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Robert L. Tanguay

Dithiocarbamates are widely used agricultural pesticides, industrial chemicals and effluent additives. DTCs and their related compounds have historical and current relevance in clinical and experimental medicine. DTC developmental toxicity is well established, but poorly understood. Dithiocarbamates according to the U.S. EPA have a mechanism of action involving, "the inhibition of metal-dependent and sulfhydryl enzyme systems in fungi, bacteria, plants, as well as mammals." We hypothesized that by using the zebrafish development model we could better define the mechanism of action of DTCs and for the first time establish a molecular understanding of DTC developmental toxicity in vertebrates. We have established that all types of dithiocarbamate pesticides and some degradation products have the potential to elicit a common toxic effect on development resulting in a distorted notochord and a significant impact to the body axis. We provide evidence to support the hypothesis that metal chelation is not the primary mechanism of action by which DTCs impact the developing vertebrate. By manipulating the exposure window of zebrafish we hypothesized that somitogenesis was the targeted developmental process. We tested this by using the

Affymetrix microarray to observe gene expression induced by the N-methyl dithiocarbamate, metam sodium (NaM). Throughout this process it is clear that genes related to muscle development are perturbed. These gene signatures are consistent with the morphological changes observed in larval and adult animals and that somitogenesis is the developmental target. Novel findings include the targeting of many redox sensitive targets and a possible role for the TGFβ signaling pathway. Thiol status is a critical modifying factor in DTC developmental toxicity but this toxicity does not result from dramatic cell death. It is possible this outcome is reached by DTCs and its primary degradation products through several pathways. Taken together we can hypothesize that the development mechanism of action of DTCs involves the depletion, oxidation, or adduct formation of critical thiols in the somites of developing vertebrates. It is likely copper plays some role but it is not the target. The proximate toxicant is likely a DTC mixture of parent and degradation products acting to alter the redox thiol state of the animal.

Developmental Ramifications of Dithiocarbamate Pesticide Exposure in Zebrafish

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APPROVED:

Redacted for Privacy

Major Professor, representing Toxicology

Redacted for Privacy

Head of the Department of Environmental and Molecular Toxicology

Redacted for Privacy

Dean of the Graduate School

I understand that my dissertation will become part of the permanent collection of Oregon State University libraries. My signature below authorizes release of my dissertation to any reader upon request.

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Fred Tilton, Author

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provided a research environment where my only limitation was myself. This was despite the difficult task of moving and establishing his laboratory at Oregon State University during my time in his laboratory. I am thankful he mentored me as a colleague and friend and this was my greatest lesson. Finally, I would like to thank my wife and colleague Susan C. Tilton. This dissertation and so many other things would not have been possible without Susan's love, support, and understanding.

CONTRIBUTION OF AUTHORS

This dissertation provided opportunities for two Oregon State University undergraduate to gain state-of-the-art laboratory experience. Meng Vue, Zoology, was a work study student that maintained our zebrafish colony in 420 Weniger Hall and expressed a desire for doing work at the bench. During the Summer of 2004 Meng assisted me as an hourly employee in the collection, scoring, and exposure set-up of the initial metal-dithiocarbamate exposures. Over 3 terms Noor Alzaarban, Biochemistry and minor in Toxicology, assisted me in the completion of the in situ comparisons of dithiocarbamates, neucuproine, and *leviathon* animals. Noor was also instrumental in the screening and establishment of the *leviathon* mutant zebrafish line in the Tanguay Laboratory. Jane LaDu contributed directly by conducting the micro-injections of GCLc morpholino experiments. As the lab coordinator of the Tanguay Laboratory, Jane was the primary contact regarding the stocks of in situ probes, immunohistochemistry antibodies, and zebrafish used in this dissertation. Jane and I were trained in 2003 by Drs. Melissa Haendel (post-doctoral researcher, Bailey Laboratory) and Robert L . Tanguay on many of the staple methods of the Tanguay Laboratory. Dr. Melissa Haendel and I co-authored the first manuscript on this subject from Dr Tanguay's laboratory. Succinctly, my contributions were to the pesticide toxicology aspects of those studies, while Dr. Haendel's contributions were the molecular biology of the zebrafish model. Finally, as the PI of these projects, Robert L. Tanguay was integral in the direction and focus of the studies found in this dissertation. These data were used as the principal support toward the submission of Northwest Health Foundation and NIH grants from his

laboratory. This project also aided the collaborative integration of the Tanguay Laboratory into the large number of investigators affiliated with the Department of Environmental and Molecular Toxicology at Oregon State University and other multi-institutional collaborations with the laboratory.

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Chapter 1. Introduction

The dithiocarbamate (DTC) chemical class has many important uses as agricultural pesticides, chemical precursors and effluent additives as well as its use in experimental and clinical medicine. Industries use DTCs for rubber vulcanization and as chemical precursors in large quantities (WHO 1998). Nearly 30 million pounds of DTC are applied annually to apple, strawberry and potato fields alone in addition to many other sites of application (U.S.G.S. 2006). Two DTCs, metam sodium (NaM) and mancozeb, were the third and twentieth most used pesticides in the United States in 2001 (U.S.G.S. 2006). Currently, many DTC pesticides are close to completing pesticide re-registration eligibility decision (RED) as mandated by the Food Quality Protection Act in 1996. However, there are regulatory data gaps related to reproductive and developmental endpoints for many DTCs in U.S. EPA Integrated Risk Information System (IRIS) and Environmental Fate and Effects documents (U.S.EPA 2005a, b).

Our laboratory observed that exposure of zebrafish to NaM during specific developmental stages resulted in a developmental abnormality that was a rapid and easily identifiable biomarker of effect (Haendel *et al.* 2004). We further exploited this toxic effect by comparing subclasses of DTCs and their metabolites to help determine the likely proximate toxicants (Chapter 2). From those studies we described a common toxic effect on development for DTCs and then moved forward by identifying molecular

targets of toxicity using NaM (Chapters 3 and 4). NaM provided a useful DTC surrogate because, while complex, there are fewer potential NaM degradation products compared to other DTC subclasses. The primary degradation product of NaM is methylisothiocyanate (MITC; Fig 1-1). Many DTCs and isothiocyanates are naturally occurring and are under active investigation as potent anti-inflammatory and anticarcinogenic agents (Callaway *et al.* 2004; Nurmi *et al.* 2004; Parodi *et al.* 2005; Zhang 2000). This allowed us to explore the toxic effects of these two distinct chemical classes in the zebrafish development model in order to better understand how DTCs perturb normal development.

Dithiocarbamates

The focus of studies found in Chapter 2 was on the three subclasses of DTC pesticides which can be segregated based on their unique degradation pathways (U.S.EPA 2001). The most well known of these subclasses are the ethylene-bis-dithiocarbamates (EBDC; Fig 1-2). EBDCs are polymeric structures and this feature increases their relative environmental stability and lowers the acute toxicity relative to other DTC pesticides. EBDCs form numerous unique degradation products one of which, ethylenethiourea (ETU), has garnered significant attention due its wide environmental occurrence. ETU occurrence was an important consideration in the U.S. EPA designation of the EBDC subclass as having a common mechanism leading to thyroid cancer (U.S.EPA 2001) (Fig 1-2). Thus, all EBDCs are regulated based on their ablity to degrade into ETU.

The alkyl-dithiocarbamates have the most number of uses and these compounds have a wide variety of toxicities mediated by the parent compounds and degradation products (Fig 1-3) (U.S.EPA 2004a, b). The alkyl-dithiocarbamates are used in either their parent or disulfide forms and are also generally considered to be of low acute toxicity and of limited environmental persistence (Extoxnet 1996). Interestingly, several of these DTCs are also known to cause thyroid cancer in laboratory animals but are not considered to have a common mechanism because their mechanism of toxicity is unknown and they do not form ETU (U.S.EPA 2001). Several examples of this subclass were investigated and will be discussed in Chapter 2.

The N-methyl dithiocarbamate, NaM, forms the third unique subclass because as a pro-pesticide it forms methylisothiocyante (MITC) when applied, for example, as a fumigant to pre-plant fields (Greenbook 2000). This primary degradation product is unlike other DTCs because of the presence of a single subsistent on the nitrogen in the DTC moiety (Fig 1-1). All the subclasses have the ability to form carbon disulfide (CS_2) in various quantities so this was also evaluated. CS_2 (or its adducts) are typically measured in determining DTC exposure in humans because of its shared nature to DTCs and many analytical limitations (U.S.EPA 2001).

Isothiocyanates

Our first collaborative investigation on the developmental effects of DTCs began with NaM and MITC (Haendel *et al.* 2004). We found that both NaM and MITC exhibited near identical dose-response curves for an endpoint involving a distortion of the embryonic notochord in zebrafish. Preliminary studies were unsuccessful at

differentiating the toxic effects of the two compounds by manipulating experimental conditions (Fig 1S-1, Table 1S-1). Therefore, we examined a structurally diverse set of isothiocyanates (ITCs) in Chapter 2 (Fig 1-4). ITCs are naturally occurring in several plant species often consumed in the human diet (e.g. allyl ITC and sulforaphane). Cover crops such as mustard which produce ITCs have also been considered as alternatives to fumigants such as NaM (McGuire 2003). ITCs are also under extensive study as cancer chemopreventative agents for some forms of cancer (Callaway *et al.* 2004). There is an inversely proportional relationship between the complexity of the ITC structure and their ability to form superoxide by inhibiting NADPH oxidase in cell culture. The much larger benzyl-ITC also has the ability to act as a strong electrophile toward sulfyhdryl groups (Miyoshi *et al.* 2004). Considering these two chemicals come from structurally distinct chemical classes and pesticide regulation of this class is heavily based on structural similarity, then improving our understanding of the proximate toxicant is important for regulatory interests and necessary to better define the mechanism of development toxicity from DTCs.

Dithiocarbamate Toxicological Effects

Acute and Chronic Toxicity

DTCs are considered *practically non-toxic* to *low in toxicity* through oral, dermal, and inhalation routes under acute exposure to adult animals (Extoxnet 1996; U.S.EPA 2001). Acutely intoxicated rats and mice exhibit poor skeletal muscle control, hyperactivity followed by inactivity, loss of muscle tone, convulsions and death. The

most likely acute hazard to humans from a DTC exposure comes from the corrosive and irritant properties of DTCs, which is reflected in the regulations of these compounds (CAL-EPA 2002). DTC immunotoxicity is one of the more active research areas of DTC toxicity. A recent study has revealed that DTC inhibits cytokine production by a MAP kinase-dependent mechanism and may prove important to our future studies (Pruett *et al.* 2005b). Data in Chapter 3 highlights some effects of NaM on immunotoxicity and perhaps provide an avenue for future investigations between these two fields.

In chronic exposure scenarios, DTCs had little effect on non-cancerous endpoints in mammals and those effects that were observed were at doses that are not considered to pose a human health risk under most circumstances (Extoxnet 1996). Several epidemiology reports, however, have suggested a link between occupational DTC use and a variety of outcomes including neuropathies, immune dysfunction, Parkinson's disease and risks of spontaneous abortions (Cory-Slechta *et al.* 2005; Garry *et al.* 2002a; Garry *et al.* 2002b; HSDB). In regulatory studies with the DTC disulfide, thiram, and the EBDC, mancozeb, rats treated with 52 to 67 mg/kg/day for 80 weeks produced symptoms of muscle incoordination and paralysis which has been shown to be associated with degeneration of nerves (Extoxnet 1996). DTC and the major degradation product, CS_2, independently forms cysteine-thiol adducts *in vivo* (Tonkin *et al.* 2004). However, direct cysteine carbamolyation of proteins does not appear to be associated with disulfuram-induced myelin lesions found in DTC-induced peripheral sensorimotor neuropathies (Valentine *et al.* 2006).

Many studies have focused on the ability of DTCs to chelate metals, (e.g. copper) possibly leading to metal toxicity, perturbation of metal-containing enzymes, and/or the

creation of reactive oxygen species (ROS) (Fitsanakis *et al.* 2002; Furuta *et al.* 2002; Heikkila *et al.* 1976; Valentine *et al.* 2006). Still others have shown thiols to be a probable component of the mechanism (Calviello *et al.* 2005; Cheng and Trombetta 2004; Chung *et al.* 2000; Corsini *et al.* 2005; Nobel *et al.* 1997; Tonkin *et al.* 2000; Valentine *et al.* 2006). It is likely that both metals and thiol status are important in the manifestation of DTC toxicities (Burkitt *et al.* 1998; Chen and Liao 2003; Cheng and Trombetta 2004; Pruett *et al.* 2005a).

EBDCs have been implicated as an environmental factor related to the development of Parkinson's disease and fetal exposures are suspected to increase the risk for adult onset (Barlow *et al.* 2005). To date the mechanistic association between exposure and Parkinson's disease is largely controversial. At this time, it is believed that DTCs generate reactive oxygen species (ROS) in combination with potent ROS generators, such as paraquat, leading to neuronal damage and the development of Parkinson's disease (Cory-Slechta *et al.* 2005). Currently, there are only 4/2180 papers using zebrafish published in the area of 'parkinson's disease and development' (McKinley *et al.* 2005; Son *et al.* 2003).

Developmental Effects

DTC developmental toxicity is well established and poorly understood. The majority of information regarding these effects comes from the publicly available pesticide registration documents. Currently, regulatory data gaps exist for reproductive and developmental endpoints for six DTCs (HSDB; U.S.EPA 2004a, b). One example of where data is available is NaM. NaM was delivered by gavage (50 to 60 mg/kg) during

gestational days 7-16 of rats and the pups were analyzed at gestational day 22. The authors report an increase in malformations including hydrocephaly, anophthalmia, and skeletal developmental delays (Tinston 1993). In rabbits receiving NaM by gavage (up to 60 mg/kg/day) on gestation days 8-20, a number of adverse effects were reported. At doses exceeding 20 mg/kg/day there were skeletal variations and at the 60 mg/kg/day dose there was an increase in cleft palate and meningocele (Hodge 1993). Similar effects were seen with other DTCs including fetal reabsorptions, fetal loss and decreased liter size (Extoxnet 1996; HSDB; U.S.EPA 2004a).

Fish are particularly sensitive to DTCs with LC_{50}'s from 130 ppb to the lower ppm range (Extoxnet 1996). Other aquatic organisms are equally sensitive with comparable acute and chronic toxicity values. In the environmentally relevant rainbow trout model, several DTCs caused a distinctive distortion of the notochord with concentrations in the low ppb range or lower. These studies largely described the notochord distortion and demonstrated effects to the surrounding muscle and collagen (Van Leeuwen *et al.* 1986). The authors also observed ectopic osteogenesis among the animals that were raised to adulthood along with compression and fusion of vertebrae and various twisted skeletal elements.

Our initial report demonstrated the NaM and MITC elicited a similar distortion at concentrations in the low ppb range and perturbed the expression of transcriptional markers important in the regulation of muscle, axial development and notochord differentiation. These initial studies established zebrafish as a viable model to dissect the molecular mechanisms of environmentally relevant DTC exposure (Haendel *et al.* 2004) (Fig 1-5, 1- 6, 1-7). By manipulating the exposure period, in addition to other

observations, we were able to test the hypothesis that DTCs target somitogenesis in Chapter 3.

Environmental Fate and Exposure Potential

Environmental fate monitoring of DTCs has lagged significantly behind other pesticide classes. For example, it is reported in the Hazardous Substance Data Bank that NaM was examined in an Ontario, Canada agricultural area where only 2 of 100 farms had a reported usage using methods with detections limits of 100 ppb (HSDB). Clearly, NaM was not detected in this scenario. NaM and several other DTCs do not appear on the National Ambient Water Quality Assessment Program (NAWQA) and are not included in the National Pesticide Survey (U.S.EPA 2005a). However, environmental fate prediction using models, chemical characteristics and pesticide regulatory studies provide a method to assess pesticide environmental fate (Extoxnet 1996; Vogue *et al.* 1994). Reflecting their water solubility and low potential for binding organic matter, DTCs are generally considered to be moderately mobile in soils (Vogue *et al.* 1994). There is at least one report in 5 years of DTC run-off from potato fields which was toxic to aquatic life (P.E.I.Canada 2000). DTCs have been detected in trace quantities below regulatory limits on several food commodities mostly outside the United States (Dogheim *et al.* 1999; Ripley *et al.* 2000). Where DTC data is available, U.S. EPA PRZM/EXAMS models for acute, 21 day and 60 day Estimated Environmental Concentrations (EEC) in surface and groundwater range from 0.0 to 98 μg/L (U.S.EPA 2004a, b, 2005a).

In mammals intentionally exposed to dithiocarbamates, the majority of these large doses were excreted as both parent and metabolites rather quickly (Extoxnet 1996;

U.S.EPA 2004a)). Dithiocarbamates would not be expected to accumulate in tissues, however, DTCs are known to 'linger' with certain target tissues in the adult animal (e.g. blood, thryroid, liver, kidney) (Extoxnet 1996). DTCs such as zineb and ziram are also known to linger in fetal tissue (Extoxnet 1996; HSDB).

Available evidence suggests there is substantial risk for sublethal exposure to aquatic organisms in areas where DTCs are utilized through an unknown mechanism. Humans are exposed to DTCs in occupational settings, food residues and in agricultural areas from familial contact (Dogheim *et al.* 1999; Gladen *et al.* 1998; HSDB; Ripley *et al.* 2000). It is also likely that uncharacterized exposures occur through contaminated groundwater and pesticide drift in areas of significant DTC applications. While there is a wide margin of safety for DTC toxicity in humans, the DTC toxicity assessment is incomplete because there is no clear mechanism of action known for these broad spectrum toxicants. The mechanism of action for DTCs according to the U.S. EPA is, "the inhibition of metal-dependent and sulfhydryl enzyme systems in fungi, bacteria, plants as well as mammals." Without an understanding of the mechanism of toxicity, it is impossible to perform an appropriate risk assessment because, for example, the factors that would define a susceptible population are not known (e.g. lifestyle habits, race, and nutritional status).

The weight of evidence suggests an increased risk for congenital malformations including nervous system and musculoskeletal defects from agricultural work (Arbuckle and Sever 1998; Garcia *et al.* 1999; Hanke and Jurewicz 2004). Several studies have shown increased odds ratios for reproductive and developmental outcomes related to fungicide use (i.e. mancozeb) (Garry *et al.* 2002a; Garry *et al.* 2002b; Garry *et al.* 1996).

These studies are the first concerted effort to link developmental biology with human health risk assessment at the molecular level in order to better understand the mechanism of toxicity of DTCs during development.

Zebrafish and Developmental Toxicology

Developmental Toxicology has its beginnings in teratology. However, because of our fragmentary understanding of normal development and the inaccessibility of the mammalian embryo as a research model, the field of Developmental Toxicology is only now maturing. These impediments have been greatly diminished with the availability of genetic and molecular techniques in models such as zebrafish (N.R.C. 2000). The zebrafish model provides a simple *in vivo* vertebrate system which, particularly during development, is tractable at the molecular and genetic level. Zebrafish development is well characterized at all levels of organization and is generally conserved with higher vertebrates (Fig. 1-8) (N.R.C. 2000; Westerfield 1995). Zebrafish colonies are easy to maintain and the model has a robust resource base of molecular tools, transgenic animals and genomic information (e.g. Zfin, Sanger). In addition, they develop externally and are optically transparent giving them many unique technical advantages in the whole animal such as the ability to perform *in situ* hybridization, immunohistochemistry and other visual labeling of molecular targets throughout development in real time. Multiplying these technical advantages by several fold is easily attainable considering the zebrafish broadcast spawning habits producing hundreds of embryos a day. Zebrafish are poised to contribute to society's many needs for high throughput studies as a rapid and informational tool in the assessment of developmental toxicants.

As a brief overview, many of the developmental milestones in mammals are also observed in zebrafish (Table 1-1). Development in the mammal begins with fertilization, followed closely by a series of cell divisions (cleavage) as the zygote travels down the fallopian tube to be implanted in the uterus. The embryo then cavitates and begins to specialize its cells in a organized and sequential fashion (blasulation). As the blastocyst transitions into gastrulation the three primary germ layers, ectoderm, mesoderm and endoderm, begin to form. This allows for organogenesis and the creation of the neuronal and circulation networks. Near the end of organogenesis the general body plan of the adult is in place and the fetus begins to grow and mature until gestation is complete (Fig. 1-8). While grossly generalized here, these different stages of development are susceptible to toxicants for unique reasons. Therefore, windows of sensitivity within developmental likely exist dependent on the ability of the toxicant to interact with the target and the availability of that target during development (Rogers and Kavlock 2001).

Considering most of a vertebrate's basic biochemistry is created, organized, and initiated during development, the number of available targets and the ramifications of toxic insult can be varied, latent, and seemingly unrelated. The results of somitogenesis are plain to see in the skeletal-muscle muscle system. Somitogenesis is the first significant division of the body plan which is easily detectable. This process allows for further specializations resulting in a variety of different structures depending on their origin along the central vertebrate axis. In zebrafish it is clear that muscle and skeletal elements derive from the somites and that this process is generally conserved among vertebrates. In our initial study we published evidence that the appearance of the distorted notochord coincided with developmental stage and somite formation (Fig 1-9).

Many dithiocarbamates cause a similar developmental toxicity in amphibian, fish, avian, and mammalian species. This strongly suggests a conserved developmental toxicity that is completely unstudied at the molecular level. The fact that aquatic organisms are particularly susceptible to DTCs compared to mammals further supports the use of the zebrafish developmental model. Zebrafish are ideally suited to help define the mechanisms of DTC toxicity in development and, in doing so, fulfill many of the recommendations put forth by the NRC regarding Developmental Toxicology and Risk Assessment (N.R.C. 2000).

We hypothesized that the zebrafish development model was responsive to DTCs in a manner that would allow us to better define the DTC mechanism of developmental toxicity at the molecular level. In Chapter 2 we tested the hypothesis that a targeted structure-activity study of DTCs and related products in the zebrafish model would be sensitive enough to identify the likely proximate toxicant and differences among DTCs, ITCs and CS_2. In Chapter 3, we tested the hypothesis that somitogenesis was the key developmental process targeted by NaM (and DTCs) by measuring transcriptional changes using a microarray and extrapolating these findings to observations in the older impaired animal. In Chapter 4, we tested the hypothesis that the unknown mechanism(s) by which DTCs elicit their developmental toxicity follow molecular pathways leading to cell death that is sensitive to known antioxidants.

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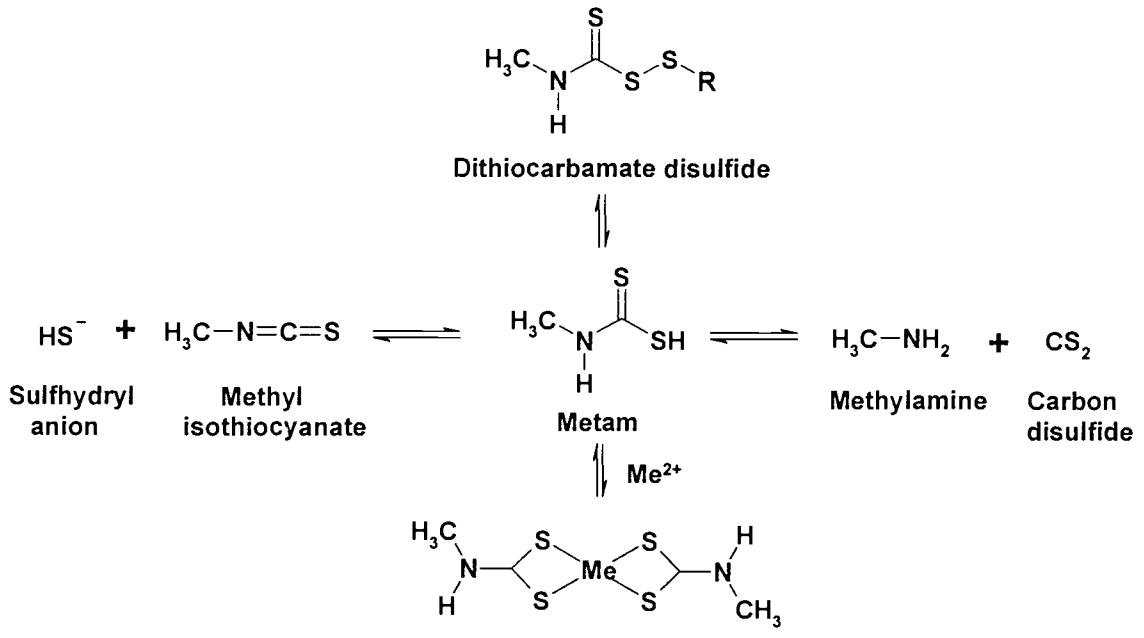


Figure 1-1. Possible pathways of metam sodium degradation. Decomposition is dependent on concentration, pH, temperature and oxygen content. The predominate route is through the acid hydrolyzed decomposition to methylisothiocyanate. Adapted from Haendel *et. al* (2004).

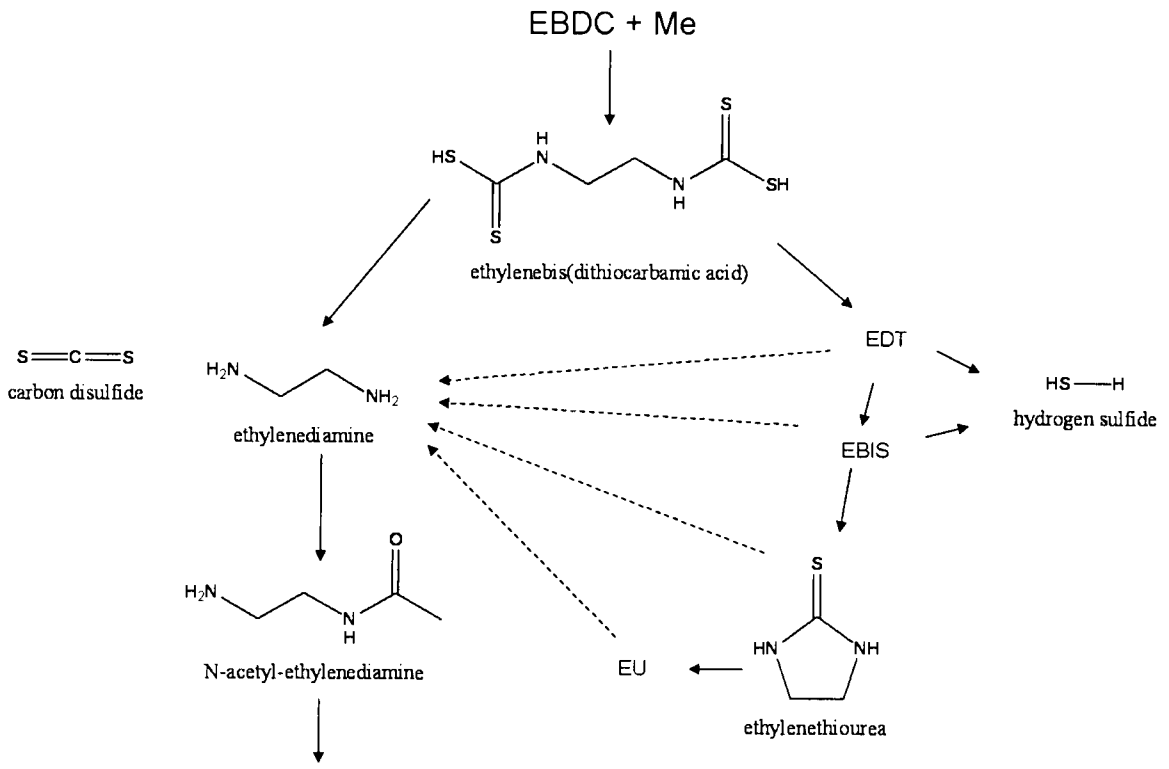


Figure 1-2. Biotransformation pathways of ethylene bis-dithiocarbamates (EBDCs). Adapted from U.S. EPA memo December 19, 2001.

