003. A profile of putative parasitism genes expressed in the esophageal gland cells of the root-knot nematode *Meloidogyne incognita*. *Mol Plant Microbe Interact* 16: 376-381.
- Jentsch, T. J., V. Stein, F. Weinreich, and A. Zdebik. 2002. Molecular structure and physiological function of chloride channels. *Physiol Rev* 82: 503-568.
- Li, X., K. Shimada, L. A. Showalter, and S. A. Weinman. 2000. Biophysical properties of CLC-3 differentiate it from swelling-activated chloride channels in Chinese hamster ovary-K1 cells. *J Biol Chem* 275: 35994-35998.
- Machaca, K., L. J. Defelice, and S. W. L. Hernault. 1996. A novel chloride channel localizes to *Caenorhabditis elegans* spermatids and chloride channel blockers induce spermatid differentiation. *Develop Biol* 176: 1-16.
- Maduke, M., C. Miller, and J. A. Mindell. 2000. A decade of CLC chloride channels: structure, mechanism, and many unsettled questions. *Annu Rev Biophys Biomol Struct* 29: 411-438.
- Makhijani, A., and K. R. Gurney. 1995. Mending the ozone hole: science, technology and

- policy. MIT Press, Cambridge, MA.
- Martin, F. N. 2003. Development of alternative strategies for management of soil borne pathogens currently controlled with methyl bromide. *Annu Rev Phytopathol* 41: 325-350.
- McCarter J. P., M. D. Mitreva, J. Martin, M. Dante, T. Wylie, U. Rao, D. Pape, Y. Bowers, B. Theising, C. V. Murphy, A. P. Kloek, B. J. Chiapelli, S. W. Clifton, D. M. Bird, and H. Waterston. 2003. Analysis and functional classification of transcripts from the nematode *Meloidogyne incognita*. *Geno Biol* 4: R26.1-R26.19.
- Nagel, W., P. Somieski, and U. Katz. 2001. Selective inhibition of Cl<sup>-</sup> conductance in toad skin by blockers of Cl<sup>-</sup> channels and transporters. *Am J Physiol Cell Physiol* 281: C1223-C1232.
- Nehrke, K., T. Begenisich, J. Pilato, and J. E. Melvin. 2000. Model organism: new insights into ion channel and transporter function. *Caenorhabditis elegans* ClC-type chloride channels: novel variants and functional expression. *Am J Physiol Cell Physiol* 279: C2052-C2066.
- Nilius, B., and G. Droogmans. 2003. Amazing chloride channels: an overview. *Acta Physiol Scand* 177: 119-147.
- Pusch, M., A. Accardi, A. Liantonio, P. Guida, S. Traverso, D. C. Camerino, and F. Conti. 2002. Mechanisms of block of muscle type CLC chloride channels (Review). *Mol Memb Biol* 19: 285-292.
- Qiu, W., G. Hu, N. Paolocci, R. C. Ziegelstein, and D. A. Kass. 2003. Differential effects of pulsatile versus steady flow on coronary endothelial membrane potential. *Am J*

- Physiol Heart Circ Physiol* 285: H341-H346.
- Rutledge, E., L. Bianchi, M. Christensen, C. Boehmer, R. Morrison, A. Broslat, A. M. Beld, A. L. George, Jr., D. Greenstein, and K. Strange. 2001. CLH-3, a ClC-2 anion channel ortholog activated during meiotic maturation in *C. elegans* oocytes. *Current Biol* 11: 161-170.
- Salcini, A. E., M. A. Hilliard, A. Croce, S. Arbucci, P. Luzzi, C. Tacchetti, L. Daniell, P. De Camilli, P. G. Pelicci, P. P. Di Fiore, and P. Bazzicalupo. 2001. The Eps 15 *C. elegans* homologue EHS-1 is implicated in synaptic vesicle recycling. *Nature Cell Biol* 3: 755-760.
- Schriever, A. M., T. Friedrich, M. Pusch, and T. J. Jentsch. 1999. CLC chloride channels in *Caenorhabditis elegans*. *J Biol Chem* 274: 34238-34244.
- Strange, K. 2002. Of mice and worms: novel insights into ClC-2 anion channel physiology. *News Physiol Sci* 17: 11-16.
- Suga, T., S. Ohta, K. Munesada, N. Ide, M. Kurokawa, M. Shimizu, and E. Ohta. 1993. Endogenous pine wood nematocidal substances in pines, *Pinus massoniana*, *P. strobus* and *P. palustris*. *Phytochem* 33: 1395-1401.
- Suga, T. 1994. Pest control chemicals against pine wood nematodes. US Patent No. 05314693.
- Talavera, M., and T. Mizukubo. 2005. Effects of DL-methionine on hatching and activity of *Meloidogyne incognita* eggs and juveniles. *Pest Management Sci* 61: 413-416.
- Ullrich, N., and H. Sontheimer. 1996. Biophysical and pharmacological characterization of chloride currents in human astrocytoma cells. *Am J Physiol* 270: C1511-C1521.
- White, G. F. 1927. A method for obtaining infective nematode larvae from cultures.

*Science* 66: 302-303.

Table 2.1. Activity of anion transporter blockers against adults of *Caenorhabditis elegans*

Exposure time (h)	Compound	No. nematodes (N)	<sup>a</sup> LC <sub>50</sub> (ppm)	95% CI <sup>b</sup>	Slope ± SEM	$\chi^2$ (df) <sup>c</sup>
48	DIDS	467	29.6	21.7-46.4	1.2 ± 0.2	1.2 (3)
	9-AC	490	42.7	32.2-66.1	1.7 ± 0.3	1.2 (3)
	NPPB	515	38.4	29.1-58.5	1.6 ± 0.2	1.1 (3)
	IAA-94	498	46.1	33.9-78.1	1.6 ± 0.3	1.5 (3)
72	DIDS	467	8.9	5.4-13.0	1.9 ± 0.2	4.2 (3)
	9-AC	490	17.8	14.4-21.8	2.0 ± 0.3	1.7 (3)
	NPPB	515	15.9	11.7-22.6	2.0 ± 0.2	3.6 (3)
	IAA-94	498	20.4	16.8-24.6	2.3 ± 0.4	1.9 (3)
120	DIDS	467	4.5	2.6-6.2	2.4 ± 0.3	3.6 (3)
	9-AC	490	8.4	4.9-11.4	2.9 ± 0.4	4.1 (3)
	NPPB	515	7.4	4.3-10.7	2.7 ± 0.3	7.5 (3)
	IAA-94	498	11.6	6.7-15.6	3.6 ± 0.4	4.7 (3)
168	DIDS	467	3.1	1.8-4.4	2.9 ± 0.3	4.8 (3)
	9-AC	490	5.3	2.8-7.3	3.1 ± 0.4	3.5 (3)
	NPPB	515	4.6	2.5-6.5	2.9 ± 0.3	7.2 (3)
	IAA-94	498	6.7	2.9-9.6	2.7 ± 0.4	4.6 (3)

<sup>a</sup> Lethal concentration required for 50% mortality of *C. elegans* adults.

<sup>b</sup> 95% confidence intervals for LC<sub>50</sub>.

<sup>c</sup> Chi-square goodness-of-fit statistic and degrees of freedom.

Table 2.2. Activity of anion transporter blockers against second-stage larvae of *Meloidogyne incognita*

Exposure time (h)	Compound	No. nematodes (N)	<sup>a</sup> LC <sub>50</sub> (ppm)	95% CI <sup>b</sup>	Slope ± SEM	$\chi^2$ (df) <sup>c</sup>
72	DIDS	486	15.4	9.6-22.0	1.9 ± 0.3	3.2 (3)
	9-AC	499	26.8	20.8-37.0	1.6 ± 0.2	1.5 (3)
	NPPB	496	20.1	15.6-26.6	1.5 ± 0.2	1.9 (3)
	IAA-94	480	30.3	24.4-40.6	2.1 ± 0.4	2.7 (3)
120	DIDS	486	7.5	3.0-11.0	2.6 ± 0.3	5.4 (3)
	9-AC	499	11.2	5.7-16.4	2.3 ± 0.3	4.8 (3)
	NPPB	496	8.1	2.3-13.0	2.4 ± 0.3	7.6 (3)
	IAA-94	480	14.9	8.8-18.0	2.3 ± 0.3	1.5 (3)
168	DIDS	486	4.5	1.9-6.6	3.0 ± 0.4	6.0 (3)
	9-AC	499	6.3	1.5-9.3	3.0 ± 0.4	6.2 (3)
	NPPB	496	5.3	4.1-6.3	2.7 ± 0.3	1.9 (3)
	IAA-94	480	8.6	4.5-11.4	3.8 ± 0.5	4.9 (3)

<sup>a</sup> Lethal concentration required for 50% mortality of *M. incognita* second-stage larvae.

<sup>b</sup> 95% confidence intervals for LC<sub>50</sub>.

<sup>c</sup> Chi-square goodness-of-fit statistic and degrees of freedom.

Table 2.3. Activity of anion transporter blockers against *Meloidogyne incognita* eggs

Exposure time (h)	Compound	No. eggs (N)	<sup>a</sup> EC <sub>50</sub> (ppm)	95% CI <sup>b</sup>	Slope ± SEM	$\chi^2$ (df) <sup>c</sup>
120	DIDS	4004	29.1	20.8-48.4	0.9 ± 0.1	0.1 (3)
	9-AC <sup>d</sup>	4083	-	-	-	-
	NPPB	3935	46.5	29.8-100.1	0.8 ± 0.1	1.0 (3)
	IAA-94 <sup>d</sup>	3893	-	-	-	-
168	DIDS	4004	23.4	16.7-38.7	0.8 ± 0.1	1.7 (3)
	9-AC	4083	48.0	31.0-101.3	0.9 ± 0.1	0.9 (3)
	NPPB	3935	34.1	23.3-62.8	0.9 ± 0.1	2.6 (3)
	IAA-94	3893	42.3	27.5-88.7	0.8 ± 0.1	0.1 (3)

<sup>a</sup> Effective concentration required for 50% inhibition of *M. incognita* egg hatch.

<sup>b</sup> 95% confidence intervals for the EC<sub>50</sub>.

<sup>c</sup> Chi-square goodness-of-fit statistic and degrees of freedom.

<sup>d</sup> Egg hatch inhibition was <50% at the highest concentration tested (40 ppm).

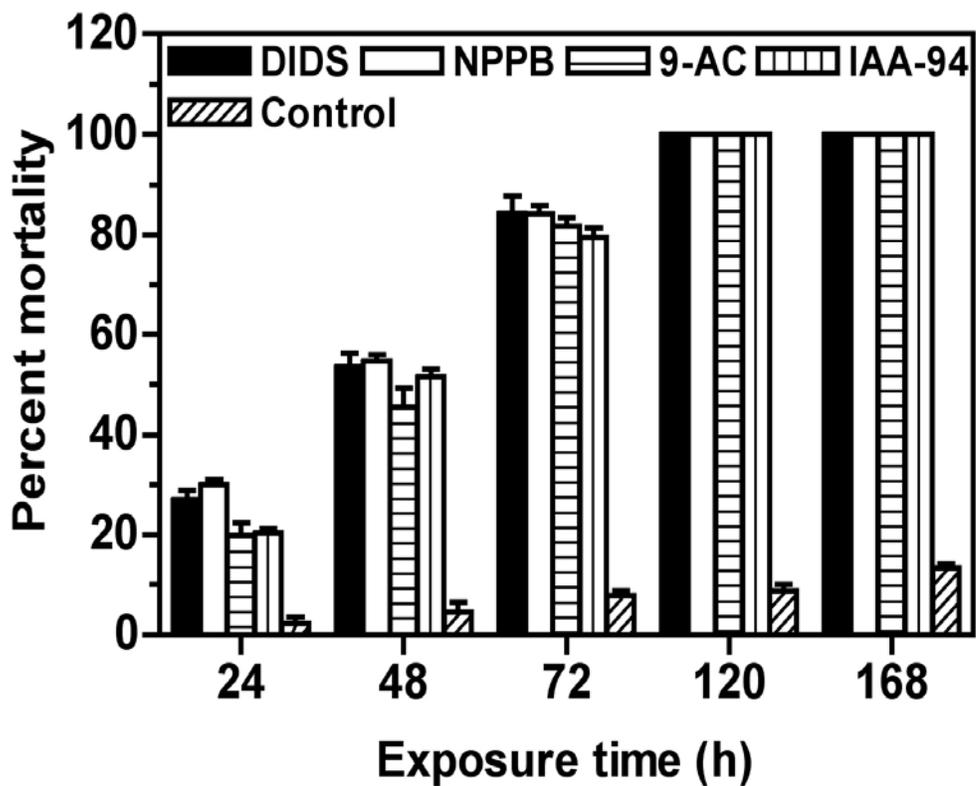


Fig. 2.1. Mortality of *Caenorhabditis elegans* adults exposed to 40 ppm of anion transporter blockers (solution pH adjusted to 7 with HEPES).

Bars are means with SEM ( $n = 2$ ). Lack of SEM for bars indicates 100% uniform lethality.

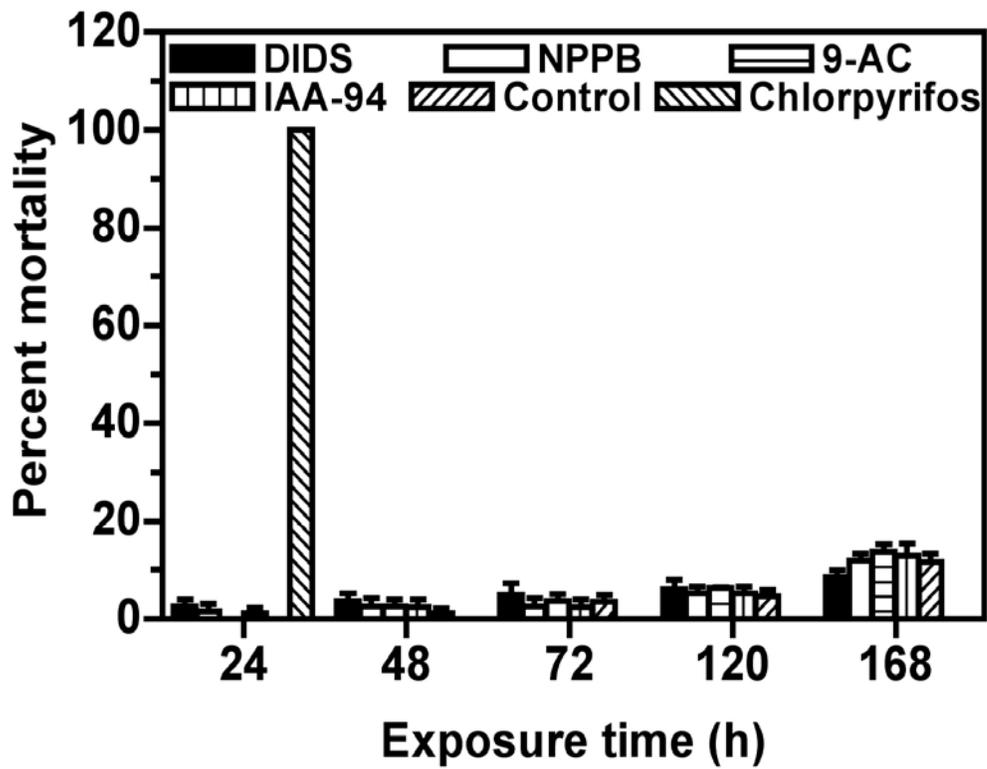


Fig. 2.2. Mortality of *Heterorhabditis bacteriophora* third-stage larvae exposed to anion transporter blockers and chlorpyrifos at 200 ppm.

Bars are means with SEM ( $n = 5$ ). Lack of SEM for chlorpyrifos indicates 100% uniform lethality.

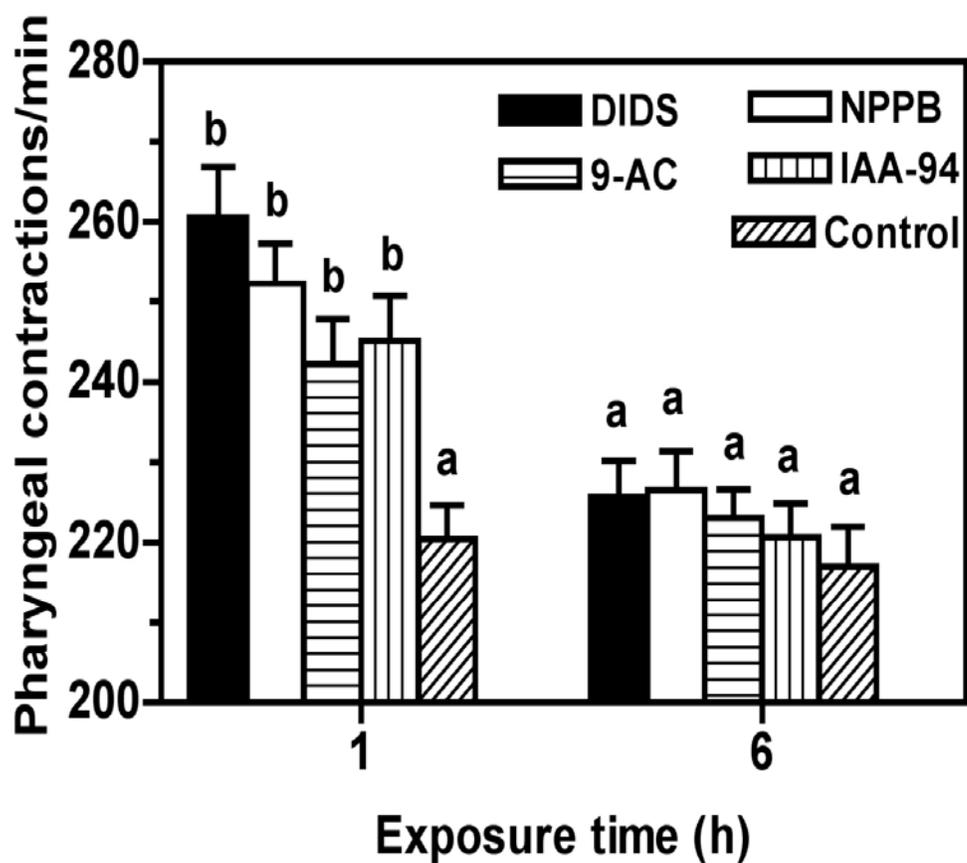


Fig. 2.3. Spontaneous contraction rates of *Caenorhabditis elegans* adult pharyngeal muscle after 1 and 6 h exposures to anion transporter blockers at 50 ppm.

Bars are means with SEM ( $n = 20$ ). Bars not labeled with same letter at a given exposure time are significantly different ( $P < 0.05$ ) from each other according to Tukey's test (SAS Institute 2005).

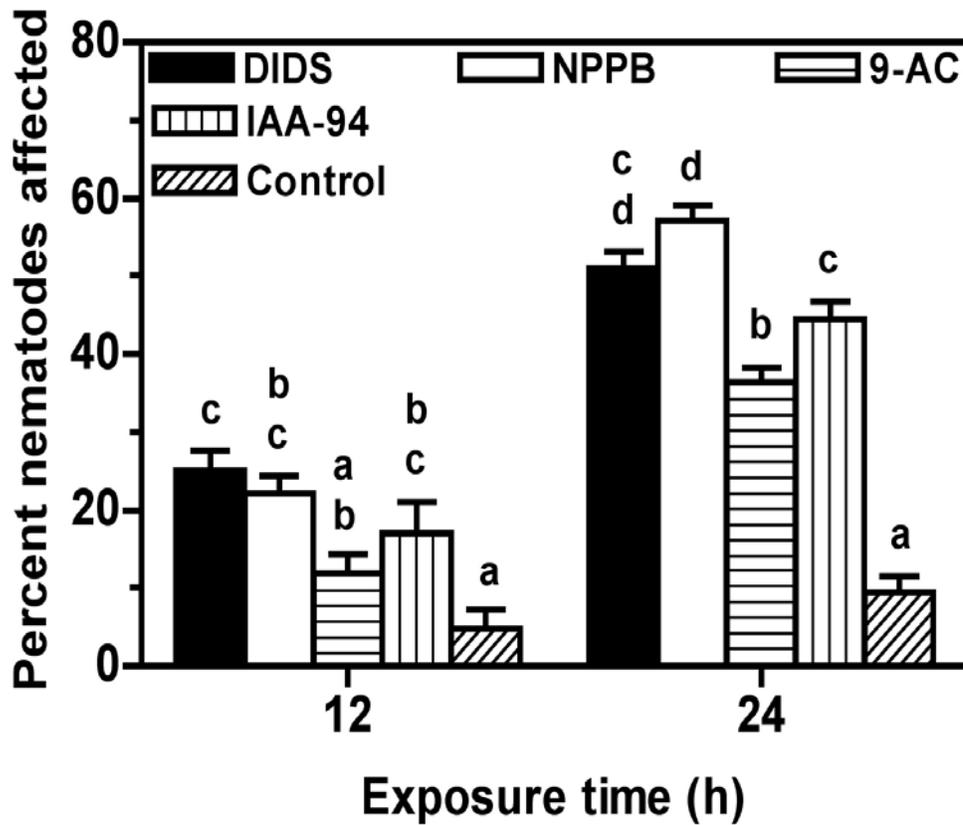


Fig. 2.4. Effect of anion transporter blockers at 50 ppm on locomotory ability of *Caenorhabditis elegans* adults. Bars are means with SEM ( $n = 3$ ). Bars not labeled with same letter at a given exposure time are significantly different from each other ( $P < 0.05$ ) according to Student Newman Keuls test (SAS Institute 2005).

## CHAPTER 3

### **Reduced Expression of Two Voltage-gated Chloride Channel Genes Affects Pharyngeal Contraction and Locomotion in *Caenorhabditis elegans* Maupas**

#### **ABSTRACT**

This study was conducted to confirm whether voltage-gated chloride channels-1 and -2 (CeCIC-1 and CeCIC-2) are target sites for anion transporter (AT) blockers in nematodes using the RNA interference technique in a model organism, *Caenorhabditis elegans* Maupas 1900. Exposure of *C. elegans* as fourth-stage larvae to long (4  $\mu\text{g}/\mu\text{L}$ ) or short (5  $\mu\text{g}/\mu\text{L}$ ) double-stranded RNA (dsRNA) of *ceclc-1* and/or *ceclc-2* for 24 h reduced the expression of *ceclc-1* and/or *ceclc-2* in F<sub>1</sub> progeny in a time-dependent manner. The maximum reduction in expression of *ceclc-1* and *ceclc-2* was seen in 48 h old worms and the gene expression was recovered to that of control worms within 72 h. F<sub>1</sub> progeny with reduced expression of *ceclc-2* showed significant phenotypic effects, such as increased pharyngeal contractions and decreased locomotion in a time-dependent manner, which were maximal in 48 h old worms. A similar reduction in expression of *ceclc-1* in F<sub>1</sub> progeny did not show any significant phenotypic effects irrespective of F<sub>1</sub> progeny age. An exception to this was, affected locomotion in 48 h old worms after exposure to short dsRNA of *ceclc-1*. F<sub>1</sub> progeny with reduced expression of both *ceclc-1* and *ceclc-2* showed increased pharyngeal contractions that was not significant from that obtained with reduced expression of *ceclc-2* alone. In contrast, F<sub>1</sub> progeny with reduced

expression of both *ceclc-1* and *ceclc-2* had a significantly higher percentage of nematodes with affected locomotion than were present with reduced expression of *ceclc-2* alone. A concentration-dependent reduction in expression of *ceclc-2* was observed after exposure to long dsRNA of *ceclc-2*. A strong negative correlation observed between long dsRNA concentration and *ceclc-2* expression level supported this finding. Further, a strong negative correlation existed between *ceclc-2* expression level and corresponding phenotypic effects. In conclusion, *ceclc-2* chloride channels (CeClC-2) are involved in pharyngeal muscle contractions and locomotion, while *ceclc-1* chloride channels do not have a role in pharyngeal muscle contractions, but could play an indirect role in nematode locomotion. These findings confirmed that the nematicidal activity of AT blockers may primarily come from the inhibition of CeClC-2.

## INTRODUCTION

Genome sequencing of *Caenorhabditis elegans* Maupas 1900 revealed the presence of six voltage-gated chloride channel (VGCC) genes viz., *ceclc-1 to -6* encoding for at least six VGCCs viz., CeCIC-1 to -6 (Schriever et al. 1999, Nehrke et al. 2000). The VGCCs are localized on either plasma or intracellular organelles membranes of body wall muscles, pharyngeal muscles, intestine lining cells, neurons, seam cells of the lateral body wall, and oocytes (Schriever et al. 1999, Petalcorin et al. 1999, Nehrke et al. 2000, Rutledge et al. 2001, Strange 2002) (see Table 1.1 for details). The proposed functions of VGCCs in *C. elegans* are stabilizing the membrane potential (CeCIC-1), chloride secretion/regulation (CeCIC-2), cell volume regulation (CeCIC-3), acidification of endocytotic vesicles (CeCIC-4 and CeCIC-5) (Franks et al. 2002, Fahlke 2001, Bloomquist 2003). Petalcorin et al. (1999) showed that mutations in the CeCIC-1 gene, *ceclc-1*, caused wider bodied worms in *C. elegans* highlighting the role of CeCIC-1 in maintaining body morphology by osmoregulation. The presence of VGCCs in nerve and muscle cells suggests they may be involved in nematode locomotion and pharyngeal pumping.