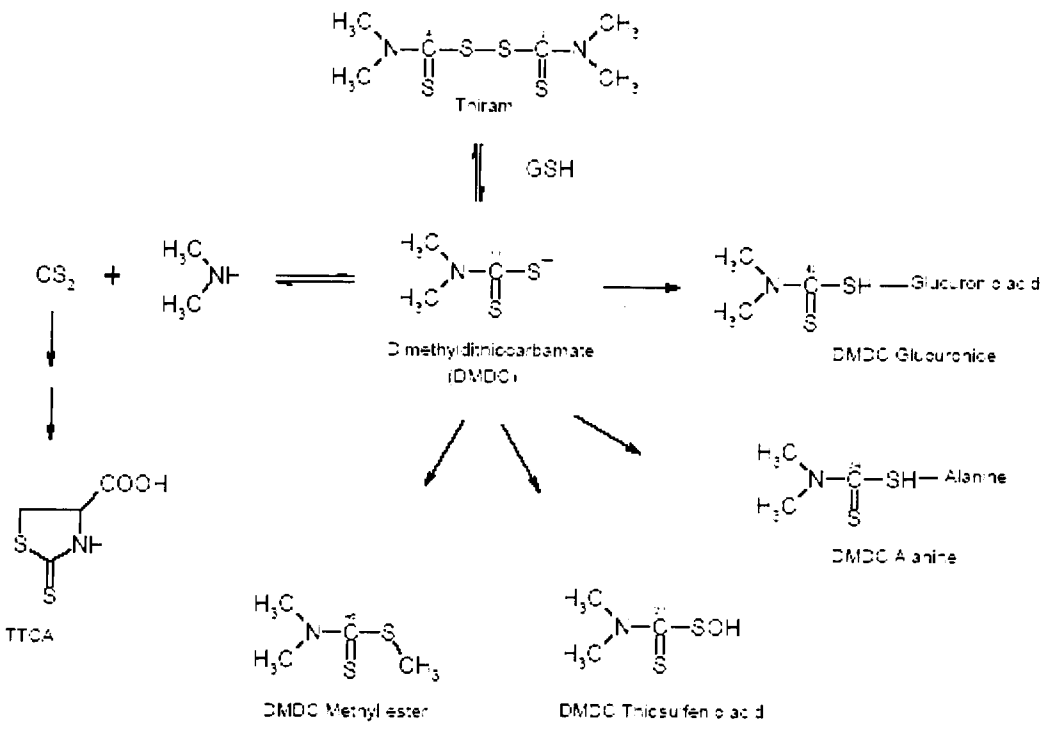


Figure 1-3. Degradation pathways of alkyl dithhiocarbamates including disulfides such as thiram. Adapted from U.S. EPA memo December 19, 2001.

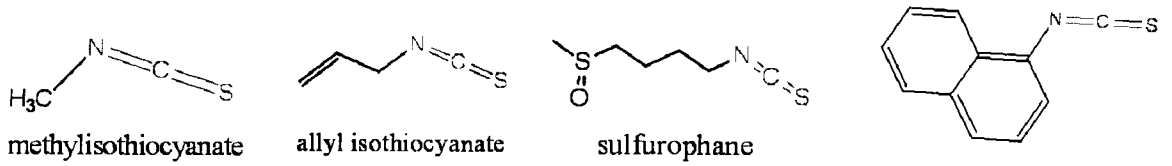


Figure 1-4. Structures of isothiocyanates used in Chapter 2.

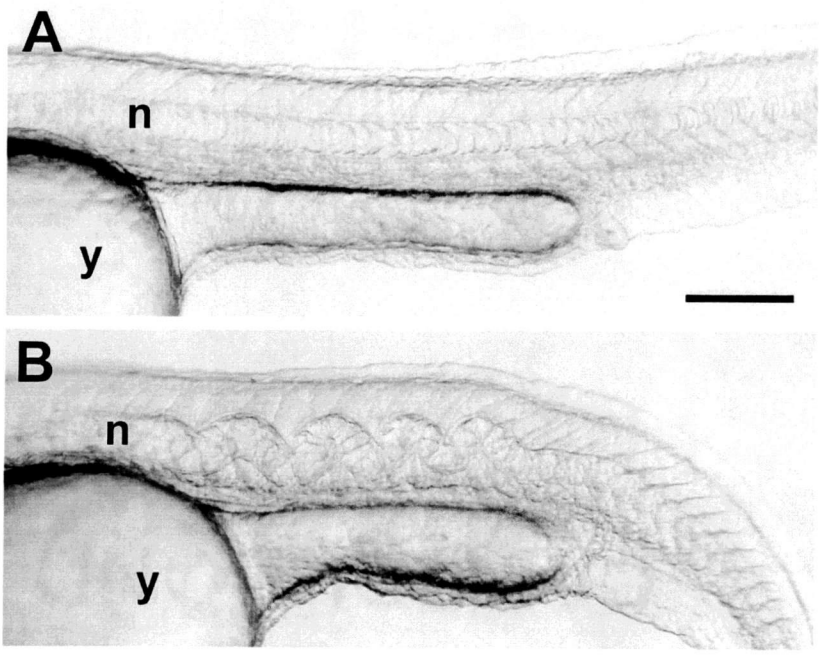
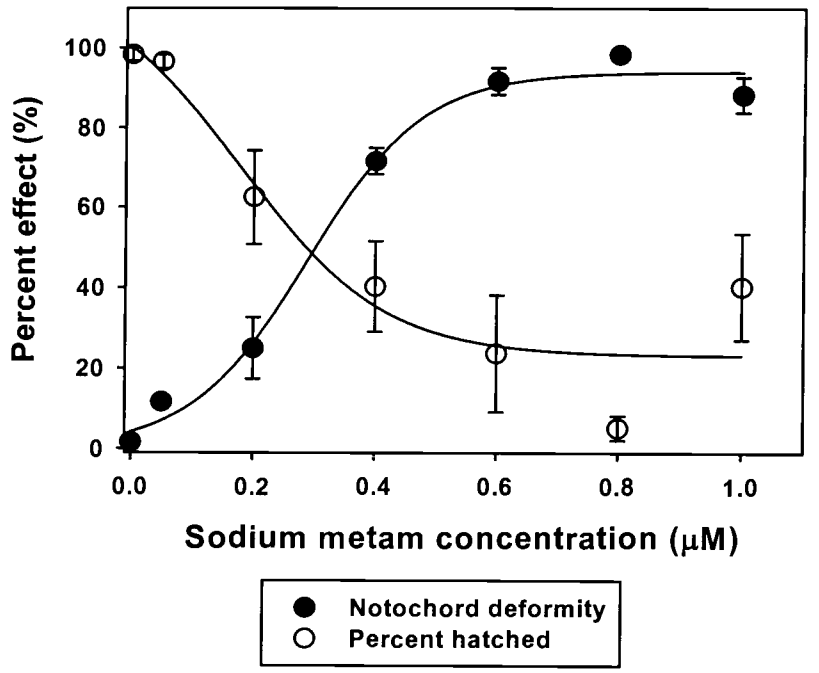


Figure 1-5. NaM distorted notochord. **(A)** Control zebrafish embryo at 24 hours post fertilization (hpf). n- notochord, y- yolk sac. **(B)** Zebrafish embryo with distorted notochord following an exposure to 0.8 μM metam sodium from 4 to 24 hpf.

A.



B.

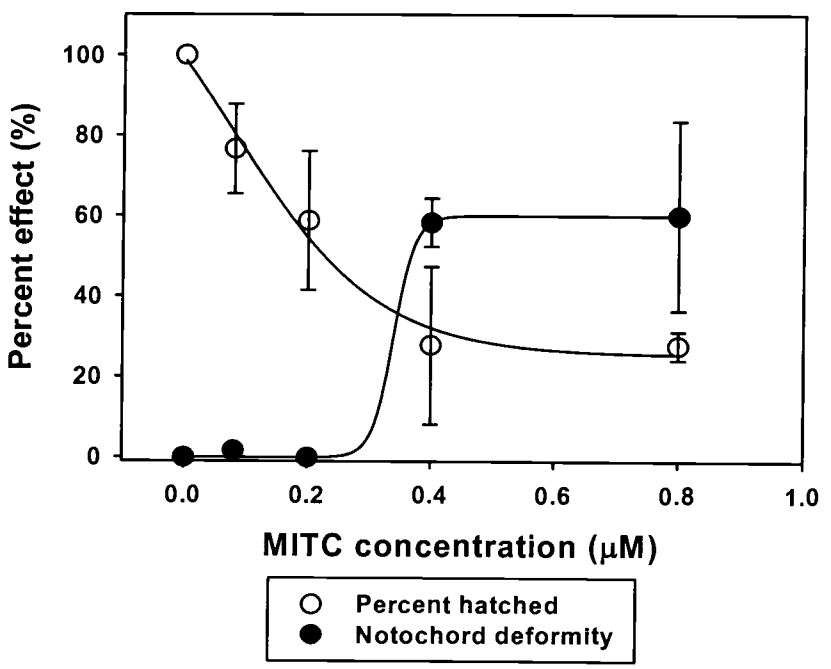


Figure 1-6. Dose response curves from a 4 to 24 hour post fertilization exposure to **(A)** sodium metam (NaM) and its major degradation product **(B)** methyl isothiocyanate (MITC).

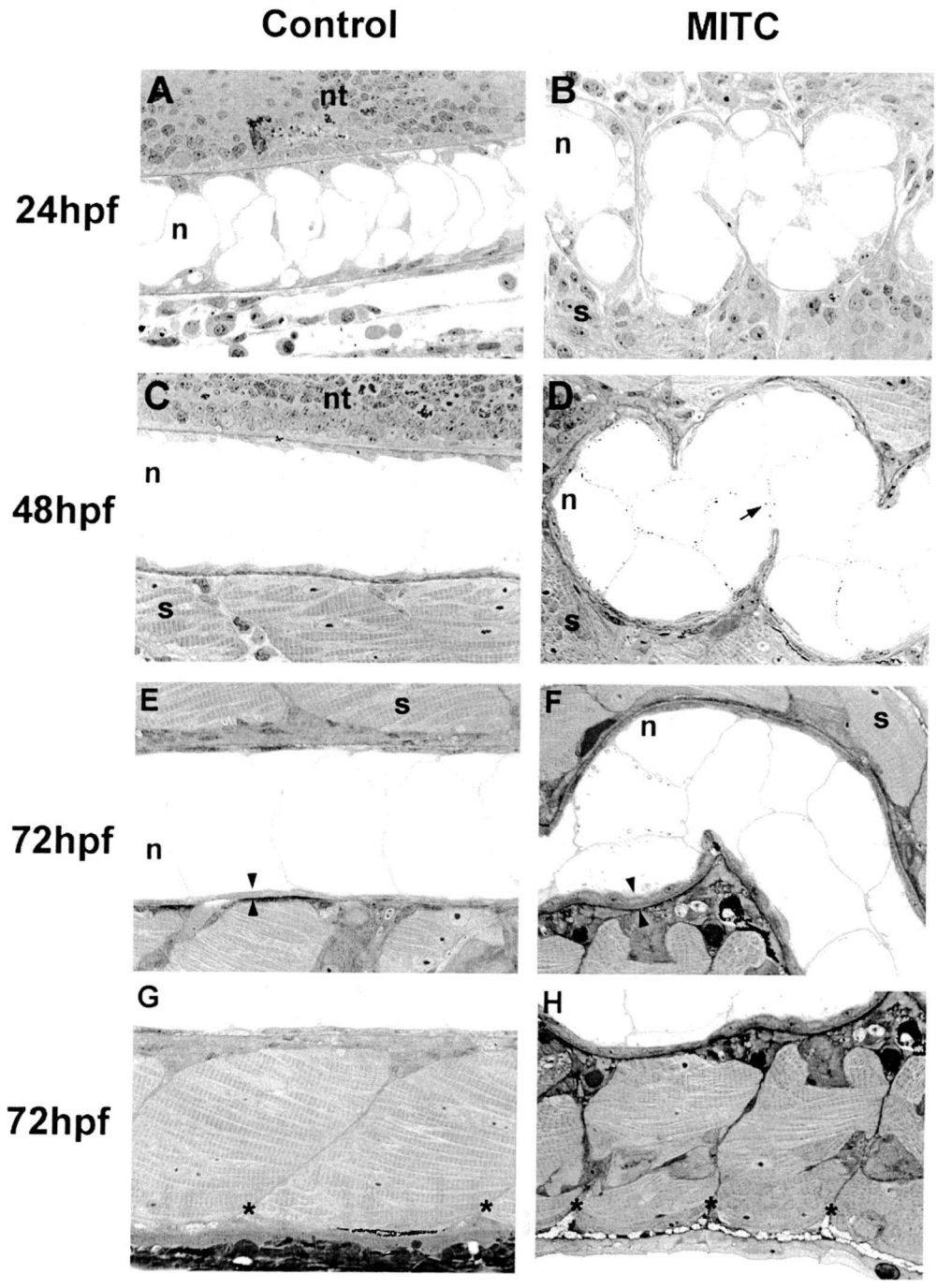


Figure 1-7. Notochord histology. Sections of embryos exposed to 0.8 mM MITC from 4 hpf to 24 hpf. **(A, C, E, G)** Control embryos at 24, 48, and 72 hpf. **(B, D, F, H)** Exposed embryos at 24, 48, and 72 hpf. **(G, H)** Views showing the muscle of the somites. Two embryos were sectioned per dose at each time point. nt,neural tube; n, notochord; s, somite; *Somite boundaries; arrows, globules; arrowheads, notochord sheath thickness.

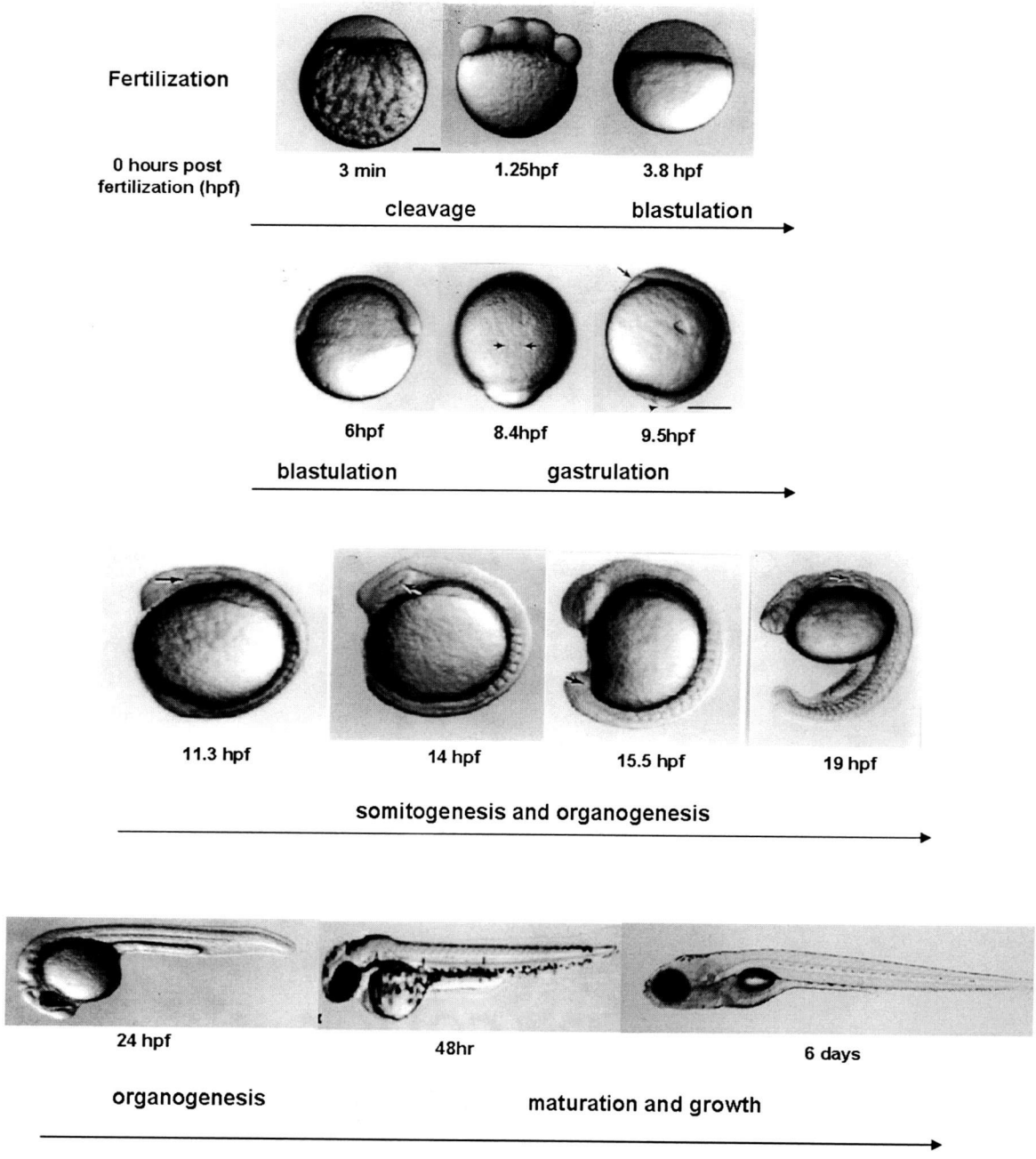
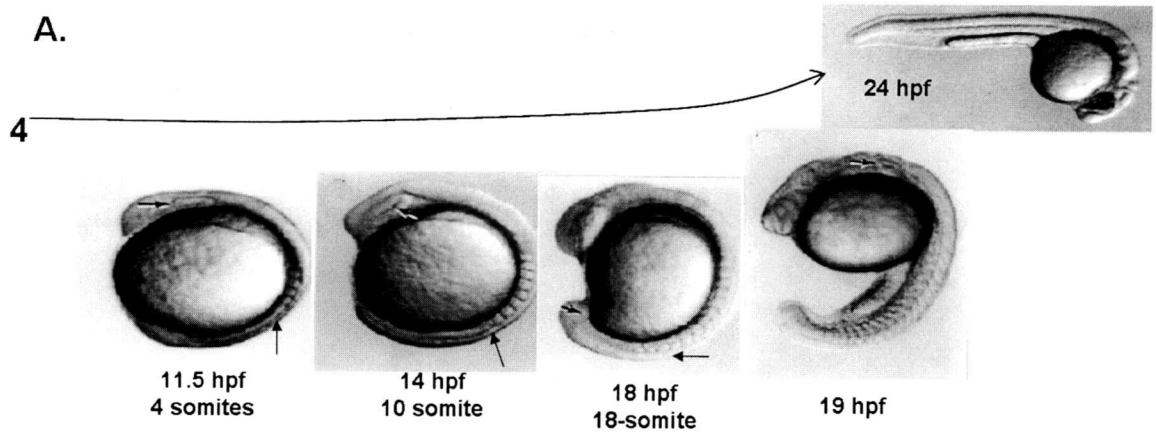
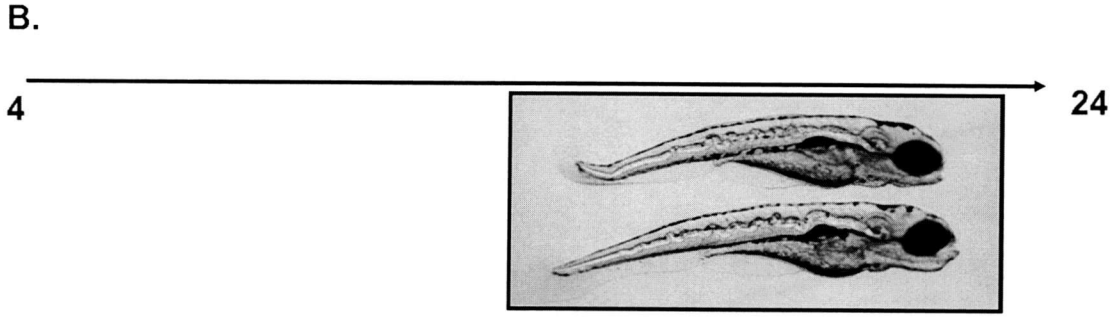
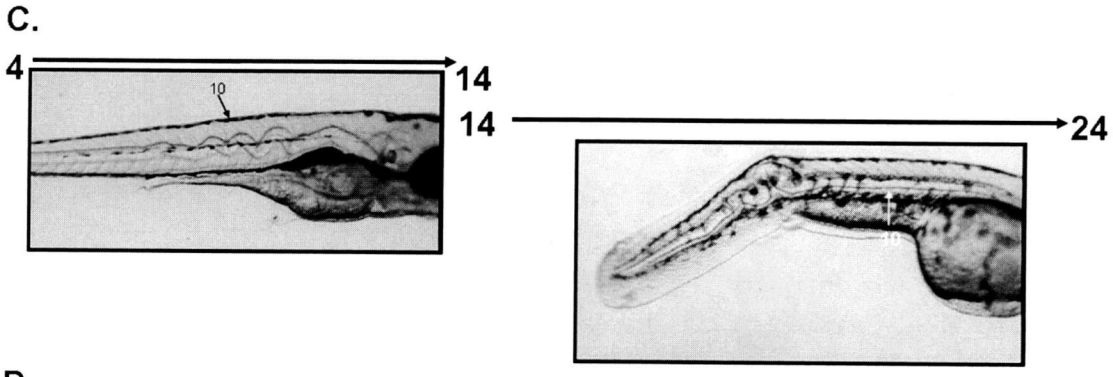


Figure 1-8. Zebrafish are used as an experimental and teaching model of vertebrate development. Development is a continuum and the major developmental stages of vertebrate development are indicated along this timeline of zebrafish development.

Figure 1-9. Somitogenesis is implicated as an important target by varying the exposure window throughout development. Somitogenesis progresses in an anterior to posterior manner (red arrows, **A**) and our standard exposure from 4 to 24 hours post fertilization (hpf) completely encompasses this process resulting in an embryo with notochord distortions along its entire axis **(B)**. If the exposure is shortened to 4 to 14 hpf (10 somite stage) the distortion is limited to the anterior portion of the notochord where during the exposure window somites 1-10 were undergoing segmentation (left). **(C).** If embryos were exposed beginning at the 10 somite stage the notochord distortion was limited to this area. Smaller windows at concentrations of 0.8 μM were not effective at causing distortions. **(D)** However at concentrations greater than 3.5 μM an exposure window of one hour was sufficient to cause distortions in the proximity of the few somites that were beginning to develop in that short period of time.


A.
4
24 hpf
11.5 hpf
4 somites
14 hpf
10 somite
18 hpf
18-somite
19 hpf


B.
4
24


C.
4
14
10
14
24

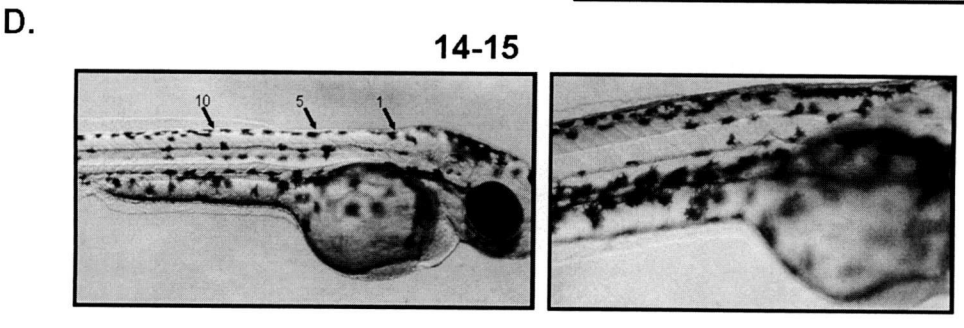

D.
14-15
10
5
1

Table 1-1. Developmental Timelines of Zebrafish and Mammals

	Zebrafish	Rat	Human
Blastula/Blastocyst	2-5 hr	3-5 days	4-6 days
Neural Plate formation	10 hrs	9.5 days	18 days
First Somite	10-11 hrs	9-10 days	20-30 days
10 Somite Stage	14 hrs	10-11 days	25-26 days
Neural Tube Formation	18-19 hrs	9-12 days	22-26 days
First Branchial arch	24 hrs	10 days	20 days
Organogenesis	48 hrs	5-6 days	21-56 days
First Heartbeat	30 hrs	10.2 days	22 days
Birth/Hatching	48-72 hrs	21 days	253 days

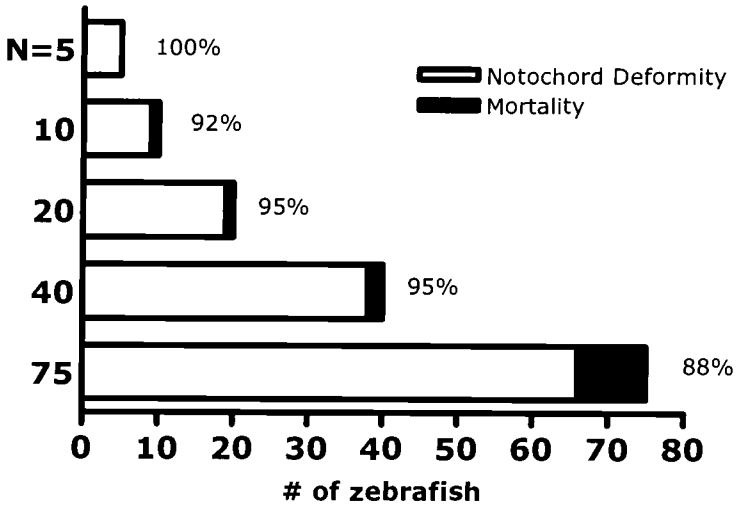


Figure 1S-1. There is no effect on the appearance of notochord distortions or mortality with increasing numbers of zebrafish embryos in the 20 mL exposure vials.

Table 1S-1. The percent of embryos with notochord distortions in 4 to 24 hpf exposures following several pre-treatment conditions with NaM and MITC spiked exposure vials.