In a previous study, we tested the biological activity of anion transporter (AT) blockers, such as DIDS, NPPB, 9-AC, and IAA-94, on adults of *C. elegans* and second-stage larvae of a plant parasitic nematode (root-knot nematode), *Meloidogyne incognita* (Kofoid and White 1919) Chitwood 1949 (Boina et al. 2007). We found that AT blockers were paralytic/lethal to these nematodes and among the symptoms noticed in *C. elegans* adults were increased pharyngeal muscle contractions (Boina et al. 2007) and decreased locomotion (chapter 2). Since AT blockers are known for inhibiting anion

transporters (ATs) such as VGCC (Cabantchik and Greger 1992), we hypothesized that inhibition of CeCIC-2 by AT blockers increased pharyngeal muscle contractions and affected locomotion in nematodes leading to paralysis. Further, inhibition of CeCIC-1 by AT blockers may have disrupted osmoregulation, leading to the formation of wider bodied worms that might affect their mobility. To confirm the above hypotheses, we wanted to reduce the expression of *ceclc-1* and/or *ceclc-2* in *C. elegans* using a technique known as RNA interference (RNAi) and observing F<sub>1</sub> progeny of treated worms for phenotypes with expected symptoms.

RNAi is a technique used to specifically reduce the expression of a gene using double-stranded RNA (dsRNA) of which one strand is complementary to the sequence of a specific gene mRNA (Fire et al. 1998, Bhaketia et al. 2005). This post-transcriptional silencing was first discovered in the free-living nematode, *C. elegans* (Fire et al. 1998). Since then, this model organism has been used extensively to study the function of genes by way of reverse genetics using RNAi and to predict the function of orthologue genes in other eukaryotes in which no clearly defined procedures for RNAi exist or where it is difficult to perform such studies.

The objectives of this study were 1) to determine the effect of soaking *C. elegans* worms in dsRNA of *ceclc-1* and/or *ceclc-2* on pharyngeal muscle contractions and locomotion; 2) to determine the effect of these treatments on expression of *ceclc-1* and/or *ceclc-2*; 3) to determine the effect of dsRNA concentration on gene expression and its corresponding phenotypic effects; and 4) to determine effect of AT blockers on locomotion of *C. elegans* adults.

## MATERIALS AND METHODS

### Preparation of dsRNA

The GenBank (<http://www.ncbi.nlm.nih.gov>) accession numbers for the two VGCC genes used in this study are AF173170 (*ceclc-1*) and AF173171 (*ceclc-2*) (Schriever et al. 1999). Since using long and short forms of dsRNA produced mixed results in reducing the target gene expression in nematodes (Parrish et al. 2000, Caplen et al. 2001, Geldhof et al. 2006), we used both long and short dsRNA molecules of target genes in this study.

Total RNA was isolated from mixed stages of *C. elegans* cultured on nematode growth medium (NGM) agar plates seeded with *Escherichia coli* (Miguel 1895) Castellani and Chalmers 1919 strain OP50 using TRIzol reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. First-strand cDNA was synthesized using Superscript III reverse transcriptase (RT) (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. In brief, 1-5 µg of total RNA was reverse transcribed using gene-specific primers (*ceclc-1R*: 5'-TCGGCTCGTGTTGATTAGTC-3', *ceclc-2R*: 5'-GTCGCGTGAAAAGTGCCTGTA-3') by incubating them at 55 °C for 45 min and then inactivating the enzyme at 70 °C for 15 min. A standard PCR was performed using first-strand cDNAs as templates to amplify fragments of 1012 bp (from 1319 to 2330 bp) and 678 bp (from 1453 to 2130) for *ceclc-1* and *ceclc-2*, respectively, using gene-specific primers with T7 promoter sequence (underlined) attached to the 5' ends (*ceclc-1T7F*: 5'-TAATACGACTCACTATAGGGCGTGAAACCCTCGTCGATT-3', *ceclc-1T7R*: 5'-TAATACGACTCACTATAGGGTCGGCTCGTGTTGATTAGTC-3'; *ceclc-2T7F*: 5'-TAATACGACTCACTATAGGGGCTGTCACTCATACTCTTTCGGTTTC-3', *ceclc-*

2T7R: 5'-TAATACGACTCACTATAGGGGTCGCGTGAAAACTGCCTGTA-3') and platinum *Taq* DNA polymerase (Invitrogen, Carlsbad, CA) according to manufacturer's instructions. The PCR conditions were 94 °C for 2 min then 37 cycles of 94 °C for 15 s, 70 °C for 30 s, 68 °C for 1.5 min and a final extension step of 68 °C for 7 min. PCR products were analyzed for their size, purity and integrity by running on 1% agarose gel. One microgram of PCR product with T7 promoter sequence flanking the 5' ends was used as a template to prepare sense and antisense strands of dsRNA in a single tube using a MEGAscript T7 in vitro transcription kit (Ambion, Inc., Austin, TX) according to the manufacturer's instructions. After reaction, template DNA was digested by adding 1 µL of TURBO DNase and incubating at 37 °C for 15 min. The sense and antisense strands were annealed by heating at 70 °C for 5 min and cooled slowly to room temperature. dsRNA was purified using spin columns (Ambion, Inc., Austin, TX) and phenol/chloroform extraction followed by isopropanol precipitation and resuspended in 50 µL of diethyl pyrocarbonate (DEPC) treated water. The amount of dsRNA was quantified by spectrophotometry and a sample was run on 1% native agarose gel for analysis of purity and integrity.

Alternatively, two commercially synthesized short-interfering dsRNA (siRNA) molecules of 21 bp for each target gene were purchased from Invitrogen (Carlsbad, CA; <http://www.invitrogen.com>) after designing them using their program, BLOCK-iT RNAi designer. The siRNA sequences used were *ceclc-1a*: 5'-CCTTCGCTCCTACCCATTTTT-3' targeting 1969 to 1987 bp and *ceclc-1b*: 5'-GCACGGCATCGGAAATCTTTT-3' targeting 2106 to 2124 bp of *ceclc-1* gene. Similarly, siRNA were *ceclc-2a*: 5'-CCATCCGATCAAGTAGAATTT-3' targeting 2016

to 2034 bp and *ceclc-2b*: 5'-CCTTCATCAGCAGATCTTTTT-3' targeting 2130 to 2148 bp of *ceclc-2* gene.

### **Soaking Worms in dsRNA Solution**

The long forms of dsRNA (long dsRNA) were diluted to a concentration of 8  $\mu\text{g}/\mu\text{L}$  with DEPC-treated water. A stock concentration of 20  $\mu\text{g}/\mu\text{L}$  was prepared for each short form of dsRNA (short dsRNA) in DEPC-treated water. A 20  $\mu\text{L}$  total volume containing long or short dsRNAs targeting *ceclc-1* and/or *ceclc-2* was placed in a 0.2 mL microfuge tube (see Table 3.1 for details). Microfuge tubes with equal volume of DEPC-treated water alone served as control for each treatment. Twenty *C. elegans* fourth-stage larvae of 2-day old (N2 Bristol wild strain) were added to each tube. Tubes were capped and placed in an incubator set at 20 °C for 24 h. After incubation, worms were transferred to NGM agar plates freshly seeded with *E. coli*. F<sub>1</sub> progeny were observed daily for phenotypes. Effect of dsRNA concentration on *ceclc-2* expression and in turn on phenotypic effects (pharyngeal muscle contractions and locomotion) was determined by soaking worms for 24 h in a lower (2  $\mu\text{g}/\mu\text{L}$ ) and a higher (8  $\mu\text{g}/\mu\text{L}$ ) concentration of long dsRNA of *ceclc-2* used in the first experiment (4  $\mu\text{g}/\mu\text{L}$ ) as described above.

### **Effect of dsRNA on Pharyngeal Muscle Contraction and Locomotion**

For measuring phenotypic effects, 15 nematodes aged 24, 48, 72 and 96 h were picked from F<sub>1</sub> progeny of treated worms in each treatment and were placed on a microscope slide with one or two drops of *E. coli* culture as a stimulus. A glass cover slip was placed on the slide with nematodes and pharyngeal contractions (pumps/min) were recorded for 1 min under a stereomicroscope (Olympus BX40).

Similarly, effect on locomotion was assessed using a radial migration assay

(Salcini et al. 2001). For locomotion assay, 10 to 19 nematodes aged 24, 48, 72 and 96 h from F<sub>1</sub> progeny of treated worms were transferred to the center of a 30-mm (inner diameter) ring made of *E. coli* lawn in the center of a NGM agar plate (90-mm diameter). One hour after nematode release, nematodes that reached the bacterial ring were counted and subtracted from the total number of nematodes released, giving the number of nematodes with affected locomotion. Experiments were replicated twice.

### **RT-PCR Quantification of Gene Expression**

Total RNA was extracted from 48 and 72 h old F<sub>1</sub> progeny of treated worms (corresponding to the maximal and minimal RNAi effect) using TRIzol reagent (Invitrogen, Carlsbad, CA). Total RNA was quantified by spectrophotometry and two or three aliquots of total RNA (1 µg each) from each treatment were reverse primed with oligo dT(20) primers using superscript III RT (Invitrogen, Carlsbad, CA). A standard PCR was performed on 2 µL cDNA using internal gene-specific forward and reverse primers (used in PCR for dsRNA preparation but without T7 promoter sequence) and platinum *Taq* DNA polymerase (Invitrogen, Carlsbad, CA), according to the manufacturer's instructions. A sample of 4 µL from each treatment was taken after 32 cycles of PCR and analyzed by running on 1% native agarose gel followed by ethidium bromide staining. A house keeping gene, isocitrate dehydrogenase (IDH), was used as loading control by amplifying a 236 bp fragment with 5'-GCTTTGAAGGGAAACATTG-3' and 5'-GATTCGACAACACGAGGAT-3' as forward and reverse primers, respectively (Zhai et al. 2006).

To determine mRNA transcript levels (gene expression) in F<sub>1</sub> progeny of treated and control worms, the DNA content in the RT-PCR samples run on 1% agarose gels was

determined based on relative intensities of gel picture bands using Kodak EDAS 290 image analysis system (New Haven, CT) and expressed as percent control.

### **Statistical Analyses**

Mean ( $\pm$ SEM;  $n = 15$ ) pharyngeal contractions/min and mean ( $\pm$ SEM;  $n = 2$ ) percent nematodes with decreased locomotion in F<sub>1</sub> progeny were calculated for each treatment in dsRNA soaking experiments. Mean ( $\pm$ SEM;  $n = 2-3$ ) mRNA transcript levels in F<sub>1</sub> progeny of treated worms were also calculated. Data were subjected to one-way ANOVA using PROC GLM program (SAS Institute 2005) for statistical significance between treatment means and means were separated using Student Newman Keuls test at  $\alpha=0.05$  level of significance. The relationship between dsRNA concentration and mRNA transcript level in F<sub>1</sub> progeny of treated worms for *ceclc-2* was determined by simple linear regression (GraphPad prism software) and the relationship between *ceclc-2* mRNA transcript level and phenotypic effects was determined by correlation analysis (SAS Institute 2005).

## **RESULTS**

### **Effect of dsRNA Solution on Pharyngeal Muscle Contractions**

A 24 h exposure to long dsRNA of *ceclc-1* had no effect on pharyngeal contractions in 24, 48, and 72 h old F<sub>1</sub> progeny of treated worms (Fig. 3.1a). In contrast, exposure to long dsRNA of *ceclc-2* significantly increased pharyngeal contractions in 24 and 48 h (14% over control) old worms (Fig. 3.1a). Further, exposure to dsRNA of *ceclc-1* and *ceclc-2* together, gave the same effect as *ceclc-2* alone in 24 (increase by 12%) and 48 h (increase by 13%) old worms. After 72 h, all effects had reversed

themselves to control levels (Fig. 3.1a).

Exposure to short dsRNA of either of the genes alone or together had no significant effect on pharyngeal contractions in 24 h old worms (Fig. 3.1b). Similarly, exposure to short dsRNA of *ceclc-1* had no effect on pharyngeal contractions in 48 h old worms. In contrast, exposure to short dsRNA of *ceclc-2* alone or together with *ceclc-1* had equal and significant effect on pharyngeal contractions (increase by 14%) in 48 h old worms. Similar to long dsRNA, exposure to short dsRNA of either of the genes alone or together had no effect on pharyngeal contractions in 72 h old worms (Fig. 3.1b)

Exposure to long dsRNA of *ceclc-2* at different concentrations increased pharyngeal contractions in a concentration-dependent manner in 24 and 48 h old F<sub>1</sub> progeny. Exposure to 2, 4, and 8 µg/µL of long dsRNA increased pharyngeal contractions by 7, 11, and 14%, respectively, in 24 h old worms and the increase was significant in 4 and 8 µg/µL (Fig. 3.1c). All the concentrations of long dsRNA significantly increased pharyngeal contractions (12 to 21%) in 48 h old worms and the increase in 8 µg/µL was significantly higher than in 2 and 4 µg/µL (Fig. 3.1c). None of the concentrations of long dsRNA, had any effect on pharyngeal contractions in 72 h old worms (Fig. 3.1c).

### **Effect of dsRNA Solution on Locomotion**

Similar to its null effect on pharyngeal contractions, a 24 h exposure to long dsRNA of *ceclc-1* did not have any effect on locomotory ability of 24 or 48 h old F<sub>1</sub> progeny of treated worms (Fig. 3.2a). In contrast, exposure to long dsRNA of *ceclc-2* decreased locomotion in a significantly higher percentage of nematodes in both 24 and 48 h old worms (Fig. 3.2a). Further, exposure to long dsRNA of *ceclc-1* and *ceclc-2*

together had the same effect on locomotion as *ceclc-2* alone in 24 h old worms, but a significantly higher effect in 48 h old worms. Exposure to long dsRNA of either of the genes alone or together had no effect on locomotion in 72 h old worms (Fig. 3.2a).

Exposure to short dsRNA of *ceclc-1* or *ceclc-2* alone did not have any effect on locomotion in 24 h old worms, while exposure to short dsRNA of *ceclc-1* and *ceclc-2* together decreased locomotion in significantly higher percent nematodes (Fig. 3.2b). Exposure to short dsRNA of either of the genes alone or together had a significant effect on locomotory ability of 48 h old worms with *ceclc-1+2* > *ceclc-2* > *ceclc-1*. Exposure to short dsRNA of either of the genes alone or together did not affect locomotion in 72 h old worms (Fig. 3.2b).

Similar to the effects on pharyngeal contractions, exposure to different concentrations of long dsRNA of *ceclc-2* decreased locomotory ability in F<sub>1</sub> progeny of treated worms in a concentration-dependent manner (Fig. 3.2c). Exposure to 4 and 8 µg/µL of long dsRNA significantly decreased locomotion in 24 h old worms. Similar to 24 h old worms, exposure to 4 and 8 µg/µL significantly decreased locomotion in 48 h old worms, but this decrease was significantly greater in 8 µg/µL than in 4 µg/µL (Fig. 3.2c). Exposure to any of the concentrations did not decrease locomotion in a significantly higher percentage of 72 h old worms (Fig. 3.2c).

### **RT-PCR Quantification of Gene Expression**

Figures 3.3 and 3.4 show the gel pictures of RT-PCR products from 48 and 72 h old F<sub>1</sub> progeny of worms exposed to dsRNA or DEPC-treated water (control). RT-PCR products from F<sub>1</sub> progeny of treated and control worms showed bands of the expected size (Figs. 3.3 and 3.4). It was clear that exposure of *C. elegans* fourth-stage larvae to

long or short dsRNA targeting *ceclc-1* and/or *ceclc-2* for 24 h degraded the respective mRNA transcripts.

Densitometry analysis of gel pictures (Fig. 3.3) revealed that exposure to long dsRNA of *ceclc-1* reduced its expression to 34% of control in 48 h old worms, while exposure to *ceclc-2* reduced its expression to 51% of control (Fig. 3.5a). Exposure to long dsRNA of *ceclc-1* and *ceclc-2* together, reduced their expression to 32 and 51% of control, respectively. In 72 h old worms, expression of *ceclc-1* was 75% of control, while expression of *ceclc-2* was 96% of control in worms exposed to long dsRNA of *ceclc-1* and *ceclc-2* separately (Fig. 3.5a). Expression levels of *ceclc-1* and *ceclc-2* were 68 and 83% of control, respectively, in worms exposed to long dsRNA of *ceclc-1* and *ceclc-2* together.

Exposure of *C. elegans* L4 stage worms to short dsRNA of *ceclc-1* (Fig. 3.3) reduced its expression to 31% of control, while exposure to short dsRNA of *ceclc-2* reduced its expression to 52% in 48 h old worms (Fig. 3.5b). Expression levels of *ceclc-1* and *ceclc-2* were 26 and 52% of control, respectively, in worms exposed to short dsRNA of *ceclc-1* and *ceclc-2* together. In 72 h old worms, expression level of *ceclc-1* was 87% of control in worms exposed to short dsRNA of *ceclc-1*, while it was 96% of control for *ceclc-2* in worms exposed to short dsRNA of *ceclc-2*. Expression levels of *ceclc-1* and *ceclc-2* were 96 and 89% of control, respectively, in worms exposed to short dsRNA of *ceclc-1* and *ceclc-2* together (Fig. 3.5b).

The densitometry analysis of gel pictures (Fig. 3.4) showed that exposure to long dsRNA of *ceclc-2* reduced its expression in a concentration-dependent manner (Fig. 3.5c). Exposure to long dsRNA at 2, 4, and 8  $\mu\text{g}/\mu\text{L}$ , reduced expression levels of *ceclc-*

2 to 82, 50, and 10% of control, respectively, in 48 h old F<sub>1</sub> progeny, while expression levels were only reduced to 91, 86, and 72% of control in 72 h old worms (Fig. 3.5c). A strong relationship was observed between long dsRNA concentration and *ceclc-2* expression level in 48 ( $R^2 = 0.98$ ;  $P = 0.08$ ) and 72 h ( $R^2 = 0.98$ ;  $P = 0.08$ ) old worms (Fig. 3.6). However, from regression equations it can be inferred that every 1  $\mu\text{g}/\mu\text{L}$  increase in dsRNA concentration leads to a 12% reduction in *ceclc-2* mRNA transcript level in 48 h old worms, while only 3% reduction in 72 h old worms. Further, a highly strong negative correlation existed between *ceclc-2* expression level and locomotion ( $r = -0.99$ ;  $P = 0.02$ ) or pharyngeal contractions ( $r = -0.96$ ;  $P = 0.16$ ).

## DISCUSSION

In this study, exposure of *C. elegans* as fourth-stage larvae to either long or short dsRNA targeting VGCC genes, *ceclc-1* and *ceclc-2*, for 24 h decreased the expression of target genes in a time dependent manner, which was maximal in 48 h old worms. The reduction in expression of genes for a given age of F<sub>1</sub> progeny was strongly dependent on dsRNA concentration, which was evident from concentration-regression analysis for *ceclc-2*. Further, this reduction in expression of *ceclc-2* for each dsRNA unit increase was very high in 48 h old worms compared to that in 72 h old worms. The reduction in expression of target genes but not in a house-keeping gene, IDH (non-target gene), indicates that the reduction in mRNA level of genes was specific. The magnitude of reduction in expression for a given target gene was similar for both forms of dsRNA used, indicating that the same RNAi effect can be obtained by using either long or short forms of dsRNA in this soaking method. The higher degree of phenotypic effect

observed in 48 h old F<sub>1</sub> progeny of treated worms gradually disappeared in 72 h old worms and corresponded with gene expression observed, especially, for *ceclc-2*. This finding suggests that the observed phenotypic effects were due, mainly, to specific degradation of *ceclc-2* mRNA. The existence of a highly strong correlation between *ceclc-2* expression and phenotypic effects in 48 h old worms quantified this relationship. This result indicates that an increase in pharyngeal contractions/min or a decrease in locomotion of nematodes was directly related the expression of *ceclc-2*, i.e. fewer number of CeClC-2 channels expressed in 48 h old worms, while restoring their number to near normal level in 72 h old worms led disappearance of phenotypic effects. The y-axis intercept value of about 100 observed in regression analyses corresponds to the maximum gene expression indicating the accuracy of prediction.

Expression of *ceclc-2* in muscles of pharynx and body wall (Schriever et al. 1999, Nehrke et al. 2000) suggests a possible role in pharyngeal muscle contractions and locomotion, respectively, and reducing its expression in respective tissues could have led to the observed phenotypic effects. Franks et al. (2002) reported that decreasing extracellular Cl<sup>-</sup> concentration of pharyngeal muscle cells caused a transient excitation and burst of action potentials. This experimental approach is analogous to preventing influx of Cl<sup>-</sup> by blocking membrane Cl<sup>-</sup> channels. No significant phenotypic effects observed even after a nearly 70% reduction in expression of *ceclc-1* in 48 h old F<sub>1</sub> progeny suggested no role for CeClC-1 channels in pharyngeal contractions and locomotion. Further, no greater effect on pharyngeal contractions was seen with reduced expression of *ceclc-1* and *ceclc-2* together than with *ceclc-2* alone. However, a significantly higher percentage of F<sub>1</sub> progeny worms with affected locomotion were

observed with reduced expression of *ceclc-1* and *ceclc-2* together, than with *ceclc-2* alone. It can be inferred from this finding that though reduction in expression of *ceclc-1* alone had no significant effect on nematode locomotion, when combined with that of *ceclc-2*, it enhanced impairment of locomotory ability in nematodes. Expression of *ceclc-1* in seam cells of body wall (Schriever et al. 1999, Nehrke et al. 2000) but not in pharyngeal muscle cells could explain, partly, the above results.

Similar to electrophysiological experiments on pharyngeal muscle (Franks et al. (2002), reducing the expression of VGCC genes should have a physiological impact similar to the inhibition of Cl<sup>-</sup> entry through VGCCs by AT blockers. Reducing expression of *ceclc-1* and *ceclc-2* decreases the number of corresponding channels in cell membranes of pharynx and body wall muscles and seam cells of body wall, respectively. Decrease in CeCIC-2 number may cause enhanced excitation of muscle cells in body wall that compromises locomotion in nematodes. Electrophysiological studies of nematode muscle treated with AT blockers are required to confirm this hypothesis. Similarly, excitation of pharyngeal muscle cells due to decreased CeCIC-2 number manifested itself as increased pharyngeal contractions in F<sub>1</sub> progeny of RNAi treated worms. Likewise, a decrease in CeCIC-1 number might have disrupted the osmoregulation process in seam cells of lateral body wall causing wider bodied worms, as described by Petalcorin et al. (1999). Worms with increased body diameter may initiate fewer sinusoidal movement/min and/or experience a reduction in amplitude, leading to further impairment of locomotory ability in nematodes already impacted by reduced expression of *ceclc-2*. However, we did not measure the width of affected nematodes or analyze sinusoidal movements in our experiments. Prolongation of the above effects could eventually lead

to the paralysis in worms but the gene expression levels went up in 72 h old worms suggesting fading of RNAi and recovery of worms.

Results from a previous study indicate that incubation of *C. elegans* adults in 50 ppm of AT blocker solution for 1 h significantly increased pharyngeal contractions (Boina et al. 2007), while incubation for 12 or 24 h decreased locomotion in exposed worms (Chapter 2). Since AT blockers are known for inhibiting VGCCs and toxic symptoms of AT blockers in *C. elegans* are similar to the phenotypic effects of reduced expression of *ceclc-1* and *ceclc-2*, it is logical to conclude that CeCIC-1 and especially CeCIC-2 are indeed the target sites for AT blockers in *C. elegans*.

In conclusion, we have shown that exposure of *C. elegans* as fourth-stage larvae to either long/short dsRNA of *ceclc-1* and *ceclc-2* either alone or together reduced the expression of *ceclc-1* and *ceclc-2* to about 30 and 50% of control, respectively, in a time dependent manner, in F<sub>1</sub> progeny. This reduction in expression of *ceclc-2* was consistent with observed significant phenotypic effects, while reduction in *ceclc-1* expression did not show any phenotypic effects. Nevertheless, when expressions of both *ceclc-1* and *ceclc-2* were reduced, locomotion was decreased in a significantly higher percentage of worms. These results confirmed that CeCIC-2 channels are major target sites for AT blockers in *C. elegans* and probably in other non-entomopathogenic nematodes.