	Normal conditions	AGED 14 hrs at 28°C	AGED 14 hrs at 37°C	AGED Fluorescent λ 14 hrs & 10 min @ 2400 µW/cm2	No light exposure
0.8 uM NaM	100%	100	100	100	100
0.8 uM MITC	100	100	100	100	100
Control	0	0	0	0	0

Chapter 2. Dithiocarbamates have a common toxic effect on zebrafish body axis formation

Fred Tilton[1,2,3], Jane K. La Du[1,2], Meng Vue[1], Noor Alzarban[1] and Robert L. Tanguay[1,2,3]

[1]Oregon State University Corvallis, Oregon 97331, [2]Department of Environmental & Molecular Toxicology,[3]Environmental Health Sciences Center and Marine & Freshwater Biomedical Sciences Center

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Supplemental material from a collaboration with Dr. Randy Peterson

Abstract

We previously determined that the dithiocarbamate pesticide, sodium metam (NaM), and its active ingredient, methylisothiocyanate (MITC) were developmentally toxic causing notochord distortions in the zebrafish. In this study developing zebrafish were exposed to isothiocyanates (ITCs), dithiocarbamates (DTCs) and several degradation products to determine the teratogenic relationship of these chemical classes at the molecular level. All dithiocarbamates tested elicited notochord distortions with notochord NOELs from < 4 to 40 ppb, while none of the ITCs caused notochord distortions with the exception of MITC. Carbon disulfide (CS_2), a common DTC degradate, also caused distortions at concentrations > 200 times the DTCs. Whole mount in situ hybridization of developmental markers for collagen (*collagen2a1*), muscle (*myoD*), and body axis formation (*no tail*) were perturbed well after cessation of treatment with pyrolidine-DTC (PDTC), dimethyl-DTC (DMDTC), NaM, MITC, and CS_2. Therefore distinct albeit related chemical classes share a common toxic effect on zebrafish notochord development. To test the responsiveness of the distortion to metal perturbation, five metal chelators and 2 metals were studied. The membrane permeable copper chelator, neocuproine (NCu), was found to cause notochord distortions similar to DTC-related molecules. DMDTC and NCu treated animals were protected with copper and *collagen 2a1* and *no tail* gene expression patterns were identical to controls in these animals. PDTC, NaM, MITC, and CS_2 were not responsive to copper indicating that the chelation of metals is not the primary means by which these molecules elicit their developmental toxicity. Embryos treated with DMDTC, NaM, and NCu were rescued by

adding tricaine (MS-222) which abolishes the spontaneous muscle contractions that begin at 18 hpf. In these animals, only *collagen 2a1* expression showed a similar pattern to the other notochord distorting molecules. This indicates that the perturbation of *no tail* expression is in response to the muscle contractions distorting the notochord, while *collagen 2a1* is associated with the impact of these molecules on much earlier developmental processes.

Introduction

The dithiocarbamate (DTC) chemical class has many important uses as chemical precursors, effluent additives, agricultural pesticides, and in experimental and clinical medicine (WHO 1998). Some DTCs, such as sodium metam (NaM) are unique because when applied, for example as a fumigant to pre-plant potato fields, they are pro-pesticides which form methylisothiocyante (MITC) (Greenbook 2000). In our previous study both NaM and MITC were shown to cause a distortion of the developing notochord in zebrafish with similar dose-response curves (Haendel *et al.* 2004). Isothiocyanates (ITCs) are naturally occurring in several plant species often consumed in the human diet (e.g. allyl ITC and sulforaphane). Cover crops such as mustard which produce ITCs have also been considered as alternatives to fumigants such NaM (McGuire 2003). ITCs are also under extensive study as cancer chemopreventative agents for some forms of cancer (Callaway *et al.* 2004). Due to these important uses it is essential to determine if, dithiocarbmates or isothiocyanates, are the primary developmental toxicants.

Currently, many of the DTCs such as NaM, maneb, and mancozeb are close to completing pesticide re-registration eligibility decision (RED) as mandated by the FQPA 1996 and NRDC Consent Decree. Also DTCs such as thiram (DTC disulfide), macozeb, and maneb have undergone recent voluntary cancellations of some of their agricultural uses (U.S.EPA 2005a, c). For NaM, the most recent data available from U.S. EPA PRZM/EXAMS models for acute, 21 day, and 60 day Estimated Environmental Concentrations (EEC) in surface and groundwater range from 0.0 to 0.02 g/L. Its primary degradation product, MITC, had predicted concentrations of 0.12 to 35.11 ppb

(U.S.EPA 2005b). The REDs available for ziram and thiram report modeled surface and groundwater levels between 0.03 and 98 ug/L (U.S.EPA 2004a, b). This suggests that the risk for exposure may not be limited to the high volume DTC pesticides such as NaM. Furthermore, these modeled values likely do not take into account studies demonstrating the stabilization of DTCs in the environment particularly when co-applied with metals such as copper (Weissmahr 2000).

Only the ethylene bis dithiocarbamate (EBDC) degradation product, ethylene thio urea (ETU), is considered to share a common mechanism of toxicity (thyroid cancer) by the US EPA (U.S.EPA 2001b). Significant analytical and exposure assessment challenges make it difficult to determine if other DTCs share a common mechanism through metabolism or degradation products such as carbon disulfide (U.S.EPA 2001b). There are also regulatory data gaps related to reproductive and developmental endpoints for NaM, ziram, thiram, maneb, zineb, and diethyldithiocarbamate in IRIS and U.S. EPA EFED documents (IRIS 1992c, d, e; U.S.EPA 2005b). Analysis of the literature reveals that many dithiocarbamates cause a similar developmental toxicity in amphibian, fish, avian, and mammalian species (Fishbein 1976), Table 2-1). This strongly suggests a conserved developmental toxicity that is completely unstudied at the molecular level.

Humans are certainly exposed to DTCs through occupational settings and food residues (Caldas *et al.* 2004; Cole 1998; Panganiban *et al.* 2004). However, despite anecdotal reports of adverse developmental outcomes in humans, developmental toxicity in mammalian studies require DTC doses of g/kg body weight which greatly diminishes the human health risk (Helmbrecht and Hoskins 1993; Kreutzer *et al.* 1996; WHO 1998). Aquatic organisms, on the other hand, appear particularly susceptible to DTC

developmental exposure. This may provide insight into the mechanism of toxicity in addition to supporting the use of the zebrafish developmental model. More importantly, little is known about the etiology of DTC induced toxicity, the ramifications of sublethal and mixture exposure, or the reasons for differences in species susceptibility.

While evidence for several mechanisms leading to DTC toxicity have been proposed in adult neuro- and immunotoxicity models, much remains to be determined particularly in vertebrate development (Calviello *et al.* 2005; Corsini *et al.* 2005; Pruett *et al.* 2005; Valentine *et al.* 2006). The thiol containing DTCs will interact with sulfhydryl groups forming thiol protein adducts and disrupt cellular antioxidant levels (Cheng and Trombetta 2004; Chung *et al.* 2000; Nobel *et al.* 1997; Tonkin *et al.* 2000). Many studies have focused on the ability of DTCs to chelate metals, (e.g. copper) possibly leading to metal toxicity, perturbation of metal containing enzymes, and/or the creation of reactive oxygen species (ROS) (Fitsanakis *et al.* 2002; Furuta *et al.* 2002; Heikkila *et al.* 1976; Valentine *et al.* 2006). It is likely that both metals and thiol status are important in the manifestation of DTC toxicities (Burkitt *et al.* 1998; Chen and Liao 2003; Cheng and Trombetta 2004; Pruett *et al.* 2005).

In previous work from our laboratory the proper formation of muscle and the tissues surrounding the notochord of the zebrafish were shown to be impaired by NaM during early somitogenesis (4 to 14 hpf). It was recently reported using the tetramethyldithiocarbamate disulfide (i.e. thiram) that spontaneous muscle contractions, which begin at 18 hpf in the zebrafish, are required to distort the notochord (Teraoka *et al.* 2005)(Fig 2S-1). Therefore the developmental target appears to be involved with proper formation of the notochord or more likely the surrounding tissues that interact

with the notochord. Considering many transcription factors, collagen forming enzymes and antioxidant enzymes are dependent on biological metals we tested the hypothesis that this morphological marker (i.e. distorted notochord) is responsive to manipulations with metals and chelators.

In this study we examined 11 dithiocarbamates, 4 isothiocyanates, 5 metal chelators, 2 metals and one common DTC degradation product for proper axis formation in the developing zebrafish using notochord formation as a morphological marker. It is clear from this comprehensive approach that most DTCs, MITC, and CS_2 have the potential to elicit a common toxic effect on zebrafish notochord development. These studies also revealed neocuproine, a phenanthroline copper chelator, was effective at inducing a similar distortion. PDTC, DMDTC, NaM, MITC, and CS_2 all caused distortions and this response was tested against copper addition. Copper could only protect embryos from DMDTC and NCu induced distortions and *collagen 2a1* and *no tail* expression patterns were comparable to controls. Protection of the distortion through muscle paralysis (tricaine) showed that *collage 2a1* remained perturbed while *no tail* resembled controls. This suggests that *collagen 2a1* is linked to the effects which occur much earlier in development while the persistence of *no tail* expression is a consequence of the muscle contractions which induced notochord distortion.

Materials and Methods

Zebrafish maintenance and collection of embryos

Adult AB strain zebrafish (*Danio rerio*) were raised and kept at standard laboratory conditions of 28°C on a 14 hr light/10 hr dark photoperiod (Westerfield 1995). Fish were maintained in reverse osmosis water supplemented with a commercially available salt solution (0.6% Instant Ocean®) and is herein referred to as 'normal fish water'. Normal fish water had a pH and conductivity range of 6.8 to 7.0 and 450 to 520 μS respectively. Embryos were collected from group spawns and staged as previously described (Westerfield 1995). All photographs were taken of intact live animals and the colorimetric whole mount in situ hybridizations using a Nikon SMZ1500 microscope and a Nikon Coolpix 5000 digital camera. All animal protocols were performed in accordance with Oregon State University Institutional Animal Care and Use Committee guidelines.

Molecules of interest

DTCs and ITCs were dissolved in dimethyl sulfoxide (DMSO) at a concentration of 8 mg/ml immediately prior to dilutions in the carrier solvent and addition to vials containing embryos and normal fish water (Table 2-2). NaM and MITC were prepared as described previously (Haendel *et al.* 2004). Cupric sulfate pentahydrate (VWR International) and zinc chloride (Sigma Chemical) stocks were prepared in normal fish

water at 20 mg/mL. The initial comparison studies were conducted at nominal concentrations of 4, 40, 400 ppb and 4 ppm. Further studies requiring other concentrations are noted in the results. Chelators were prepared in the same manner and at the same concentrations as the DTCs and ITCs. EDTA was prepared at 1 mM in normal fish water. All molecules with the exception of Pyrrolidine DTC (Fluka), NaM/MITC (Chem Service Inc.), and carbon disulfide (Omni-Solve) were purchased from Sigma Chemical. Sulforaphane and metam disulfide were gifts from the Dashwood Laboratory and Beckman Laboratories at Oregon State University.

Embryo exposures

Embryos showing proper and sequential development in the first 3 hours post fertilization (hpf) were selected for exposures and were placed in Teflon sealed clear glass vials (25 mL capacity) when they reached 4 hpf. All exposures were in 20 mL normal fish water from 4 to 24 hpf in order to capture the major early developmental milestones. The specific number of animals and replicates are described within each table or figure. In toxicant and copper co-exposures, embryos were added to vials which contained normal fish water and the appropriate concentration of copper. The second test molecule was added within 20 minutes of the addition of embryos. When embryos were removed from the exposure vials they were rinsed three times in clean water before being placed in 60 x 15 mm Petri-dishes and grown-out through hatch (day 5) using our standard protocol. For the tricaine protection studies, NaM, DMDTC, and NCu induced distortions were protected with tricaine following the methods outlined in Teraoka 2005 (Teraoka *et al.* 2005). At 17 hpf the embryos were removed from exposure vials and

placed in tricaine pH 7.0 0.04% (0.4 mg/ml). Positive control exposures were also terminated at this time. Previous studies show that this has no effect on the percentage of animals exhibiting notochord distortions. Animals were then scored at 24 hpf.

Whole mount in situ hybridization

Whole embryos were fixed overnight in 4% paraformaldehyde at the appropriate hpf. In situ hybridization was performed as described with minor modifications (Westerfield 1995). Briefly, embryos were stored in 100% methanol at -20°C until use. The embryos were rehydrated in PBST and treated with proteinase K at 2 mg/ml in PBST for varying lengths of time depending on the stage of development. The embryos were prehybridized in 50% formamide, 5X SSC, and 0.1% Tween for 1 h and then hybridized overnight at 70°C with digoxigenin labeled antisense probe in 50% formamide, 5X SSC, 0.1% Tween, 500 mg/ml yeast RNA and 50 mg/ml heparin at pH 6.0. The embryos were first washed at 70°C in 2X SSC, 0.2X SSC, and 0.1X SSC and then at 25°C in PBST. Digoxigenin was detected with an anti-DIG-AP Fab fragments antibody (Roche, Indianapolis, IN) in a blocking solution containing 1% DMSO, 2% sheep serum and 2 mg/ml bovine serum albumin in PBST. Finally, the embryos were developed with 20 ml NBT/BCIP per ml (Roche) in color buffer containing 100 mM Tris-Cl, pH 9.5, 50 mM MgCl2, 100mM NaCl and 0.1% Tween-20. The collagen 2a1, no tail and myoD antisenseRNA probes have been described (Weinberg *et al.* 1996; Yan *et al.* 1995).

Statistics

Data are illustrated as the mean with standard error of the mean (SEM) using GraphPad Prism v4.0 for Windows (GraphPad Inc). ANOVA statistical analysis was performed to test significance of the effect (SigmaStat Version 2.03 for Windows software; (SPSS, Inc., Chicago, IL). Where treatment effects were shown to be significant ($p<0.05$) the specific statistical treatments are detailed in the figure legends where they were applied.

Results

Initial dose-response studies were conducted to determine the developmental toxicity of the molecules of interest with a nominal range of 4 ppb, 40 ppb, 400 ppb, and 4 ppm (Table 2-2). With the exception of CS_2, PDTC, nabam, and sulforaphane, this range was sufficient to determine approximate LC_{50}'s and the induction of notochord distortions (Table 2-3)(Appendix A). Further study of CS_2 determined a lethal threshold between 31 to 62 ppm (400-800 uM) and while it also caused notochord distortions it was several orders of magnitude less potent than any other molecule tested. None of the structurally diverse ITCs, except for MITC, caused notochord distortions. The DTCs used in this study were chosen to represent each DTC subclass, all of which caused similar notochord distortions in zebrafish (Fig. 2-1 for representative pictures of distortions). DTC disulfides and ferbam were significantly more potent than the other molecules (Table 2-3). With the exception of the pharmaceutical antabuse (disulfiram) there were no discernable differences in the notochord distortions among molecules and

the animals developed normally without other overt morphological defects. Disulfiram exposed embryos exhibited a concurrent presence of yolk sac and cardiac edema in over half of the embryos and this effect was exacerbated when ethanol was used as a carrier solvent (data not shown). Surprisingly, when the number of experimental animals in PDTC follow-up studies was reduced, the dose-response curve shifted proportionately to the left. Presumably PDTC partitioned in the zebrafish embryo, although we made no attempts to measure the molecule.

To further evaluate this common effect three diverse DTCs (i.e. PDTC, DMDTC, NaM) in addition to CS_2 and MITC were selected for more detailed studies. Refined notochord dose-responses with a narrowed concentration range for CS_2, PDTC, and DMDTC were determined to complement what was already known for NaM and MITC (Fig. 2-1). These molecules have a similar steep dose-response curve over a narrow range of concentrations as observed previously (Haendel *et al.* 2004). Relative to the smaller alkyl DTCs, PDTC and the ethylene-bis-DTCs elicit a response over a broader range of concentrations. CS_2 had the broadest range but the threshold for the notochord distortion was rather dramatic beginning at 103 μM (Table 2-3, Fig. 2-1).

In order to investigate the role of metals and the DTC metal chelating properties in this developmental toxicity, a series of metal chelators were tested under similar conditions for their ability to produce notochord distortions (Table 2-4). Only the membrane permeable copper chelator, neocuproine (NCu) (2,9-dimethyl, 1,10-phenathroline), had an adverse effect on notochord development (Fig 2-2). NCu elicited the same notochord distortion beginning at concentrations of 38 uM which is 100 times less potent than the DTCs tested (Fig 2-2).

This led us to investigate notochord and muscle specific transcriptional markers for five representative molecules (NaM, MITC, CS_2, PDTC, and DMDTC) and the chelator NCu. These probes were selected because they are well characterized developmental markers and are expressed in the developing muscle and notochord. The expression pattern of the well characterized notochord specific collagen transcript, *collagen 2a1*, remained elevated in the notochord through the last development timepoint evaluated (36 hpf) which is 12 hours after the cessation of the exposure. In control embryos collagen 2a1 expression is dramatically decreased by 24 hpf. (Fig 2-3a; DMDTC shown as representative). *No tail,* an orthologue to the mouse gene *brachyury*, is important for proper axis formation and by 24 hpf is restricted to the tail bud. However, in treated animals expression persisted well after the cessation of exposure throughout the notochord (Fig 2-3b).

The myogenic determination factor, *myoD*, which is a transcription factor important in muscle cell proliferation and differentiation, was also altered compared to controls. *myoD* expression appeared less organized in the myotomes of treated animals compared to controls, particularly in areas where the notochord was malformed (Fig 2-3c). Unlike the other two markers, by 36 hpf the expression of *myoD* was absent in both control and treated animals.

The role of copper was investigated by measuring the copper dependent mortality and notochord distortion potential (Fig 2-4a). Significant lethality appeared between 20 and 200 μM (1 and 10 ppm) copper and there were no overt morphological developmental defects at 48 hpf. When copper and NCu were added as a mixture to the vials, there was a complete absence of NCu induced notochord distortions (Fig 2-4b).

Moreover, there was a significant decrease in mortality which would be expected from the 200 μM (1 ppm) concentration of copper.

When the DTC related molecules were added with copper under identical conditions (at the minimal required DTC concentration to cause 100% distorted notochords) only DMDTC-induced notochord distortions were reduced (Fig 2-4c). In additional studies, the DTC disulfide thiram induced notochord distortions were also greatly diminished (data not shown). However, in both of these exposures there was no change in the toxicity of copper at any concentration.

A small percentage of the animals treated with PDTC and 200 μM copper were normal (6 % ± 4), however this was not statistically significant and the copper lethality remained unchanged (Fig. 2-5a). There was no protection of NaM, CS_2 and MITC-induced notochord distortions with copper co-treatment (Fig. 2-5b). There was an apparent increase in the mortality expected from 20 and 200 μM $CuSO_4$ alone in both CS_2 and MITC co-treatments; however this was not statistically significant (Fig. 2-5b). In addition the apparent copper protection of CS_2 induced distortions was also not statistically significant from the CS_2 controls.

The gene expression of *collagen 2a1* and *no tail* were evaluated in animals treated with DMDTC, NCu and NaM in combination with copper or tricaine (Fig 2-6 a, b, c). It is clear in the DMDTC and NCu copper co-treatments that both *no tail* and *collagen 2a1* expression resemble that of the controls, while NaM, which is not protected with copper, maintains the prolonged expression of both of these genes. When following the tricaine protection protocol of Teraoka 2005 to see if muscle relaxation would protect against chemical-induced notochord distortions, it was found that DMDTC, NCu, and NaM were

responsive to this treatment in a manner in complete agreement with studies using thiram. Interestingly, in these animals *no tail* expression is normal at 24 hpf while *collagen 2a1* expression remains perturbed regardless of the state of the notochord.

Discussion

This study reports that representative molecules from all DTC subclasses and several degradation products have the potential to cause a similar developmental toxicity during zebrafish development (Table 2- 3). The DTC concentrations required to elicit notochord distortions in zebrafish under controlled laboratory conditions are sufficiently low to warrant concern for DTC-related developmental health risk from environmental exposure and necessitate more detailed study of the environmental fate and effects of these molecules.

DTC-disulfides and ferbam, which contains multiple DTC moieties, are much more potent relative to the other molecules tested (Table 2-2). As a diethyldithiocarbamate (DEDTC) disulfide, disulfiram was the least potent of this subtype of DTC. This is likely due to free DEDTC formation which acts as a suicide substrate inhibitor of aldehyde dehydrogenase (IRIS 1992b). Considering the unique responses relative to the other DTCs (yolk sac and cardiac edema), which is characteristic of this type of enzyme inhibition, it is unlikely that aldehyde dehydrogenase is directly related to the mechanism by which DTCs induce a distorted notochord. DMDTC was the only dialkyl DTC to have comparable potency to the disulfides; perhaps these properties

are reflective of DMDTC disulfide formation. Clearly, more information is needed regarding the toxicokinetics of these molecules in zebrafish to further the understanding of these responses.

PDTC is an experimental DTC used extensively in cell culture and *in vivo* due to its potential therapeutic applications. It has been shown, depending on the conditions, to have both pro-/anti-apoptotic, and copper transport capabilities (Pruett *et al.* 2005; Valentine *et al.* 2006). As reported, there was a clear tank effect in this study with PDTC where fewer embryos in the exposure vials resulted in a shift of the dose-response curve to the left. This observation may prove to be a significant clue to the mechanism of toxicity considering PDTC is resistant to acid-catalyazed decomposition to CS_2 and, based on our observations, appears to associate strongly with the embryo unlike the other molecules under study. Overall the ethylene bis-dithiocarbamates (EBDCs) appear to be the least potent of the DTCs tested (Table 2-2). This may be due to the fact that EBDCs form many sub-class specific degradation products, none of which are MITC or other ITCs (U.S.EPA 2001a). Only the common DTC degradation product carbon disulfide was tested in this study. Therefore, without further study it is difficult to discern the proximate toxicant; however, all DTCs clearly share the ability, through a common intermediate or mode of toxicity, to induce the development of a distorted notochord.

With the exception of MITC, the range of ITC concentrations and structures was sufficient to determine a complete lack of notochord distortion potential from this important class of chemicals (Table 2-3). The ability of MITC to cause distortions suggests that it either has unique properties or is transformed into a common intermediate shared with DTCs. Evidence to support that ITCs are converted, via glutathione

conjugation, to DTCs and excreted from cells *in vitro* has been shown in several studies (Callaway *et al.* 2004; Zhang 2000; Zhang *et al.* 1996). Given that other ITCs would also be expected to conjugate with glutathione, this does not completely explain the unique MITC response. Possible explanations may lie in the rate of glutathione conjugation of the different ITCs or in MITCs affinity for the target. More importantly, MITC is expected to form from only two pro-pesticides, NaM and dazomet; and dazomet does not form a DTC in its two step transformation to MITC (BCPC 1997; U.S.EPA 2001a). Therefore, DTC-generated MITC does not explain the distorted notochords of the remaining DTCs suggesting that MITC in addition to other DTC products act independently to cause similar notochord distortions.

CS_2 was found to be several orders of magnitude less potent at causing notochord distortions compared to any other test molecule. Therefore, it seems unlikely that DTC-generated CS_2 is the causative agent. CS_2 is formed non-enzymatically from DTCs at varying rates and is the major shared environmental degradation and metabolic product. Our study did not address DTC disposition leading to CS_2 formation at the target. If poor absorption and/or distribution of CS_2 occurred, this would result in the observation of lower potency of CS_2 reported in this study. DTC-generated CS_2 is a well established neurotoxicant and both DTC-generated CS_2 and diethyl-DTC will independently form similar cysteine adducts (Tonkin *et al.* 2003; Valentine *et al.* 1995). It is difficult to predict one common intermediate from the diverse chemistries of notochord distorting molecules in this study suggesting that multiple DTC related products share, at least, a common mode of developmental toxicity. Future studies focused on the oxidative state

of these animals may provide the necessary insight to understand this common developmental toxicity.

The isolated ability of the membrane permeable copper chelator NCu to produce identical distortions as DTCs, MITC, and CS_2 infers that copper plays a role in the developmental toxicity (Fig 2-2, Table 2-4). A decrease in both copper toxicity and notochord induced distortions in copper and NCu co-exposures indicate a clear interaction, likely rendering one or both unavailable to the target (Fig 2-4). This is an odd response considering DTCs have been shown to elevate copper concentrations both *in vivo* and *in vitro* (Furuta *et al.* 2002; Tonkin *et al.* 2004). One possibility not tested was the permeability of NCu and copper chelated NCu to the zebrafish chorion. However, it has been demonstrated in other studies that NCu-induced nitric oxide relaxation of non-adrenergic non-cholinergic nerves were diminished upon NCu copper chelation. Furthermore, DEDTC also did not modulate the NCu effect in co-exposure. (De Man *et al.* 2001; Gocmen *et al.* 2005; Gocmen *et al.* 2000). Considering DTC, CS_2 or MITC formation would not be predicted from NCu degradation, this is a promising comparative tool to evaluate the mechanism of toxicity of DTCs. Therefore, NCu and DTCs may not necessarily act in a similar manner and NCu may have novel properties relative to other chelators. In support of this, Teraoka also exposed zebrafish to two metal chelators neither of which caused distorted notochords. In that study 2,2 dipyridyl did distort the notochord although there were many other effects, not least of which was the lack of responsiveness to tricaine protection through paralysis (Teraoka *et al.* 2005).

A classical explanation for DTC mediated toxicity is the interaction of DTCs with metal containing enzymes such as dopamine beta-hydroxlyase and Cu/Zn superoxide

dismutase (Heikkila *et al.* 1976; Simonian *et al.* 1992). If metal containing enzymes are the target of DTCs in this toxicity, then co-exposure with excess metal should have been sufficient to alter this effect. Considering PDTC, CS_2, NaM, and MITC clearly interacted with the target despite the presence of excess copper, this mechanism of toxicity can not be a direct explanation for the notochord distortions. Although we made no attempt to measure copper transport by DTCs there was no increase in mortality which would be predicted in our system based on the copper dose-response curve. Protection of DMDTC and the DTC-disulfide thiram (data not shown) distortions with copper without a change in copper toxicity remain to be explored in greater detail (Fig 2-4). Undoubtedly, DTCs have metal chelating and transport abilities and CS_2 is highly reactive with biological metals (Danielsson *et al.* 1990; IRIS 1992a; Oskarsson 1987; Tonkin *et al.* 2004). Perhaps the smaller alkyl DTCs are less likely to react with the target when stabilized by copper or the important intermediate is less likely to form when the parent is stabilized by copper (Weissmahr 2000). Moreover, PDTC, NaM, MITC, and CS_2 may have properties that distinguish themselves from the remaining DTCs which need to be further characterized in the whole animal.

The similar altered expression patterns of *collagen 2a1*, *no tail*, and *myoD* following exposure to DTCs, MITC, CS_2, and NCu lends support to the hypothesis that these molecules are acting on the embryo in a similar manner (Fig 2-3a, b, c). Recent studies have demonstrated that spontaneous muscle contractions in the zebrafish (beginning at 18 hpf) are necessary to reveal the notochord distortion from exposure to the DTC-disulfide thiram (Teraoka *et al.* 2005). In the study presented here the persistent expression of these genes does not become apparent until after the commencement of the

spontaneous muscle contractions. This raises the possibility that the perturbed gene expression is secondary to an altered neurological response. While the *no tail* expression is consistent with previous findings with thiram there was no reported persistent expression of *collagen 2a1* by Teraoka (Teraoka *et al.* 2005). In the detailed analysis of gene expression presented in our study, none of the six molecules were a DTC disulfide. While a developmental time series of these probes with thiram would remove the potential for bias from developmental delay and allow for more complete interpretation, we can not conclude that disulfides may be acting through alternative pathways compared to the other molecules tested in this study.

The expression of *collagen 2a1* and *no tail* were examined in animals treated with NCu, DMDTC, and NaM in combination with copper or tricaine (Fig 2-6a,b,c). The *no tail* expression in animals exposed to DMDTC and NCu in addition to either copper or tricaine was indistinguishable from controls (Fig 2-6a and 2-6b). Consistent with the morphological data reported in this study, *collagen 2a1* and *no tail* expression are not responsive to copper and NaM co-treatment (Fig 2-5 and Fig 2-6c). Tricaine exposure beginning at 17 hpf, however, is sufficient to create a *no tail* expression pattern that resembles controls in these animals. This supports the hypothesis of copper stabilization of these molecules leading to either a decrease in the bioavailability or transformation to an intermediate which is the proximate toxicant. Taken together it is clear that the prolonged expression of *no tail* is in response to the mechanical distortion of the notochord by the spontaneous muscle contractions which have been shown to reveal the underlying developmental impairment beginning at 18 hpf (Teraoka *et al.* 2005). It can then be inferred from these data that *no tail* and the molecular pathways which control its

expression are secondarily perturbed to the distortion of the notochord and are not related to the target of notochord distorting molecules.