#### LITERATURE CITED

Bhaketia, M., W. L. Charlton, P. E. Urwin, M. J. McPherson, and H. J. Atkinson. 2005.

RNA interference and plant parasitic nematodes. *Trends Plant Sci* 18: 362-367.

- Bloomquist, J. R. 2003. Chloride channels as tools for developing selective insecticides. *Arch Insect Biochem Physiol* 54: 145-156.
- Boina, D., E. E. Lewis, and J. R. Bloomquist. 2007. Nematicidal activity of anion transport blockers against *Meloidogyne incognita*, *Caenorhabditis elegans*, and *Heterorhabditis bacteriophora*. *Pest Manag Sci* (In press).
- Cabantchik, Z. I., and R. Greger. 1992. Chemical probes for anion transporters of mammalian cell membranes. *Am J Physiol* 262: C803-C827.
- Caplen, N. J., S. Parrish, F. Imani, A. Fire, and R. A. Morgan. 2001. Specific inhibition of gene expression by small double-stranded RNAs in invertebrate and vertebrate systems. *Proc Natl Acad Sci USA* 98:9742-9747.
- Fire, A., S. Xu, M. K. Montgomery, S. A. Kostas, S. E. Driver, and C. C. Mello. 1998. Potent and specific genetic interference by double-stranded RNA in *Caenorhabditis elegans*. *Nature* 391: 806-811.
- Fahlke, C. 2001. Ion permeation and selectivity in ClC-type chloride channels. *Am J Physiol Renal Physiol* 280: F748-F757.
- Franks, C. J., D. Pemberton, I. Vinogradova, A. Cook, R. J. Walker, and L. Holde-Dye. 2002. Ionic basis of the resting membrane potential and action potential in the pharyngeal muscle of *Caenorhabditis elegans*. *J Neurophysiol* 87: 954-961.
- Geldhof, P., L. Murray, A. Couthier, J. S. Gilleard, G. McLauchlan, D. P. Knox, and C. Britton. 2006. Testing the efficacy of RNA interference in *Haemonchus contortus*. *Int J Parasitol* 36: 801-810.
- Nehrke, K., T. Begenisich, J. Pilato, and J. E. Melvin. 2000. Model organism: new insights into ion channel and transporter function. *Caenorhabditis elegans* ClC-

- type chloride channels: novel variants and functional expression. *Am J Physiol Cell Physiol* 279: C2052-C2066.
- Parrish, S., J. Fleenor, S. Xu, C. Mello, and A. Fire. 2000. Functional anatomy of a dsRNA trigger: differential requirement for the two trigger strands in RNA interference. *Mol Cell* 6: 1077-1087.
- Petalcorin, M. I. R., T. Oka, M. Koga, K. Ogura, Y. Wada, Y. Ohshima, and M. Futai. 1999. Disruption of *clh-1*, a chloride channel gene, results in a wider body of *Caenorhabditis elegans*. *J Mol Biol* 294: 347-355.
- Rutledge, E., L. Bianchi, M. Christensen, C. Boehmer, R. Morrison, A. Broslat, A. M. Beld, A. L. George, Jr., D. Greenstein, and K. Strange. 2001. CLH-3, a ClC-2 anion channel ortholog activated during meiotic maturation in *C. elegans* oocytes. *Current Biol* 11: 161-170.
- Salcini, A. E., M. A. Hilliard, A. Croce, S. Arbucci, P. Luzzi, C. Tacchetti, L. Daniell, P. De Camilli, P. G. Pelicci, P. P. Di Fiore, and P. Bazzicalupo. 2001. The Eps 15 *C. elegans* homologue EHS-1 is implicated in synaptic vesicle recycling. *Nature Cell Biol* 3: 755-760.
- SAS Institute. 2005. The SAS system version 9.1 for Windows. SAS Institute, Cary, NC.
- Schriever, A. M., T. Friedrich, M. Pusch, and T. J. Jentsch. 1999. CLC chloride channels in *Caenorhabditis elegans*. *J Biol Chem* 274: 34238-34244.
- Strange, K. 2002. Of mice and worms: novel insights into ClC-2 anion channel physiology. *News Physiol Sci* 17: 11-16.
- Zhai, R., G. Meng, Y. Zhao, B. Liu, G. Zhang, and X. Zheng. 2006. A novel nuclear-

localized protein with special adenylate kinase properties from *Caenorhabditis elegans*. *FEBS Lett* 580: 3811-3817.

Table 3.1. Concentrations of double-stranded RNA used for targeting *ceclc-1* and *ceclc-2* in *Caenorhabditis elegans* in RNA interference experiments

dsRNA	Volume ( $\mu\text{L}$ ) of RNAi treatment (T) solutions											
	T <sub>1</sub> <sup>c</sup>	T <sub>2</sub>	T <sub>3</sub>	T <sub>4</sub>	T <sub>5</sub> <sup>d</sup>	T <sub>6</sub>	T <sub>7</sub>	T <sub>8</sub>	T <sub>9</sub> <sup>e</sup>	T <sub>10</sub>	T <sub>11</sub>	T <sub>12</sub>
<i>ceclc-1</i> <sup>a</sup>	-	10	-	10	-	-	-	-	-	-	-	-
<i>ceclc-2</i> <sup>a</sup>	-	-	10	10	-	5	10	20	-	-	-	-
<i>ceclc-1a</i> <sup>b</sup>	-	-	-	-	-	-	-	-	-	5	-	5
<i>ceclc-1b</i> <sup>b</sup>	-	-	-	-	-	-	-	-	-	5	-	5
<i>ceclc-2a</i> <sup>b</sup>	-	-	-	-	-	-	-	-	-	-	5	5
<i>ceclc-2b</i> <sup>b</sup>	-	-	-	-	-	-	-	-	-	-	5	5
DEPC-water	20	10	10	0	20	15	10	0	20	10	10	0
Final vol ( $\mu\text{L}$ )	20	20	20	20	20	20	20	20	20	20	20	20
Conc. of dsRNA ( $\mu\text{g}/\mu\text{L}$ )	0	4	4	4	0	2	4	8	0	5	5	5

<sup>a</sup> Long dsRNA and stock solution used was 8  $\mu\text{g}/\mu\text{L}$ .

<sup>b</sup> Short dsRNA and stock solution used was 20  $\mu\text{g}/\mu\text{L}$ .

<sup>c</sup> Treatments, T<sub>2</sub>, T<sub>3</sub>, and T<sub>4</sub> each had a separate control (T<sub>1</sub>).

<sup>d</sup> A common control was used for treatments, T<sub>6</sub>, T<sub>7</sub>, and T<sub>8</sub>.

<sup>e</sup> Treatments, T<sub>10</sub>, T<sub>11</sub>, and T<sub>12</sub> each had a separate control (T<sub>9</sub>).

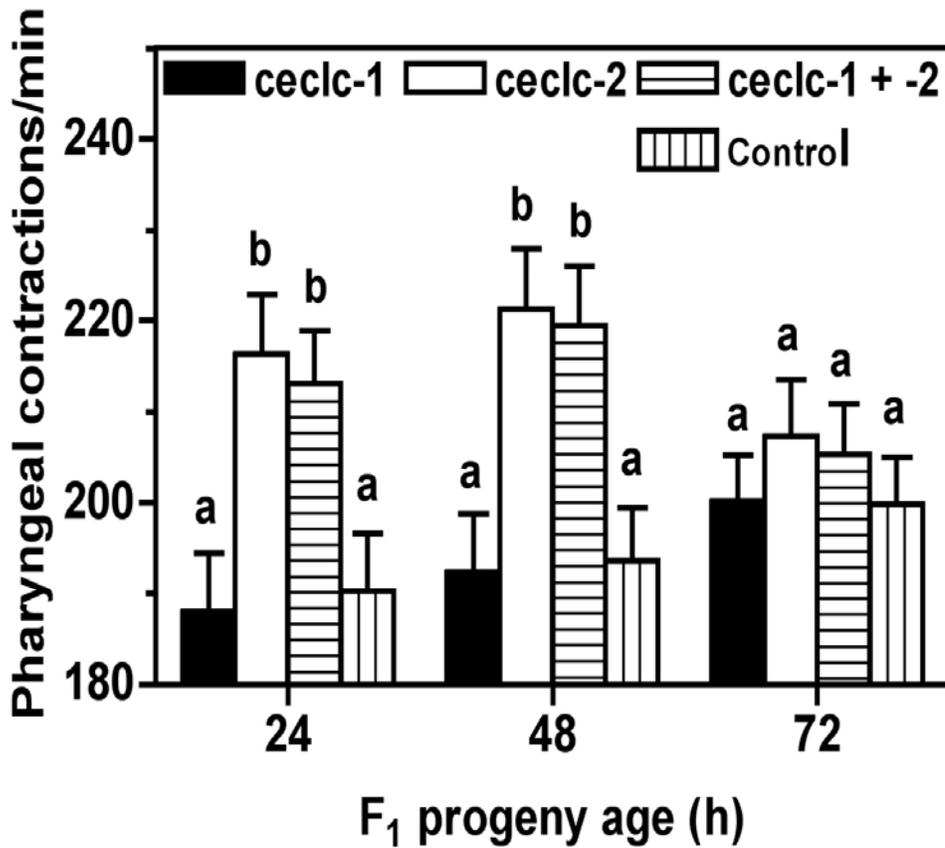


Fig. 3.1a. Spontaneous pharyngeal muscle contractions rates of F<sub>1</sub> progeny of *Caenorhabditis elegans* treated as fourth-stage larvae with long double-stranded RNA of *ceclc-1* and/or *ceclc-2* for 24 h. Bars are means with SEM ( $n = 15$ ). Bars not labeled with same letter at a given age are significantly different from each other ( $P < 0.05$ ) according to Student Newman Keuls test (SAS Institute 2005).

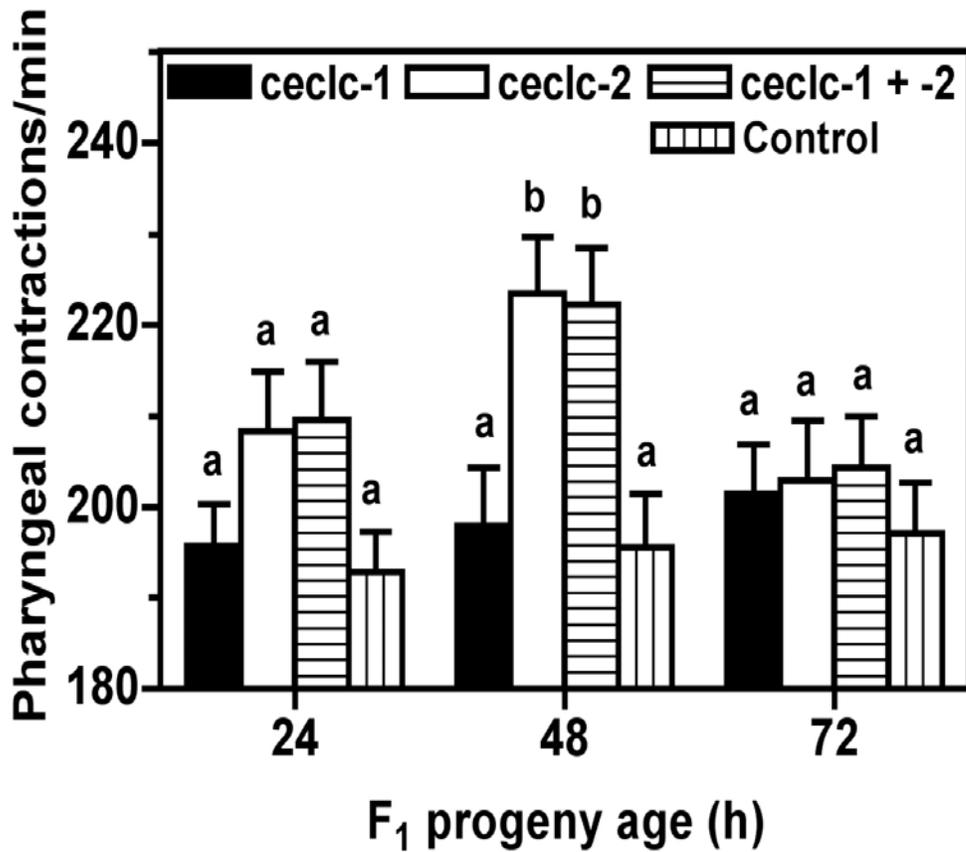


Fig. 3.1b. Spontaneous pharyngeal muscle contraction rates of F<sub>1</sub> progeny of *Caenorhabditis elegans* treated as fourth-stage larvae with short double-stranded RNA of *ceclc-1* and/or *ceclc-2* for 24 h. Bars are means with SEM ( $n = 15$ ). Bars not labeled with same letter at a given age are significantly different from each other ( $P < 0.05$ ) according to Student Newman Keuls test (SAS Institute 2005).

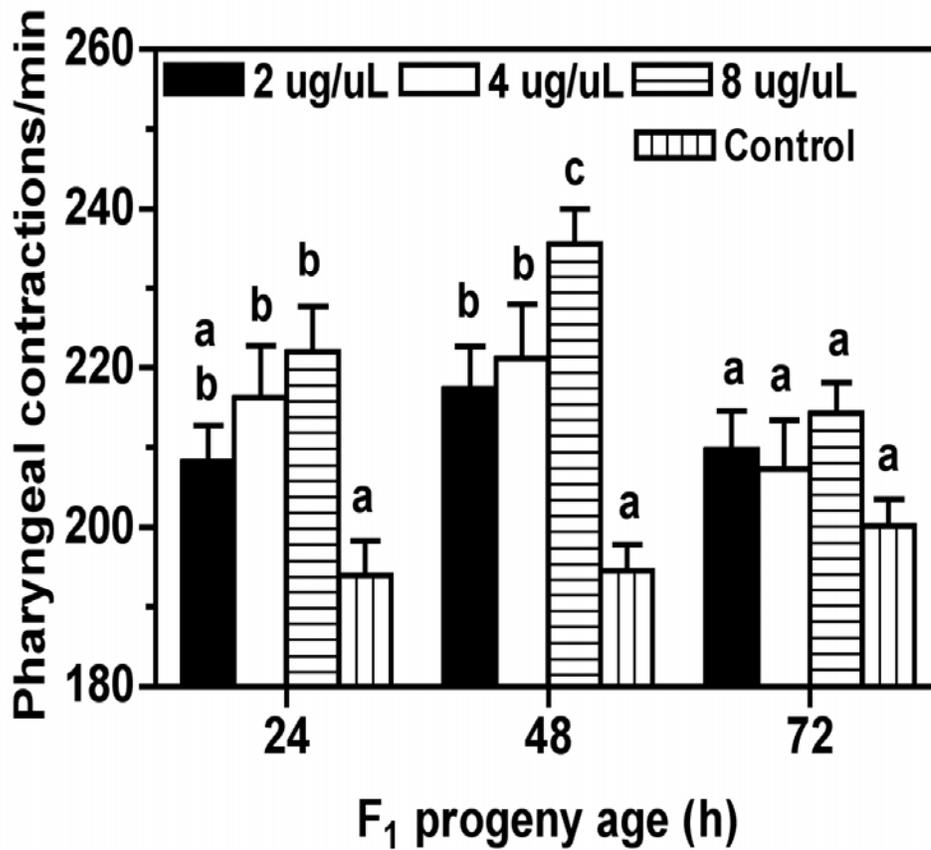


Fig. 3.1c. Concentration-dependent effect on spontaneous pharyngeal muscle contraction rates of F<sub>1</sub> progeny of *Caenorhabditis elegans* treated as fourth-stage larvae with long double-stranded RNA of *ceclc-2* for 24 h. Bars are means with SEM ( $n = 15$ ). Bars not labeled with same letter at a given age are significantly different from each other ( $P < 0.05$ ) according to Student Newman Keuls test (SAS Institute 2005).

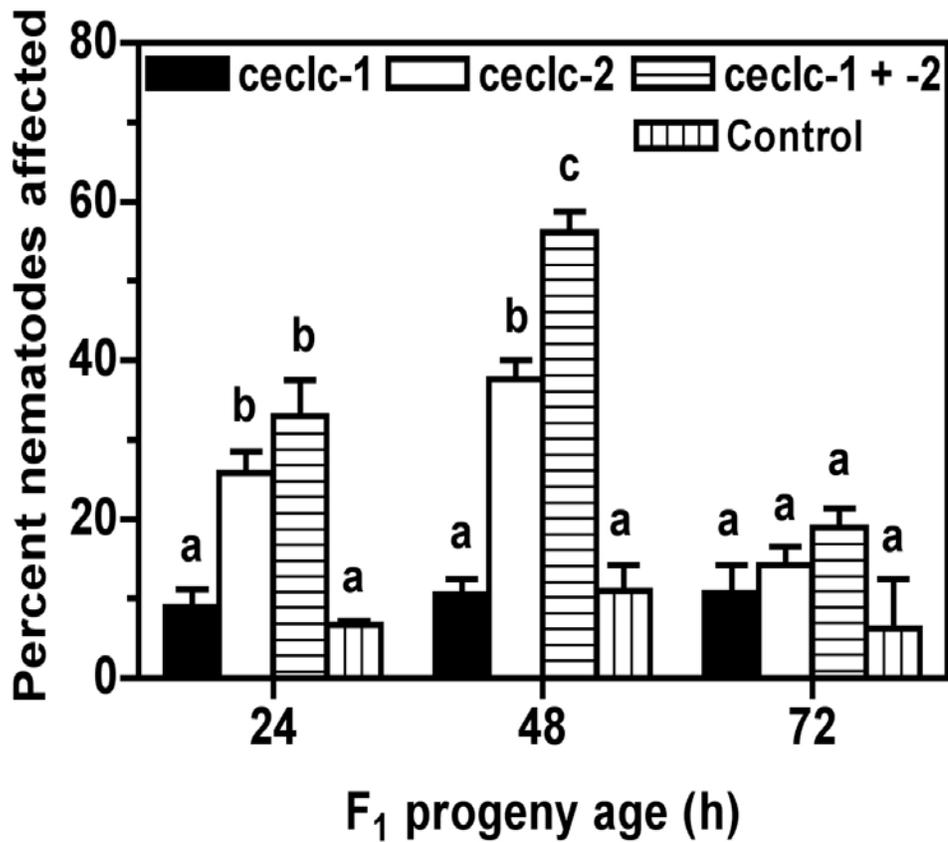


Fig. 3.2a. Percent F<sub>1</sub> progeny with decreased locomotion after fourth-stage larvae of *Caenorhabditis elegans* were treated with long double-stranded RNA of *ceclc-1* and/or *ceclc-2* for 24 h. Bars are means with SEM ( $n = 2$ ). Bars not labeled with same letter at a given age are significantly different from each other ( $P < 0.05$ ) according to Student Newman Keuls test (SAS Institute 2005).

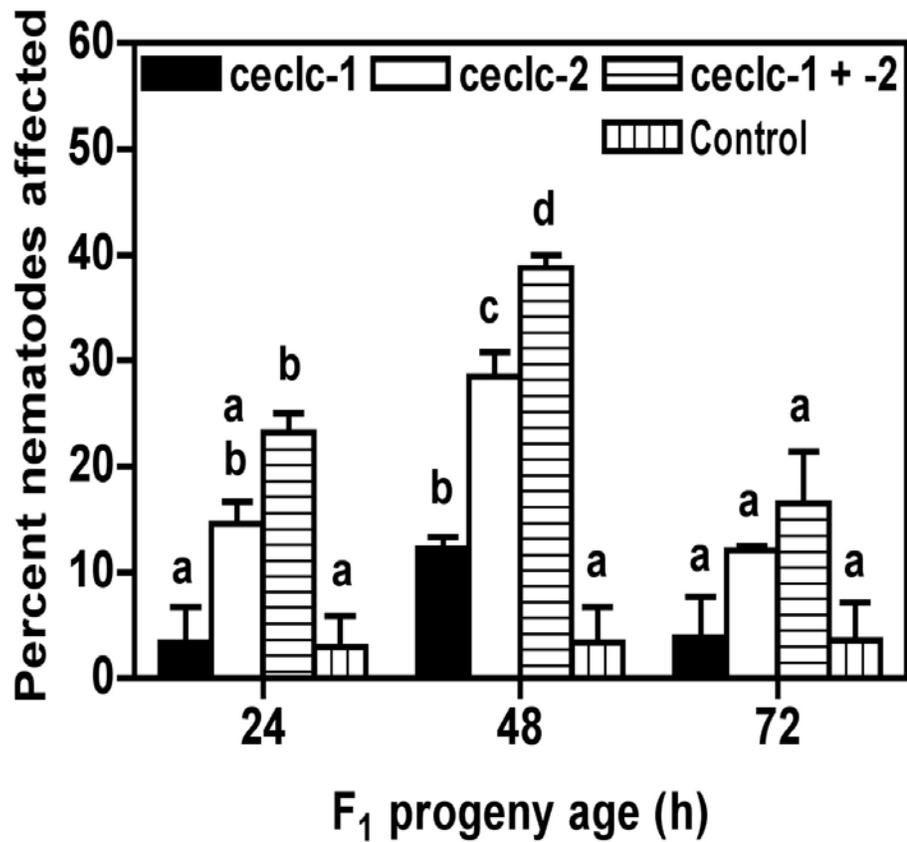


Fig. 3.2b. Percent F<sub>1</sub> progeny with decreased locomotion after fourth-stage larvae of *Caenorhabditis elegans* were treated with short double-stranded RNA of *ceclc-1* and/or *ceclc-2* for 24 h. Bars are means with SEM ( $n = 2$ ). Bars not labeled with same letter at a given age are significantly different from each other ( $P < 0.05$ ) according to Student Newman Keuls test (SAS Institute 2005).

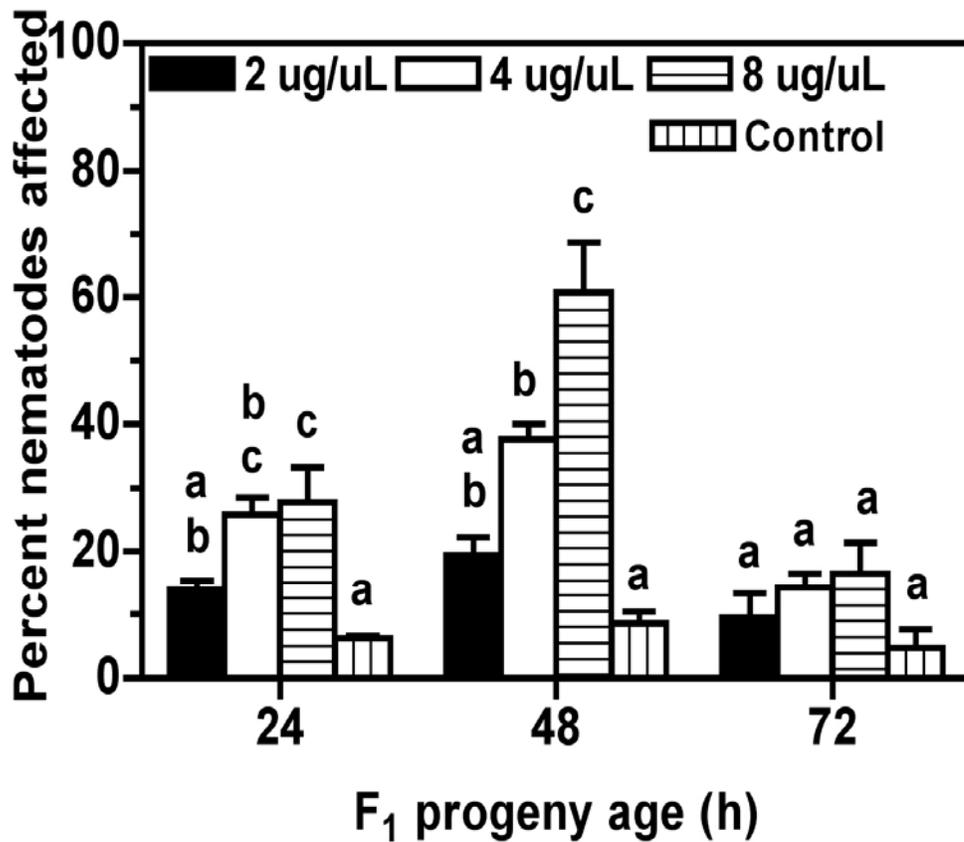


Fig. 3.2c. Concentration-dependent effect on percent F<sub>1</sub> progeny with decreased locomotion after *Caenorhabditis elegans* treated as fourth-stage larvae with long double-stranded RNA of *ceclc-2* for 24 h. Bars are means with SEM ( $n = 10-19$ ). Bars not labeled with same letter at a given age are significantly different from each other ( $P < 0.05$ ) according to Student Newman Keuls test (SAS Institute 2005).

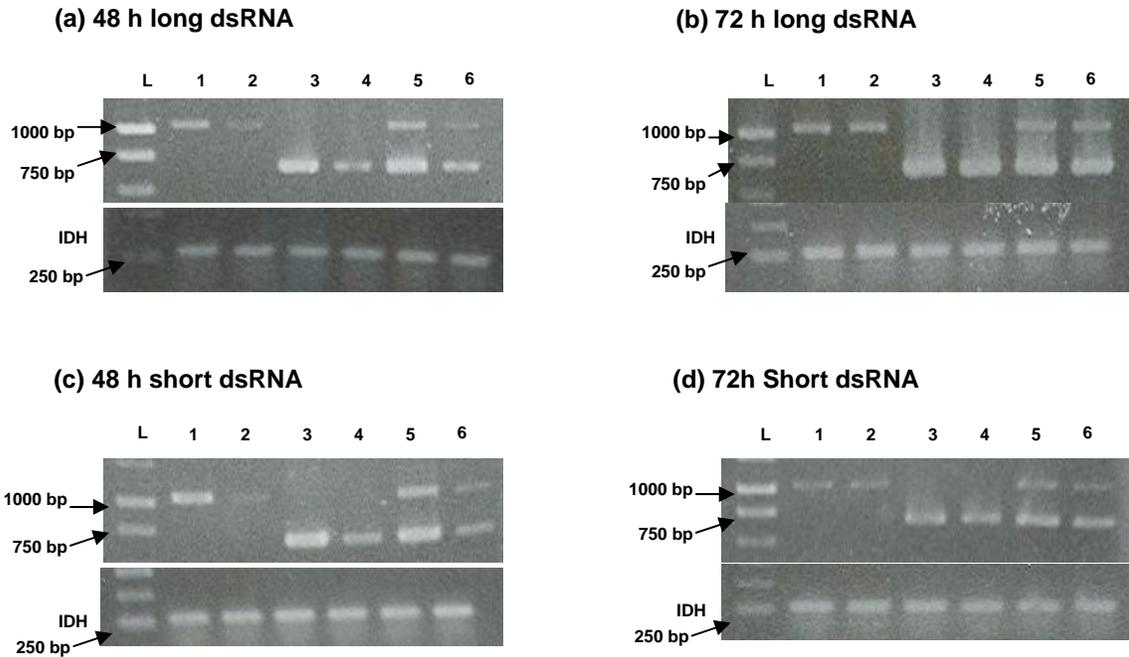


Fig. 3.3. Expression level (mRNA transcript) of *ceclc-1* and/or *ceclc-2* in 48 (a and c) and 72 h (b and d) old F<sub>1</sub> progeny of *Caenorhabditis elegans* treated as fourth-stage larvae with long (a and b) or short (c and d) double-stranded RNA of *ceclc-1* and/or *ceclc-2* for 24 h. Isocitrate dehydrogenase (IDH, 236 bp fragment), a house keeping gene of *C. elegans*, was used as a loading (internal) control. Molecular weight standard used in all the gel analyses was a 1Kb ladder (lane L). In (a), (b), (c), and (d); lanes 2, 4, and 6 indicate expression level of *ceclc-1*, *ceclc-2* and *ceclc-1+2* in treated worms with either long dsRNA at 4  $\mu\text{g}/\mu\text{L}$  (T<sub>2</sub>, T<sub>3</sub>, and T<sub>4</sub>, respectively) or short dsRNA at 5  $\mu\text{g}/\mu\text{L}$  (T<sub>10</sub>, T<sub>11</sub>, and T<sub>12</sub>, respectively), with treatments (T) as defined in Table 3.1. Lanes 1, 3, and 5 indicate expression level of *ceclc-1*, *ceclc-2* and *ceclc-1+2* in respective DEPC-water treated worms (controls, T<sub>1</sub> or T<sub>9</sub>, in Table 3.1).

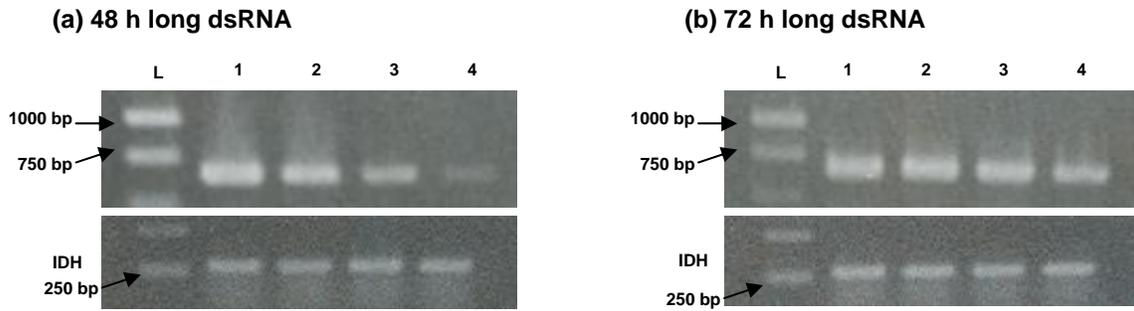


Fig. 3.4. Expression level (mRNA transcript) of *ceclc-2* in 48 (a) and 72 h (b) old F<sub>1</sub> progeny of *Caenorhabditis elegans* treated as fourth-stage larvae with long double-stranded RNA of *ceclc-2* for 24 h. Isocitrate dehydrogenase (IDH, 236 bp fragment), a house keeping gene of *C. elegans*, was used as a loading (internal) control. Molecular weight standard used in all the gel analyses was a 1Kb ladder (lane L). In (a) and (b); lanes 2, 3, and 4 indicate expression level of *ceclc-2* in treated worms with long dsRNA of *ceclc-2* at 2, 4, and 8  $\mu\text{g}/\mu\text{L}$  (T<sub>6</sub>, T<sub>7</sub>, and T<sub>8</sub>, respectively). Lane 1 indicates expression level of *ceclc-2* in respective DEPC-water treated worms (control, T<sub>5</sub>, in Table 3.1).

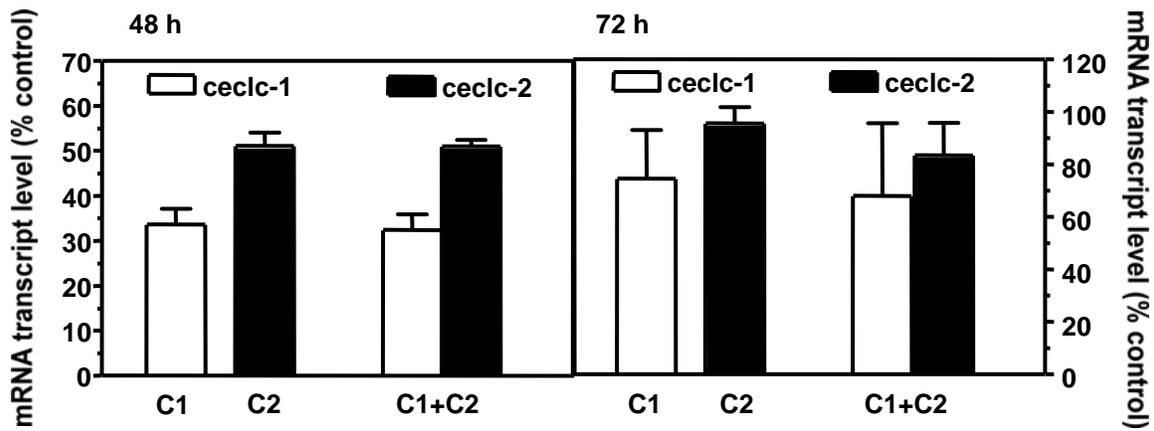


Fig. 3.5a. Densitometry analysis of mRNA transcript levels of *ceclc-1* (C1) and/or *ceclc-2* (C2) in 48 and 72 h old F<sub>1</sub> progeny of *Caenorhabditis elegans* treated as fourth-stage larvae with long double-stranded RNA of *ceclc-1* and/or *ceclc-2* for 24 h (data from fig. 3.3 [a and b]). Bars are means with SEM ( $n = 2$ ).

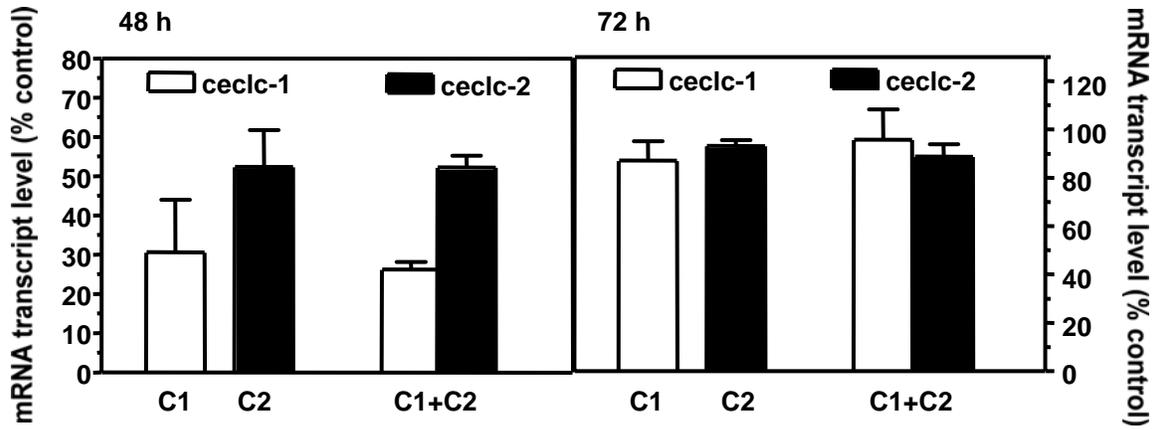


Fig. 3.5b. Densitometry analysis of mRNA transcript levels of *ceclc-1* (C1) and/or *ceclc-2* (C2) in 48 and 72 h old F<sub>1</sub> progeny of *Caenorhabditis elegans* treated as fourth-stage larvae with short double-stranded RNA of *ceclc-1* and/or *ceclc-2* for 24 h (data from fig. 3.3 [c and d]). Bars are means with SEM ( $n = 2$ ).

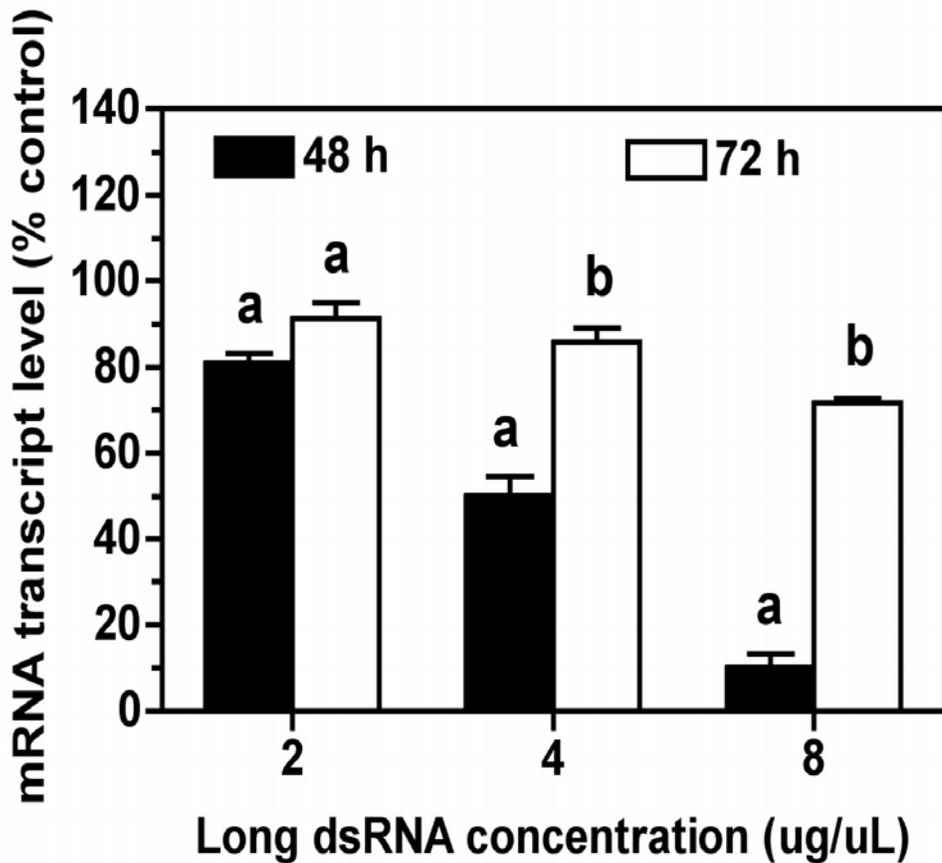


Fig. 3.5c. Densitometry analysis of mRNA transcript levels of *ceclc-2* in 48 and 72 h old  $F_1$  progeny of *Caenorhabditis elegans* treated as fourth-stage larvae with long double-stranded RNA of *ceclc-2* for 24 h (data from fig. 3.4). Bars are means with SEM ( $n = 3$ ). Bars not labeled with same letter at a given concentration are significantly different from each other ( $P < 0.05$ ) according to Student Newman Keuls test (SAS Institute 2005).

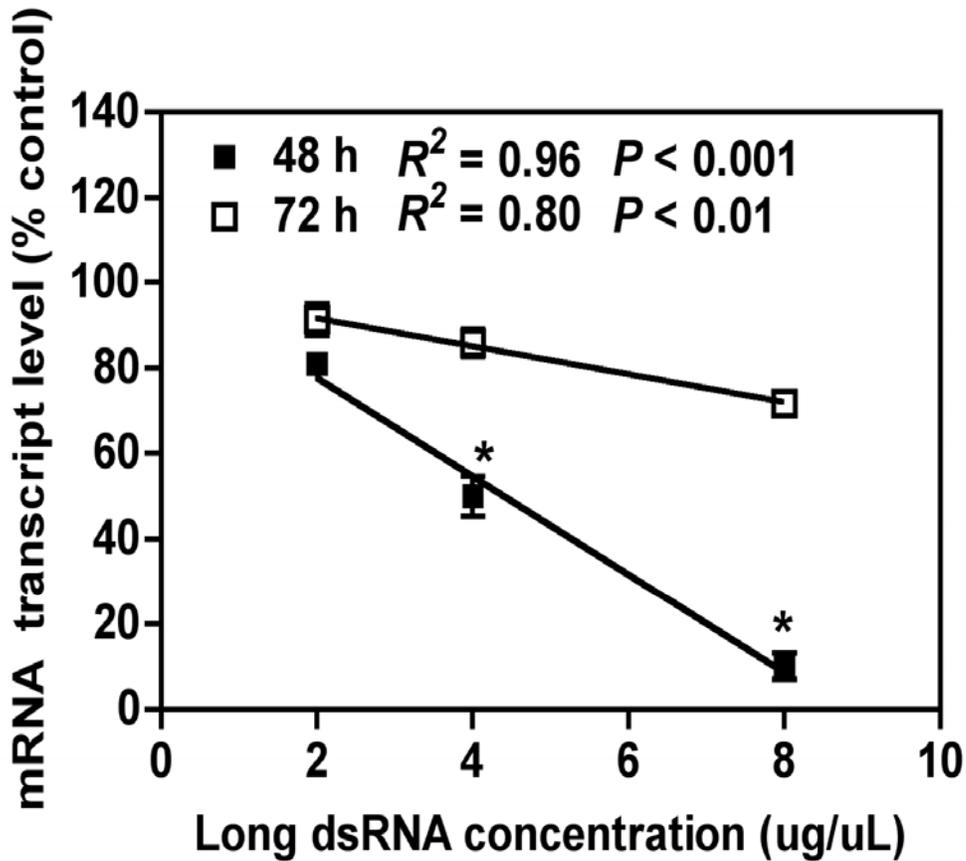


Fig. 3.6. Relationship between dsRNA concentration and *ceclc-2* expression level in 48 and 72 h old F<sub>1</sub> progeny of *Caenorhabditis elegans* treated as fourth-stage larvae with long double-stranded RNA of *ceclc-2* for 24 h. Regression equation for 48 h (*ceclc-2* expression = 100.8 - 11.5\*concentration of dsRNA). Regression equation for 72 h (*ceclc-2* expression = 98.4 - 3.3\*concentration of dsRNA)

## CHAPTER 4

### **Toxicity and Neurophysiological Effects of Anion Transporter Blockers Against *Drosophila melanogaster* Meigen**

#### **ABSTRACT**

Four anion transporter (AT) blockers, DIDS (4, 4'-diisothiocyanatostilbene-2, 2'-disulfonic acid, NPPB (5-nitro-2-(3-phenylpropylamino) benzoic acid, 9-AC (anthracene-9-carboxylic acid, and IAA-94 (indanyloxy acetic acid) were tested for toxicity against susceptible 'Oregon-R' and dieldrin-resistant *rdl* strains of *Drosophila melanogaster* Meigen 1830 using a feeding bioassay. The AT blockers showed slowly developing toxicity against adults of the susceptible strain and were equally active. The lethal concentration required for 50% mortality in susceptible adult flies after 48 h treatment ranged between 126 and 170 ppm and the toxicity was increased by 1- to 2-fold after 72 h of exposure. Adding piperonyl butoxide (PBO), a mixed function oxidase inhibitor, increased the toxicity of AT blockers by 2.0- to 3.5-fold. Resistant *rdl* flies were equally susceptible to DIDS requiring 148 and 72 ppm, respectively for 50% mortality in adults at 48 and 72 h exposures, and PBO increased the toxicity by 3.3- and 4.2-fold. Application of AT blockers at 100  $\mu$ M to desheathed central nervous system (CNS) preparations from third-instar *Drosophila* larvae produced significantly higher mean nerve firing rates than applying dimethyl sulfoxide (DMSO) alone as internal control. Further,  $\gamma$ -aminobutyric acid (GABA) application to the desheathed CNS preparations suppressed the nerve firing from the CNS, and DIDS application did not reverse this

effect. This finding indicates that AT blockers probably inhibited  $\text{Cl}^-$  currents through voltage-gated chloride channels (VGCCs) in the CNS and not GABA-gated chloride channels. In addition, application of AT blockers to intact CNS preparations did not produce mean nerve firing rate different from control, implying that AT blockers were unable to cross the blood-brain barrier. However, a modest excitatory effect of DIDS on ascending peripheral nerve activity was observed. Feeding flies on 10% sugar solution mixed with 100 ppm of DIDS for 6 h decreased the midgut pH approximately 2 units. Therefore, the slowly developing toxicity of AT blockers observed in adult flies was probably the result of their inhibitory action on ATs of non-nervous tissue, perhaps the VGCCs or chloride/bicarbonate exchangers of midgut epithelial cells.

## INTRODUCTION

Anion transporter (AT) blockers such as DIDS (4, 4'-diisothiocyanatostilbene-2, 2'-disulfonic acid; stilbene disulfonic acid); a stilbene disulfonic acid), NPPB ((5-nitro-2-(3-phenylpropylamino) benzoic acid; an anthranilic acid)), 9-AC (anthracene-9-carboxylic acid; an aromatic carboxylic acid), and IAA-94 (indanyloxy acetic acid; an indanyloxy alkanolic acid) (Fig. 1.1) belong to different classes of organic acids. These compounds are known inhibitors of voltage-gated chloride ion (Cl<sup>-</sup>) channels (VGCCs) and chloride/bicarbonate (Cl<sup>-</sup>/HCO<sub>3</sub><sup>-</sup>) exchangers (CBEs) on cell membranes of various tissues in biological systems (Cabantchik and Greger 1992, Machaca et al. 1996, Phipps et al. 1996, Ferroni et al. 1997, Tosco et al. 1998, Wang and Telfer 1998, Pusch et al. 2002, Kawamata et al. 2006, Uchiyama et al. 2006).

Very few studies have documented the activity of AT blockers against insects *in vivo*. DIDS at 100 μM reduced the highly alkaline larval midgut pH to neutral within an hour of feeding in the yellow fever mosquito, *Aedes aegypti* (Linnaeus 1762), possibly by blocking CBEs (Boudko et al. 2001). Further, DIDS at 100 μM blocked <sup>36</sup>Cl<sup>-</sup> exchange in *Manduca sexta* (Linnaeus 1763) embryo cell cultures (English and Cantley 1984). In a previous study, we have shown that DIDS and the other AT blockers mentioned here were toxic to the nematodes *Caenorhabditis elegans* Maupas 1900 and *Meloidogyne incognita* (Kofoid and White 1919) Chitwood 1949, with increased pharyngeal contraction (Boina et al. 2007) and reduced locomotion as toxic symptoms. Therefore, we wanted to see whether AT blockers have insecticidal activity, as well. Furthermore, we wanted to know whether toxicity arose from their action on ATs within the nervous system using electrophysiological methods. *Drosophila melanogaster* Meigen 1830 was

selected for these experiments, as simple and well-developed methods for bioassays and neurophysiological recordings are available (Bloomquist et al. 1991).

## **MATERIALS AND METHODS**

### **Chemicals**

All the tested chemicals (DIDS, NPPB, 9-AC, and IAA-94), piperonyl butoxide (PBO),  $\gamma$ -aminobutyric acid (GABA), and dimethyl sulfoxide (DMSO) were purchased from Sigma-Aldrich (St. Louis, MO).

### ***Drosophila melanogaster* Cultures**

The *D. melanogaster* strains, susceptible (Oregon-R) and dieldrin-resistant (*rdl*), used in the experiments were cultured and maintained on instant *Drosophila* media (Formula 4-24, Carolina Biological Supply Co., Burlington, NC).

### **Bioassays on Adult Flies**

Bioassays on adults were conducted as per the procedure described by Bloomquist et al. (1991). In brief, stock solutions of DIDS, 9-AC, NPPB, and IAA-94 were prepared in DMSO. Final concentrations of 6.3, 12.5, 25, 50, and 100 ppm were made in 10% sugar solution with or without the synergist, PBO (1:1 ratio v/v). One end of a cotton wick was dipped in 0.5 mL of sugar solution and used as a stopper for a glass vial containing 10 adult flies of mixed sexes. Vials with stopper dipped in sugar solution having DMSO/PBO alone served as controls. Each treatment was replicated three times. Mortality counts of flies were taken at 24, 48, and 72 h after treatment. Flies without activity were considered dead. Similar bioassays were performed on adults of the cyclodiene-resistant *rdl* strain using DIDS and dieldrin at 5, 10, 20, 40, and 80 ppm

concentrations and each test vial contained 20 adult flies.

Effect of AT blockers on midgut alkalinity of adult flies was determined by allowing the flies to feed on 10% sugar solution mixed with DIDS at 100 ppm for 6 h. Control flies were fed with 10% sugar solution and solvent, DMSO (0.2%), alone. After 6-h feeding, flies were dissected in saline solution, essentially as described by Shanbhag and Tripathi (2005) for *Drosophila* larvae. Separated midguts were placed on a piece of universal pH indicator paper and pressed gently to release the midgut contents. The change in pH paper color in treatments was compared with that of pH standards to approximately determine the midgut pH in adult flies.

### **Neurophysiological Activity**

Activity of AT blockers on *Drosophila* central nervous system (CNS) was recorded using the procedure described by Bloomquist et al. (1991). Briefly, susceptible *Drosophila* third-instar larval (wandering stage) CNS was dissected and placed in a small wax dish filled with 998  $\mu$ L of *Drosophila* physiological saline (NaCl 157 mM, KCl 3 mM, CaCl<sub>2</sub> 2 mM, HEPES 4 mM with pH adjusted to 7.2). The CNS was kept either intact or desheathed (disrupting the blood brain barrier [BBB] by transecting the CNS just posterior to the cerebral hemispheres and retaining only the ventral ganglion). Using a recording suction microelectrode, a few central nerves were drawn into the microelectrode and electrical signals originating from the CNS were amplified with a differential amplifier (DAM 50, World Precision Instruments, Inc., Sarasota, FL). Amplified signals were then digitized and displayed as a firing rate (frequency in Hz) on Chart software (PowerLab, ADInstruments, Inc., Colorado Springs, CO).

A baseline discharge rate (spontaneous nerve firing) was established by

monitoring the activity for 1 to 2 min. One microliter of DMSO, the solvent used to prepare solutions was applied as an internal control for each preparation and nerve activity was recorded for 1 to 2 min. The preparation was then challenged with 1  $\mu$ L of 100 mM AT blockers (1000-fold dilution giving a final concentration of 100  $\mu$ M) and nerve discharge was recorded for 5 to 10 min. For each compound, recordings were made from at least four desheathed or intact CNS preparations. In order to see if the application of DMSO (once as control and once as AT blocker solvent) had any effect on nerve firing rate in the above experiments, four separate desheathed CNS preparations were treated with 1  $\mu$ L of DMSO twice and the response was observed as described above.