By comparison only *collagen 2a1* remains persistently expressed in NCu, DMDTC, and NaM-induced distortions regardless of tricaine protection (Fig 2-6a and 2-6b). Disruption of collagen is consistent with previous reports investigating the histopathology of DTCs in fish (Birch and Prahlad 1986; Van Leeuwen *et al.* 1986). Collagen is clearly important for the proper formation and stability of the notochord among other developmental process and this is the first report to link the disruption of *collagen 2a1* expression to the early developmental response of DTC exposed zebrafish. However, the consistent expression of *collagen 2a1* is likely one of several perturbations occurring early in vertebrate development and the target may in fact remain upstream of the molecular signals which perturb collagen formation in these embryos. More detailed analysis of gene regulation early in development will be needed to identify the target and further compare these molecules.

Overall, these data demonstrate that DTCs from every subclass exert a developmental toxicity that can be mimicked with NCu. MITC and CS_2 share these properties suggesting a possible shared mode of action among these molecules rather than a common mechanism. These data further support, at the molecular level, that DTCs and related molecules perturb early developmental processes related to collagen formation and somitogenesis resulting in significant notochord distortions in the zebrafish. Taken together the zebrafish developmental assay provides a useful *in vivo* tool to elucidate the molecular mechanisms of DTC toxicity, particularly as it relates to early development.

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Supplemental Data

Collaboration with Dr. Randy Peterson, Harvard Medical School

The Peterson lab is primarily interested in the high-throughput screening of small molecules of biological interest. Currently a chemical library exists of 12,000 diverse molecules: 2,000 bioactives from MicroSource Discovery systems that are known drugs and natural products and 10,000 relatively uncharacterized molecules from Chembridge Corporation. These molecules were selected for their physiochemical properties which increase the likehood that they are bioavailable (e.g. logP 2 to 6, molecular weights 200 to 500 Da). Approximately 99% of the 12,000 compounds are commercially available in significant quantities. The primary goal of the screening is to develop resources for regenerative research.

In an initial pilot screen of approximately 2,000 molecules, 7 were identified as having notochord distorting phenotypes which were similar to those of DTCs and three were studied at Oregon State University (Fig 2S-1). Only one, 77f11, was studied in successive exposures because it appeared to be the most reasonable phenocopy of DTC

effects (Fig 2S-2). The small molecules was received at 5 mg/ml in DMSO and tested at 0.0, 0.025, to 2 uM under the same experimental conditions described in this chapter. These embryos found to phenocopy the DTC effects. It would not be predicted that 77f11 forms a dithiocarbamate or one of the numerous degradation products. Future studies will be directed toward comparing the transcriptional signature of this small molecule, neocuproine, selected DTCs, MITC, and CS_2. Taken together it will aid in determining whether these compounds are acting in a similar mode or mechanism of action.

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Zhang, Z., Balmer, J. E., Lovlie, A., Fromm, S. H., and Blomhoff, R. (1996). Specific teratogenic effects of different retinoic acid isomers and analogs in the developing anterior central nervous system of zebrafish. *Dev Dyn* **206**, 73-86.

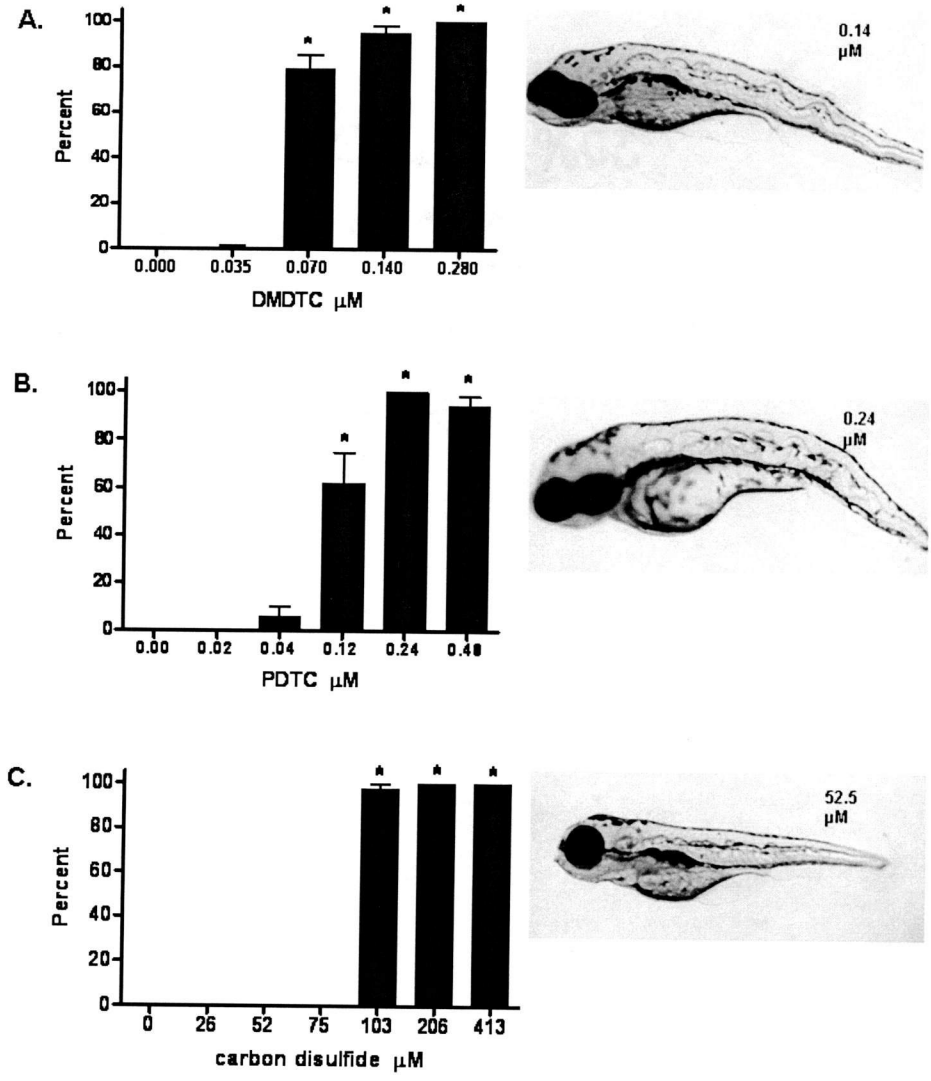


Figure 2-1. The percentage of embryos exhibiting notochord distortions with treatment to DMDTC, PDTC, and CS_2. **(A)** DMDTC, concentrations greater than 0.12 µM were statistically different from controls ($P < 0.001$). **(B)** PDTC, concentrations greater than 0.24 µM were statistically different from controls ($P < 0.001$). **(C)** CS_2, concentrations greater than 103 µM were statistically different from controls ($P < 0.001$). Kruskal-Wallis one way analysis of variance on ranks, pairwise comparisons performed using Tukey Test. N=5 per nominal concentration with 10 animals per vial.

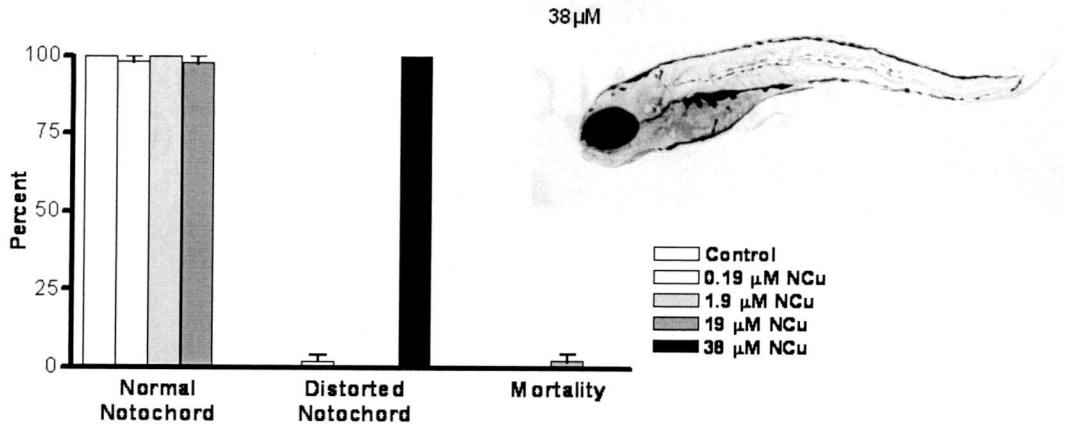
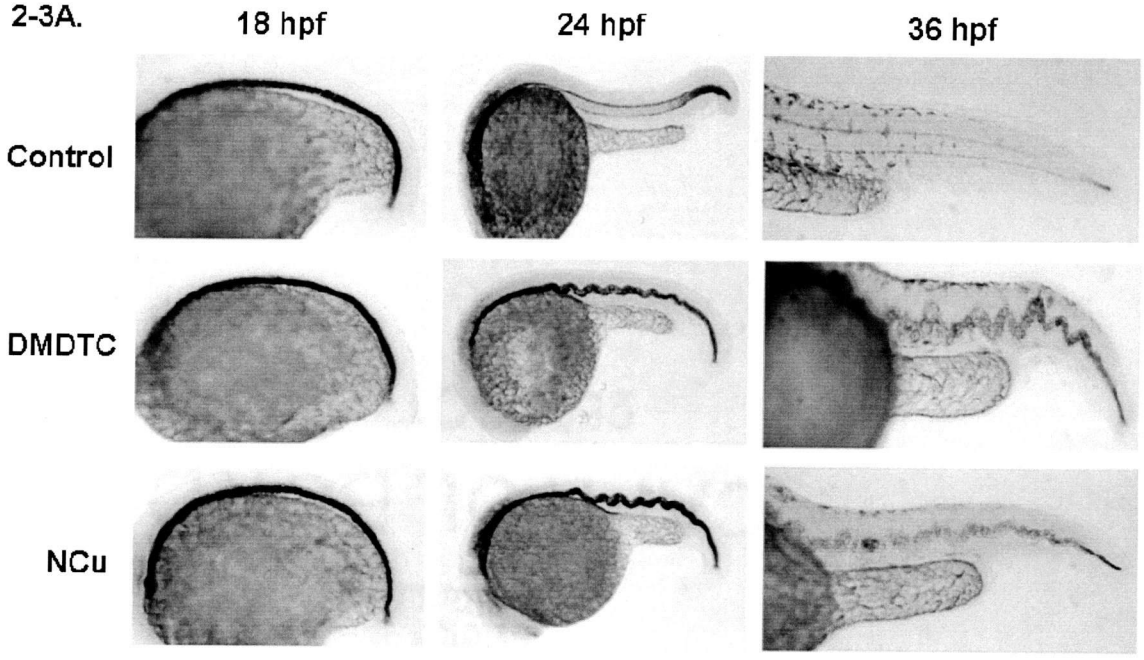


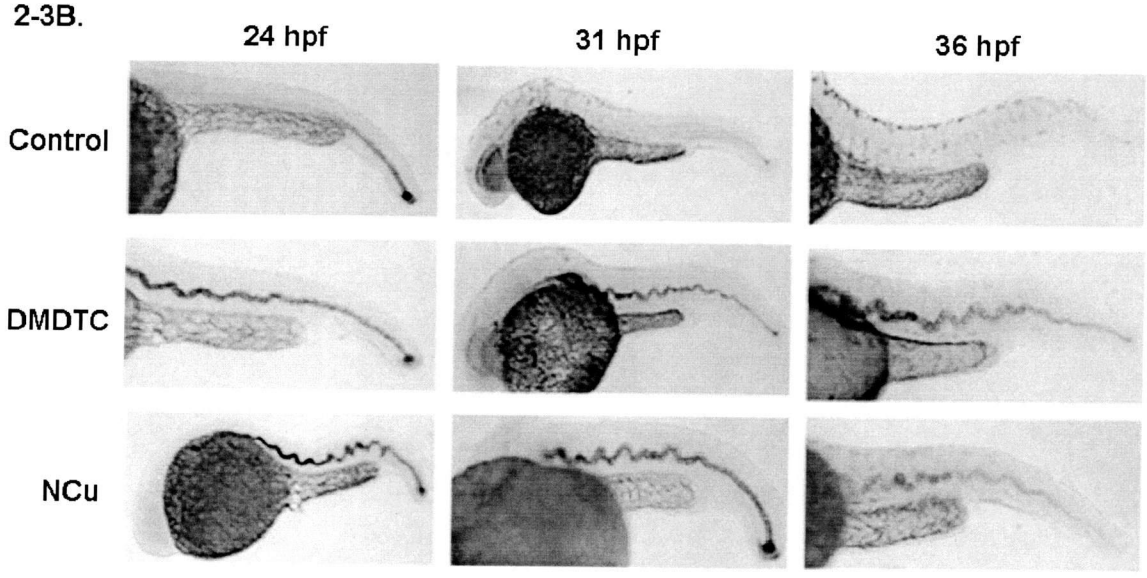
Figure 2-2. Concentration dependent responses to the membrane permeable copper chelator neocuproine. N=5 per nominal concentration with 10 animals per vial.

Figure 2-3. Whole animal in situ hybridization of zebrafish embryos. **(A)** Collagen 2a1. Top panel (left to right) untreated embryos at 18, 24, and 36 hpf. Middle panel (left to right) 0.12 μM DMDTC treated embryos at 18, 24, and 36 hpf, representative of all DTC, MITC responses. Bottom panel (left to right) 0.38μM neocuproine treated embryos at 18, 24, and 36 hpf. **(B)** no tail. Top panel (left to right) untreated embryos at 24, 31, and 36 hpf. Middle panel (left to right) 0.12 μM DMDTC treated embryos at 24, 31, and 36 hpf. Bottom panel (left to right) 0.38μM neocuproine treated embryos at 24, 31, and 36 hpf. **(C)** myoD. Top panel (left to right) untreated embryos at 24 and 36 hpf. Middle panel (left to right) 0.12 μM DMDTC treated embryos at 24 and 36 hpf. Bottom panel (left to right) 0.38μM neocuproine treated embryos at 24 and 36 hpf.

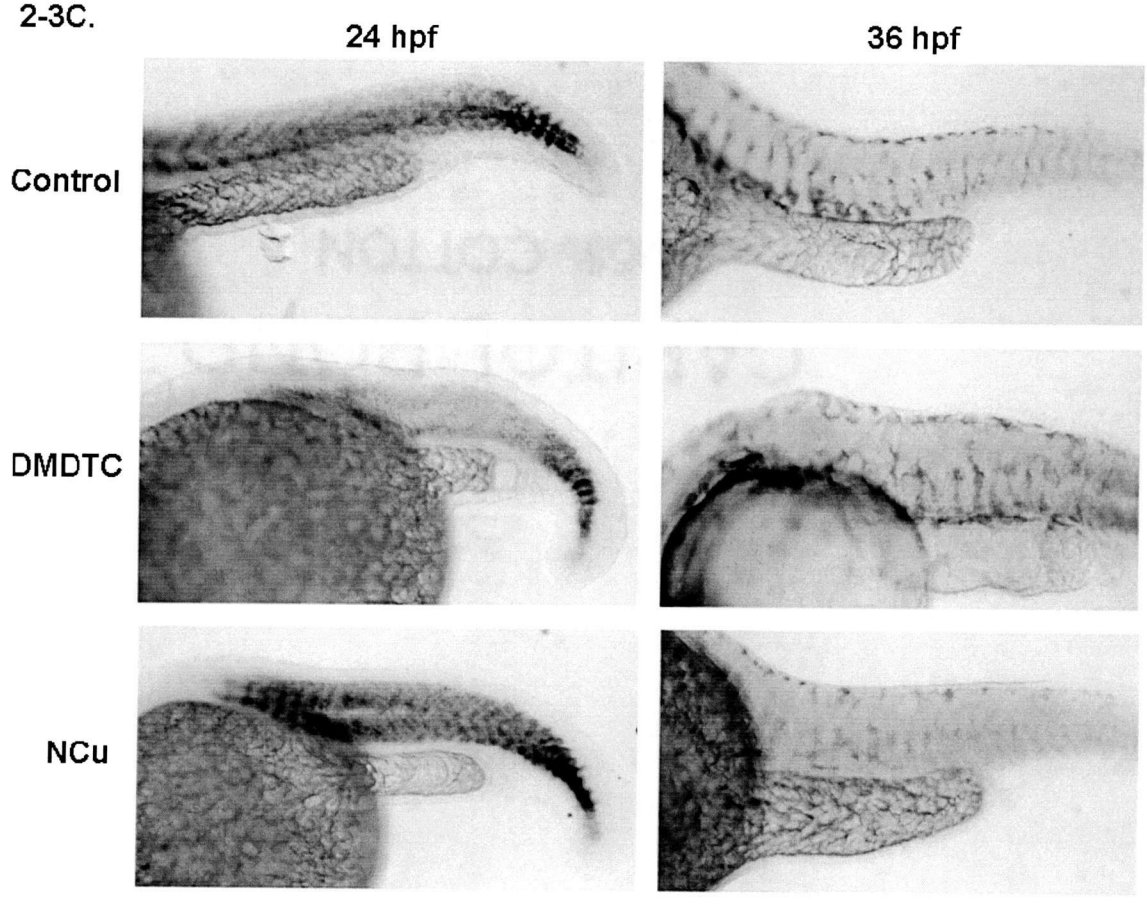
2-3A.


18 hpf
24 hpf
36 hpf
Control
DMDTC
NCu

2-3B.


24 hpf
31 hpf
36 hpf
Control
DMDTC
NCu

2-3C.



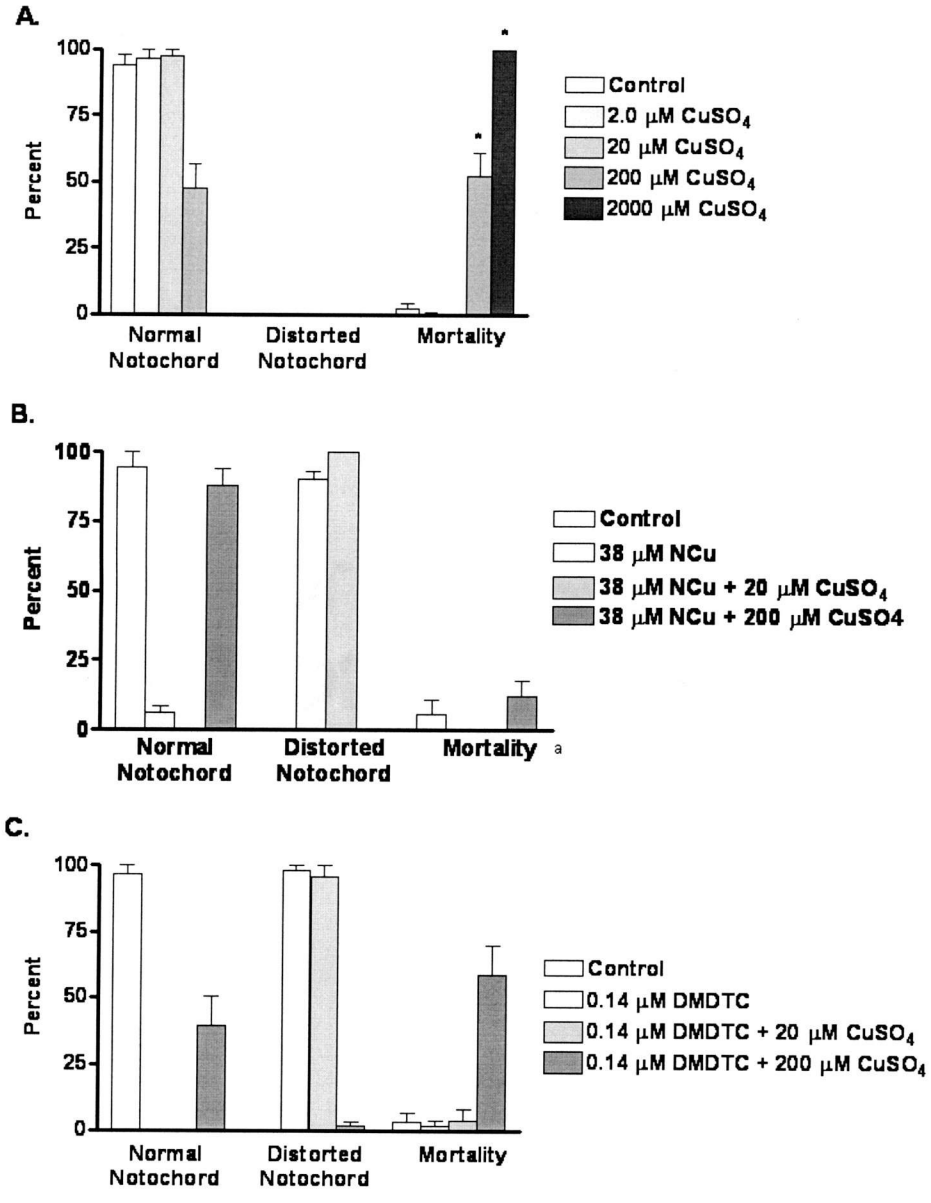


Figure 2-4. Protection of notochord distortion with copper in the developing embryo. **(A)** Copper sulfate dose-response. (*) Statistically significant increase in mortality from controls ($P < 0.001$) by Kruskal-Wallis one way analysis of variance on ranks with pairwise comparisons performed using Tukey test. **(B)** Neocuproine and copper sulfate co-exposure concentration dependent responses. (a) Statistically significant decrease in mortality compared to 200 μM copper sulfate ($P < 0.001$). Comparisons performed using Tukey Test. **(C)** DMDTC and copper sulfate co-exposure concentration dependent responses. N=5 per nominal concentration with 10 animals per vial.

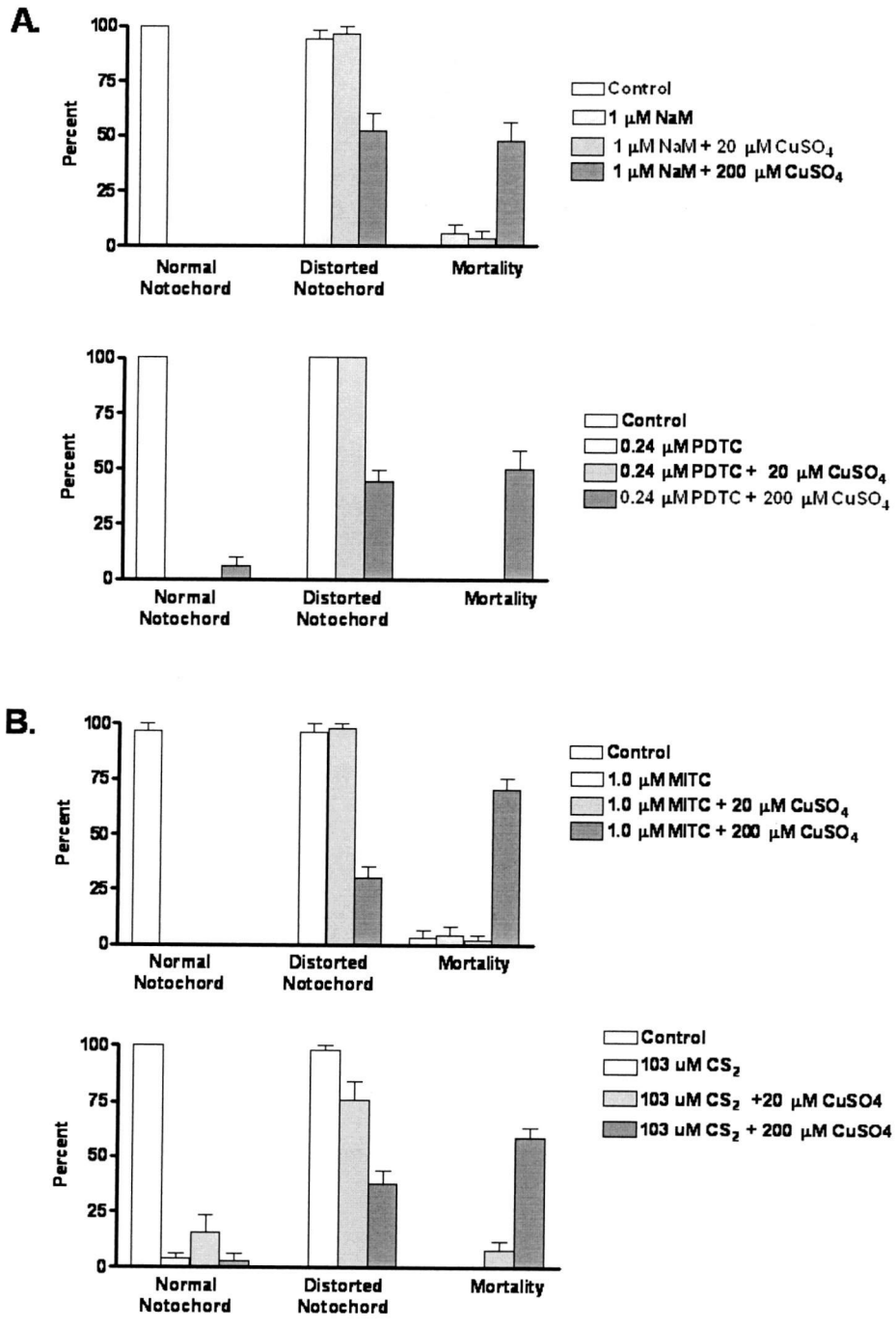
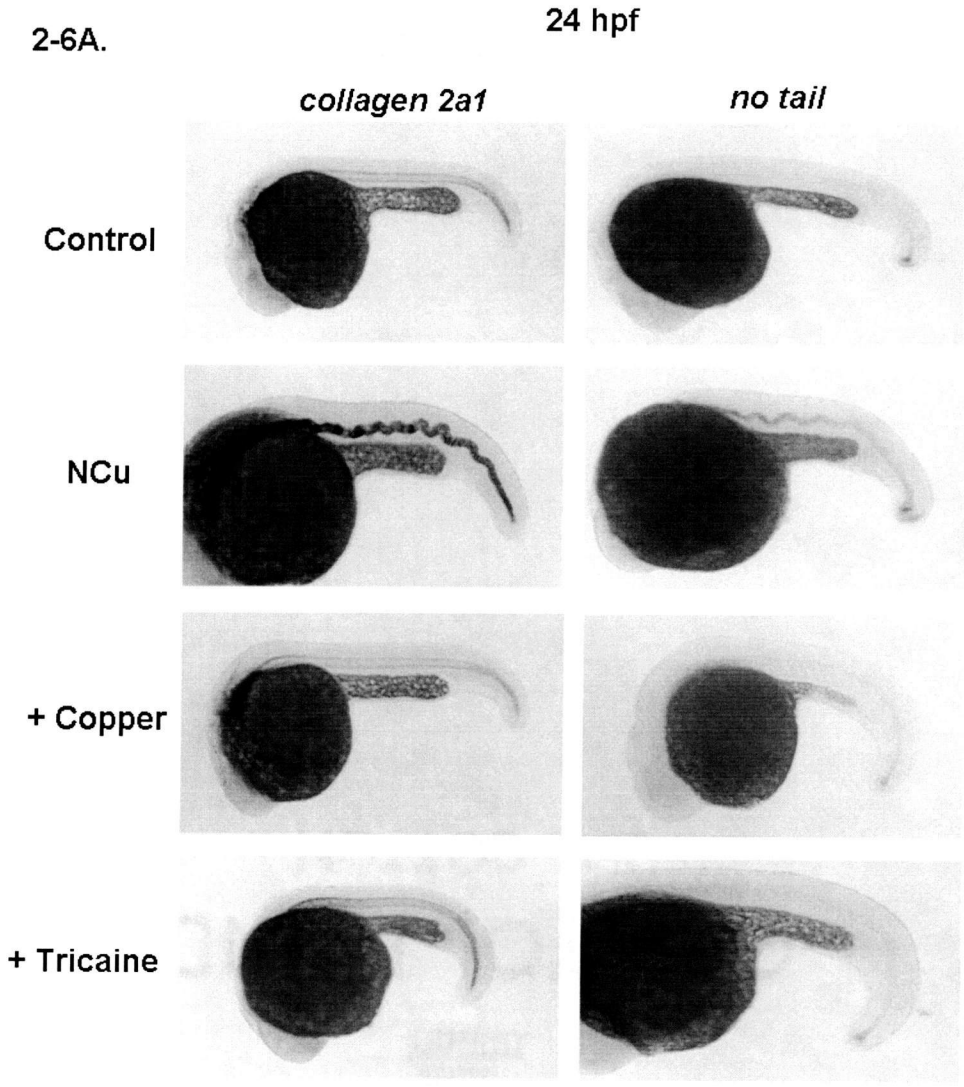


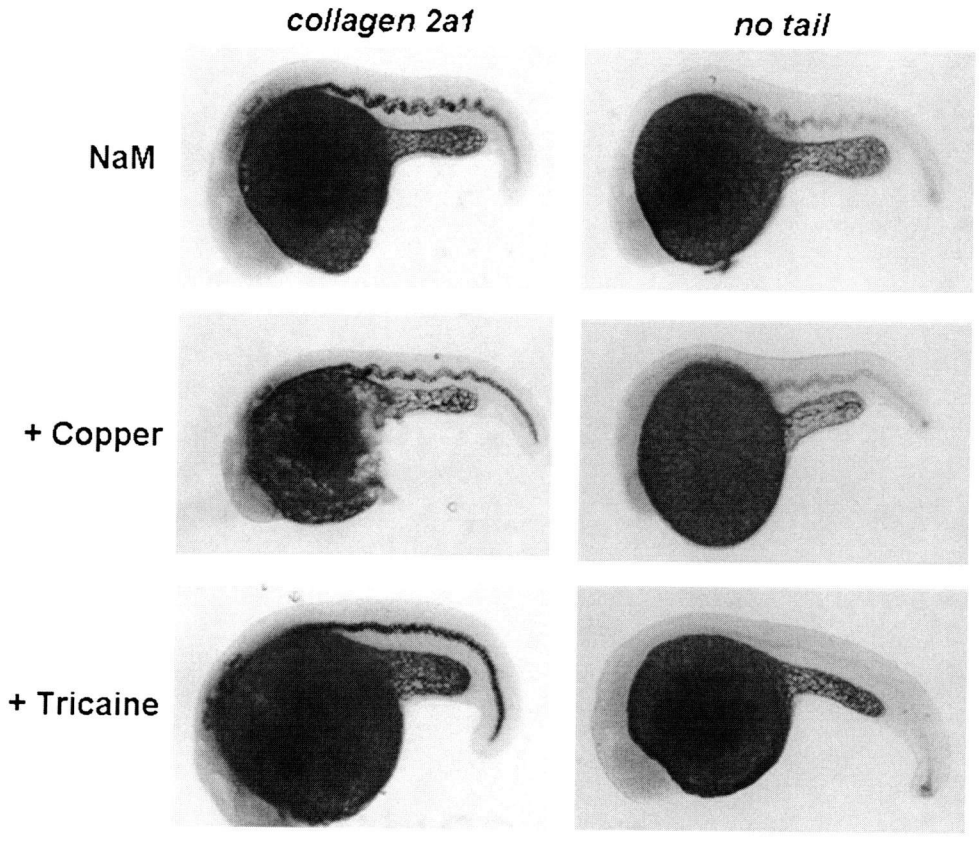
Figure 2-5. Lack of notochord malformation protection with copper co-exposure. **(A)** NaM top graph, PDTC bottom graph. **(B)** MITC, top graph, CS_2 bottom graph. N=5 per nominal concentration with 10 animals per vial.

Figure 2-6. Whole animal in situ hybridization of zebrafish embryos. **(A)** Top panel (left to right) no treatment *collagen 2a1* and *no tail* controls embryos at 24 hpf. (representative of copper, tricaine, and carrier controls). Bottom panel (first row) 24 hpf *collagen 2a1* and *no tail* 0.38μM neocuproine treated embryos. (second row) 24 hpf 0.38μM neocuproine + 0.04% tricaine (at 17hpf) treated embryos . (third row) 24 hpf 0.38μM neocuproine + 200 uM copper sulfate treated embryos. **(B)** (first row) 24 hpf *collagen 2a1* and *no tail* 1.0μM metam treated embryos. (second row) 24 hpf 1.0μM metam + 0.04% tricaine (at 17hpf) treated embryos . (third row) 24 hpf 1.0μM metam + 200 uM copper sulfate treated embryos. **(C)** (first row) 24 hpf *collagen 2a1* and *no tail* 0.12 μM DMDTC treated embryos. (second row) 24 hpf 0.12 μM DMDTC + 0.04% tricaine (at 17hpf) treated embryos . (third row) 24 hpf 0.12 μM DMDTC + 200 uM copper sulfate treated embryos.

2-6A.



2-6B. 24 hpf



2-6C.

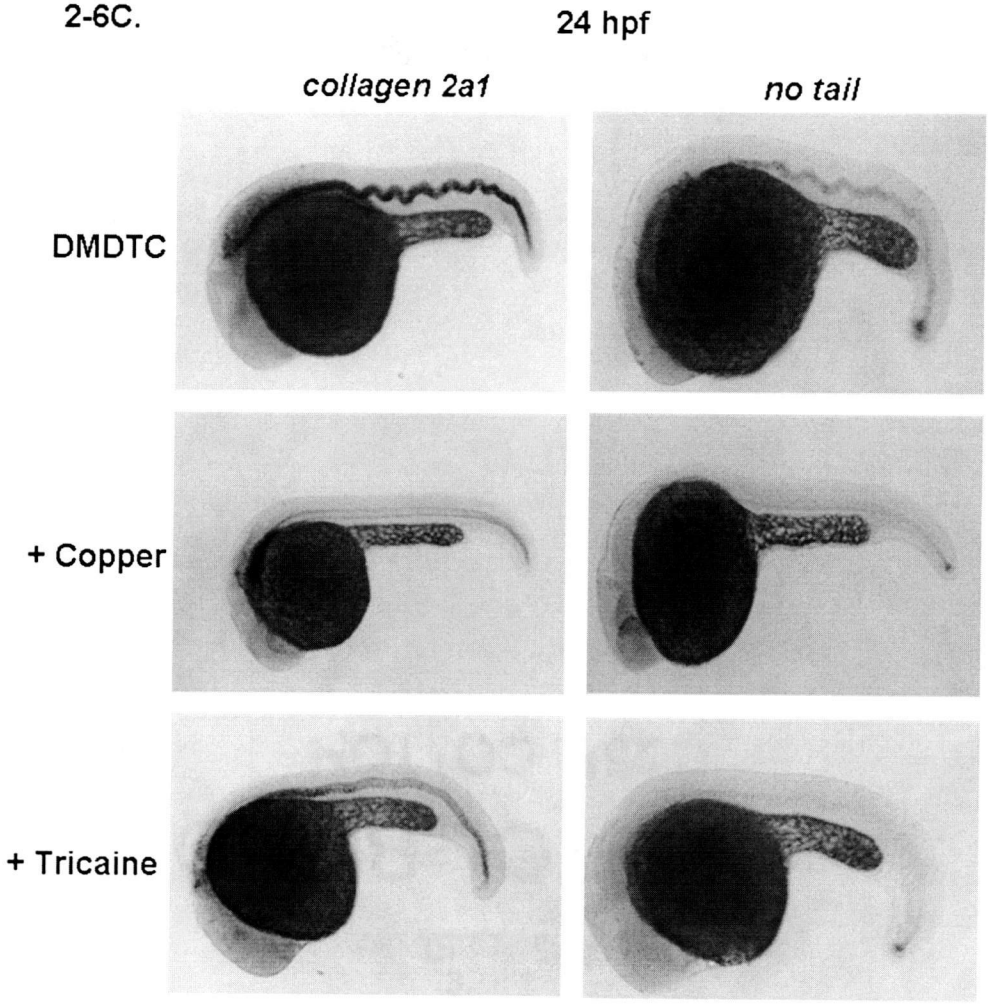


Table 2-1: Developmental toxicity from dithiocarbamate exposure is conserved across several

Author	Year	Species	Developmental toxicity
Zavanella *et. al.*	1984	newt	abnormal and malformed limb regeneration
Birch and Prahlad	1986	Xenopus	malformed notochord
Ghate	1983	Xenopus	malformed notochord
Van Leeuwen *et al.*	1986	rainbow trout	malformed notochord
Marsh-Armstrong *et. al.*	1995	zebrafish	malformed notochord
Suzuki *et. al.*	2001	flounder	malformed notochord
Korhonen *et al.*	1983	chick	death, skelatal anomolies, cranio-facial defects
Rath *et. al.*	2004	chick	tibia hyperplasia
Hodge	1993	rabbit	skeletal anomolies, cleft palate, meningocele
Tinston	1993	rat	skeletal anomolies, hydrocephaly, anophthalmia
Matthiaschk	1973	mice	skeletal anomolies, cleft palate, micrognathia
Roll	1971	mice	skeletal anomolies, cleft palate, micrognathia

Table 2-2. Chemicals under study

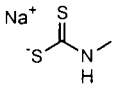
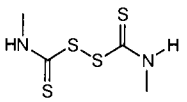
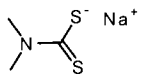
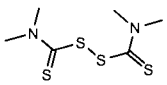
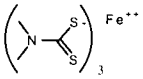
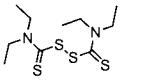
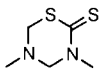
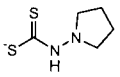
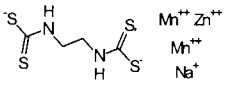
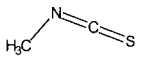
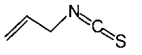
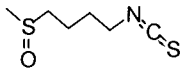
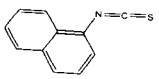
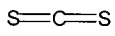
Common Names	Structure	Chemical Name	Uses
Dithiocarbamates			
metam		sodium monomethyl dithiocarbamate	fumigant, herbicide, insecticide
metam disulfide		monomethyl dithiocarbamate disulfide	metam environmental degradation product
DMDTC		dimethyl dithiocarbamate, sodium	experimental compound
thiram		dimethyl dithiocarbamate disulfide	fungicide
ferbam		poly-dimethyldithiocarbamate, iron	fungicide
disulfiram		diethyl dithiocarbamate disulfide	pharmaceutical, industry
dazomet		2-Thio-3,5-dimethyltetrahydro-1,3,5-thiadiazine	fumigant, insecticide
PDTC		pyrrolidne dithiocarbamate	experimental compound
mancozeb, maneb, nabam		ethylene-bis-dithiocarbamate	fungicide
Isothiocyanates			
MITC		methyl isothiocyanate	pesticide, degradation product
AITC		allyl isothiocyanate	mustard oil, pesticide
sulforaphane		4-Methylsulfinylbutyl isothiocyanate	found in cruciferous vegetables
ANIT		alpha-1-napthylisothiocyanate	experimental compound
Dithiocarbamate degradation product			
carbon disulfide		carbon disulfide	chemical precusor, alkyl dtc degradation product

Table 2-3: Dithiocarbamates and not isothiocyanates cause notochord distortions

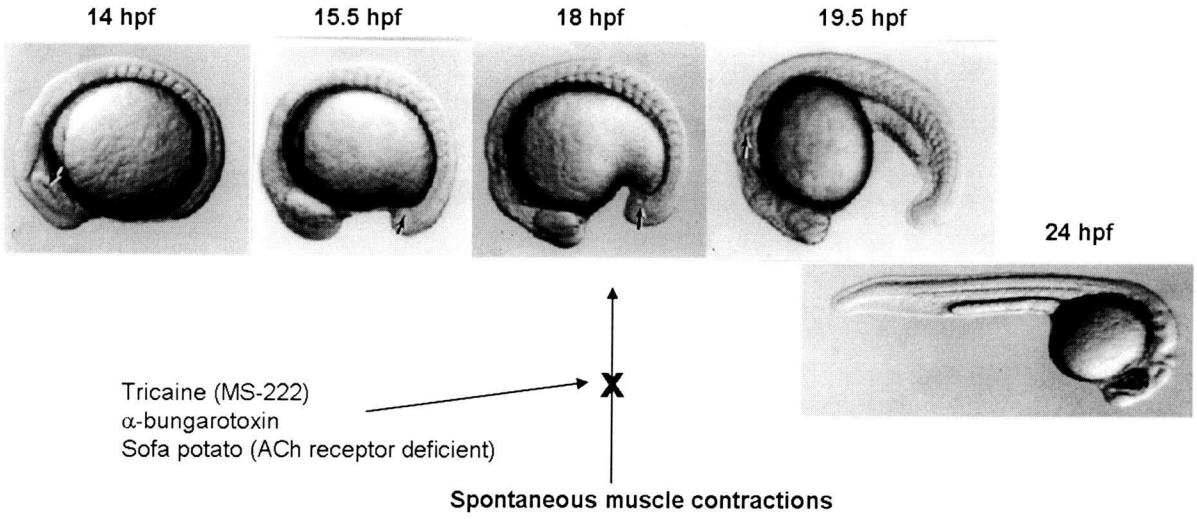
Compound	Notochord Distortion	Notochord NOEL ppb (µM)	LC_{50} ppb (µM)	
Dithiocarbamates				
metam (a)	Yes	13 (0.1)	250	(2.0)
metam disulfide	Yes	4 (0.02)	40-400	(0.19-1.88)
DMDTC	Yes	4 (0.03)	400-4000	(2.79-27.9)
thiram	Yes	< 4 (< 0.02)	40-400	(0.17-1.66)
disulfiram	Yes	40 (0.13)	400-4000	(1.35-13.5)
ferbam	Yes	< 4 (< 0.01)	40-400	(0.1-0.96)
dazomet	Yes	< 40 (< 0.25)	160-400	(1.0-2.46)
PDTC	Yes	40 (0.24)	> 4000	(> 23.6)
mancozeb	Yes	40 (0.15)	400-4000	(1.5-14.8)
maneb	Yes	40 (0.15)	400-4000	(1.5-15.1)
nabam	Yes	40 (0.16)	> 4000	(>15.6)
Isothiocyanates				
MITC (a)	Yes	16 (0.1)	137	(1.87)
allyl isothiocyanate	No	n/a	40-400	(0.4-4.0)
sulforaphane	No	n/a	> 40K	(> 220)
ANIT	No	n/a	400-4000	(2.1- 21.6)
Dithiocarbamate degradation product				
carbon disulfide	Yes	3930 (52)	31-62K	(400-800)

(a) data taken from Haendel *et. al.* 2004. All other exposures were from 4 to 24 hours post fertilization. 20 animals per vial and 3 replicates per concentration. Nominal concentrations ranged from 4 ppb to 4 ppm and were sufficient to determine complete mortality and an approximate LC_{50} in test embryos unless otherwise noted.

Table 2-4 Metals and chelators tested for notochord distortions

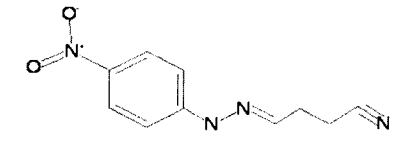
Abbreviations	Chemical Names	Chelator type	Lethality Threshold
Membrane permeable chelators			
DFOM	desferrioxamine mesylate	Fe, Cu, & Zn divalent metals	> 2.4 mM
NCu	neocuproine	Cu (II)	> 38 µM
TPEN	NNNN-tetrakis-(2-pyridylmethyl ethylenediamine)	divalent metals	> 1.9 mM
Membrane impermeable chelators			
BPDS	bathophenanthroline disulfonic acid	Fe (II)	12 to 23 mM
BCPS	bathocuproine disulfonic acid, disodium salt	Cu (II)	> 8.8 mM
Non-specific chelators			
EDTA	ethylenediaminetetraacetic acid	multi-valent metals	> 1mM
Metals			
Zn	zinc (II) chloride	n/a	35 to 350 mM
Cu	cupric sulfate pentahydrate	n/a	0.02 to 0.2 mM

Exposures were from 4 to 24 hours post fertilization with nominal concentrations between 4 ppb and 4 ppm. 15 to 20 animals per vial and 2 to 3 replicates per concentration. Follow-up exposures were conducted with zinc, copper, EDTA, and BPDS to determine the relative concentration at which mortality increased above control values.

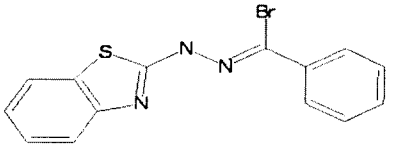


Adapted from Teraoka *et. al* 2005

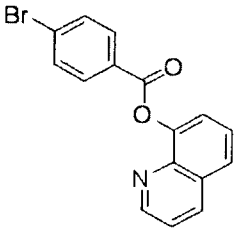
Figure 2S-1. Schematic depicting the timing of spontaneous muscle contractions and the inhibition of these contractions by a variety of methods demonstrated that these contractions reveal the underlying impairment to the notochord and surrounding tissue.



3c7



4j3



77f11

Figure 2S-2. The structures of three chemicals identified in large scale screens to have notochord distorting potential.

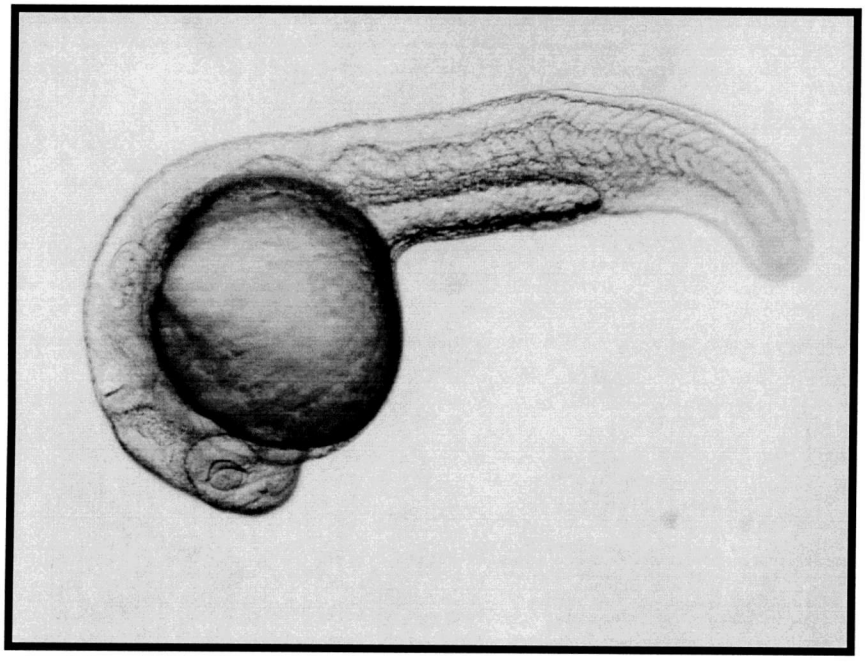


Figure 2S-3. Compound 77f11 phenocopies DTC developomental toxicity in morphology at 24 hpf.

Chapter 3. Gene expression changes resulting from exposure to the N-methyl dithiocarbamate pesticide, metam sodium during zebrafish somitogenesis.

Fred Tilton[*,†] and Robert L. Tanguay [*,†,‡]

[*]Oregon State University, Corvallis, Oregon 97331, [†]Department of Environmental & Molecular Toxicology, [‡]Environmental Health Sciences Center and Marine & Freshwater Biomedical Sciences Center.

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Abstract

Dithiocarbamates (DTCs) share a common toxic effect on development through an undetermined mechanism of action. In the zebrafish model, DTCs appear to alter development during somitogenesis resulting in obvious impairment of the notochord and muscle by 24 hours post fertilization (hpf). To understand the changes that occur during development, gene expression was examined in developing embryos exposed to metam sodium (NaM) using the Affymetrix zebrafish microarray (Zebrafish430_2) and compared to biochemical and morphological endpoints. Zebrafish embryos were exposed to NaM beginning at 4 hpf (1000 cells) and total RNA was isolated from 80 embryos at the 3 somite (11 hpf), 10 somite (14 hpf), 18 somite (18 hpf) and prim-6 (24 hpf) stages of development. It was clear that gene expression was perturbed throughout somitogenesis. Genes differentially regulated at least 2-fold ($P<0.05$) were examined at each time point (11 hpf-104 genes, 14 hpf-151; 18 hpf-154; 24 hpf-26) and few genes present indicated specific developmental effects rather than overt toxicity from NaM. The normal tightly controlled sequence of vertebrate muscle development was impaired. Several transcripts important for neuronal function were perturbed and it is clear from NBT transgenic, Zn12 and AAT whole mount immunohistochemistry that there are significant alterations to neuronal structures. These transcriptional and structural changes may help better explain the role of neuromuscular communication in revealing DTC-induced notochord distortions. Transcripts encoding proteins relevant for degradation and stabilization, axis formation, and muscle were significantly altered by NaM. This gene signature correlated well with previous observations in older animals and may

support a role for TGFβ signaling in DTC toxicity. In particular, genes within the activin pathway were altered, such as lefty which influences proper axis formation and acts as a competitive inhibitor to TGFβ signaling. Misregulation of TGFβ factors that are muscle specific, e.g. myostatin-1, may play an important role in the expression of fast muscle specific genes present in our gene lists. Further, ubiquitination pathways can be inhibited with over stimulation of TGFβ signaling and is reflected in the gene expression changes observed in this study. Overall, the transcriptional alterations caused by NaM in early development indicate that somitogenesis is targeted resulting in the molecular, biochemical and morphological changes observed in the animal.

Introduction

The dithiocarbamate (DTC) chemical class has many important uses as chemical precursors, effluent additives and agricultural pesticides as well as its use in experimental and clinical medicine (WHO 1998). Significant work has been conducted on the mechanisms of neuropathy and immunotoxicity in adults following exposure to DTCs, including the N-methyl dithiocarbamate, metam sodium (NaM) (Pruett *et al.* 2005; Thompson *et al.* 2002). NaM is unique to the dithiocarbamate class because it is a pro-pesticide. In a typical application to pre-plant fields, NaM is watered into the soil where it hydrolyzes into methylisocyanate (MITC) in turn acting as an insecticide (e.g. nematocide) as it dissipates into the atmosphere. Effective and safe fumigation requires a firm understanding of many local conditions, including weather, wind and soil conditions

across the entire acreage. Ground and surface water in areas of DTC use, including NaM, have the potential to receive concentrations which could pose a significant developmental risk to aquatic organisms and unintended exposure (U.S.EPA 2005).

DTCs are potential developmental toxicants, although little is known about the developmental processes targeted or the mechanism(s) by which DTCs interact with the developing embryo. While mammals are at a significantly lower risk, DTC developmental toxicity is conserved across species and appears to be shared among the entire class of compounds (Van Leeuwen *et al.* 1986a; Van Leeuwen *et al.* 1986b, Chapter 1). Zebrafish exhibit a pronounced and reproducible notochord distortion that has been studied at the molecular level (Haendel *et al.* 2004). Previous studies found that neuromuscular function during spontaneous muscle contractions at 18 hours post fertilization (hpf) was required to distort the notochord (Teraoka *et al.* 2005). Work in our laboratory established that the restricted exposure windows coincided with the process of somitogenesis which begins 7 hours prior to the onset of spontaneous muscle contractions (Haendel *et al.* 2004, Chapter 2).

The objective of the current study was to identify key transcriptional changes occurring during zebrafish somitogenesis while under insult from the dithiocarbamate, NaM. Somitogenesis is the first easily visualized specialization of the developing embryo and is the continuation of a process which is well understood from the study of cell fate maps of early development in the zebrafish model (Schier 2001; Stemple 2004). The eventual outcome of somitogenesis is plain to see in the architecture of the vertebrate muscular-skeletal system. By following the induction of paraxial mesoderm (i.e. future somites) using such well known factors as sonic hedgehog, it is possible to measure the

appearance of the first somites and the beginning of myogenesis at approximately 11 hpf (Drapeau *et al.* 2002). The somites then differentiate through several cellular transformations until the appearance of the first fibers and muscle pioneer cells.

One method to visualize neurogenesis is with antibodies directed at surface proteins associated with the peripheral nervous system, such as alpha-acetelyated tubulin (AAT), and early 'primary' neurons, such as zn 12 (Metcalfe *et al.* 1990). In addition, transgenic animals generated to contain a fluorescent protein driven by a neural-specific beta tubulin promoter (NBT) can be used to visualize primary motor neuron development (Thisse *et al.* 2001). There is a clear interdependence of muscle and neuronal development that is under active investigation by many laboratories (Drapeau *et al.* 2002; Eisen and Melancon 2001). Toxicants as diverse as cadmium and fipronil have recently been shown to impact this delicate relationship (Chow and Cheng 2003). It is unclear how DTCs elicit their mechanism of toxicity during development. The current studies were designed to capture major transcriptional changes in a population of zebrafish embryos exposed to NaM. The resulting transcriptional profiles have allowed us to generate an interesting molecular hypothesis for DTC developmental toxicity that is supported by biochemical and morphological changes observed later in development.

Materials and Methods

Zebrafish maintenance and collection of embryos. Adult AB strain zebrafish (*Danio rerio*) and (neural specific beta tubulin promoter) NBT transgenics were raised and kept

at standard laboratory conditions of 28°C on a 14hr light/10hr dark photoperiod (Westerfield 1995). Fish were maintained in reverse osmosis water supplemented with a commercially available salt solution (0.6% Instant Ocean®) at a pH and conductivity range of 6.8 to 7.0 and 450 to 520 μS respectively. Embryos were collected from group spawns and staged as previously described (Westerfield 1995). All photographs were taken of intact animals the immunohistochemistry using Axiovision software, Axiocam HR (Zeiss) mounted to a Zeiss Axiovert 200M motorized inverted microscope. All animal protocols were performed in accordance with Oregon State University Institutional Animal Care and Use Committee guidelines.

Embryo exposures. Embryos showing proper and sequential development in the first 3 hpf were selected for exposures and were placed in Teflon sealed clear glass vials (25 mL capacity) when they reached 4 hpf. One-hundred embryos were placed in a vial per treatment. The concentration of NaM utilized in these studies was 1.0 μM. The NaM dose-response is well characterized and this concentration will produce 100% notochord distortions in embryos by 24 hpf. We have also established that there is no tank effect with NaM and no increased mortality in this system with 100 embryos/ 20 mL (Chapter 2). For the immunohistochemistry, transmission electron microscopy (TEM) and transgenic exposures, our standard protocol of 4 to 24 hpf at 1 μM NaM was followed until embryos were sampled at the appropriate time. For the array experiments, animals were removed from their exposure vials at the appropriate time, washed, and 80 embryos were selected by counting the number of visible somites. The remaining embryos were incubated in petri-dishes to confirm the presence of notochord distortions after 24 hpf

(with the exception of 11 hpf which is not a sufficient period of time at this concentration to elicit notochord distortions). Two separate experiments were conducted in groups of 4 (Exp 1: 18/24 hpf,; Exp 2: 11/14 hpf) with control and treated animals. Consequently, these timepoints span somitogenesis including the period before, during, and after the notochord distortions become visible.