To assess effects on ascending sensory nerve activity, *Drosophila* third-instar larvae were dissected longitudinally in a small dish filled with physiological saline. After clearing the gut and fat tissue, the CNS was removed carefully leaving behind peripheral nerves with the body. Old saline was replaced with 998  $\mu$ L of fresh saline and using a recording suction microelectrode, one or two peripheral nerves were drawn into microelectrode. Nerve activity was recorded from the preparations as per the procedure described above.

To establish that the observed nerve excitation with AT blockers were due to VGCCs inhibition and not due to a GABAergic mechanism, a desheathed CNS preparation was placed in 499.5  $\mu$ L of saline. After observing the baseline response for 2 to 3 min, 499.5  $\mu$ L of 2 mM GABA (final concentration of 1 mM) was applied and the response was recorded for 2 to 4 min. The preparation was then treated with 1  $\mu$ L of DIDS (100 mM) in DMSO and activity was recorded for a minimum of 5 min.

Recordings were made from nine independent CNS preparations. If GABA-induced suppression of nerve firing from the CNS is restored by AT blocker, it suggests the inhibitory action of AT blockers was on the GABA-gated chloride channels (Bloomquist et al. 1991).

### **Statistical Analyses**

Adult fly mortality data from three replications of each dosage was pooled and subjected to probit analysis using the POLO PLUS program (LeOra Software, Berkeley, CA) to determine the lethal concentration required for 50% mortality of test insects ( $LC_{50}$ ) with corresponding 95% confidence intervals for each compound. For analyzing nerve activity, the mean ( $\pm$ SEM;  $n = 4-10$ ) firing rates (Hz) were calculated from a 1 min duration sample encompassing the highest observed nerve firing rate before (DMSO) and after AT blocker application for central and peripheral nerve preparations. For GABA experiments, the mean ( $\pm$ SEM;  $n = 9$ ) nerve firing rates before (baseline), during, and after (DIDS) GABA application for the entire treatment duration were calculated. Data were analyzed by one-way ANOVA using the PROC GLM program (SAS Institute 2005) for significant differences and means were separated using Tukey's test at the  $\alpha = 0.05$  level of significance.

## **RESULTS**

### **Bioassays on Adult Flies**

None of the blockers at the highest concentration tested, with or without PBO, caused  $\geq 50\%$  mortality in susceptible adults at 24 h exposure. Therefore,  $LC_{50}$ s were not calculated at this time point. At 48 and 72 h of exposure, all the AT blockers with or

without PBO caused more than 50 percent mortality. Based on the calculated  $LC_{50}$  values, DIDS was the most active compound at 48 and 72 h of exposure followed by NPPB, 9-AC, and IAA-94 in that order of activity (Table 4.1). At 72 h of exposure, the activity of AT blockers increased by 1- to 2-fold compared to 48-h exposure. However, overlap of 95% CIs indicated no significant difference between their activities at a given exposure time. Addition of PBO synergized AT blockers activity by 2- to 3.4-fold and 2.8- to 3.5-fold, respectively, at 48 and 72 h of exposure (Table 4.1).

As was observed for the susceptible strain, the highest concentration of DIDS tested did not give  $\geq 50\%$  mortality in cyclodiene-resistant flies at 24 h of exposure. At 48 and 72 h of exposure, 148 and 72 ppm of DIDS, respectively, were required for 50% mortality in resistant flies, while addition of PBO synergized DIDS toxicity by 3.3- and 4.2-fold, respectively (Table 4.2). Mortality in resistant flies was only 17% with the highest concentration of dieldrin (80 ppm) tested at 72 h of exposure.

The change in the color of universal pH paper due to the midgut contents from adult flies fed on control (DMSO) or DIDS mixed sugar solution is shown in Figure 4.1. Based on pH paper color change, the midgut pH of control flies was ca. 8.0 ( $n = 3$ ), while the midgut pH of flies fed on DIDS at 100 ppm for 6 h decreased by approximately 2 pH units to 6.0 ( $n = 3$ ).

### **Neurophysiological Activity**

Nerve activities recorded from desheathed and intact CNS preparations of third-instar *Drosophila* larva after challenge with AT blocker (DIDS) are presented in figures 4.2 and 4.3, respectively. Application of DMSO twice to the CNS preparations had no effect on nerve firing (Fig. 4.4) indicating that any change in nerve firing rate observed

after application of AT blocker was due to only AT blocker. Except for 9-AC, application of AT blockers at 100  $\mu$ M to desheathed CNS preparations produced significantly higher mean firing rates compared to the application of DMSO alone ( $P < 0.05$ ) (Fig. 4.5). In contrast, challenging intact CNS preparations with any of the AT blockers at 100  $\mu$ M did not produce a mean firing rate that was significantly different from DMSO application ( $P > 0.05$ ) (Fig. 4.6). Not all desheathed preparations responded to AT blockers by increased nerve discharge. The proportion of responding preparations was 0.75, 0.75, 0.67, and 0.83 for DIDS, NPPB, 9-AC, and IAA-94, respectively.

Peripheral nerve activity recordings from *Drosophila* third-instar larvae showed a modest response of DIDS on nerve excitation in 63% of the preparations, though not significant from control (DMSO) (Fig. 4.7). Figure 4.8 shows the nerve firing pattern when desheathed CNS preparations were challenged with GABA and DIDS. Application of GABA to the desheathed CNS preparations suppressed the nerve firing significantly and application of DIDS to the same preparation did not restore nerve firing in majority of the preparations (67%) (Figs. 4.8 and 4.9).

## DISCUSSION

All the AT blockers showed slowly developing insecticidal activity against susceptible *D. melanogaster* adults, as greater than 50% mortality could only be observed 48 h after treatment at the highest concentration of AT blockers tested. This effect is similar to the slowly developing toxicity of these compounds observed against nematodes, where 48 and 72 h, respectively, were required for obtaining 50% mortality in *C. elegans* and *M. incognita* (Boina et al. 2007). In general, nematode species seem more

susceptible to these compounds than *D. melanogaster*. *Caenorhabditis elegans* adults were ~4 and 6-8 times more sensitive after 48 and 72 h exposure, respectively, while *M. incognita* second-stage larvae were ~4 times more sensitive after 72 h exposure compared to *D. melanogaster* (Boina et al. 2007). In the present study, toxicity of DIDS against *rdl* flies was similar to that of susceptible flies, suggesting that the dieldrin-resistant flies were equally susceptible to these compounds.

Addition of PBO synergized the toxicity of AT blockers to varying degrees against both susceptible (2- to 3.5-fold) and *rdl* resistant (3.3- to 4.2-fold) flies. This finding suggests P<sub>450</sub> monooxygenases are involved in metabolizing these compounds. Increased toxicity of AT blockers with PBO explains, partly, the slowly developing toxicity of these compounds against *D. melanogaster* in our study as P<sub>450</sub> monooxygenases may aid in rapid detoxification of AT blockers. Though effect of PBO on AT blockers toxicity against nematodes was not tested in our previous study (Boina et al. 2007), the same phenomenon might hold true for nematodes as well. Similar to our study, PBO at 10 µg/4 mL vial increased the toxicity of some volatile liquid insecticides belonging to heterobicyclic groups (menthofuran) and formate esters (methyl formate and ethylene glycol diformate) by 1- to 2-fold against a susceptible *D. melanogaster* strain ‘Canton-S’ through inhibition of P<sub>450</sub> monooxygenases (Nguyen et al. 2007). Numerous studies have shown a synergistic effect of PBO, which prevents the detoxification of compounds by inhibiting P<sub>450</sub> monooxygenases (for example, Wu et al. 2007).

The AT blockers were neuroexcitatory on desheathed CNS preparations; however, DIDS showed only a small excitatory effect on peripheral nerves. Higher mean CNS firing rates observed with 9-AC and IAA-94 compared to DIDS and NPPB (Figs.

4.3 and 4.4) were due to the higher baseline nerve firing rates in some of the CNS preparations used for 9-AC and IAA-94. Greater mean firing rates observed with the AT blockers in the desheathed CNS suggests their neurophysiological activity was the result of VGCCs inhibition in the CNS leading to nerve depolarization and subsequent hyperexcitation. In a previous study, Clark et al. (1998) showed that DIDS (1 mM), NPPB (200  $\mu$ M) and 9-AC (1 mM) significantly inhibited the  $\text{Cl}^-$  current through VGCCs (CIC-2) in dissociated rat superior cervical ganglion neurons and inhibition of  $\text{Cl}^-$  current by 9-AC was slower compared to DIDS and NPPB. Similarly, intracellular application of DIDS at 200  $\mu$ M blocked irreversibly voltage-dependent  $\text{Cl}^-$  conductance via  $\text{Cl}^-$  channels in squid axon plasma membrane (Inoue 1985).

Application of DIDS to the CNS preparation did not restore the nerve firing suppressed by GABA, an agonist of the GABA-receptor chloride channel complex, supports the above conclusion. In this experiment, application of GABA activated CNS GABA receptors, causing influx of  $\text{Cl}^-$  and leading to hyperpolarization. Apparently, the magnitude of hyperpolarization by GABA was greater than the depolarization by DIDS, resulting in a slight or no increase in nerve firing. If AT blockers were GABA antagonists, a significantly higher nerve firing rate with DIDS compared to GABA would have been observed.

Absence of increased nerve firing with AT blockers in intact CNS preparations could be due to the protective role played by the BBB of the CNS that prevents the entry of some chemicals from hemolymph. The CNS is surrounded by a layer comprised of an outer non-cellular neural lamella and an inner cellular perineurium, collectively called the nerve sheath (Chapman 1969). The neural lamella is secreted by perineural cells and is

permeable to ions and other molecules. Perineural cells are specialized glial cells and are held together by tight junctions and intercellular septate junctions with anionic sites and act as the BBB (Chapman 1969, Juang and Carlson 1992). The septate junctions make long paracellular pathways and their anionic sites attract positively charged molecules and retain them for a prolonged time (Juang and Carlson 1992). All these strategies help to prevent certain harmful chemicals in the hemolymph from reaching central neurons as desheathing the abdominal ganglia in *Periplaneta americana* (Linnaeus 1758) greatly facilitated the entry of positively charged molecules such as quaternary ammonium salts (Eldefrawi et al. 1968, Thomas 1976). Further, liposolubility of molecules can be the primary factor in determining BBB penetration (Eldefrawi et al. 1968), and most insecticides are lipophilic.

Therefore, although AT blockers are not positively charged molecules, the presence of the BBB in intact *Drosophila* CNS preparations may have prevented AT blocker entry into the CNS and the disruption of which is required for neurophysiological activity. If AT blockers inhibit VGCCs in the CNS, it would cause hyperactivity in flies and lead to their rapid death. In contrast, little effect on peripheral nerve was noted, toxicity development was slow, and no apparent hyperactivity symptoms were observed, suggesting VGCCs of the nervous system were not the target sites for AT blockers and their insecticidal activity was the result of inhibition of ATs located in non-neural tissue. Though information is lacking on neuromuscular effects of these compounds in *Drosophila*, the slowly developing toxicity suggests that these may not be target sites. Nevertheless, electrophysiological recordings from neuromuscular junctions of *Drosophila* larvae will shed light on this aspect. A decrease in midgut alkalinity in flies

fed with DIDS supported that VGCCs and/or CBEs of midgut epithelial cells are the target sites in *Drosophila*. The evidence for the presence of CBEs in midgut of insects such as larvae of *A. aegypti* (Boudko et al. 2001) and *M. sexta* (Chao et al. 1989) and their role in luminal alkalization has been documented. Boudko et al. (2001) reported that exposing *A. aegypti* larvae to 100  $\mu$ M DIDS decreased the highly alkaline midgut pH to neutral pH within an hour.

Similarly, feeding AT blockers at various concentrations to the second-instar larvae of the European corn borer, *Ostrinia nubilalis* (Hübner 1796), affected their growth, development, and survival in a dose-dependent manner (see chapter 4). Subsequent experiments found a significant decrease in midgut alkalinity and Cl<sup>-</sup> transport from midgut lumen into epithelia in AT blocker-fed larvae. These findings suggest that inhibition of CBEs disrupted the digestion and absorption of dietary proteins required for growth and development (see chapter 4). Similarly, in the present study changes that occurred in midgut alkalinity due to AT blocker inhibition of CBEs could have disturbed the gut leading to disruption of digestion and starvation of flies to death. The small excitation seen with DIDS in peripheral nerves could have aided to the above effects in decreasing fly survival. Direct cellular effects of AT blockers on the VGCCs and CBEs underlying toxicity remain to be determined.

In conclusion, we have shown that AT blockers were toxic to both susceptible and dieldrin-resistant adult flies of *D. melanogaster* and toxicity increased with time. PBO synergized the toxicity of AT blockers in both strains. All AT blockers, except 9-AC, at 100  $\mu$ M showed significant neuroexcitatory effect on desheathed CNS of third instar larvae of *Drosophila*, while DIDS at 100  $\mu$ M modestly increased the nerve firing rates

from ascending peripheral nerves. Feeding on DIDS at 100 ppm decreased midgut alkalinity of adult flies by 2 pH units suggesting inhibition of VGCCs and/or CBEs of midgut epithelial cells probably responsible for slowly developing insecticidal activity of AT blockers.

### LITERATURE CITED

- Bloomquist, J. R., R. H. French-Constant, and R. T. Roush. 1991. Excitation of central neurons by dieldrin and picrotoxinin in susceptible and resistant *Drosophila melanogaster* (Meigen). *Pesti Sci* 32: 463-469.
- Boina, D., E. E. Lewis, and J. R. Bloomquist. 2007. Nematicidal activity of anion transport blockers against *Meloidogyne incognita*, *Caenorhabditis elegans*, and *Heterorhabditis bacteriophora*. *Pest Manag Sci* (In press).
- Boudko, D. Y., L. L. Moroz, W. R. Harvey, P. J. Linser. 2001. Alkalinization by chloride/bicarbonate pathway in larval mosquito midgut. *Proc Natl Acad Sci USA* 98: 15354-15359.
- Cabantchik, Z. I., and R. Greger. 1992. Chemical probes for anion transporters of mammalian cell membranes. *Am J Physiol* 262: C803-C827.
- Chapman, R. F. 1969. Nervous system. In: RF Chapman (Ed), The insects structure and function. Fourth edition, Cambridge university press, New York, pp. 533-569.
- Chao, A. C., A. R. Koch, and D. F. Moffett. 1989. Active chloride transport in isolated posterior midgut of tobacco hornworm (*Manduca sexta*). *Am J Physiol* 257: R752-R761.
- Clark, S., S. Jordt, T. J. Jentsch, and A. Mathie. 1998. Characterization of the

- hyperpolarization-activated chloride current in dissociated rat sympathetic neurons. *J Physiol* 506: 665-678.
- Eldefrawi, M. E., A. Topozada, M. M. Salpeter, and R. D. O'Brien. 1968. The location of penetration barriers in the ganglia of the American cockroach, *Periplaneta americana* (L.). *J. Exp Biol* 48: 325-338.
- English, L. H., and L. C. Cantley. 1984. Characterization of monovalent ion transport systems in an insect cell line (*Manduca sexta* embryonic cell line CHE). *J Cellular Physiol* 121: 125-132.
- Ferroni, S., C. Marchini, M. Nobile, and C. Rapisarda. 1997. Characterization of an inwardly rectifying chloride conductance expressed by cultured rat cortical astrocytes. *Glia* 21: 217-227.
- Inoue, I. 1985. Voltage-dependent chloride conductance of the squid axon membrane and its blockade by some disulfonic stilbene derivatives. *J. Gen Physiol* 85: 519-537.
- Juang, J., and S. D. Carlson. 1992. Fine structure and blood-brain barrier properties of the central nervous system of a dipteran larva. *J. Comp Neurol* 324: 343-352.
- Kawamata, K., H. Hayashi, and Y. Suzuki. 2006. Chloride-dependent bicarbonate secretion in the mouse large intestine. *Biomed Res* 27: 15-21.
- Machaca, K., L. J. Defelice, and S. W. L. Hernault. 1996. A novel chloride channel localizes to *Caenorhabditis elegans* spermatids and chloride channel blockers induce spermatid differentiation. *Develop Biol* 176: 1-16.
- Nguyen, S. N., C. Song, and M. E. Scharf. 2007. Toxicity, synergism, and neurological effects of novel volatile insecticides in insecticide-susceptible and -resistant

- Drosophila* strains. *J. Econ Entomol* 100: 534-544.
- Phipps, D. J., D. R. Branch, and L. C. Schlichter. 1996. Chloride-channel block inhibits T lymphocyte activation and signaling. *Cell Signal* 8: 141-149.
- Pusch, M., A. Accardi, A. Liantonio, P. Guida, S. Traverso, D. C. Camerino, and F. Conti. 2002. Mechanisms of block of muscle type CLC chloride channels (Review). *Mol Memb Biol* 19: 285-292.
- SAS Institute. 2005. The SAS system version 9.1 for Windows. SAS Institute, Cary, NC.
- Shanbhag, S., and S. Tripathi. 2005. Electrogenic H<sup>+</sup> transport and pH gradients generated by a V-H<sup>+</sup>-ATPase in the isolated perfused larval *Drosophila* midgut. *J Membr Biol* 206: 61-72.
- Thomas, M. V. 1976. Insect blood-brain barrier: a radioisotope study of the kinetics of exchange of a liposoluble molecule (*n*-butanol). *J. Exp Biol* 64: 119-130.
- Tosco, M., M. N. Orsenigo, G. Gastaldi, and A. Faelli. 1998. pH dependence of Cl<sup>-</sup>/HCO<sub>3</sub><sup>-</sup> exchanger in the rat jejunal enterocyte. *Biochem et Biophysic Acta* 1372: 323-330.
- Uchiyama, H., H. Hayashi, and Y. Suzuki. 2006. Functional characterization of Cl<sup>-</sup>/HCO<sub>3</sub><sup>-</sup> exchange in villous cells of the mouse ileum. *Biomed Res* 27: 265-274.
- Wang, Y., and W. H. Telfer. 1998. Cyclic-AMP-induced water uptake in a moth ovary: inhibition by bafilomycin and anthracene-9-carboxylic acid. *J Exp Biol* 201: 1627-1635.
- Wu, G., T. Miyata, C. Y. Kang, and L. H. Xie. 2007. Insecticide toxicity and synergism by enzyme inhibitors in 18 species of pest insect and natural enemies in crucifer

vegetable crops. *Pest Manag Sci* 63: 500-510.

Table 4.1. Effect of AT blockers against susceptible *Drosophila melanogaster* adults (Oregon-R)

Exposure time (h)	Treatment	No. insects (N)	<sup>a</sup> LC <sub>50</sub> (ppm)	<sup>b</sup> 95% CI	Slope ± SEM	$\chi^2$ (df) <sup>c</sup>	Synergism ratio <sup>d</sup>
48	DIDS	150	126	80-341	1.7 ± 0.4	1.8 (3)	
	DIDS + PBO	150	44	33-60	2.3 ± 0.4	1.5 (3)	2.8
	9-AC	150	151	92-500	1.7 ± 0.4	1.3 (3)	
	9-AC + PBO	150	74	53-128	1.9 ± 0.5	1.8 (3)	2.0
	NPPB	150	137	61-427	1.4 ± 0.5	1.1 (3)	
	NPPB + PBO	150	40	28-72	2.1 ± 0.5	1.4 (3)	3.4
	IAA-94	150	170	98-711	1.7 ± 0.4	1.4 (3)	
	IAA-94 + PBO	150	65	46-104	1.9 ± 0.4	1.2 (3)	2.6
72	DIDS	150	73	50-126	1.9 ± 0.4	1.3 (3)	
	DIDS + PBO	150	26	17-32	3.6 ± 0.8	2.5 (3)	2.8
	9-AC	150	111	74-262	1.9 ± 0.5	1.3 (3)	
	9-AC + PBO	150	34	25-45	2.4 ± 0.4	1.6 (3)	3.2
	NPPB	150	70	49-122	1.8 ± 0.4	1.3 (3)	
	NPPB + PBO	150	20	15-25	3.2 ± 0.5	1.6 (3)	3.5
	IAA-94	150	116	74-296	1.7 ± 0.4	1.7 (3)	
	IAA-94 + PBO	150	35	26-47	2.3 ± 0.4	1.2 (3)	3.3

<sup>a</sup> Lethal concentration required for 50% mortality.

<sup>b</sup> 95% confidence intervals for LC<sub>50</sub>.

<sup>c</sup> Chi-square goodness-of-fit statistic and degrees of freedom.

<sup>d</sup> Synergism ratio =  $LC_{50}$  without piperonyl butoxide/ $LC_{50}$  with piperonyl butoxide.

Table 4.2. Effect of DIDS against dieldrin-resistant *Drosophila melanogaster* adults (*rdl*)

Exposure time (h)	Compound	No. insects (N)	<sup>a</sup> LC <sub>50</sub> (ppm)	<sup>b</sup> 95% CI	Slope ± SEM	$\chi^2$ (df) <sup>c</sup>	Synergism ratio <sup>d</sup>
48	DIDS	300	148	90-392	1.3 ± 0.3	1.5 (3)	
	DIDS + PBO	300	44	33-67	1.3 ± 0.2	1.1 (3)	3.3
72	DIDS	300	72	52-121	1.5 ± 0.3	1.2 (3)	
	DIDS + PBO	300	17	13-22	1.7 ± 0.2	1.9 (3)	4.2

<sup>a</sup> Lethal concentration required for 50% mortality.

<sup>b</sup> 95% confidence intervals for LC<sub>50</sub>.

<sup>c</sup> Chi-square goodness-of-fit statistic and degrees of freedom.

<sup>d</sup> Synergism ratio = LC<sub>50</sub> without piperonyl butoxide/LC<sub>50</sub> with piperonyl butoxide.

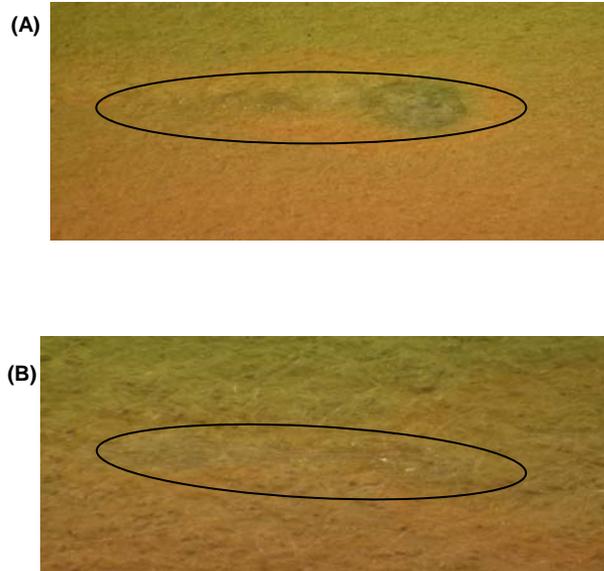


Fig. 4.1. Effect of DIDS on *Drosophila* adult midgut pH determined by universal pH indicator paper. Color change in universal pH indicator paper due to contents from midgut of flies fed for 6 h on sugar solution mixed with dimethyl sulfoxide (solvent) (A) and DIDS at 100 ppm (B) indicated pH of 6.0 ( $n = 3$ ) and 8.3 ( $n = 3$ ), respectively. Ellipses indicate the rough outline of the midgut silhouette.

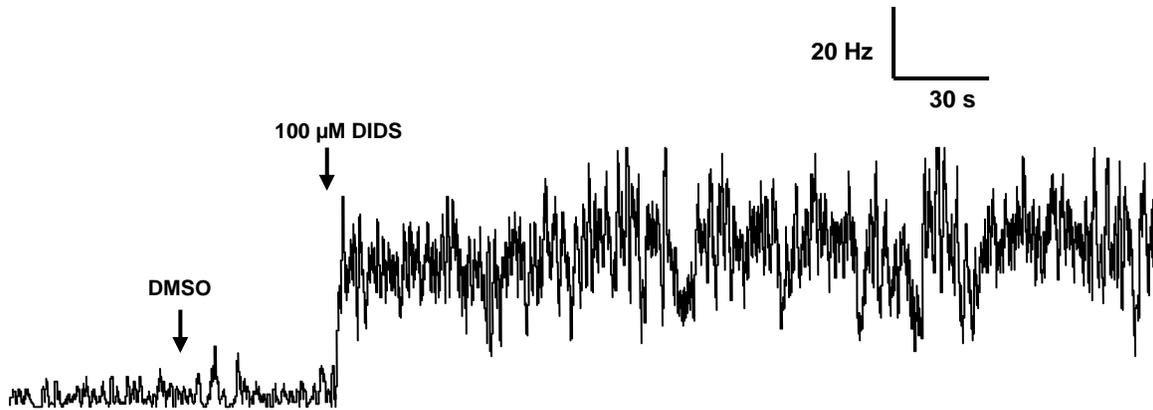


Fig. 4.2. Central nerve firing rate before and after application of DIDS to desheathed central nervous system preparations from third-instar *Drosophila* larvae. After a baseline response observation for 1 to 2 min, 1  $\mu$ L of dimethyl sulfoxide was applied as control and the activity was observed for 1 min. The preparation was then challenged with 1  $\mu$ L of 100 mM DIDS (final concentration 100  $\mu$ M), mixed well and the activity was observed for a minimum of 5 min.