Total RNA isolation. Whole embryos (80) showing proper development were rinsed and placed in polypropylene tubes with their chorions intact. The surrounding water was completely removed with a narrow glass pipet gently worked to the bottom. TRIzol Reagent (500 µl; Invitrogen, Carlsbad, CA) was placed on the embryos and they were quickly homogenized with a hand-held tissue tearer. An additional aliquot of TRIzol (Invitrogen) was added, mixed and the samples were snap frozen in liquid nitrogen followed by storage at –80°C. Total mRNA was isolated from whole emybro homogenates using TRIzol (Invitrogen) followed by cleanup with Rneasy Mini Kits (Qiagen, Valencia, CA) according to manufacturer's instructions. Total RNA quantity and quality was determined by UV absorbance at 260/280 nm using a Nanodrop spectrophotometer before submission to the Center for Gene Research and Biotechnology (CGRB) at Oregon State University. Ribosomal RNA abundance and the degree of degradation was determined in electropherogram patterns using the 2100 Bioanalyzer and RNA 6000 Nano chips by the CGRB (Agilent Technologies, Palo Alto, CA).

Affymetrix microarray. Total RNA was isolated from 80 embryos per treatment in three separate experiments. Hybridizations were conducted for samples from all timepoints and treatments concurrently in each experiment. Probe synthesis, hybridization and scanning was conducted by the CGRB using standard Affymetrix procedures (Santa Clara, CA). Total RNA (2.5 μg) was used to generate biotinylated complementary RNA (cRNA) for each treatment group using the One-Cycle Target Labeling kit (Affymetrix). Briefly, RNA was reverse transcribed using a T7-(dT)24 6primer and Superscript II reverse transcriptase (Invitrogen) and double stranded cDNA was synthesized and purified. Biotinylated cRNA was synthesized from the double stranded cDNA using T7 RNA polymerase and a biotin-conjugated pseudouridine containing nucleotide mixture provided in the IVT Labeling Kit (Affymetrix). Prior to hybridization, the cRNA was purified, fragmented and 10 μg from each experimental sample was hybridized to zebrafish genome arrays (Zebrafish430_2) according to the Affymetrix GeneChip Expression Analysis Technical Manual (701021Rev. 5). Affymetrix fluidics station 400 was used to wash the arrays and arrays were scanned with an Affymetrix scanner 3000. The Affymetrix eukaryotic hybridization control kit and Poly-A RNA control kit were used to ensure efficiency of hybridization and cDNA amplification. All cRNA for each experiment was synthesized at the same time. Each array image was visualized to discount artifacts, scratches or debris. Experiments were MIAME certified. The Affymetrix zebrafish genome array was designed to detect over 14,900 transcripts. Probe sets comprising 16 different oligonucleotides were designed to detect an individual transcript. Sequences used to design the probe sets were found in dbEST (July 2003), GenBank (release 136.0, June 2003), RefSeq (July 2003), and UniGene (Build 54, June

2003). Sequence similarity to known genes was determined by identifying the full length mRNA sequence for each zebrafish probe set by conducting a BLAST search of each Affymetrix probeset against Genbank (http://www.ncbi.nlm.nih.gov/BLAST/), TIGR (http://tigrblast.tigr.org/tgi/), and Sanger (http://www.sanger.ac.uk/Projects/D_rerio/) databases. Affymetrix .CEL files were imported into GeneSpring 7.1 software (AgilentTechnologies, Palo Alto, CA) and GC-RMA processed to correct for background signal across arrays. Each transcript was normalized to the median signal on the array and all arrays were normalized to the 50^{th} percentile of signal. All statistical analysis of microarray data was conducted using GeneSpring 7.1 software. Only genes at least 2-fold differentially expressed from control transcript levels were considered in the annotations from Experiments 1 and 2. When comparing the similar timepoints of 11 and 14 hpf a 1.7 fold cut-off was assigned. With only duplicates for the early time points, statistical significance could not be determined using analysis of variance (ANOVA) methods. However, significance was determined using the cross gene error model in GeneSpring 7.1 for all arrays and all assumptions of variance resulting in a t-test ($P < 0.05$) identifying signals which were significantly different across all three experiments. This list was then matched to those genes which were also greater than 1.5 fold differentially regulated for the respective timepoints.

qRT-PCR validation of mRNA abundance. Prior to the creation of cDNA, total mRNA was DNase-treated with RQ1 DNase (Promega, Madison, WI) according to the manufacture's protocol. cDNA was prepared from 1 μg RNA per group using Superscript II (Life Technologies, Gaithersburg, MD) and oligo(dT) primers in a final 50

μl volume. Quantitative PCR using gene specific primers was conducted using the Opticon 3 real-time PCR detection system (MJ Research, Waltham, MA). Specifically, 1 μl of each cDNA pool was used for each PCR reaction in the presence of SYBR Green, using DyNAmo SYBR Green qPCR kit according to the manufacturer's instructions (Finnzymes, Espoo Finland). All experimental samples were run on the same plate as β-actin along with an internal calibrator for each plate. C(t) values for control and treated samples were taken by choosing a barline across all samples before calculating the relative difference of each sample compared to the internal calibrator. Samples were then normalized to the corresponding β-actin value and calculated as ratios of treated compared to control samples. Negative controls for each experiment consisted of RNA without reverse transcriptase and another without template. All oligonucleotide primers were synthesized by MWG-Biotech (High Point, NC; Table 3-1). Primers were designed to amplify a sequence within the Affymetrix probe set sequence. Agarose gel electrophoresis and thermal denaturation (melt curve analysis) were conducted to ensure formation of specific products.

Whole-mount neuronal labeling. Monoclonal antibodies generated against acetylated tubulin (mouse anti-AT, 1:1000; Sigma) label most axons and major peripheral processes in the developing embryo. The Zn-12 antibody (goat anti-Zn12 1:500) is a monoclonal antibody recognizing L2/HNK-1 tetrasaccharide which is associated with the early 'primary' neurons of the zebrafish embryo and is a well established zebrafish marker for motor neurons (Metcalfe *et al.* 1990). Animals were fixed overnight in 4% paraformaldehyde in phosphate-buffered saline (PBS) and washed in PBS + 0.1% Tween

20 (PBST). The larvae were permeablized with 0.005% trypsin (4°C) in PBS on ice for 5 min, rinsed in PBST and postfixed in 4% paraformaldehyde. Permeablized larvae were blocked in 10% normal goat serum in PBS + 0.5% Triton X-100 for an hour at 22°C and incubated with the primary antibody overnight at 4°C in 1% normal goat serum-PBS + 0.5% Triton X-100. After four 30-min washes in PBST, the larvae were incubated with a secondary antibody (1:1000 Alexa-546 conjugated goat anti-mouse; Invitrogen) for 5 h at 22°C. The larvae were then washed four times for 30 min in PBST and visualized by fluorescence microscopy. All animals were examined and representative pictures were taken. The experiment was repeated for all the developmental time points. Z-stacks were acquired varied depth increments to capture the breadth of the animal (12-30 stacks).

Transmission Electron Microscopy (TEM). Zebrafish embryos were placed in a one-half strength Karnovsky's fixative consisting of a solution of 2% paraformaldehyde and 3% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.4. After fixing overnight, fish were surrounded by 1% agarose (low melting temperature), allowed to set, cut into blocks approximately 2x2 mm and placed back into fixative until processed. Fish were processed in a Lynxel Microscopy Tissue Processor with a processing schedule of: 0.1 M cacodylate buffer 2x 30 min RT; 1% osmium tetroxide in 0.1 M cacodylate buffer 1 hr RT; 0.1 M cacodylate buffer 2x 30 min RT; acetone (10%, 30%, 50%, 70%, 80%, 95%, 100%, 100%, 100%) all 10 min RT, 3:1 acetone: resin 30 min RT, 1:1 acetone: resin 30 min RT, 1:3 acetone: resin 30 min RT, 100% resin 1hr 30°C, 100% resin 1hr 30°C, 100% resin 20 hr RT. Fresh resin was placed in flat embedding silicone molds, fish were orientated and were polymerized for 24 hr at 60°C. Resin was a modified Mollenhour

formula of Epon-Araldite (10 ml Embed 812, 10 ml Araldite 502, 24 ml DDSA, 0.9ml DMP-30; Electron Microscopy Sciences, Fort Washington, PA). Blocks were sectioned on a MT-5000 ultramicrotome using a diamond knife and sections were placed on 300 mesh copper grids. Grids were stained with saturated aqueous uranyl acetate and Reynolds lead citrate and viewed using a Zeiss 10A TEM.

Results

There were fewer than 150 genes 2-fold differentially regulated at any one time in both Experiments 1 and 2 (Fig. 3-1). Only 2 genes were shared between 11, 14 and 18 hpf. These genes are for the muscle fiber, titin, and an unidentified transcript. From an array with nearly 15,000 oligonucleotides representing almost the same number of unique genes this response indicates a specific target and mechanism rather than a general toxicosis induced by DTCs. Transcriptional responses from the Affymetrix array were validated with real time qPCR using primers designed to emcompass the array oligonucleotide sequence, thereby directly confirming the array response (Table 3-2). The fold induction between the array and qPCR confirm the trends observed with the Affymetrix array. In addition, two other independent samples were collected at the 11 and 14 hpf timepoints and were used as biological replicates in determining error for some qPCR samples (Fig 3-3). These primer pairs were selected for their presence across all experimental samples and timepoints and are relevant to the types of transcriptional changes observed.

Early developmental transcriptional responses to NaM encode for genes important in neurogenesis, myogenesis and transcription and/or translational control. There were only two notable mesodermal inducers differentially regulated in our studies: *lefty* 1 (14 hpf) and *sizzled* (24 hpf). *Lefty* belongs to the TGFβ family of factors which act as antagonists on nodal signaling. Mutants related to *lefty* develop no somites except for the most posterior (Schier 2001). Sizzled has a negative impact on BMP signaling and its expression is spatially restricted to the most posterior somites. The *bhikhari* and *smad 3a* transcripts were upregulated at 11 and 14 hpf, respectively. Both play critical roles at different junctions in the TGFβ signaling pathway. The expression patterns of these related gene products suggests increased TGFβ signaling in response to NaM developmental exposure in zebrafish.

Not immediately apparent from these illustrations is the number of differentially regulated genes throughout the three earliest time points related to oxidative stress pathways, including the upregulation of *glutathione transferase pi (14/24), glutathione reductase(24),* and *hypoxia-inducible factor1(18),* suggesting these genes are under inductive cues from DTCs. The upregulation of genes encoding fast muscle fibers was interesting in its specificity, but not surprising due to previous observations in the older animal. Across all timepoints, *titins* were consistently upregulated (Table 3-2 to 3-6). At 14 hpf myosin light and heavy chains are initially down regulated, but from 18 through 24 hpf the expression of these muscle genes and many others remain elevated (Fig 3-2b). This timing correlates well with the expected timing of myogenesis in the developing embryo and suggests an impact to key regulatory processes.

Considering Teraoka *et al.* (2006) demonstrated a requirement for neuro-muscular function to distort the notochord; it is possible that the spontaneous motor neurons were either malformed or improperly functioning. We present evidence for both in the transcriptional signature observed during early development and in biochemical endpoints measured in older animals using whole mount immunohistochemistry and transgenic animals to follow the development of pheripheral, motor, and early 'primary' neurons in control and treated embryos (Fig 3-5). From the alpha acetylated and ZN-12 immunohistochemistry it is clear that distortion of the notochord results in a perturbation of spinal interneurons and 'primary' neuronal network. Compared to control animals, the normal straight tracts making up the spinal cord are pulled out of place in NaM-treated animals and it is clear that there are more branches in the axons (Figure 3-5). Within the gene lists there are many genes related to the proper function of the glutamate and glycine signaling. Expression data for these genes places them in the CNS and spinal chord supporting the clear perturbations illustrated in Fig 3-3 (Thisse *et al.* 2001). For example, SCAMP 5 was upregulated in response to NaM at 14 hpf and this is a marker for the maturation of the synapse. Acetylcholinesterase was 3-fold down regulated at 11 hpf (Table 3-5). In Zn-12 animals, the 'primary' neurons appear to extend down in a regular repeating pattern to innervate muscle in exposed embryos albeit increased branching in these neuronal tracts. These neurons appear to reach their target in NBT-animals treated with NaM despite their convoluted pathways around the distorted notochord (Figure 3-6). This may indicate that normal neural crest cell migration, which precedes these labeling events by several hours, is unaltered by DTCs.

In TEM images of 48 hpf MITC-exposed animals, it is clear that there is a distinct disruption of collagen (Fig. 3-4). This is consistent with previous reports of collagen IIa miss-expression where collagen and collagen processing gene expression is perturbed (Table 3-5). It was also of interest that we observed a large number of substance-filled vacuoles in the notochord cells of MITC-treated zebrafish at 48 hpf that may be explained by the miss-regulation of protein processing and degradation pathway (Table 3-6). In the many laminin mutants there is a mutation in the genes related to structure as well as the filling of notochord vacuoles which exhibits a similar but distinctive phenotype (Eisen and Melancon 2001). Additionally, we have some limited evidence that there is little difference between control and treated 24 hpf embryos proteome (Figure 3S-1).

Discussion

Skeletal muscle genes begin to be expressed as early as 10 hpf in the zebrafish and when normalized to developmental stage these patterns compare favorably with the mouse (Xu *et al.* 2000a). There is a clear gradient of muscle gene expression that follows the wave of somitogenesis and maturation of the somites. Muscle related genes are known to be derived from cells present in these somites. Slow muscle formation is directly influenced in part by the notochord due to the proximity of adaxial cells to the notochord (Devoto *et al.* 1996). The presomitic mesoderm, which gives rise to fast muscle, is closer to the surface ectoderm. This process is preceded and reinforced by

expression of several muscle regulatory factors (MRFs) including MyoD, for which we have shown impairments of expression using in situ hybridizations pattern (Chapter 2)(Hsiao *et al.* 2003; Xu *et al.* 2000a). Besides MyoD there are other muscle regulatory factos identified which are involved in the determination and differentiation of muscle cells for which expression patterns are known (Hsiao *et al.* 2003; Xu *et al.* 2000b). In this study the muscle genes perturbed by NaM exposure were largely restricted to the fast muscle type (Table 3-2/6). Therefore, the early developmental sequence involved in the formation of skeletal muscle is targeted by DTCs. It is unclear how these cell types differentiate and how NaM may perturb this process (Devoto *et al.* 1996). Myostatin-1 is a TGFb transcription factor that functions as a negative regulator of skeletal muscle development and growth (Amali *et al.* 2004). Myostatin knock down in zebrafish resulted in upregulation of IGF-1 and muscle specific genes including other metal regulatory factors. IGF-1 is upregulated at 14 hpf in these studies and we have previously demonstrated impacts to myoD expression. We have also identified two other factors, one unknown, possibly involved in muscle development up-regulated at 11, 14, and 18 hpf.

Both heat shock protein 70 (hsp70) and heat shock protein 90 (hsp90) are down regulated at 14 hpf. Hsp90 and hsp70 family of molecular chaperones play important roles in maintaining the conformation of proteins as well as chaperoning their degradation (Cyr *et al.* 2002). Pharmacological inhibition of hsp90 resulted in the absence of engrailed-2 expressing muscle pioneer cells which was stage specific (Hartson *et al.* 1999). The down regulation of hsp70 and hsp90 may play a role in the muscle fiber types perturbed in this study.

Bhikhari is a zebrafish LTR-retroelement transcribed in early mesendodermal cells which was shown to form complexes with Smad proteins and FAST-1 proteins (Vogel and Gerster 1999). It is interesting then that Bhikhari is upregulated rather dramatically for this study at 14 and 18 hpf. Smad has been shown to be associated with neuronal and synaptic plasticity (Zhou *et al.* 2003). Ubiquitiation is down regulated in response to increased TGFbeta signaling through Smad proteins (Ma *et al.* 2003). Of the 9 genes related to the processing and degradation of proteins found in the gene lists of Experiments 1 and 2, 6 are down regulated and they are all within the 14 hpf timepoint. Ubiquitation involves several enzymes with the intent of tagging the protein for further processing. The rate limiting enzyme in this process and the critical active site is a cysteine dependent (Pickart and Eddins 2004). TGF-beta family proteins such as lefty contain a cysteine knot motif which serves as ligand for many types of plasma membrane receptors. Several cysteine knot motifs have been evaluated for their ability to function as BMP antagonists (Avsian-Kretchmer and Hsueh 2004).

Members of the transforming growth factor (TGF)-beta family of cell-signaling molecules have been implicated recently in mammalian left-right (LR) axis development Analysis of 126 human cases of LR-axis malformations showed one nonsense and one missense mutation in LEFTY A. Both mutations lie in the cysteine-knot region of the protein LEFTY A, and the phenotype of affected individuals is very similar to that typically seen in Lefty1-/- mice with LR-axis malformations (Kosaki *et al.* 1999). Lefty 1 antagonizes TGFb signaling, specifically squint, and this pathway has been identified in zebrafish (Bisgrove *et al.* 1999; Constam and Robertson 2000; Feldman *et al.* 2002; Hashimoto *et al.* 2004)). Interestingly, no zebrafish mutant for this antagonist of Nodal

signaling is yet to be identified (Schier 2001).

Teraoka *et al.* (2006) reported that the abolishment of neuromuscular junctional function resulted in a straight notochord. This raised the possibility that deficits in neural crest cell migration would result in structural abnormalities such as excessive branching of motor neurons in the role in notochord distortions. AAT and Zn-12 whole mount immunohistochemistry labeled motor neurons in both control and treated embryos (Fig 3-4). It is clear that neural crest cells migrated along mostly normal tracts because the labeled neurons are derived and localized by these cell movements. Furthermore, the transcriptional responses characteristic of this pathway (e.g. Notch, Sonic Hedgehog) are not observed in the transcriptional responses to NaM reported here. In support, the numerous mutants related neural crest cells do not exhibit any reported notochord distortions (Coles *et al.* 2006; Honjo and Eisen 2005; Parsons *et al.* 2002). Lamanin mutants have some similarity to DTC induced distortions in the secretion of cell products into the notochord resulting in some animals with waves in the notochord sheath cells (Supplemental EM fig) (Coles et al 2006). Still the phenotypes are dramatically different and this may indicate only minor contributions of Shh signaling and related pathways in DTC developmental toxicity.

The adaxial cells which become slow muscle pioneer cells are adjacent to the notochord during early somitogenesis before migrating out to the periphery are also not present in the NaM-induced gene lists (Devoto *et al.* 1996). Furthermore, this slow muscle has been shown to direct neural crest cell movements when forming motor neurons (Honjo and Eisen 2005). This may explain why motor neurons appear to largely reach their target unlike other neurotoxities such as cadmium (Chow and Cheng 2003).

TGFbeta signaling has a positive impact on lysyl oxidase and other collagen related enzymes (Koslowski *et al.* 2003). One possible explanation for the developmental neurotoxicity reported here may lie in improperly formed collagen networks inhibiting proper neural crest cell movement. Consideration of neuronal function during development will need to be considered in future studies.

One example is the early down regulation of acetylcholinesterase. NaM is cited as having tested positive in acetylcholinesterase assays (U.S.EPA 2001). While important in its own right, this may also indicate impairment at the neuromuscular junction decreasing the stability of neuromuscular synapse stability during early development (Downes and Granato 2004). DTCs, such as Propineb, have been shown to facilitate acetylcholine signaling by increased pre-synaptic release (Marinovich *et al.* 2002). It would be predicted that neuronal function is impaired by developmental DTC exposure and further study is warranted.. It will require more cell specific studies to understand when neural tissue is impacted by developmental exposure to NaM or if these alterations reflect secondary effects. It would seem logical from the genes perturbed involving neuronal functional that behavioral deficits likely exist.

The expressions of genes known to be have restricted expression in the brain and spinal chord were differentially regulated in treated animals throughout exposure (Thisse *et al.* 2001). From the AAT immunohistochemistry it is clear that treated embryos have significant distortions in the patterning of spinal interneurons. It is unclear whether these effects are secondary to the distorted notochord. In addition, expression of Napor/CUG-BP2, a key regulator of neuronal development, was elevated at 11 hpf. The expression of this RNA-binding protein is restricted to the CNS and adaxial mesoderm cells during

early somitogenesis (Choi *et al.* 2003). Several other interesting targets were identified including up regulation of NMDA receptors, Pax6 (GABA neuron maturity). These gene products are involved in the coordinated movement of swimming behavior; (opposed muscle movements) which is a well established behavioral endpoint in zebrafish (Hanson and Landmesser 2003; Higashijima *et al.* 2004). In normal development these neurons play no role in the early spontaneous muscle contractions. It would not be predicted that they would be involved then in the distortion of notochord, although this requires additional studies.

It has recently been demonstrated in zebrafish that the pi class glutathione-S-transferase (GSTpi) genes are regulated by Nrf2 through a conserved regulatory element (suzuki et al 2005). In response to electrophilic compounds, Nrf2 will mediate the expression of GSTpi resulting in the clearance of the toxicant. GSTpi is upregulated early (14 hpf) and stays overexpressed for the remainder of early development in response to NaM. Glutathione reductase also appears on the gene list at 24 hpf as do other redox sensitive genes related to apoptosis (Tables). The expression profile of these genes suggests that DTCs may be altering cellular thiol levels, acting to induce this pathway, or creating a state of oxidative stress. This possibility will be begin to be tested in Chapter 4.

We used several lines of evidence to relate the observed gene expression changes during somitogenesis to the eventual ramifications seen in the older embryo. For yet unknown reasons somitogenesis appears to be a susceptible period of development for DTC insult. The major developmental processes (i.e. muscle) that derive from somites were reflected in the genes and observations in the whole animal supporting the

hypothesis that DTCs target somitogenesis. Upon annotation and analysis it became clear that a transcription signature for activin signaling was present and is a likely candidate as a molecular target for DTC developmental toxicity. The transcriptional profile and other observations made in this study lead us to hypothesize that DTCs are acting to suppress inhibitory cysteine knot TGFb transcription factors resulting in an overactive TGFb signaling pathway leading to increased muscle growth and perturbed protein degradation pathways. Additional understanding of the molecular ramifications of DTC insult during development.

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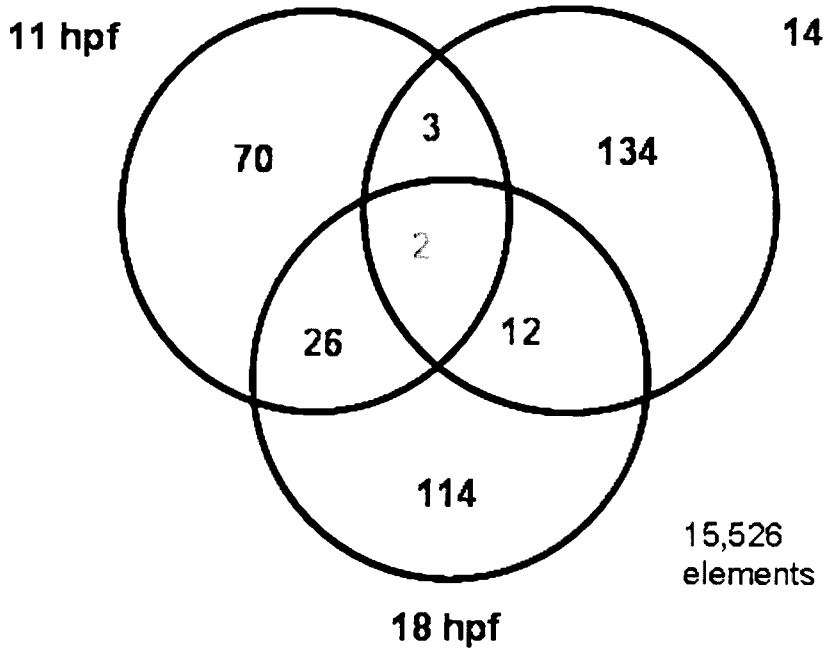

Figure 3-1. Venn Diagram of 2-fold differentially regulated genes (up and down) at 11, 14, and 18 hour post fertilization (hpf) in metam sodium exposed zebrafish embryos.

Table 3-1 **Primers used to validate the Affymetrix zebrafish arrays used in this study**

Annotated Gene Name	GenBank	Forward Primer	Reverse Primer
Danio rerio tryptophan hydroxylase 1, like	BQ092469	5' TCAGAATTGGACGCAGATCA	5' TGTTCAACTCCCGAAAC
Danio rerio similar to titin isoform N2-B	AI601291	5' AAACCTGAAACGAAAGA	5' AGTTGTCCTGCTTACATGAG
mouse Grid2ip (ionotropic glutamate receptor delta 2 interacting protein 1)	BQ284721	5' AGTACACATGCGTCCATC	5' ACTAAATTCACAAGCGTGTC
vertebrate discoidin domain receptor family, member 2	BI888730	5' TCTGACACGGTGACTATTG	5' GGCATTGTAAAAAGTAGATG
Danio rerio secretory carrier membrane protein 5 (scamp5)	AL907213	5' GCCCCTCAATAGGAA	5' TGAATATGTAAGCCAACCAA
Danio rerio myosin, heavy polypeptide 2, fast muscle specific (myhz2)	NM_152982.1	5' GCCTGCAGCACCGTC	5' TCTTGTAAGCCTTGACCTTC
Danio rerio SRY-box containing gene 31	BM402113	5' TCTTCGCTGTCAGTCAATG	5' GCAGGAGTTAAAACTAACC
Danio rerio glutathione S-transferase pi (gstp1)	NM_131734.1	5' TTCAGTCCAACGCCATGC	5' ATGAGATCTGATCGCCAACC
Danio rerio sizzled	NM_181663.1	5' CGCATCATTCACTAGTAGC	5' TAAATCATATCTAGGGCACA

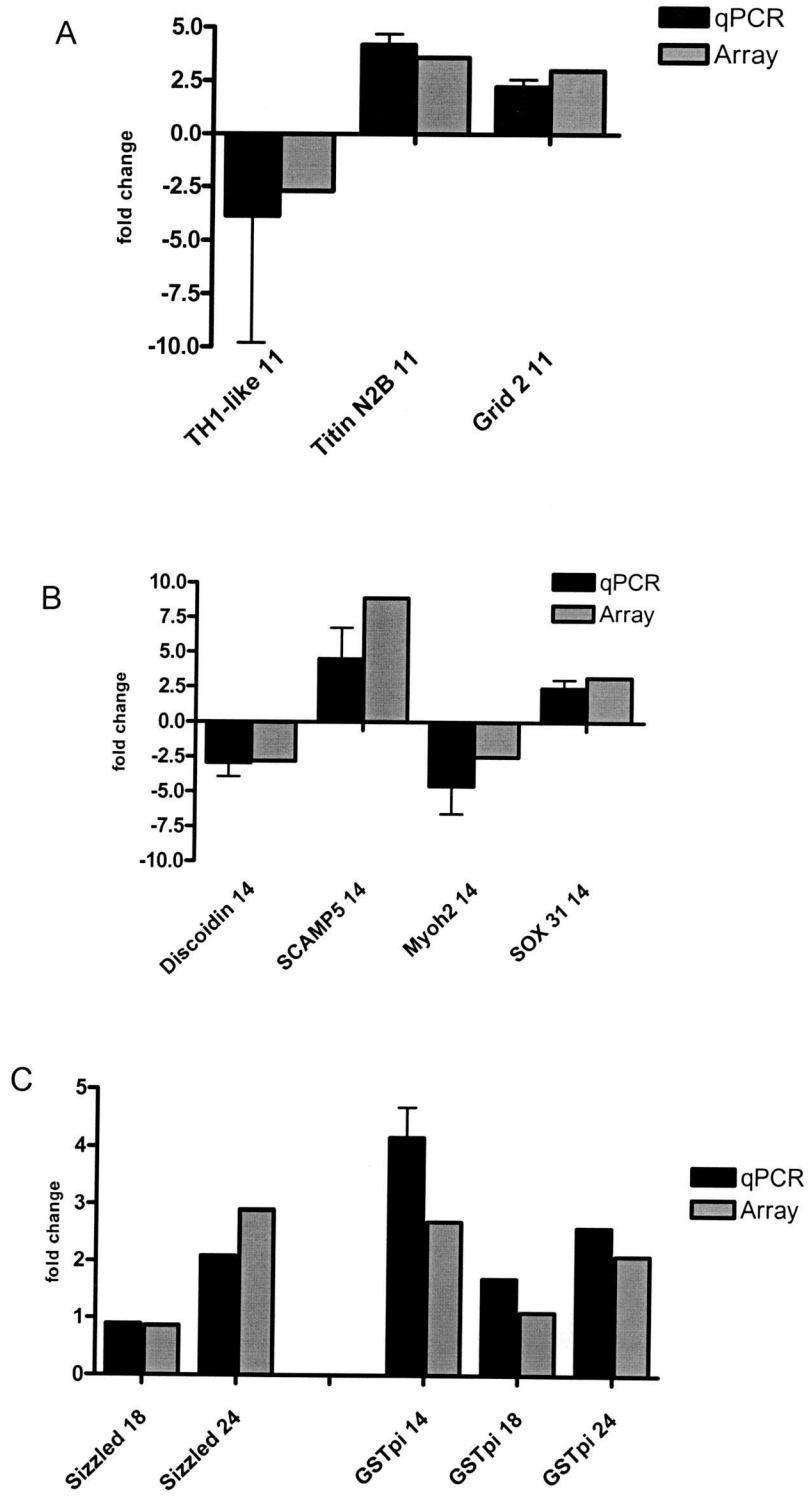

Figure 3-2. Real time Q-PCR of selected genes from all samples. 11 and 14 hpf N=3 in duplicate from total RNA from the two experiments and a third independent replicate. 18 and 24 N=1 in duplicate.

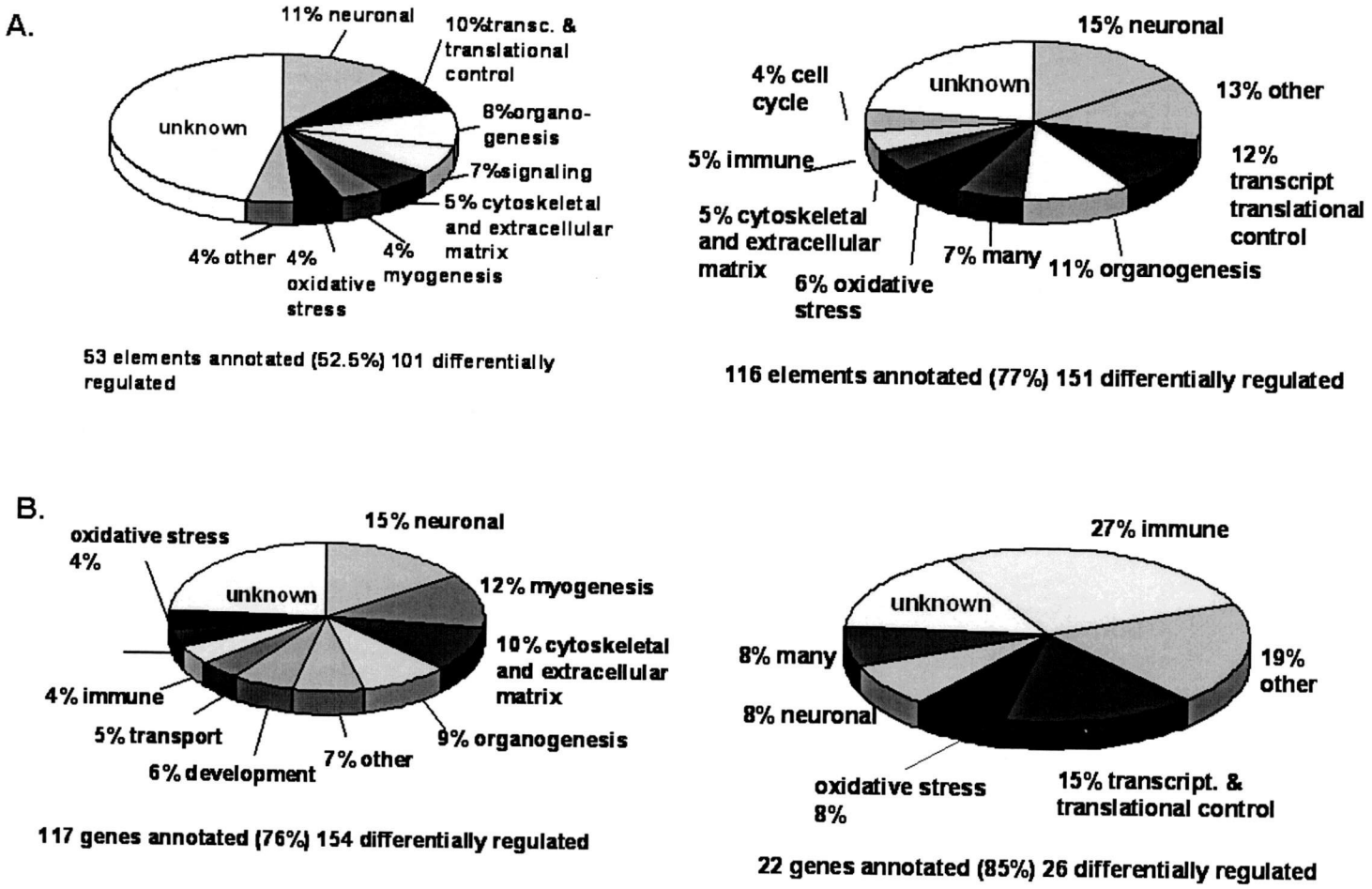

Figure 3-3. Pie graphs showing annotation groupings of genes from the 2-fold lists of Experiments 1 and 2. A) Grouping of genes at 11 and 14 hours post fertilization (hpf) from Experiment 2. B) Grouping of genes at 18 and 24 hpf.

Table 3-2 Genes differentially regulated from controls greater than 3.0 fold at 11 hpf

fold change	GenBank	annotation	GenBank	Bits	E-value	length	homology %
MYOGENESIS							
3.6	AI601291	Danio rerio similar to titin isoform N2-B, mRNA	BC095863	290	8e-76	152	98
3.2	AY081167.1	Danio rerio titin mRNA, partial cds	AY081167	3489	0.0	1760	100
TRANSCRIPTION							
7.4	AI793374	Gallus gallus Jumonji, AT rich interactive domain 2 (JARID2), mRNA	NM_001012862	313	2e-81	506	82
6.5	BG306111	Danio rerio kheper mRNA, transcription factor with zinc finger domain and homeo domain, complete cds. Danio rerio zinc finger homeobox 1 (zfhx1), mRNA.	AB016799 NM_131709	6950	0.0	3542	99
3.3	AI964806	Zebrafish DNA sequence from clone DKEYP-6C7 in linkage group 20 Contains the 5' end of the gene for a novel protein similar to vertebrate mannosidase alpha class 1A member 1 (MAN1A1) and two CpG islands, complete sequence.	BX465187	270	9e-70	152	98
-3.0	NM_131789.1	Danio rerio cryptochrome 1a (cry1a), mRNA	AB042248	3313	0.0	1671	100
NEUROGENESIS							
-3.1	NM_131846.1	Danio rerio acetylcholinesterase (ache), mRNA	NM_131846	5051	0.0	2548	100
2.0	BM025940	Danio rerio cullin 3 (CUL3) mRNA, complete cds.	AY423034	418	e-114	238	96
3.1	AI942866	Mus musculus tetraspanin 9 (Tspan9), mRNA.	NM_175414	236	5e-59	255	86
3.0	AI667492	Zebrafish DNA sequence from clone CH211-114C9 in linkage group 20 Contains the gene for a novel protein similar to vertebrate NIMA (never in mitosis gene a)-related kinase 1 (NEK1), the gene for a novel protein similar to vertebrate SH3 multiple domains 2 (SH3MD2), a novel gene and a CpG island, complete sequence. Zebrafish DNA sequence from clone CH211-87E4 in linkage group 20 Contains the 5' end of a novel gene, a novel gene, the gene for a novel protein similar to vertebrate SH3 multiple domains 2 (SH3MD2), a novel gene (zgc:56141) and a CpG island, complete sequence. Zebrafish DNA sequence from clone DKEY-15J16 in linkage group 20 Contains the 3' end of the gene for a novel protein similar to human MDN1, midasin homolog (yeast) (MDN1), the gene for a novel protein similar to human and rodent SH3 multiple domains 2 (SH3MD2) , two novel genes, the gene for a novel protein similar to vertebrate katanin p60 (ATPase-containing) subunit A 1 (KATNA1), the 3' end of the gene for a novel protein similar to human and mouse NIMA (never in mitosis gene a)-related kinase 1 (NEK1) and a CpG island, complete sequence.	BX276086 BX255904 CR388170	131 123 131	2e-27 4e-25 2e-27	183 183 183	84 84	84
3.1	AI942824	Zebrafish DNA sequence from clone DKEY-264K15 in linkage group 4 Contains the ascl1a gene for achaete-scute complex-like 1a (Drosophila), the gene for a novel protein similar to vertebrate RecQ protein-like DNA helicase Q1-like (RECQL), the gene for a novel protein similar to tyrosine hydroxylase (th), the pah gene for phenylalanine hydroxylase, the 5' end of the gene for a novel protein similar to vertebrate characterized hematopoietic stem/progenitor cells protein MDS028 (MDS028) and a CpG island, complete sequence.	BX511171	99.6	6e-18	102	88

Table 3-3 Genes differentially regulated from controls greater than 3.5 fold at 14 hpf

fold change	GenBank	cgrb annotation	GenBank annotation	Bits	E-value	length	homology %
SKELETAL AND EM							
3.9	AI396977	unknown					
IMMUNE							
3.7	CD594679	Cyprinus carpio mRNA for natural killer cell enhancing factor, complete cds. Oncorhynchus mykiss clone Arlee natural killer cell enhancement factor (Nkef) mRNA, complete cds.	AB010959 AF250195	557 246	e-155 6e-62	517 472	88 81
ORGANOGENESIS							
ERYTHROPOIESIS							
3.5	BI979764	Danio rerio bloodthirsty (bty) mRNA, complete cds.	AY454307	981	0.0	555	97
HEPATIC							
315.2	NM_131108.1	Danio rerio type I cytokeratin (cki), mRNA	AF197880	2716		1391	99
MYOGENESIS							
3.8	AY081167.1	Danio rerio titin mRNA, partial cds	same	3489	0.0	1760	100
TRANSCRIPTION							
5.1	NM_131803.1	Danio rerio bhikhari (bhik), mRNA	same	3669	0.0	1851	100
5.0	AW165103	Rainbow trout histone H2A and H3 genes.	SGHIS2A3	190	3e-45	186	88
NEURONAL							
8.9	AL907213	Danio rerio secretory carrier membrane protein 5 (scamp5), mRNA.	NM_205547	438	e-120	257	96
4.3	BC048896.1	Danio rerio ubiquitin conjugating enzyme E2D mRNA, partial cds. Mus musculus in vitro fertilized eggs cDNA, RIKEN full-length enriched library, clone:7420474N15 product:ubiquitin-conjugating enzyme E2D 2, full insert sequence.	AF510491 AK136197	837 315	0.0 2e-82	422 443	100 83
4.3	AA497258	Zebrafish DNA sequence from clone DKEY-90M5 in linkage group 20 Contains the gene for a novel protein similar to vertebrate solute carrier family 10 (sodium/bile acid cotransporter family) member 4 (SLC10A4), the zar1 gene for zygote arrest 1, the 5' end of the gene for a novel protein similar to vertebrate palladin, the gene for a novel protein similar to human and mouseanaphase promoting complex subunit 4 (ANAPC4), the 3' end of a novel gene, two novel genes and four CpG islands, complete sequence. fa04e08.s1 Zebrafish ICRFzfls Danio rerio cDNA clone 1O24 3', mRNA sequence.	BX537133	103	1e-19	135	84
UNDETERMINED							
3.9	AW128368	unknown					
3.6	BM775264	unknown					
10.2	AFFX-Dr-U43284-1	Cloning vector phGFP-S65T, complete sequence, green fluorescent protein (gfp) gene, complete cds.	CVU43284	1176	0.0	593	100

Table 3-5 Genes differentially regulated from controls greater than 3.5 fold at 18 hpf

fold change	GenBank	cgrb annotation	GenBank annotation	Bits	E-value	length	homology %
NEURONAL							
5.4	BQ450306	Rattus norvegicus peptidylglycine alpha-amidating monooxygenase (Pam), mRNA.	NM_013000	42.1	0.69	21	100
MYOGENESIS							
4.0	AY081167.1	Danio rerio titin mRNA, partial cds	AY081167	3489	0.0	1760	100
3.9	AI601291	Danio rerio similar to titin isoform N2-B, mRNA (cDNA clone IMAGE:7158960), partial cds.	BC095863	285	2e-74	152	98
5.4	BI878949	Zebrafish DNA sequence from clone BUSM1-258D18 in linkage group 9 Contains the 3' end of a novel gene similar to TTN (titin), complete sequence. Zebrafish DNA sequence from clone BUSM1-167C3 in linkage group 9 Contains the 3' end of a novel gene similar to TTN (titin), a novel gene similar to FKBP (FK506-binding protein) and the 5' part of a novel collagen gene, complete sequence. Danio rerio similar to titin isoform N2-B, mRNA (cDNA clone IMAGE:7158960), partial cds.	AL772356 AL714003 BC095863	1332 133 127	0.0 6e-28 4e-26	691 126 80	99 88 96
4.3	AI793374	Gallus gallus Jumonji, AT rich interactive domain 2 (JARID2), mRNA.	NM_001012862	313	2e-81	506	82
OXIDATIVE STRESS							
4.0	BG306111	Zebrafish DNA sequence from clone CH211-146F4 in linkage group 12 Contains a novel gene similar to human BAP28, a putative novel gene, a novel gene similar to human ARG1 (liver arginase), a novel gene similar to human and rodent P4HA1 (procollagen-proline, 2-oxoglutarate 4-dioxygenase (proline 4-hydroxylase), alpha polypeptide I), a novel gene for a protein with a NUDIX domain and four CpG islands, complete sequence.	AL732629	135	1e-28	109	90
DEVELOPMENT							
3.7	AA497258	Zebrafish DNA sequence from clone DKEY-90M5 in linkage group 20 Contains the gene for a novel protein similar to vertebrate solute carrier family 10 (sodium/bile acid cotransporter family) member 4 (SLC10A4), the zar1 gene for zygote arrest 1, the 5' end of the gene for a novel protein similar to vertebrate palladin, the gene for a novel protein similar to human and mouseanaphase promoting complex subunit 4 (ANAPC4), the 3' end of a novel gene, two novel genes and four CpG islands, complete sequence.	BX537133	103	1e-19	135	84
UNDETERMINED							
4.5	AA495044	unknown					

Table 3-6 Genes differentially regulated from controls greater than 3.0 fold at 24 hpf

Fold Change	Genbank	CGRB Annotation	GenBank	Bits	E-value	length	homology %
TRANSPORT							
3.055	AW232676	ferritin middle subunit [Salmo salar=Atlantic salmon, liver, mRNA, 1010 nt].	S77386	216	5e-53	229	86
ORGANOGENESIS							
6.946	BE201834	unknown					
NEURONAL							
0.488	AF124396.1	Danio rerio R-cadherin precursor, mRNA, partial cds	AF124396	4375	0.0	2252	99
0.496	BM342706	unknown					

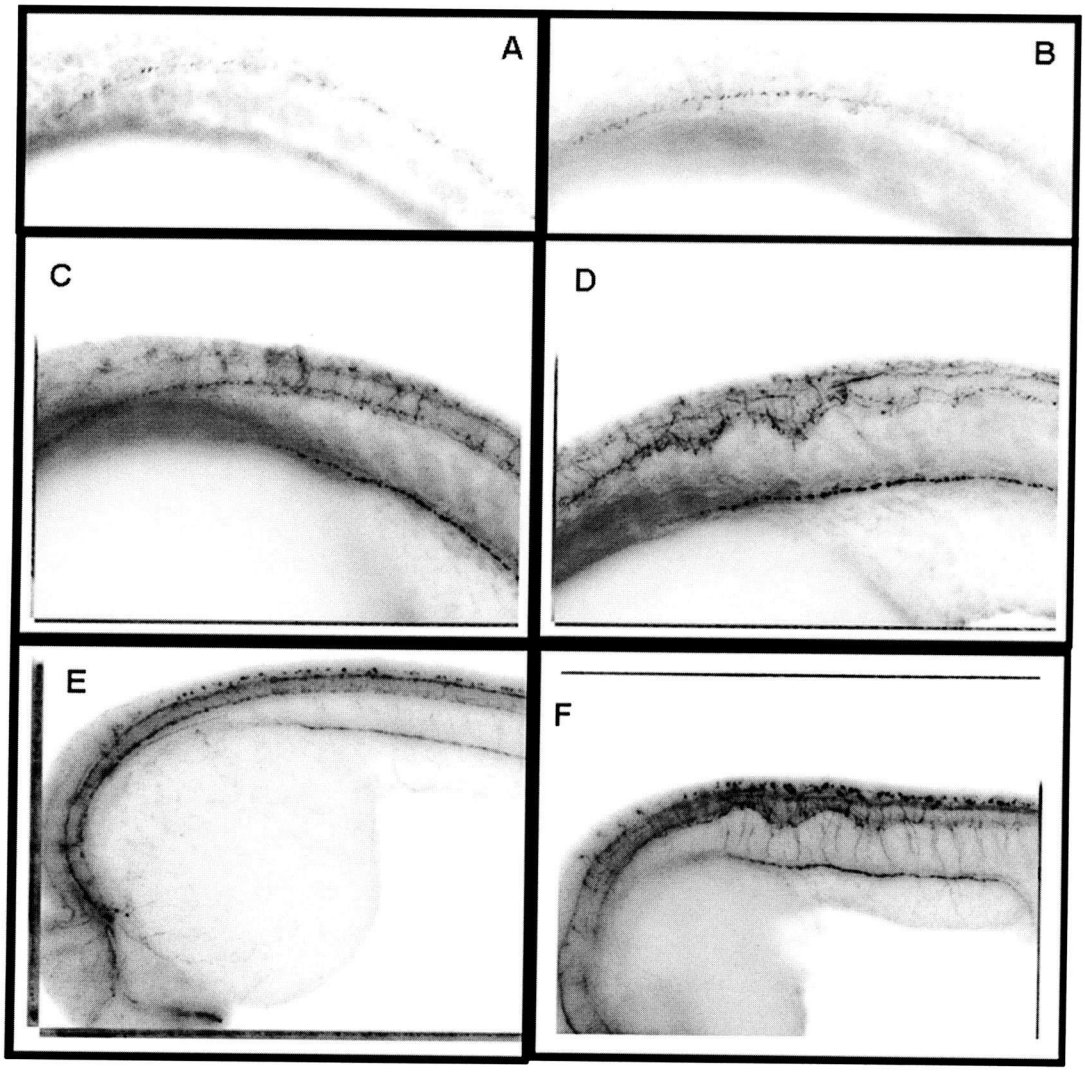

Figure 3-4. Whole embryo immunohistochemistry showing the labeling alpha-acetylated tubulin in the peripheral nervous system of control and exposed embryos at 18, 19.5, and 24 hours post fertilization (hpf). Control (A) and (B) 1.0 uM metam sodium treated embryos at 18 hpf. Control (A) and (B) 1.0 uM metam sodium treated embryos at 19.5hpf.. Control (A) and (B) 1.0 uM metam sodium treated embryos at 24 hpf.

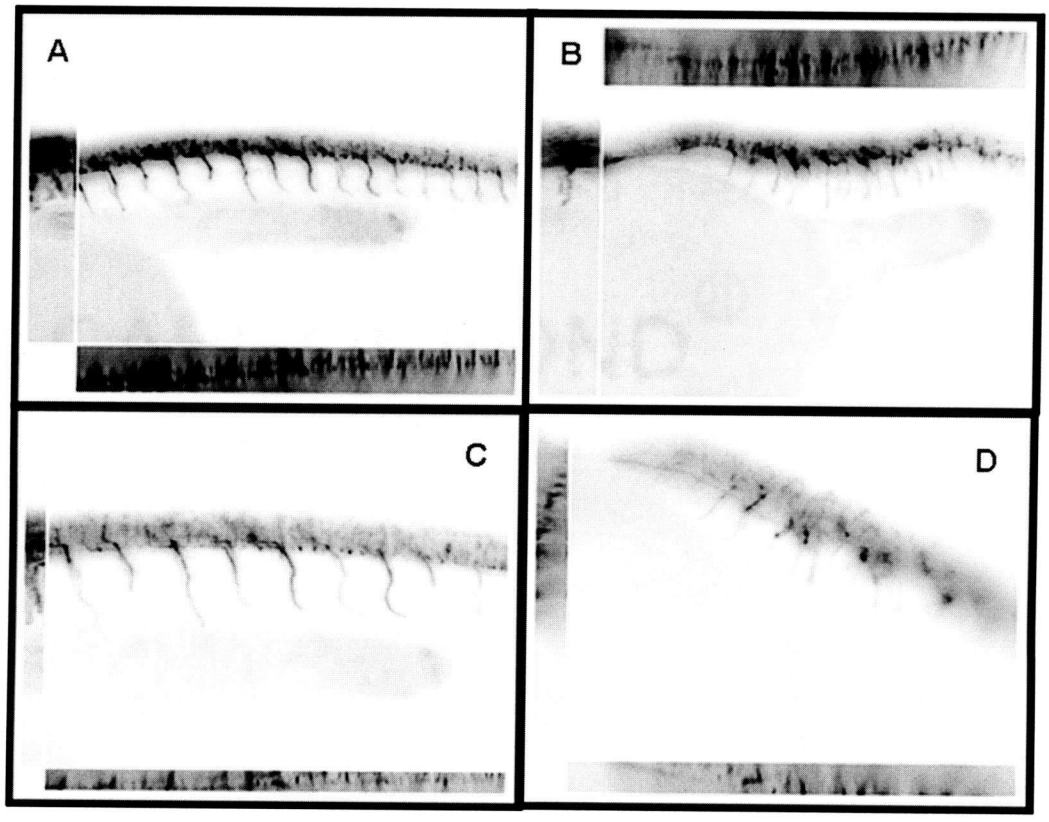

Figure 3-5. NBT transgenic animals with neuronal fluorescence in primary motor neurons exposed to metam sodium. A(8.5x), C(10x). Control NBT embryos at 24 hours post fertilization. (hpf). B(8.5x), D(10x). NBT embryos exposed to metam sodium from 4 to 24 hpf.

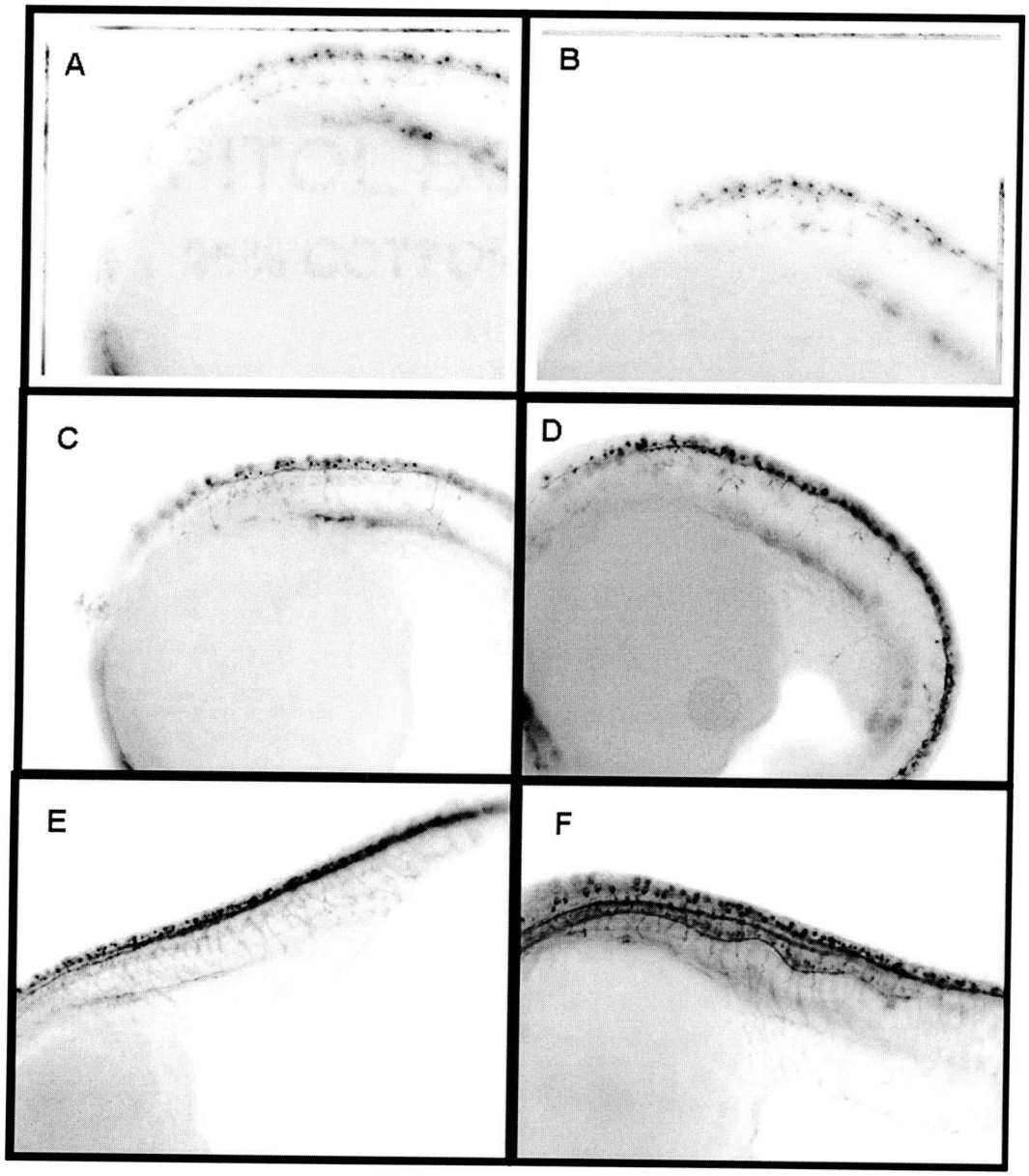

Figure 3-6. Whole mount immunohistochemistry using the Zn-12 antibody recognizing the 'primary' neurons in development. A(10x) control animal and B (10x) metam treated embryos at 18.5 hours post fertilization (hpf). C(8.5x) control and B (8.5x) metam sodium treated embryos at 21 hpf. E(5x) control and F(8.5x) metam sodium treated embryos at 24 hpf.