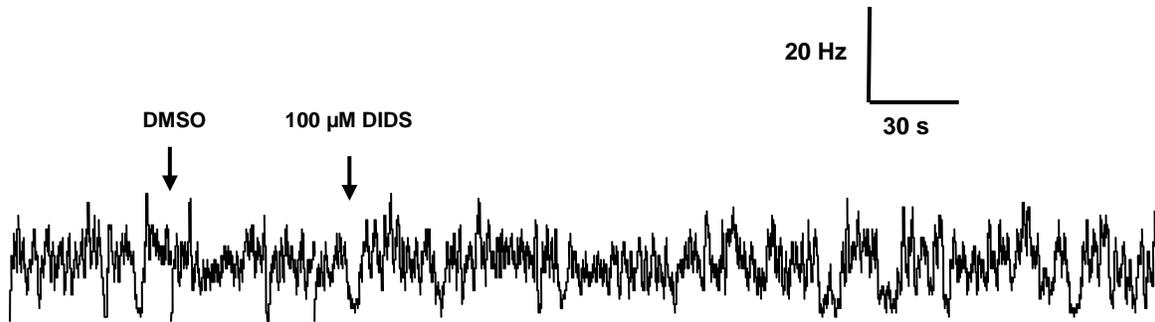


Fig. 4.3. Central nerve firing rate before and after application of DIDS to intact central nervous system preparations from third-instar *Drosophila* larvae. After a baseline response observation for 1 to 2 min, 1  $\mu$ L of dimethyl sulfoxide was applied as control and the activity was observed for 1 min. The preparation was then challenged with 1  $\mu$ L of 100 mM DIDS (final concentration 100  $\mu$ M), mixed well and the activity was observed for a minimum of 5 min.

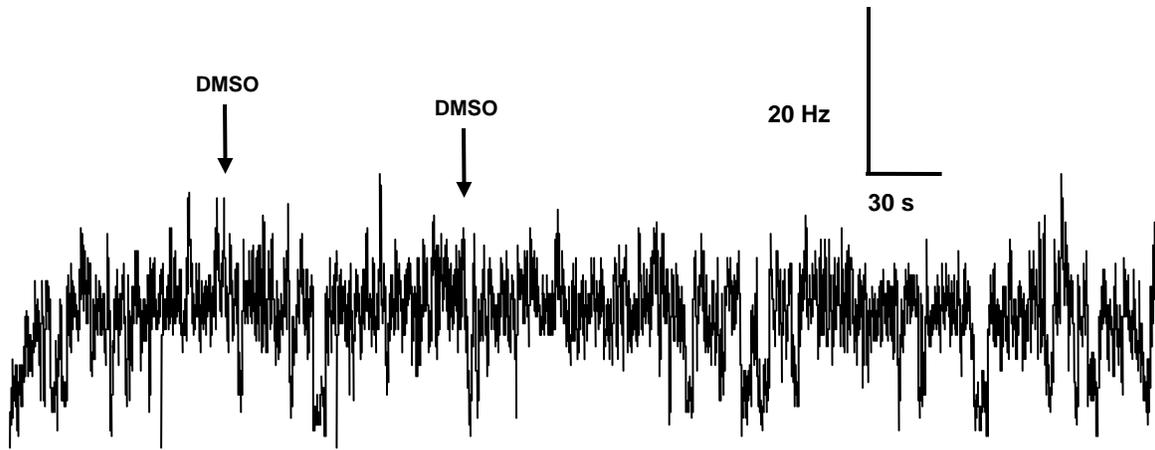


Fig. 4.4. Central nerve firing rate after application of dimethyl sulfoxide twice to desheathed central nervous system preparations ( $n = 3$ ) from third-instar *Drosophila* larvae. After a baseline response observation for 1 to 2 min, 1  $\mu\text{L}$  of dimethyl of sulfoxide was applied and the activity was observed for 1 to 2 min. One microliter of dimethyl sulfoxide was applied again, mixed well and the activity was observed for a minimum of 5 min.

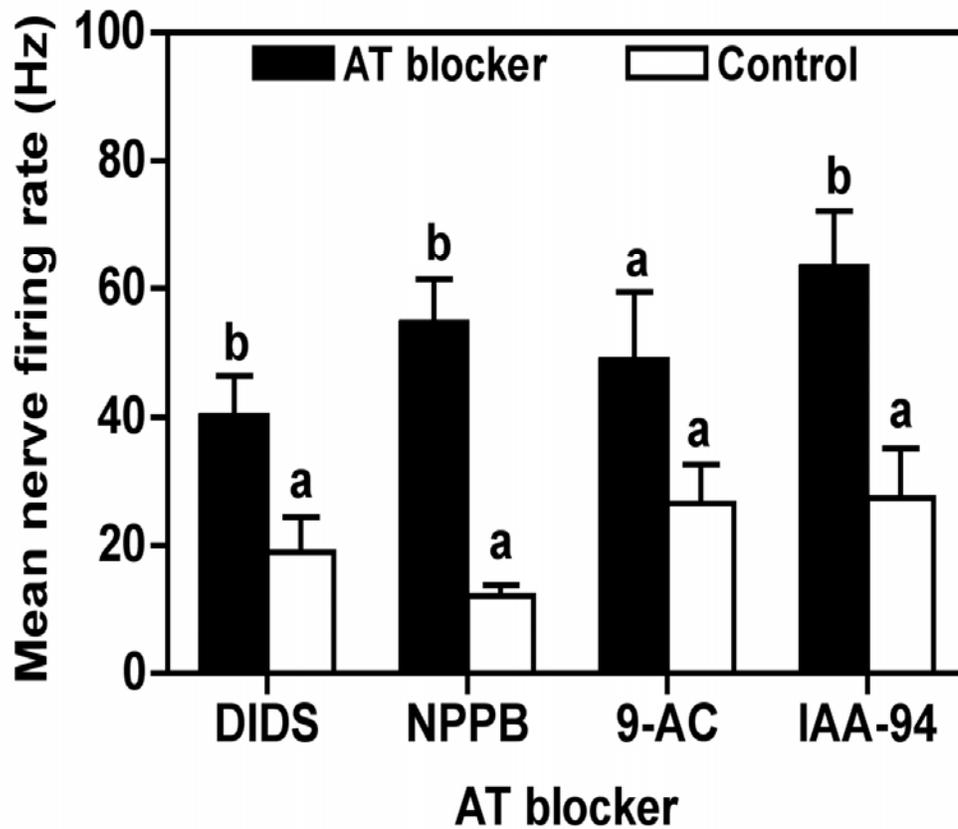


Fig. 4.5. Quantified neurophysiological activity of anion transporter blockers on desheathed central nervous system of *Drosophila* third-instar larvae. Bars are means with SEM ( $n = 8, 4, 10,$  and  $6$  for DIDS, NPPB, 9-AC, and IAA-94, respectively). Bars not labeled with same letter for a given anion transporter blocker are significantly different from matched control ( $P < 0.05$ ) according to Tukey's test (SAS Institute 2005).

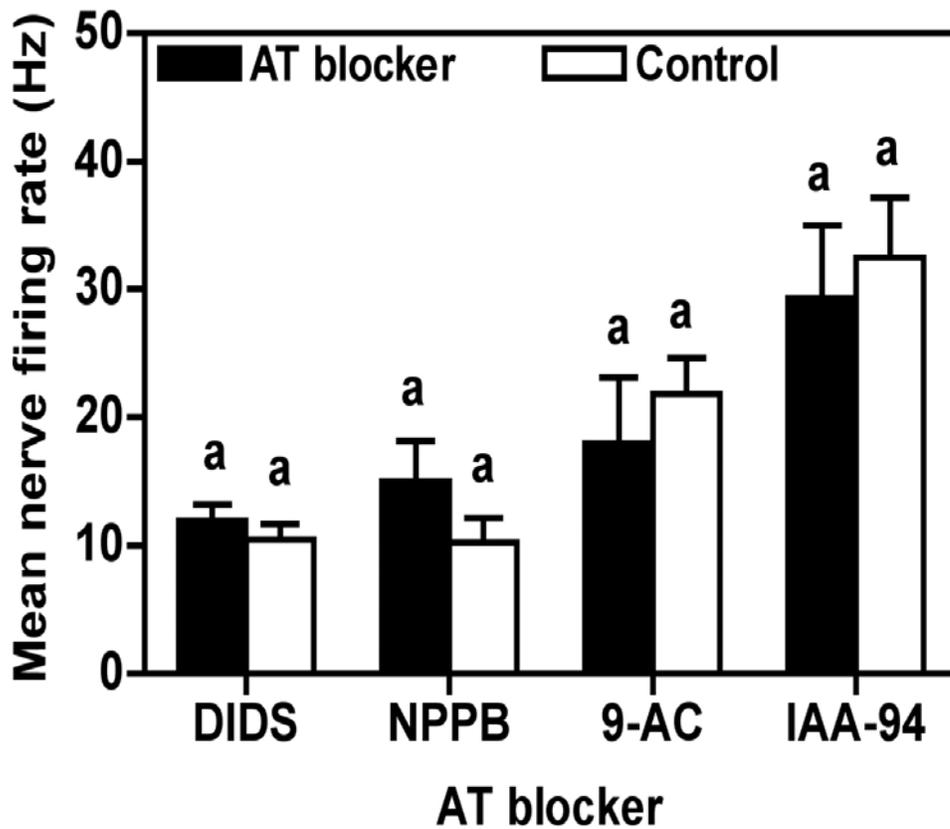


Fig. 4.6. Quantified neurophysiological activity of anion transporter blockers on intact central nervous system of *Drosophila* third-instar larvae. Bars are means with SEM ( $n = 4, 4, 4,$  and  $7$  for DIDS, NPPB, 9-AC, and IAA-94, respectively). Bars not labeled with same letter for a given anion transporter blocker are significantly different from matched control ( $P < 0.05$ ) according to Tukey's test (SAS Institute 2005).

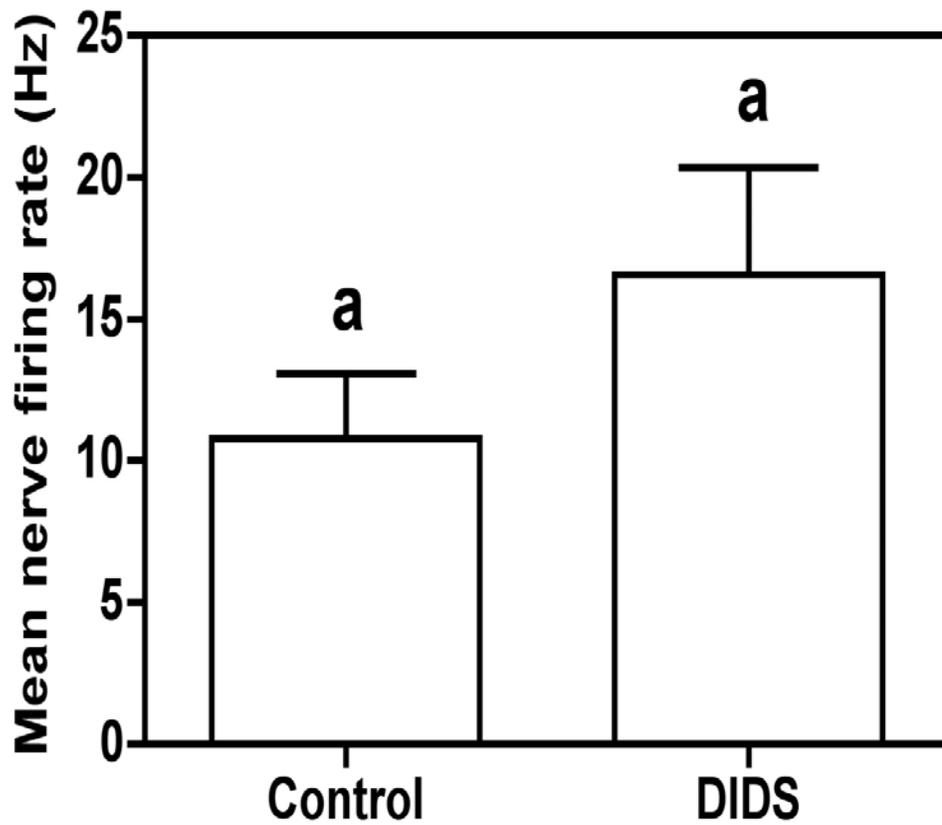


Fig. 4.7. Quantified neurophysiological activity of peripheral sensory nerves from *Drosophila* third-instar larvae treated with DIDS. Bars are means with SEM ( $n = 8$ ). Bars labeled with same letter are not significantly different from each other ( $P > 0.05$ ) according to Tukey's test (SAS Institute 2005).

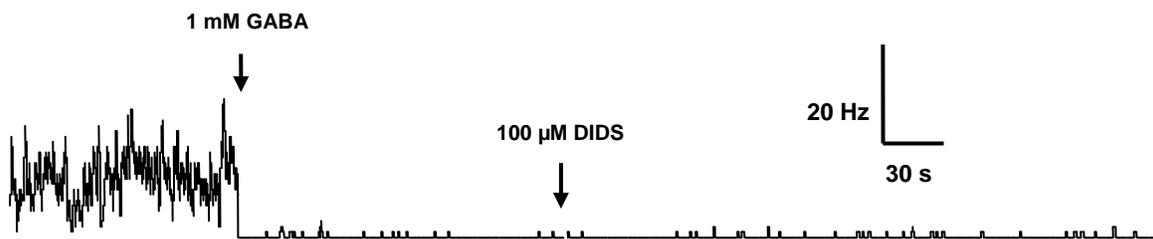


Fig. 4.8. Central nerve firing rate after application of  $\gamma$ -amino butyric acid and DIDS to desheathed central nervous system preparations from third-instar *Drosophila* larvae. After a baseline response observation for 2 to 3 min, 499.5  $\mu$ L of 2 mM GABA (final concentration 1 mM) was applied, mixed well and the activity was observed for 2 to 4 min. The preparation was then challenged with 1  $\mu$ L of 100 mM DIDS (final concentration 100  $\mu$ M), mixed well and the activity was recorded for a minimum of 5 min.

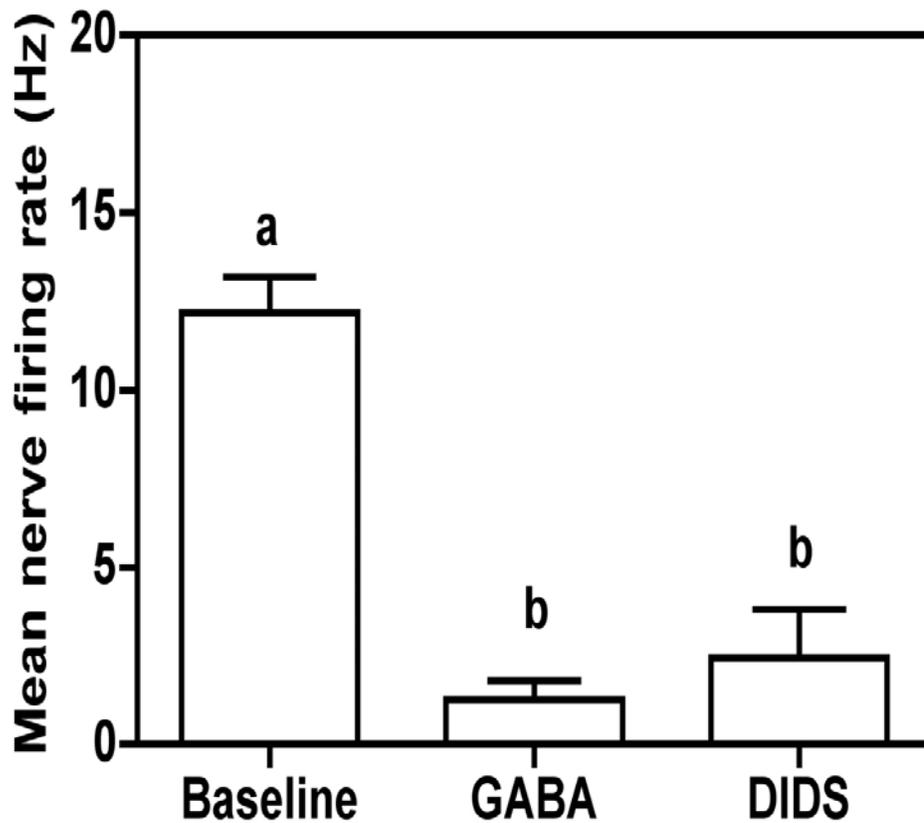


Fig. 4.9. Quantified neurophysiological activity from desheathed central nervous system of *Drosophila* third-instar larvae treated with  $\gamma$ -aminobutyric acid and DIDS. Bars are means with SEM ( $n = 9$ ). Bars not labeled with same letter are significantly different ( $P < 0.05$ ) from each other according to Tukey's test (SAS Institute 2005).

## CHAPTER 5

### **Effects of Anion Transporter Blockers on Growth, Development, and Survival of Larvae of the European Corn Borer, *Ostrinia nubilalis* (Hübner)**

#### **ABSTRACT**

In this study, four blockers of anion transporters (ATs) belonging to four different classes of organic acids, including DIDS (4, 4'-diisothiocyanatostilbene-2, 2'-disulfonic acid; stilbene disulfonic acid), NPPB [(5-nitro-2-(3-phenylpropylamino) benzoic acid; anthranilic acid)], 9-AC (anthracene-9-carboxylic acid; aromatic carboxylic acid), and IAA-94 (indanyloxy acetic acid; indanyloxy alcanoic acid) were tested for their toxicity against the European corn borer (ECB), *Ostrinia nubilalis* (Hübner 1796). All the AT blockers inhibited the growth of larvae (in weight), increased the developmental time, and decreased survival compared to control, when ECB second-instar larvae were fed for seven days on treated diet. In general, DIDS and NPPB were the most active compounds, with the rank order of activity being DIDS > NPPB > IAA-94 > 9-AC. All the AT blockers decreased the midgut alkalinity in fifth-instar larvae when fed for 3 h on treated diet. Effective concentrations required for 50% decrease in midgut alkalinity (EC<sub>50</sub>) ranged between 29.1 and 41.2 ppm and the rank order of activity was NPPB > DIDS > IAA-94 > 9-AC. Similarly, all the tested AT blockers inhibited chloride ion (Cl<sup>-</sup>) transport from midgut lumen into epithelia in fifth-instar larvae when fed for 3 h on treated diet. Concentrations required for 50% inhibition of Cl<sup>-</sup> uptake from midgut lumen into epithelia (IC<sub>50</sub>) ranged between 7.4 and 11.0 ppm and the rank order of activity was

DIDS > NPPB > 9-AC > IAA-94. Modest to highly strong positive correlations observed among growth, midgut alkalinity, and midgut Cl<sup>-</sup> transport in AT blocker-fed larvae suggested that these are connected to each other. Finally, the AT blockers have the potential to become good candidates for development of insecticides with a unique mode of action.

## INTRODUCTION

Caterpillar midgut alkaline pH, the highest (pH >12) known to date in any biological system, plays a major role in protein digestion and absorption necessary for growth and survival (Berenbaum 1980, Dow 1984, 1992, Johnston and Felton 1996). For instance, midgut alkalinity in caterpillars enhances the solubility of dietary proteins by preventing the formation of protein-tannin complexes and reduces the adverse effects of secondary metabolites from plant material they feed upon (Berenbaum 1980, Dow 1984, 1992, Govenor et al. 1997, Johnston and Felton 1996). Further, caterpillar digestive enzymes, mainly serine proteinases, have adapted to and show maximal activity at high alkaline pH (Anwar and Saleemuddin 2001, Applebaum 1985, Christeller et al. 1992, Houseman and Chin 1995, Houseman et al. 1989, Johnson and Felton 1996). On the other hand, high midgut alkalinity of caterpillars makes them susceptible to the action of biopesticides such as *Bacillus thuringiensis* Berliner 1915 (*Bt*) and baculoviruses (Appel and Schultz 1994, Keating et al. 1990).

Caterpillars maintain metabolically costly midgut lumen alkalinity by continuously transporting acid/base ions across the apical membrane of epithelial cells. Several studies presented evidence for exchange of luminal chloride ion (Cl<sup>-</sup>) for epithelial HCO<sub>3</sub><sup>-</sup> via Cl<sup>-</sup>/HCO<sub>3</sub><sup>-</sup> exchangers (CBEs), in parallel with K<sup>+</sup> transport, for maintaining high alkalinity in midgut (Chamberlin, 1990a, 1990b, Chao et al. 1989, Clark et al. 1998, Coddington and Chamberlin 1999, Dow 1984, 1992, Dow and O'Donnell 1990, Moffett and Cummings 1994). A similar system is present in larvae of the yellow fever mosquito, *Aedes aegypti* (Linnaeus 1762) (Boudko et al. 2001). Previous reports found that insect midgut alkalinity could be decreased by blocking transporters involved in the luminal

alkalization process (Boudko et al. 2001) and that this action adversely affected caterpillar survival (Skibbe et al. 1996). Although effects of decreased gut alkalinity on larval survival were studied with dietary allelochemicals or baculovirus (Govenor et al. 1997, Keating et al. 1990), a detailed investigation on decreased gut alkalinity on larval growth, development, and survival is lacking in the literature.

A wealth of information is available on anion transporter (AT) blockers interaction with Cl<sup>-</sup> channels and exchangers, *in vitro* (Cabantchik and Greger 1992, Kawamata et al. 2006, Machaca et al. 1996, Pedersen et al. 2005, Phipps et al. 1996, Pusch et al. 2002, Spiegel et al. 2003, Uchiyama et al. 2006), but less is known about the activity of AT blockers on insects, *in vivo* (Boudko et al. 2001). Therefore, in the present study, the activity of four AT blockers belonging to four different classes of organic acids and known for blocking Cl<sup>-</sup> channels and exchangers, including DIDS (a stilbene disulfonic acid), NPPB ((5-nitro-2-(3-phenylpropylamino) benzoic acid; an anthranilic acid)), 9-AC (anthracene-9-carboxylic acid; an aromatic carboxylic acid), and IAA-94 (indanyloxy acetic acid; an indanyloxy alkanolic acid) (Fig. 1.1), was tested against an important lepidopteran pest of field and vegetable crops, the European corn borer (ECB), *Ostrinia nubilalis* (Hübner 1796) in no-choice feeding bioassays. The objectives of this study were 1) to determine AT blocker effects on larval growth, development and survival; 2) to measure any AT blocker effect on larval midgut pH; 3) to assess the AT blocker effect on Cl<sup>-</sup> transport in larval midgut; and 4) to determine the relationship among larval growth, midgut alkalinity, and midgut Cl<sup>-</sup> transport in AT blocker-treated ECB larvae.

## MATERIALS AND METHODS

### Chemicals

All the tested AT blockers, DIDS, NPPB, 9-AC, and IAA-94, and dimethyl sulfoxide (DMSO) were purchased from Sigma-Aldrich (St. Louis, MO).

### Effect on ECB Larval Growth, Development, and Survival

For bioassays, ECB larvae (French Agricultural Research, Inc, Lamberton, MN) and MULTIPLESPECIES diet (Southland Products, Inc, Lake Village, AR) were purchased. Larval diet was prepared by adding 162 g of diet mix to 930 ml of boiling water and stirring for 4 min. Three to four milliliter of diet was poured into 45-ml cups and allowed to cool for 30 min under a fume hood. Stock solutions of DIDS, NPPB, 9-AC, and IAA-94 were prepared in DMSO and final concentrations of 6.25, 12.5, 25, and 50 ppm were prepared in 95% ethanol (EtOH). The final DMSO:EtOH ratio was 1:950. One hundred microliters of treatment solution/cup was layered over the diet and the solvent was allowed to evaporate. Cups with solvent (DMSO/EtOH) alone served as control. One ECB second-instar larva per cup was placed on the diet and cups were covered with a lid having perforations for aeration. Cups with larvae were placed in a growth chamber set at  $25 \pm 1^\circ\text{C}$  and  $70 \pm 5\%$  RH and larvae were allowed to feed on treated diet for seven days. After seven days, surviving larvae were transferred to new cups with sufficient untreated diet and incubated until pupation. Each treatment had ten cups. One and 14 days after transferring to normal diet, dead larvae were counted and weights of surviving larvae were recorded. Finally, the number of days for pupation of surviving larvae was recorded. The whole experiment was conducted twice.

### **Effect on ECB Larval Midgut Alkalinity**

Since it is difficult to determine midgut alkalinity precisely in second-instar larvae (used in bioassays) due to their small size, fifth-instars were used in midgut pH measurement experiments. Nevertheless, second-instar larval midgut pH was also estimated using a pH indicator paper method (Shanbhag and Tripathi 2005). In brief, second-instar larvae fed on treated or control diet for 3 h were chilled on ice before dissection. Midguts were removed in physiological saline and placed on a piece of universal pH indicator paper and pressed gently to release the midgut contents. Each pH paper with changed color was scanned and photographed, and compared with pH paper color change obtained with pH standards (1-12). Fifth-instar larval midgut pH was determined following the procedure described by Gringorten et al. (1993) using a combination microelectrode, Model MI-415 (Microelectrodes, Inc., Londonderry, NH). In brief, fifth-instar larvae were starved for 6 h then allowed to feed on treated (12.5, 25, 50, or 100 ppm of AT blocker) or control (DMSO/EtOH) diet in 12-well cell culture plates. After 3 h of feeding, larvae were placed on a filter paper and transected just posterior to the third pair of thoracic legs. The anterior portion of the larva was discarded and a pH microelectrode probe (pre-calibrated with pH standards 4 and 7) was inserted into the midgut, with pH recordings made using a digital pH meter, Model 05669-20 (Cole-Parmer Instrument and Equipment, Co, Vernon Hills, IL). Three pH readings were taken at different points along the midgut from each larva and were averaged. Each treatment pH reading came from five to ten larvae.

### **Effect on ECB Larval Midgut Cl<sup>-</sup> Transport**

Chloride ion transport into midgut epithelia from the lumen and its inhibition by AT

blockers was determined using radioisotope ( $^{36}\text{Cl}^-$  as sodium chloride with 9.3  $\mu\text{Ci}/\text{mg}$  specific activity, purchased from American Radiolabeled Chemicals, Inc., St. Louis, MO) mixed in the diet. For this assay, fifth-instar larvae were starved for 24 h and allowed to feed on treated (3.12, 6.25, 12.5, 25, 50, or 100 ppm of AT blocker) or control (DMSO/EtOH) diet containing 2  $\mu\text{Ci}$  of  $^{36}\text{Cl}^-$  isotope in 6-well cell culture plates. After 3 h of feeding, larvae were chilled on ice, dissected and midguts were removed. Dissected midguts were cut open longitudinally and were washed three times with physiological saline to remove the gut contents and any unbound  $^{36}\text{Cl}^-$ . Washed midguts were individually placed in 7-ml plastic scintillation vials and 0.5 ml of Beckman tissue-solubilizer-450 (Beckman Instruments, Inc, Fullerton, CA) was added and incubated overnight. Then 6 ml of Scintiverse E scintillation cocktail (Fisher Scientific, Pittsburgh, PA) was added to the vials and radioactivity in samples was quantified using a Beckman Coulter LS 6500 multi-purpose scintillation counter (Beckman Coulter, Inc, Fullerton, CA). Each treatment was replicated with three to six larvae.

### **Statistical Analyses**

Mean ( $\pm\text{SEM}$ ;  $n=2$ ) larval weight (in mg) data at 1 and 14 days after feeding on treated diet were converted to percent control and were subjected to probit analysis using the PROC PROBIT program (SAS Institute 2005) to determine the concentration for 50% growth inhibition ( $\text{IC}_{50}$ ). Mean ( $\pm\text{SEM}$ ;  $n=2$ ) percent larval survival and mean ( $\pm\text{SEM}$ ;  $n=2$ ) developmental time (days to pupation) for surviving larvae for each treatment were calculated and were subjected to one-way ANOVA using the PROC GLM program (SAS Institute 2005). Means were separated using Tukey's studentized range test (HSD) at a significance level  $\alpha = 0.05$ .

Midgut Cl<sup>-</sup> uptake (<sup>36</sup>Cl<sup>-</sup> uptake into midgut epithelia in CPM) for each treatment was calculated and converted to percent control. Midgut alkalinity and Cl<sup>-</sup> uptake data were subjected to non-linear regressions using a four parameter logistic equation (Prizm, GraphPad software, San Diego, CA) to estimate the concentration required for 50% decrease in midgut alkalinity (EC<sub>50</sub>), and Cl<sup>-</sup> uptake (IC<sub>50</sub>), respectively, with corresponding 95% confidence intervals (CIs) and hill slopes etc. For Cl<sup>-</sup> uptake, the maximum and minimum values were fixed at 100 and 0, respectively, while for midgut alkalinity, the maximum and minimum pH values were fixed at 9.84 (control larval pH) and 7, respectively, to give the most accurate curve fits. Statistical differences in activity between AT blockers, if any, were determined based on non-overlap of 95% CIs. The relationship among growth, midgut alkalinity, and Cl<sup>-</sup> transport in AT blocker-fed larvae was determined by correlation analysis (SAS Institute 2005) of their respective EC<sub>50</sub> or IC<sub>50</sub> values.

## RESULTS

### **Effect of AT blockers on ECB Larval Growth, Development, and Survival**

All the AT blockers, when fed for seven days, inhibited larval growth. One day after feeding on treated diet, the IC<sub>50</sub> values indicated that DIDS was the most potent for inhibiting the larval growth followed by NPPB, IAA-94, and 9-AC in decreasing order of activity (Table 5.1). Both DIDS and NPPB were significantly more active than 9-AC and IAA-94 based on non-overlap of the 95% CIs (Table 5.1). At 14 days post-feeding, DIDS was still the most active compound and the rank order of activity was similar to that observed 1 day post-feeding (Table 5.1), with DIDS and NPPB being significantly

more active than 9-AC. Nevertheless, 9-AC and IAA-94 were more active than at one day after feeding. Figures 5.1a and 5.1b show the difference in the size of the larvae fed on diet mixed with DIDS at various concentrations or DMSO/EtOH (control) at 1 and 14 days after feeding on treated diet.

All the AT blockers increased the pupation time in treated larvae significantly from control ( $P < 0.05$ ) at 25 ppm (Fig. 5.2), with the exception of 9-AC. Feeding with AT blockers not only affected larval growth and development but also the survival, though at higher concentrations (25 and 50 ppm) (Fig. 5.3). One day post-feeding at 25 ppm, all AT blockers decreased the larval survival, but only NPPB was significantly different from control (Fig. 5.3) and its effect was not significant from the other AT blockers. However, at 14 days post feeding all the AT blockers decreased larval survival significantly from control ( $P < 0.05$ ) with NPPB significantly different from all other AT blockers except DIDS. Decrease in larval survival by DIDS at 14 days was significantly higher than 9-AC (Fig. 5.3). At 50 ppm, all the AT blockers, except 9-AC, decreased larval survival significantly at 1 day post-feeding compared to controls. At 14 days post-feeding, both 9-AC and IAA-94 decreased larval survival significantly from control ( $P < 0.05$ ) but were on par with each other, while no larvae survived in the DIDS and NPPB treatments (Fig. 5.3).

### **Effect on ECB Larval Midgut Alkalinity**

Midgut alkalinity of second-instar larvae ranged between 9-10, 8-9, and 7-8 pH units, respectively, at 12.5, 25, and 50 ppm of AT blocker, while in control larvae it was ~10 pH units (data not shown). In fifth-instar control larvae it was 9.84 ( $\pm 0.07$ ;  $n = 31$ ) pH units. This value was not different from the pH values observed at the lowest

concentration of AT blockers, which were slightly higher (Fig. 5.4). Fifth-instar larval midgut alkalinity in AT blocker-treated larvae was decreased by 0 to 2.6 pH units, depending upon the concentration of AT blocker used (Fig. 5.4). Based on the  $EC_{50}$  values calculated from non-linear regressions (Fig. 5.4), it was clear that DIDS and NPPB were significantly more active in decreasing the midgut alkalinity than 9-AC and IAA-94 (Table 5.2). Based on non-overlapping of 95% CIs, IAA-94 was significantly superior to 9-AC in decreasing midgut alkalinity (Table 5.2).

### **Effect on ECB Larval Midgut $Cl^-$ Transport**

Midguts from control larvae exposed to solvent accumulated, on average, 46.1 ( $\pm 8.4$ ;  $n = 9$ ) nmoles  $^{36}Cl^-$  over the 3 h feeding period. All the tested AT blockers reduced  $Cl^-$  uptake from the lumen to the epithelia in the midgut in a concentration-dependent manner (Fig. 5.5).  $IC_{50}$  values were calculated by non-linear regression and this analysis indicated that DIDS was the most potent blocker of  $Cl^-$  transport, followed by NPPB, 9-AC, and IAA-94 in that order of activity (Table 5.2). However, based on 95% CI overlap, all the AT blockers showed similar potency in decreasing  $Cl^-$  uptake (Table 5.2).

### **Relationship Among Larval Growth, Midgut Alkalinity, and Midgut $Cl^-$ Transport**

In AT blocker-fed larvae, a highly strong positive correlation was observed between growth and midgut alkalinity ( $r = 0.93$ ;  $P = 0.06$ ), while modest positive correlations were seen between midgut alkalinity and midgut  $Cl^-$  uptake ( $r = 0.43$ ;  $P = 0.56$ ) and between growth and midgut  $Cl^-$  uptake ( $r = 0.72$ ;  $P = 0.27$ ). Modest to strong correlations observed between growth or midgut alkalinity and midgut  $Cl^-$  uptake in AT blocker-fed larvae was mainly due to outlying nature of 9-AC. Highly strong positive

correlations observed between growth and midgut Cl<sup>-</sup> uptake ( $r = 0.99$ ;  $P = 0.03$ ) and midgut alkalinity and midgut Cl<sup>-</sup> uptake ( $r = 0.95$ ;  $P = 0.18$ ) without 9-AC supported this conclusion.

## DISCUSSION

In the present investigation, we demonstrated that all the tested AT blockers, when fed for seven days in no-choice feeding bioassays, inhibited the growth, decreased larval survival, and prolonged the developmental time in ECB larvae. The general trend of AT blockers activity against ECB larvae was DIDS > NPPB > IAA-94 > 9-AC, with DIDS and NPPB significantly more active than 9-AC.

In our study, dietary exposure to AT blockers caused growth inhibition in ECB larvae. Since AT blockers are known for inhibiting Cl<sup>-</sup> channels and exchangers (Cabantchik and Greger 1992) and causing a decrease in midgut alkalinity in mosquito larvae (Boudko et al. 2001), we wanted to see if feeding of AT blockers caused any changes in ECB larval midgut alkalinity. All AT blockers tested in our study decreased ECB larval midgut alkalinity in a concentration-dependent fashion. Further, the order of activity of AT blockers (based on IC<sub>50</sub> values) in decreasing midgut alkalinity was more or less similar to that observed in growth inhibition studies. Although for ease of handling we used fifth-instar larvae in pH studies, we found no appreciable difference in midgut alkalinity between second and fifth instars. Similar to the midgut alkalinity decrease observed in ECB larvae with DIDS in our study, exposure to DIDS at 100 μM decreased the highly alkaline midgut pH to neutral within 30 min in *A. aegypti* larvae (Boudko et al. 2001). This study, however, did not report the effects of midgut alkalinity

decrease on larval biology. Similarly, feeding tobacco cutworm, *Spodoptera litura* (Fabricius 1775), larvae with 1 mM valinomycin, a cation transporter blocker, reduced the midgut pH and resulted in 36% mortality in larvae (Skibbe et al. 1996).

Many studies have shown that insect growth and development depends on critical dietary protein digestion and absorption, and inhibiting the activity of digestive proteinases limits larval growth and survival, and increases developmental time (Duncan et al. 2006, Zhu et al. 2007). In caterpillars, availability of dietary proteins for digestion (Berenbaum 1980, Johnson and Felton 1996) and the activity of midgut proteinases were found to be maximal at alkaline midgut pH (Berenbaum 1980, Anwar and Saleemuddin 2001), which favors protein digestion and absorption. Houseman and Chin (1995) reported that in ECB larva the two main serine proteinases involved in protein digestion are trypsin (form I & II), and chymotrypsin. As trypsin I activity is maximal at pH 10 and its function is critical for initial digestion (breaking larger proteins into smaller peptides which later serve as substrates for other proteinases) (Houseman and Chin 1995, Houseman et al. 1989), decreasing the midgut alkalinity results in poor digestion and absorption of dietary proteins. This proposed mechanism explains why at lower concentrations only the growth, and at higher concentrations both growth and survival were affected in ECB larvae, as effects on midgut alkalinity by AT blockers was concentration-dependent. Existence of a highly positive correlation between growth inhibition and midgut alkalinity decrease in AT blocker-treated larvae further corroborates this conclusion.

The necessity of luminal  $\text{HCO}_3^-$  transport for maintaining highly alkaline pH and electroneutrality in caterpillar (*M. sexta*) midgut (Dow 1984, 1992, Chao et al. 1989,

Coddington and Chamberlin 1999, Jungreis et al. 1981, Ridgeway and Moffett 1986) as well as evidence for the role of CBEs in transporting  $\text{HCO}_3^-$  into the midgut lumen in *M. sexta* (Chao et al. 1989) have been presented. Taken together, the present study results suggest that exchange of luminal  $\text{Cl}^-$  for epithelial  $\text{HCO}_3^-$  may be taking place and is a component of midgut luminal alkalization in ECB larvae, although other mechanisms such as  $\text{H}^+$  V-ATPase and  $\text{nH}^+/\text{K}^+$  antiporter complex have been reported in caterpillars (*M. sexta*) elsewhere (Wieczorek et al. 1991, Azuma et al. 1995). Therefore, blocking any of the transporters in these mechanisms could result in midgut alkalinity reduction in caterpillars and mosquito larvae (Boudko et al. 2001, Skibbe et al. 1996). It appears that the observed reduction in ECB larval midgut alkalinity in our study is probably the result of CBEs inhibition by DIDS and other AT blockers. Similar to our study results, blocking of CBEs in *A. aegypti* larvae caused a rapid reduction in midgut alkalinity (Boudko et al. 2001).

Since CBEs are involved in the exchange of luminal  $\text{Cl}^-$  for cellular  $\text{HCO}_3^-$ , we expected that AT blockers would block this  $\text{Cl}^-$  exchange. Accordingly, our feeding experiments with radioisotope ( $^{36}\text{Cl}^-$ ) have shown that all the AT blockers indeed blocked  $\text{Cl}^-$  transport from lumen into epithelia in a concentration-dependent manner. Our feeding experiments, however, do not define whether the observed  $\text{Cl}^-$  transport inhibition was due to the blocking of  $\text{Cl}^-$  channels or exchangers or both. Given the non-specific nature of AT blockers (Cabantchik and Greger, 1992), we assume both  $\text{Cl}^-$  channels as well as exchangers may have been inhibited in  $^{36}\text{Cl}^-$  feeding studies. A positive correlation between midgut alkalinity decrease and  $\text{Cl}^-$  transport inhibition in this investigation suggested the observed alkalinity decrease was due to the inhibition of  $\text{Cl}^-$

/HCO<sub>3</sub><sup>-</sup> exchange between the midgut lumen and epithelia. In a previous study also, DIDS at 100 μM was shown blocking <sup>36</sup>Cl<sup>-</sup> exchange in *M. sexta* embryo cell culture (English and Cantley 1984).

In summary, inhibition of midgut CBEs by AT blockers caused the decrease in ECB larval midgut alkalinity. This in turn probably inhibited digestive proteinases activity, leading to poor digestion and assimilation of dietary proteins and affecting larval growth, development, and survival. These conclusions are based on modest to highly strong positive correlations observed among growth, midgut alkalinity, and midgut Cl<sup>-</sup> transport in AT blocker-treated larvae. Finally, the results of this study indicate that AT blockers are possible leads for developing insecticides for controlling lepidopteran larvae by means of nutrient deficiency.

#### LITERATURE CITED

- Anwar, A., and M. Saleemuddin. 2001. Regulation of digestive proteolytic activity in the larvae of *Spilosoma oblique* (Lep., Arctiidae). *J Appl Entomol* 125: 577-582.
- Appel, H. M., and J. C. Schultz. 1994. Oak tannins reduce effectiveness of Thuricide (*Bacillus thuringiensis*) in gypsy moth (Lepidoptera: Lymantridae). *J Econo Entomol* 87: 1736-1742.
- Azuma, M., W. R. Harvey, and H. Wiczorek. 1995. Stoichiometry of K<sup>+</sup>/H<sup>+</sup> antiport helps to explain extracellular pH 11 in a model epithelium. *FEBS Lett* 36: 153-156.
- Berenbaum, M. 1980. Adaptive significance of midgut pH in larval lepidoptera. *Am Nat* 115: 138-146.

- Boudko, D. Y., L. L. Moroz, W. R. Harvey, and P. J. Linser. 2001. Alkalinization by chloride/bicarbonate pathway in larval mosquito midgut. *Proc Natl Acad Sci USA* 98: 15354-15359.
- Cabantchik, Z. I., and R. Greger. 1992. Chemical probes for anion transporters of mammalian Cell membranes. *Am J Physiol* 262: C803-C827.
- Chamberlin, M. E. 1990a. Ion transport across the midgut of the tobacco hornworm (*Manduca sexta*). *J Exp Biol* 150: 425-442.
- Chamberlin, M. E. 1990b. Luminal alkalinization by the isolated midgut of the tobacco hornworm (*Manduca sexta*). *J Exp Biol* 150: 467-471.
- Chao, A.C., A. R. Koch, and D. F. Moffett. 1989. Active chloride transport in isolated posterior midgut of tobacco hornworm (*Manduca sexta*). *Am J Physiol* 257: R752-R761.
- Christeller, J. T., W. A. Laing, N. P. Markwick, and E. P. J. Burgess. 1992. Midgut protease inhibitors in 12 phytophagous lepidopteran larvae: dietary and protease inhibitor interactions. *Insect Biochem Mol Biol* 22: 735-746.
- Clark, T. M., A. R. Koch, and D. F. Moffet. 1998. Alkalinization by *Manduca sexta* anterior midgut in vitro: requirements and characteristics. *Comp Biochem Physiol A* 121: 181-187.
- Coddington, E. J., and M. E. Chamberlin. 1999. Acid/base transport across the midgut of the tobacco hornworm, *Manduca sexta*. *J Insect Physiol* 45: 493-500.
- Dow, J. A. T. 1984. Extremely high pH in biological systems: a model for carbonate transport. *Am J Physiol* 246: R633-R635.
- Dow, J. A. T. 1992. pH gradients in lepidopteran midgut. *J Exp Biol* 172:355-375.

- Dow, J. A. T., and M. J. O'Donnell. 1990. Reversible alkalization by *Manduca sexta* midgut. *J Exp Biol* 150: 247-256.
- Duncan, A., H. Ren, F. Bound, J. Tully, D. S. Chandler, and R. M. Sandeman. 2006. Assessment of novel inhibitors of *Helicoverpa* aminopeptidases as anti-insect agents. *Pest Manag Sci* 62: 1098-1108.
- Gringorten, J. L., D. N. Crawford, and W. R. Harvey. 1993. High pH in the ectoperitrophic space of the larval lepidopteran midgut. *J Exp Biol* 183: 353-359.
- English, L. H., and L. C. Cantley. 1984. Characterization of monovalent ion transport systems in an insect cell line (*Manduca sexta* embryonic cell line CHE). *J Cellular Physiol* 121: 125-132.
- Govenor, H. L., J. C. Schultz, and H. M. Appel. 1997. Impact of dietary Allelochemicals on gypsy moth (*Lymantria dispar*) caterpillars: importance of midgut alkalinity. *J Insect Physiol* 43: 1169-1175.
- Houseman, J. G., and P. S. Chin. 1995. Distribution of digestive proteinases in the alimentary tract of the European corn borer, *Ostrinia nubilalis* (Lepidoptera: Pyralidae). *Arch Insect Biochem Physiol* 28: 103-111.
- Houseman, J. G., B. J. R. Philogene, and A. E. R. Downe. 1989. Partial Characterization of proteinase activity in the larval midgut of the European corn borer, *Ostrinia nubilalis* Hübner (Lepidoptera: Pyralidae). *Can J Zool* 67: 864-868.
- Johnson, K. S., and F. W. Felton. 1996. Potential influence of midgut pH and redox potential on protein utilization in insect herbivores. *Arch Insect Biochem Physiol* 32:85-105.

- Jungreis, A. M., N. D. Barron, and J. W. Johnston. 1981. Comparative properties of tobacco hornworm, *Manduca sexta*, carbonic anhydrases. *Am J Physiol* 241: R92-R99.
- Kawamata, K., H. Hayashi, and Y. Suzuki. 2006. Chloride-dependent bicarbonate secretion in the mouse large intestine. *Biomed Res* 27: 15-21.
- Keating, S. T., J. C. Schultz, and W. G. Yendol. 1990. The effect of diet on gypsy moth (*Lymantria dispar*) larval midgut pH, and its relationship with larval susceptibility to a baculovirus. *J Invert Pathol* 56: 317-326.
- Machaca, K., L. J. DeFelice, and S. W. L'Hernault. 1996. A novel chloride channel localizes to *Caenorhabditis elegans* spermatids and chloride channel blockers induce spermatid differentiation. *Dev Biol* 176: 1-16.
- Moffett, D. F., and S. Ann Cummings. 1994. Transepithelial potential and alkalization in an *in situ* preparation of tobacco hornworm (*Manduca sexta*) midgut. *J Exp Biol* 194: 341-345.
- Pedersen, P. H., F. D. Paoli, and O. B. Nielsen. 2005. Increased excitability of acidified skeletal muscle: role of chloride conductance. *J. Gen Physiol* 125: 237-246.
- Phipps, D. J., D. R. Branch, and L. C. Schlichter. 1996. Chloride-channel block inhibits T lymphocyte activation and signaling. *Cell Signal* 8: 141-149.
- Pusch, M., A. Accardi, A. Liantonio, P. Guida, S. Traverso, D. C. Camerino, and F. Conti. 2002. Mechanisms of block of muscle type CLC chloride channels (Review). *Mol Membr Biol* 19: 285-292.
- Ridgeway, R. L., and D. F. Moffett. 1986. Regional differences in the histochemical localization of carbonic anhydrase in the midgut of tobacco hornworm (*Manduca*

- senta*). *J Exp Zool* 237: 407-412.
- SAS Institute. 2005. The SAS system version 9.1 for Windows. SAS Institute, Cary, NC.
- Shanbhag, S., and S. Tripathi. 2005. Electrogenic H<sup>+</sup> transport and pH gradients generated by a V-H<sup>+</sup>-ATPase in the isolated perfused larval *Drosophila* midgut. *J Membr Biol* 206: 61-72.
- Skibbe, U., J. T. Christeller, P. T. Callaghan, C. D. Eccles, and W. A. Laing. 1996. Visualization of pH gradients in the larval midgut of *Spodoptera litura* using <sup>31</sup>P-NMR microscopy. *J Insect Physiol* 42: 777-790.
- Spiegel, S., M. Phillipper, H. Rossmann, B. Riederer, M. Gregor, and U. Seidler. 2003. Independence of apical Cl<sup>-</sup>/HCO<sub>3</sub><sup>-</sup> exchange and anion conductance in duodenal HCO<sub>3</sub><sup>-</sup> secretion. *Am J Physiol Gastrointest Liver Physiol* 285: G887-G897.
- Uchiyama, H., H. Hayashi, Y. Suzuki. 2006. Functional characterization of Cl<sup>-</sup>/HCO<sub>3</sub><sup>-</sup> exchange in villous cells of the mouse ileum. *Biomed Res* 27: 265-274.
- Wieczorek, H., M. Putzenlechner, W. Zeiske, and U. Klein. 1991. A vacuolar-type proton pump energizes K<sup>+</sup>/H<sup>+</sup> antiport in an animal plasma membrane. *J Biol Chem* 266: 15340-15347.
- Zhu, Y. C., C. A. Abel, and M. S. Chen. 2007. Interaction of Cry1 Ac toxin (*Bacillus thuringiensis*) and proteinase inhibitors on the growth, development, and midgut proteinase activities of the bollworm, *Helicoverpa armigera*. *Pesti Biochem Physiol* 87: 39-46.

Table 5.1. Effect of anion transporter blockers on *Ostrinia nubilalis* larval growth

Days after feeding	Compound	No. insects (N)	<sup>a</sup> IC <sub>50</sub> (ppm)	<sup>b</sup> 95% CI	Slope ± SEM	$\chi^2$ (df) <sup>c</sup>
<sup>d</sup> 1	DIDS	70	17.0	15.1-19.2	2.7 ± 0.2	0.8 (2)
	NPPB	70	18.3	16.1-20.8	2.6 ± 0.2	0.5 (2)
	9-AC	76	31.6	27.8-36.8	2.7 ± 0.3	0.2 (2)
	IAA-94	72	25.0	21.7-29.3	2.2 ± 0.2	1.9 (2)
<sup>d</sup> 14	DIDS	62	18.0	15.8-21.0	3.0 ± 0.4	1.4 (1)
	NPPB	61	18.9	16.3-22.6	2.7 ± 0.3	0.1 (1)
	9-AC	72	26.8	24.1-30.1	3.4 ± 0.3	2.0 (2)
	IAA-94	68	19.6	12.6-32.2	3.0 ± 0.4	4.9 (2)

<sup>a</sup> Inhibitory concentration required for 50% reduction in growth (weight).

<sup>b</sup> 95% confidence intervals for IC<sub>50</sub>.

<sup>c</sup> Chi-square goodness-of-fit statistic and degrees of freedom.

<sup>d</sup> Growth inhibition measured at one and 14 days after second-instar larvae were fed on AT blocker-treated diet for seven days.

Table 5.2. Effect of anion transporter blocker on *Ostrinia nubilalis* larval midgut alkalinity and chloride ion transport

Parameter	Compound	No. insects ( <i>N</i> )	<sup>a</sup> EC <sub>50</sub> or IC <sub>50</sub> (ppm)	<sup>b</sup> 95% CI	Hill Slope ± SEM	<i>R</i> <sup>2</sup> (df) <sup>c</sup>
<sup>d</sup> Alkalinity	DIDS	32	29.3	27.3-31.5	2.5 ± 0.2	0.94 (30)
	NPPB	31	29.1	26.1-32.5	2.0 ± 0.2	0.86 (29)
	9-AC	31	41.2	38.5-44.0	2.4 ± 0.2	0.95 (29)
	IAA-94	32	35.1	32.7-37.7	2.4 ± 0.2	0.94 (30)
<sup>d</sup> Cl <sup>-</sup> transport	DIDS	21	7.4	6.3-8.8	1.3 ± 0.1	0.91 (19)
	NPPB	21	8.2	6.2-10.8	1.3 ± 0.3	0.77 (19)
	9-AC	21	9.7	7.7-12.2	1.5 ± 0.3	0.84 (19)
	IAA-94	21	11.0	8.5-14.1	1.4 ± 0.3	0.80 (19)

<sup>a</sup> Effective or inhibitory concentration required for 50% decrease in midgut alkalinity or 50% inhibition in midgut Cl<sup>-</sup> uptake.

<sup>b</sup> 95% confidence intervals for IC<sub>50</sub>.

<sup>c</sup> Goodness-of-fit statistic and degrees of freedom.

<sup>d</sup> Midgut alkalinity and Cl<sup>-</sup> uptake from midgut lumen into epithelia were measured in fifth-instar larvae 3 h after feeding on AT blocker-treated diet.

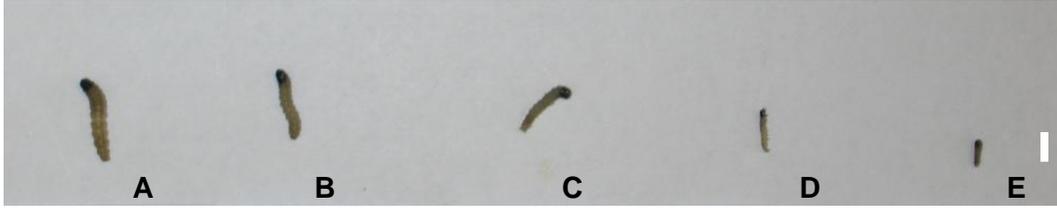


Fig. 5.1a. Reduction in *Ostrinia nubilalis* larval size fed with DIDS-amended diets. Second-instar larvae were fed with diet containing either control (A) or DIDS at 6.25 (B), 12.5 (C), 25 (D), and 50 ppm (E) for seven days and larval size was measured one day after feeding. Bar = 3 mm.

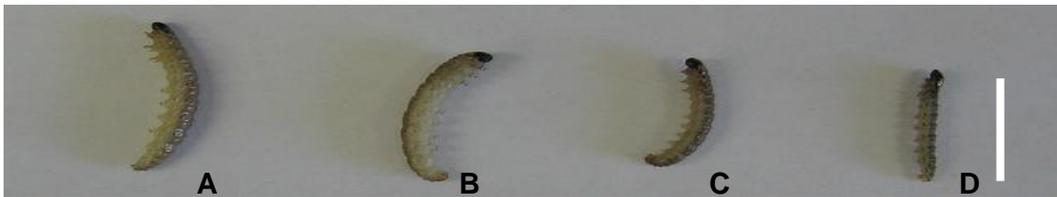


Fig. 5.1b. Reduction in *Ostrinia nubilalis* larval size fed with DIDS-amended diets. Second-instar larvae were fed with diet containing either control (A) or DIDS at 6.25 (B), 12.5 (C), 25 (D), and 50 ppm for seven days and larval size was measured 14 days after feeding. Bar = 10 mm. Larvae fed with 50 ppm of DIDS did not survive the 14 days period after feeding.

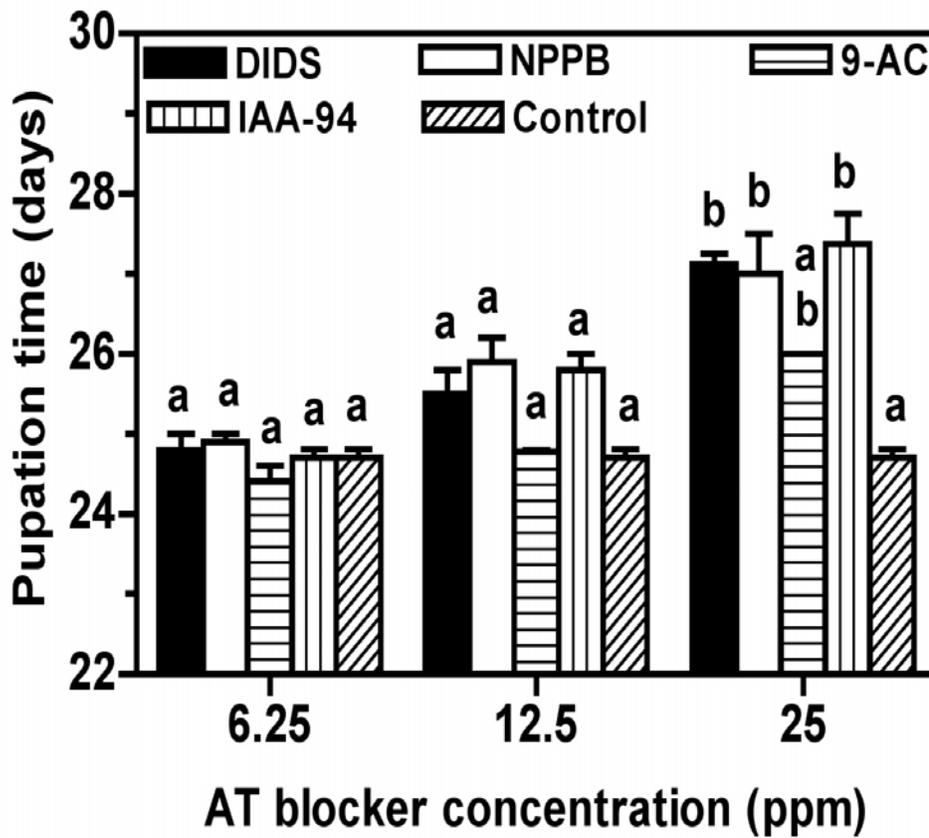


Fig. 5.2. Effect of anion transporter blockers on *Ostrinia nubilalis* larval development (pupation time). Bars are means with SEM ( $n = 2$ ). Bars not labeled by the same letter at a given concentration are significantly different ( $P < 0.05$ ) from each other according to Tukey's test (SAS Institute 2005).

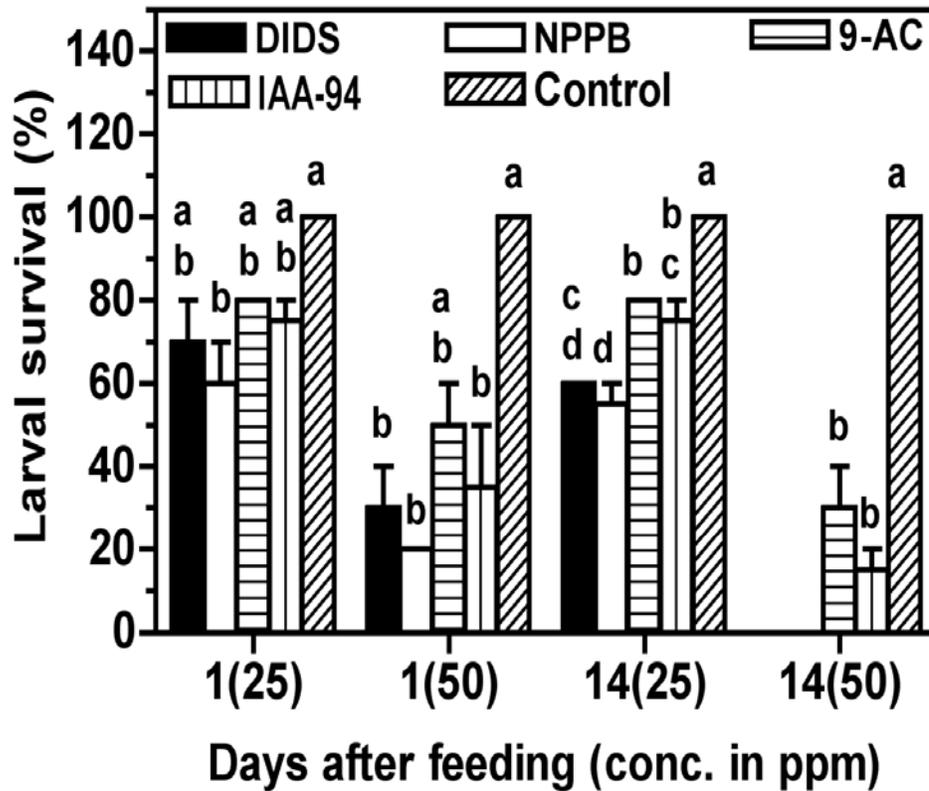


Fig. 5.3. Effect of anion transporter blockers on *Ostrinia nubilalis* larval survival. Bars are means with SEM ( $n = 2$ ). Bars without SEM indicate identical % survival. Absence of bar indicates 0% survival. Bars not labeled by the same letter at a given time and concentration are significantly different ( $P < 0.05$ ) from each other according to Tukey's test (SAS Institute 2005).

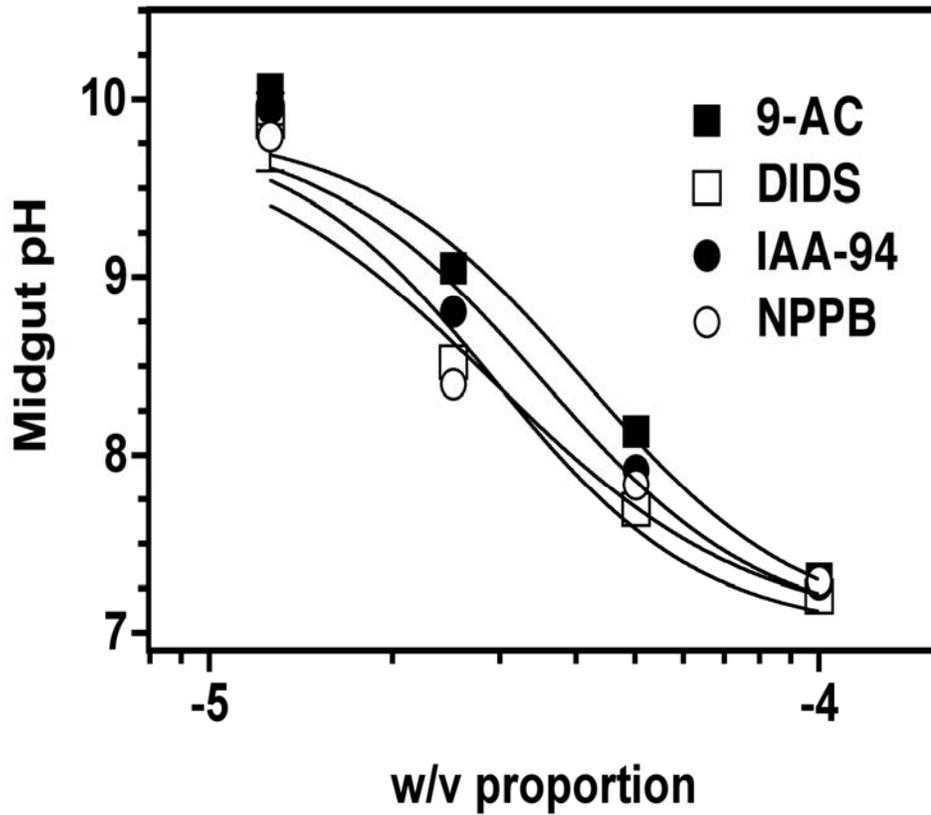


Fig. 5.4. Dose-dependent effect of anion transporter blockers based on *Ostrinia nubilalis* larval midgut alkalinity. Symbols are means with SEM ( $n = 5-10$ ). In almost all cases, SEM bars are so small that they reside within the size of the respective symbol. For reference,  $10^{-5}$  w/v proportion = 10 ppm and  $10^{-4}$  w/v proportion = 100 ppm.

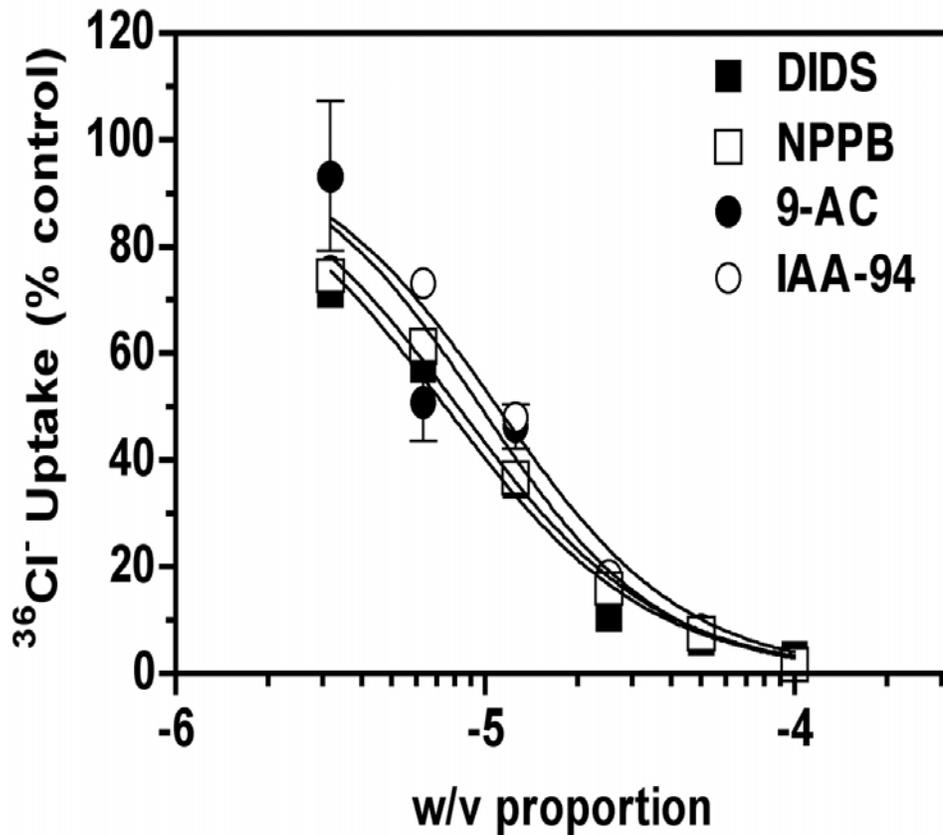


Fig. 5.5. Dose-dependent effect of anion transporter blockers based on *Ostrinia nubilalis* larval midgut chloride ion transport. Symbols are means with SEM ( $n = 3-6$ ). For clarity of presentation, SEM bars were removed except for those associated with 9-AC, which produced the most variable responses. The data variability for the other compounds was less. For reference,  $10^{-5}$  w/v proportion = 10 ppm and  $10^{-4}$  w/v proportion = 100 ppm.

## CHAPTER 6

### SUMMARY

The present investigations were aimed at evaluating anion transporters (ATs) such as voltage-gated chloride channels (VGCCs) and chloride/bicarbonate ( $\text{Cl}^-/\text{HCO}_3^-$ ) exchangers (CBEs) in insects and nematodes as potential target sites for developing new pesticides. Four AT blockers belonging to four different chemical classes of organic acids were selected to work with in my dissertation research. These were DIDS (4, 4'-diisothiocyanatostilbene 2, 2'-disulfonic acid), NPPB (5-nitro-2-[3-phenylpropylamino] benzoic acid), 9-AC (anthracene-9-carboxylic acid), and IAA-94 (indanyloxy acetic acid) (Fig. 1.1). Toxicity and site/mode of action of these four AT blockers in insects and nematodes were determined using toxicological, electrophysiological, and molecular techniques.

In the first objective of my dissertation, the toxicity of AT blockers against a plant-parasitic root-knot nematode, *Meloidogyne incognita* (Kofoid and White 1919) Chitwood 1949, a free-living soil nematode, *Caenorhabditis elegans* Maupas 1900, and an entomopathogenic nematode, *Heterorhabditis bacteriophora* Poinar 1975 was determined in whole organism bioassays. All the four AT blockers showed slowly developing nematocidal activity against second-stage larvae of *M. incognita* and adults of *C. elegans* with increased pharyngeal muscle contractions and decreased locomotion as toxic symptoms in adult *C. elegans*. The concentration required for 50% mortality was <10 ppm in all the AT blockers for both sensitive species after 168 h of incubation. In contrast, none of the AT blockers were toxic to third-stage larvae of *H. bacteriophora* at

200 ppm. Based on the toxic symptoms observed, it was hypothesized that among the six types of VGCCs expressed in *C. elegans*, CeCIC-1 and CeCIC-2 could be the target sites for AT blockers in nematodes. Since AT blockers were not toxic to third-stage larvae of *H. bacteriophora*, cloning and sequencing of *ceclc-2*, which codes for CeCIC-2 channel protein, the main proposed target site for AT blockers, in *H. bacteriophora* would shed some light on factors that contribute to its resistance to AT blockers i.e. any gene mutations. Increased electrophysiological activity from pharyngeal muscle cells of *C. elegans* treated with AT blockers would further confirm that AT blockers inhibitory action on CeCIC-2 channels of pharynx led to an increase in pharyngeal muscle contractions.

The second objective of my dissertation was focused on confirming the above hypothesis that VGCCs, CeCIC-1 and CeCIC-2 are the target sites for AT blockers in nematodes using RNA interference (RNAi) studies. Reducing the expression of *ceclc-1* alone (gene coding for CeCIC-1 channel protein) had no effect on pharyngeal muscle contractions and locomotion. In contrast, reducing the expression of *ceclc-2* alone (gene coding for CeCIC-2 channel protein) increased pharyngeal muscle contractions and affected locomotion significantly in a concentration-dependent manner. Reducing the expression of both the genes together produced same effect on pharyngeal contractions as *ceclc-2* alone. In contrast, reducing the expression of both the genes together showed significantly higher effect on locomotion than *ceclc-2* alone. These experiments revealed that CeCIC-2 channels are necessary for nematode pharyngeal contractions and locomotion, while CeCIC-1 channels may have an indirect role in nematode locomotion. These findings supported that CeCIC-2 channels are the major target sites for AT

blockers. Further, observed nematicidal activity of AT blockers was the result of CeCIC-2 channels inhibition as toxic symptoms of AT blocker were comparable to phenotypic effects observed with reduced expression of *ceclc-2*. Performing similar RNAi experiments targeting CeCIC-1 and CeCIC-2 channel genes in *M. incognita* and observing phenotypic effects (locomotion) similar to that in *C. elegans* would tell that these genes are present in *M. incognita* too, and have the same physiological roles.

In the third objective of my dissertation, insecticidal and neurophysiological activities of AT blockers were determined using pomace fly, *Drosophila melanogaster* Meigen 1830. Similar to their nematicidal activity, all the AT blockers showed slowly developing toxicity against adults of a susceptible *D. melanogaster* strain (Oregon-R) as the mortality crossed 50% only 48 h after treatment. DIDS showed a similar toxicity against adults of a dieldrin-resistant *D. melanogaster* strain (*rdl*). The AT blockers were mainly detoxified by the P<sub>450</sub> monooxygenases in *D. melanogaster* as addition of a mixed function oxidases inhibitor, piperonyl butoxide (PBO), increased the toxicity of AT blockers by 2.0- to 3.5-fold. At 100  $\mu$ M, all the AT blockers, except 9-AC significantly increased central motor nerve firing in third-instar larvae but disruption of the blood brain barrier (BBB) was required for this action. Further, DIDS did not inhibit  $\gamma$ -aminobutyric acid (GABA) receptor Cl<sup>-</sup> channel complex. These findings suggested that inhibition of neither ATs nor GABA receptors in central nervous system (CNS) was responsible for AT blockers toxicity. However, a modest excitatory effect of DIDS on peripheral sensory nerves of third-instar larvae and a decrease in midgut alkalinity of DIDS-fed flies were observed. These findings indicated that the observed toxicity of AT blockers against adult flies was mainly the result of their inhibitory action on ATs of non-CNS

tissue. ATs, probably CBEs, present on the midgut epithelial cell membranes could be the major target sites for AT blockers in *D. melanogaster* and inhibition of which might have decreased midgut alkalinity. Changes in midgut alkalinity could lead to disruption of digestion and starvation of flies to death. Recording electrophysiological activity from neuromuscular junctions (NMJs) of *Drosophila* larvae treated with AT blockers would tell whether AT blockers acting on NMJs or not.

In the fourth objective of my dissertation, the toxicity and site/mode of action of AT blockers were determined against an important lepidopteran pest, the European corn borer, *Ostrinia nubilalis* (Hübner 1796). Feeding second-instar larvae on AT blocker-treated diet for seven days showed dose-dependent lethal and sublethal effects by inhibiting growth (weight gain), increasing developmental time (no. days for pupation), and decreasing larval survival at the higher concentrations tested. The general rank order of activity was DIDS > NPPB > IAA-94 > 9-AC. Similarly, feeding fifth-instar larvae on AT blocker-treated diet for 3 h decreased midgut alkalinity and inhibited chloride ion ( $\text{Cl}^-$ ) uptake from midgut lumen into epithelia. Further, positive associations were observed among growth, midgut alkalinity, and midgut  $\text{Cl}^-$  transport in AT blocker-fed larvae. These findings suggested that inhibition of CBEs on midgut epithelial cells by AT blockers prevented the exchange of luminal  $\text{Cl}^-$  for epithelial  $\text{HCO}_3^-$  that could have led to the decrease in midgut alkalinity. Decrease in midgut alkalinity may have adversely affected proteinases function disrupting the digestion and absorption of proteins. This led to observed lethal and sublethal effects on larvae. Performing anion replacement and the isotope exchange experiments in presence or absence of AT blockers on isolated midgut

tissue from *O. nubilalis* or *Drosophila* larvae using Ussing chamber would further substantiate the above conclusions.

The LC<sub>50</sub> values of AT blockers tested in this study reached below 10 ppm against sensitive nematode species with seven day exposure, while they were about 100 and 50 ppm against *D. melanogaster* adults and *O. nubilalis* larvae. In order to be used in the field as pesticides against target pests, the activity of these compounds needs to be improved. This can be achieved by chemical substitution of the molecules. For example, a stilbene compound, 2-hydroxy-4'-methoxy stilbene (Fig. 1.1), having hydroxyl group at 2 position and methoxy group on 4' position increased its toxicity enormously against pine wood nematode (Suga 1994). Therefore, chemical substitutions of AT blockers with fluorine, chlorine, bromine, hydroxyl, methoxy, and cyanide etc. groups at different position on the phenyl rings and determining the structure-activity relationships would assist in finding suitable chemical substituents that will enhance the toxicity of these compounds against target organisms.

Since these compounds can be used for controlling both below ground and above ground pests, other characteristics such as photostability, thermostability, binding to soil particles, and soil or foliage residual activity are important. All these characters of a compound help to maintain its effects longer in the field. Therefore, a formulation that satisfies all these requirements will be an ideal one and the most suitable formulation may be an emulsifiable concentrate. Since most contact nematicides are applied via drip lines, emulsifier keeps the compound in a suspended state in water, aiding the uniform spread in soil (Martin 2003). Similarly, for aerial application, apart from emulsifiers other

surfactant agents that spread and retain the compound on plant aerial parts for longer time periods could be added to improve its efficacy.

#### LITERATURE CITED

- Martin, F. N. 2003. Development of alternative strategies for management of soil borne pathogens currently controlled with methyl bromide. *Annu Rev Phytopathol* 41: 325-350.
- Suga, T. 1994. Pest control chemicals against pine wood nematodes. US Patent No. 05314693.