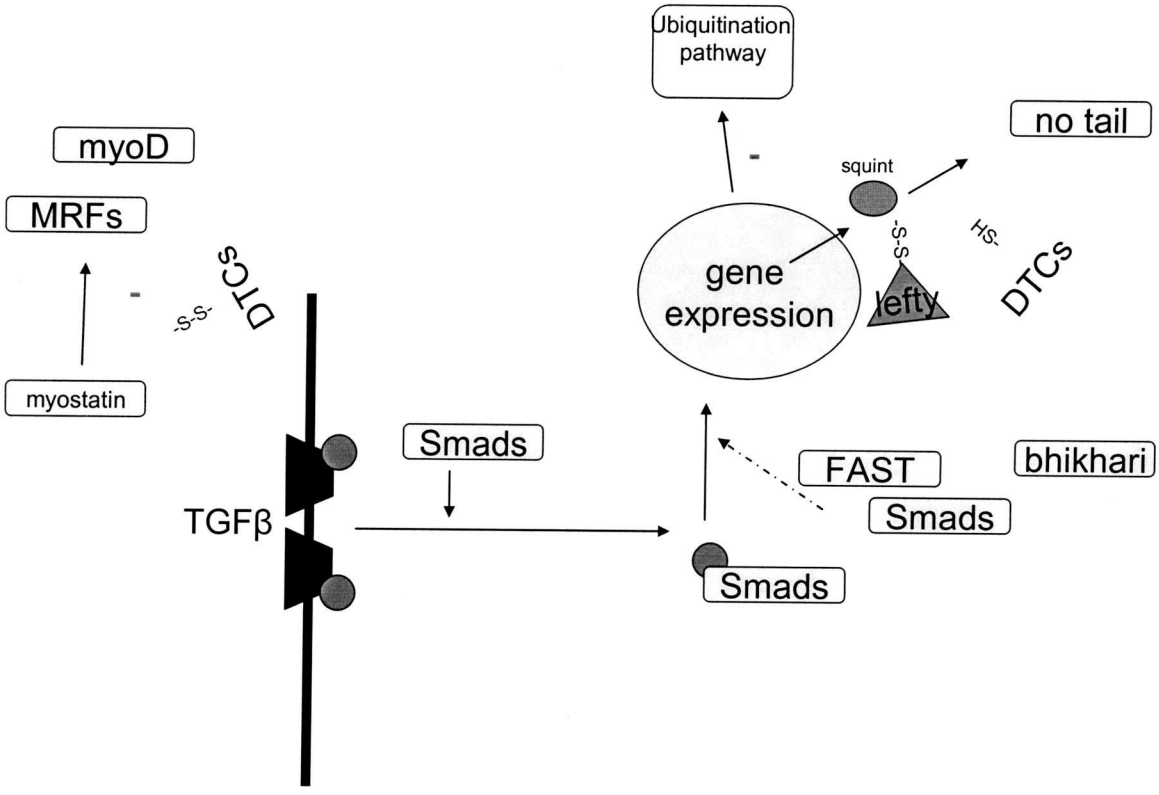

Figure 3-7. Proposed mechanism of DTC interaction with TGFβ pathway during somitogenesis. DTCs may form thiol adducts, alter thiol oxidation state, or directly compete with thiol sensitive transcription factors resulting in misregulated TGFβ signaling.

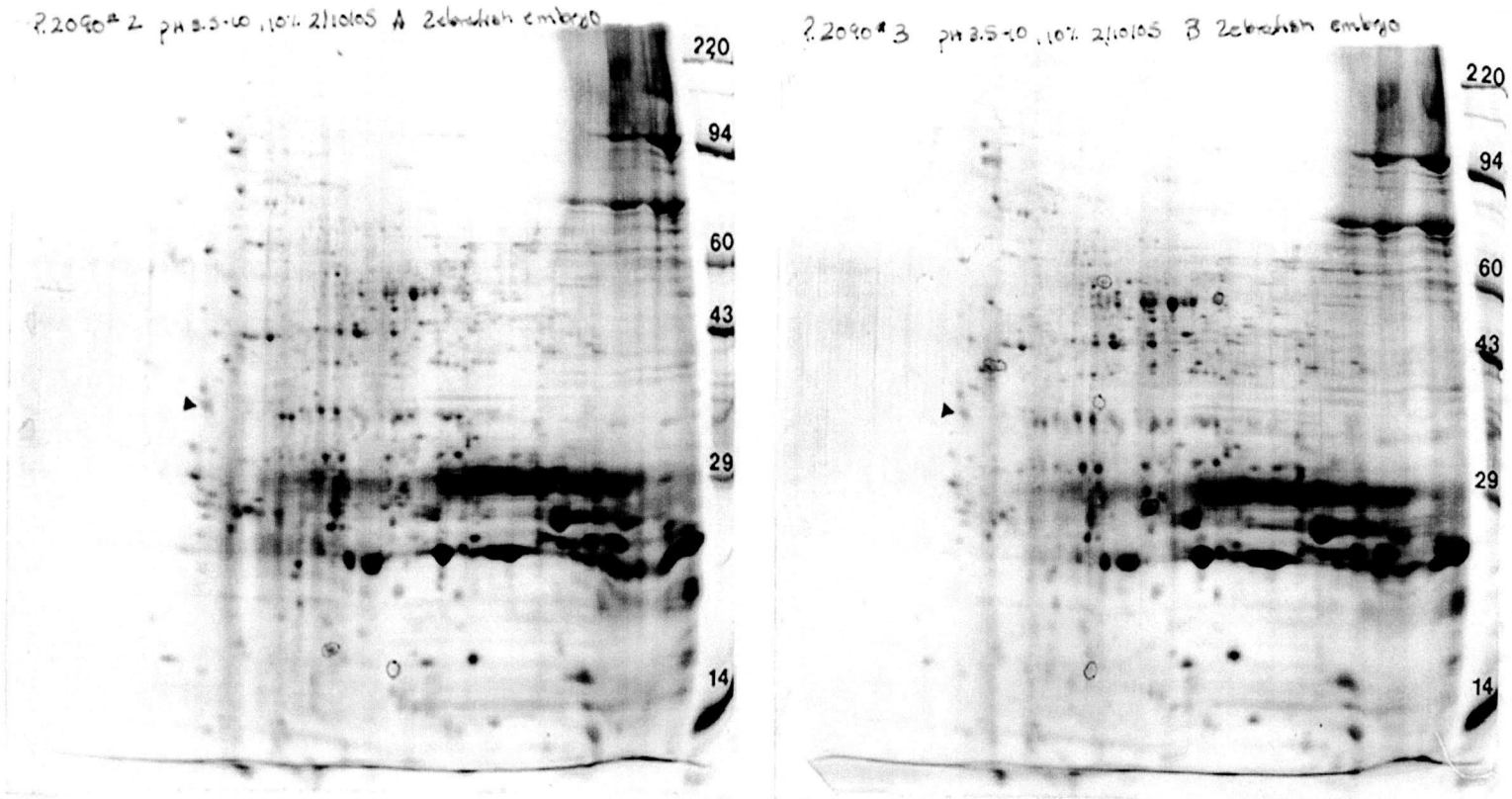

Figure 3S-1. Silver stained 2D gel electrophoresis of proteins from control and metam sodium treated zebrafish embryos at 24 hpf showing few noticeable changes to the proteome. **(A)**. Control embryos with different protein patterns relative to exposed sample are circled. Arrow is protein standard for gel comparison. **(B)**. Exposed embryos with different protein patterns relative to control sample circled. Arrow is protein standard for gel comparison.

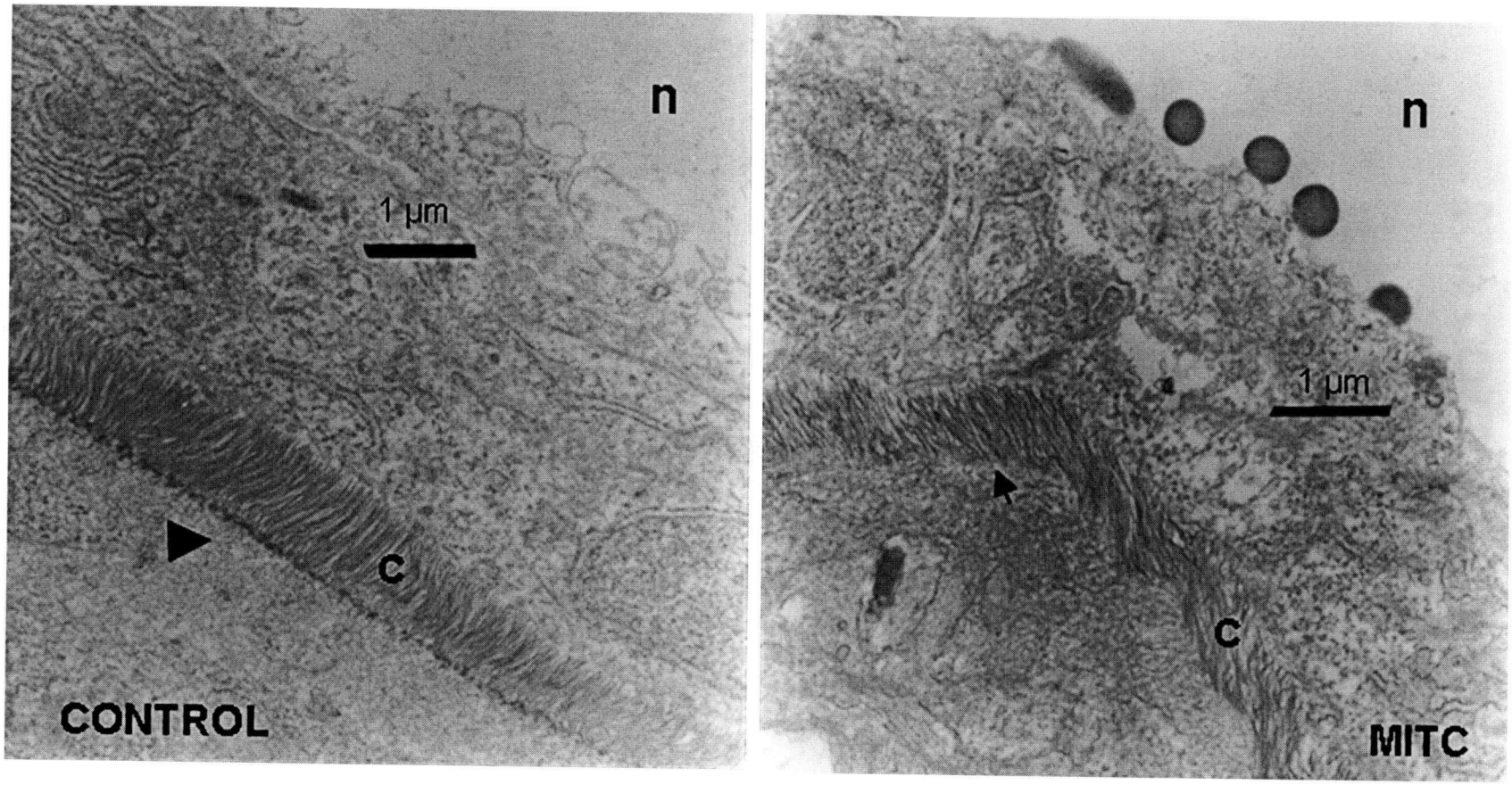

Figure 3S-2. Transmission Electron microscopy of control and methyl isothiocyanate exposed zebrafish embryos. C. collagen fibers, arrow, collagen anchor fibers or lack thereof as in MITC exposed embryos. n, notochord cell

Chapter 4. Glutathione modulates dithiocarbamate developmental toxicity in zebrafish: Implications for oxidative stress

Fred Tilton[*,†,‡], Jane K. La Du[*,†] and Robert L. Tanguay [*,†,‡]

[*]Oregon State University, Corvallis, Oregon 97331,[†]Department of Environmental & Molecular Toxicology, [‡]Environmental Health Sciences Center and Marine & Freshwater Biomedical Sciences Center,

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Abstract

Dithiocarbamates (DTCs) are known to have both anti-oxidant and pro-oxidant abilities depending on the experimental system and conditions. Considering DTCs are thiol containing compounds often associated with metals, we wanted to investigate the role of cell death in the manifestation of DTC-induced notochord distortions in the zebrafish development model. Metam sodium (NaM)-induced distortions could be rescued with the addition of glutathione and n-acetyl-cysteine while other DTCs and methylisothiocyanate were largely non-responsive. We further determined that glutathione could protect the embryos if added in restricted windows during the ten hours following a NaM exposure from 4 to 14 hours post fertilization (gastrulation to 10 somites). Injecting a morpholino at the 1 to 2 cell stage to suppress transcription of the catalytic subunit of glutymyl cysteine ligase, the rate limiting enzyme in glutathione production, produced a dose-response curve for NaM-induced notochord distortions that was shifted to the left. This indicates that glutathione production is critical in reducing susceptibility to DTC exposures. However, NaM-induced distortions could not be modulated with antioxidants, such as ascorbic acid, trolox (synthetic vitamin E), and lipoic acid, suggesting that generation of excessive reactive oxygen species is not involved. Furthermore, no indication of unusual cell death was observed in or around the notochord of NaM-exposed embryos stained with acridine orange at 11, 14, 18 and 24 hpf suggesting that the change in thiol status was not leading to cell death. Taken together, this demonstrates the importance of embryonic glutathione production and thiol status in the susceptibility to DTC-induced notochord distortions.

Introduction

Dithiocarbamates (DTCs) are thiol-containing compounds with nomenclatures derived from the metal cations with which many are associated. The current U.S. EPA mechanism of action for DTCs minimally states that they interact with biologically critical sulfyhryl and metal systems (U.S.EPA 2001). The mechanisms by which DTCs perturb normal vertebrate development are largely unknown and it is unclear whether DTCs, metals, and/or DTC-thiol interactions are responsible for the many DTC observed toxicities. Therefore, while little is known about DTC mechanisms of toxicity, it is reasonable to hypothesize that oxidative stress may play a critical role in the manifestation of DTC-mediated developmental toxicity.

In previous studies of zebrafish exposed to sodium metam (NaM) during early development, we observed significant impairment of transcription for genes, such as glutathione-S-transferase, that are controlled by the antioxidant response element (Chapter 3). These transcriptional changes correlated with NaM-induced notochord distortions, which have been described as a common developmental toxicity for all DTCs (Chapter 2). NaM makes an interesting test compound to probe the potential mechanisms that DTCs have on the oxidative state of developing zebrafish because, unlike other DTCs, NaM rapidly transforms to methylisothiocyanate (MITC) in the environment and it is associated with sodium instead of metals. There is sufficient evidence in the published literature to suspect a role for copper in some, if not all, DTC reported toxicities (Fitsanakis *et al.* 2002; Furuta *et al.* 2002; Valentine *et al.* 2006). However, we have previously demonstrated that NaM and MITC-induced notochord distortions are

unresponsive to addition of exogenous copper (Chapter 2). Therefore, it is likely that DTCs are not simply catalysts for metal redistribution in the developing embryo, but may also be causing notochord distortions in zebrafish by modulating antioxidant pools through thiol-dependent mechanisms.

Previous research indicates that DTC-disulfides can be formed through redox cycling of DTCs with copper and in turn oxidize pools of GSH possibly altering the oxidative state without the production of reactive oxygen species (ROS) (Burkitt *et al.* 1998; Nobel *et al.* 1995; Nobel *et al.* 1997). DTC disulfides can directly oxidize catalytic thiols inhibiting pro-apoptotic enzymes, such as caspase-3, and all DTCs can form *in vivo* cysteine-adducts (Nobel *et al.* 1997; Tonkin *et al.* 2003; Valentine *et al.* 1995). Moreover, several studies have demonstrated the ability of thiols to diminish DTC-induced toxicity in a variety of model systems suggesting the importance of thiol status (Chen and Liao 2003; Cheng and Trombetta 2004; Fitsanakis *et al.* 2002; Furuta *et al.* 2002; Thompson *et al.* 2002).

DTCs have been shown to increase the activity of glutathione peroxidase, superoxide dismutase and alter glutathione ratios differently than ROS generated by paraquat (Barlow *et al.* 2005). Recent evidence further indicates that DTCs may be able to alter cell signaling pathways important for processes of cell proliferation and apoptosis. For example, pyrollidine-DTC induced a bi-phasic response in cell viability and changes to the JNK/ERK system at concentrations too low to be attributable to an increase in cellular metal concentrations (Chung *et al.* 2000). Furthermore, the thiol-containing compound N-acetyl cysteine (NAC), a synthetic precursor to glutathione, had the opposite effect of pyrollidine-DTC on the activation of another cell signaling and

transcription factor, NFκB, which also influences apoptosis and proliferation (Fernandez *et al.* 1999). Given the possible link between DTCs and apoptotic pathways, it is interesting that a recent study in zebrafish reported that the dithiocarbamate thiram did not cause an increase in apoptotic cell populations in developing zebrafish using the TUNEL assay (Teraoka *et al.* 2005). Taken together the probable oxidative properties of DTCs *in vivo* may involve redox targeting of critical components in and around the somites resulting in the altered muscle and neuronal development previously reported from our laboratory (Chapter 2 and 3).

The expression patterns of many enzymes important for the glutathione system are expressed in the developing embryo as early as gastrulation (Thisse *et al.* 2001). While glutathione transferase activity is present in zebrafish development (Best *et al.* 2002; Wiegand *et al.* 2001), it is unknown what impact thiol containing toxicants such as DTCs would have on developing vertebrate systems. In this study we investigated whether developmental exposure to DTCs alters the oxidative state of embryos leading to inappropriate cell death in areas necessary for proper notochord development. We found that DTC developmental toxicity is sensitive to thiol containing antioxidants such as GSH and there are no patterns of cell death in the notochord or somites. This suggests inappropriate levels of ROS do not reach levels overtly toxic to cells and that thiol status is critically important to the susceptibility of the developing vertebrates to DTCs.

Materials and Methods

Zebrafish maintenance and collection of embryos. Adult AB strain zebrafish (Danio rerio) and NBT transgenics were raised and kept at standard laboratory conditions of 28°C on a 14 hr light/10hr dark photoperiod (Westerfield 1995). Fish were maintained in reverse osmosis water supplemented with a commercially available salt solution (0.6% Instant Ocean®) at a pH and conductivity range of 6.8 to 7.0 and 450 to 520 μS, respectively. Embryos were collected from group spawns and staged as previously described (Westerfield 1995). All animal protocols were performed in accordance with Oregon State University Institutional Animal Care and Use Committee guidelines.

Embryo exposures. Embryos showing proper development in the first 3 hours post fertilization (hpf) were selected for exposures and were placed in Teflon sealed clear glass vials when they reached 4 hpf. All exposures were in 20 mL water from 4 to 24 hpf in order to capture the major early developmental milestones. The concentrations of NaM (1.0 μM), MITC (1.0 μM), PDTC (0.24 μM) and dimethyl DTC (DMDTC) (0.24 μM) were the experimentally determined concentrations which produce notochord distortions in 100% of the embryos (Chapter 2). For NaM experiments during restricted windows, embryos were removed from the exposure vials at 14 hpf, rinsed and either placed in Petri-dishes for incubation in clean water or placed into new vials for the next treatment.

Antioxidant exposures were conducted with a range of at least four concentrations and were made up in exposure water just prior to the addition of embryos to minimize

pre-exposure degradation or oxidation (Table 4-1). Experiments were performed in duplicate with 20 animals per treatment. GSH was purchased from Sigma (#3654), Ascorbic acid powder and trolox (stock at 250 mM DMSO) were gifts from the Traber Laboatory at Oregon State University. Lipoic acid was provided by the Hagen Laboratory in a 400 mM stock. This was then followed, when appropriate, with 1 μM NaM. Animals were then scored at 24 hpf.

Acridine Orange. Embryos used in live acridine orange staining were dechorionated once they reached the desired developmental timepoint and 5 animals per treatment were placed in 2 mL eppendorf tubes. They were then incubated at 5ug/mL acridine orange for 1 hour. It is also a known mutagen requiring appropriate handling and disposal. Animals were then lightly rinsed and placed on a microscope slide for fluorescent microscopy. All animals were examined and representative pictures are shown (Fig 4-4). The experiment was repeated for all the developmental time points. Z-stacks were acquired at 10 μm increments throughout the depth of the tissue, between 8 to 12 stacks deep. Pictures and z-stacks were acquired using Axiovision software, Axiocam HR (Zeiss) mounted to a Zeiss Axiovert 200M motorized inverted microscope.

Morpholinos. We identified the zebrafish glutymyl cysteine ligase (GCL), catalytic subunit GCLc in an EST (accession number BC068331). It is 81% identical and 90% similar at the amino acid level to the human ortholog. A zebrafish morpholino (GCLc-MO) (Gene Tools, Corvallis, OR) was designed to target the translation start site beginning 4 bp upstream of the AUG codon to 19 bp downstream of the sequence. The

sequence of the GCLc-MO was 5'-CTGTGACAGCAAGCCCATAGCACAA-3' and the 3'-end was fluorescein tagged to assess microinjection success. Morpholinos were diluted to 2.8 mM in 1x Danieau's solution [58 mM NaCl, 0.7 mM KCl, 0.4 mM $MgSO_4$, 0.6 mM $Ca(NO_3)_2$, and 5 mM HEPES, pH 7.6], as described previously (Nasevicius and Ekker 2000). The embryos were injected at the one- to two-cell stage with approximately 3 nL of the appropriate morpholino solution. Embryos were screened for fluorescence at 4 hpf to reveal successful injection. Morphants, 10 per treatment, were exposed to 0.2, 0.4, and 1.0 μM NaM, no treatment controls and 0.2, 0.4, and 1.0 μM NaM uninjected controls. The morphants were raised to 24 hpf and the presence of a distorted notochord was noted.

Statitics. Data are illustrated as the mean with standard error of the mean (SEM) using GraphPad Prism v4.0 for Windows (GraphPad Inc) where appropriate. ANOVA statistical analysis was performed to test significance of the effect (SigmaStat Version 2.03 for Windows software; SPSS, Inc., Chicago, IL). Where treatment effects were shown to be significant ($p<0.001$) the Tukey Test for pairwise multiple comparison was applied.

Results

We have previously established that concentrations greater than 0.8 μM NaM elicit notochord distortions in all embryos by 24 hpf (Haendel *et al.* 2004). To test

whether this effect could be modulated by GSH, embryos were exposed with NaM in the presence of increasing concentrations of GSH. There was a concentration-dependent decrease in the number of embryos showing deformed notochords (Fig 4-1). Animals having notochord distortions were clearly diminished in severity at the 32 μM GSH and 1.0 μM NaM treatment. It was further determined that NAC, a synthetic precursor to glutathione, also protected the embryos exposed to NaM at concentrations of 25 μM and had a lethal threshold by itself between 50 to 250 μM. MITC and PDTC showed no signs of protection with GSH (N=5, over same range of GSH shown in Fig 4-1). DMDTC was also not protected with GSH, although with concentrations of 65 and 130 μM the magnitude of the distortions was diminished (N=5, over same range of GSH). The lethal threshold of reduced glutathione is 150 μM in the 4-24 hpf exposure window and limited testing higher concentrations with these compounds to test these possible differences in target affinity in detail. It is clear that for NaM exposures exogenously added glutathione protects the embryo from NaM-induced notochord distortions suggesting the ratio of reduced and oxidized glutathione is altered through some mechanism.

By decreasing the exposure windows to 10 hr periods, it was possible to stagger exposures with GSH and NaM so that the same embryos were first exposed to NaM for ten hours (4-14 hpf) followed by GSH from 14 to 24 hpf or vice versa (Fig 4-2). Animals exposed to NaM from 4-14 hpf followed by 130 μM GSH from 14-24 hpf had significantly fewer notochord distortions than those that were exposed to only NaM from 4 to 14 hpf. In animals that were pre-exposed to GSH there was a decrease in the number exhibiting notochord distortions, however this could not be separated statistically from

the positive control. This suggests that the NaM and glutathione interaction is likely taking place *in vivo* and is not simply a reflection of decreased bioavailability of DTCs to the embryo within the vial.

In order to establish a role for glutathione production in DTC developmental toxicity, transcription of glutymyl cysteine ligase (GCL), the rate limiting enzyme in glutathione production, was suppressed during early development. Animals were injected at the 1 to 2 cell stage with a morpholino designed to overalap the start codon of the catalytic subunit of GCL (GCLc) thereby suppressing transcription until the degradation resistant morpholino is degraded. Embryos were left to develop following injection until 4 hpf when they were checked for fluorescence prior to being placed in the appropriate exposure vials. From this experiment it is clear that the dose-response of NaM shifts to the left with the number of embryos having notochord distortions increasing from 0% at 0.2 μM NaM to 50% in the 0.2μM NaM-GCLc-mo (Figure 4-3). This indicates that GCLc morphants are more sensitive to the effects of NaM and that GSH production is a critical factor in determining DTC-sensitivity during development.

In order to investigate whether DTCs alter the oxidative state of zebrafish leading to cell death, several known antioxidants were evaluated by exposing the embryo concurrently to these compounds with 1.0 μM NaM. The antioxidants were tested in a range of concentrations until a lethal threshold was reached to increase confidence that they were reaching the embryo in effective quantities (Table 4-1). Ascorbic acid and trolox, a synthetic vitamin E, are known to buffer cells against the creation of ROS. However, in our studies there was no statistically significant protection of NaM-induced distortions with these two compounds (Table 4-1). Protection of NaM exposed embryos

with ascorbic acid was observed in some studies; however, it could not be separated from mortality or reliably repeated. Lipoic acid is a compound that contains thiols, has ROS buffering capabilities and has a known ability to induce glutathione systems in mammals. This compound was also unable to modulate the developmental effects of NaM. Although, it did have a lethality threshold of 12.5 μM under the exposure conditions limiting the range of concentrations that could tested for protection.

To address whether the observed thiol sensitivity was an indication that cell death was a possible cause of the notochord distortions, we evaluated the *in vivo* cellular uptake of acridine orange, a general indicator of cell death, in the living embryo at 11, 14, 18 and 24 hpf in control and exposed embryos. Non-specific fluorescence from the yolk obscured the view of cells in 11 hpf animals. At the 14, 18, 24 hpf timepoints, there was no clear cell death in exposed animals distinguishable from controls in the somites or surrounding tissue (Figure 4-4). Of interest was the possible clustering of fluorescent cells in the spinal interneurons or Rohon-Beard cells near the location of the first three somites (11hpf) and the somitogenic front (18hpf) . Future studies are necessary to confirm and identify the cell population(s) involved. It has been previously reported that thiram-exposed embryos show no changes in apoptotic cells using the TUNEL method (Teraoka *et al.* 2005). We also confirmed this in NaM, DMDTC, MITC and carbon disulfide (CS_2) exposed animals (Fig 4S-1).

Discussion

NaM-induced notorchord distortions are sensitive to exogenously added glutathione. Similarly, the production of glutathione *in vivo* plays a role in the susceptibility of zebrafish embryos to DTC insult during development. The manner in which NaM causes impairment or interacts with the developmental target is reversible with thiols. From our previous studies, somitogenesis is the most likely targeted developmental process in DTC exposures (Chapter 3) suggesting that further investigation into the role of thiol status in proper somite formation and differentiation is warranted. Furthermore, thiol status likely plays a significant role in the increased margin of safety observed for DTC developmental toxicity in higher vertebrates. For example, in previous studies of reproductive tract secretions of mammalian embryos, GSH levels measured at 51 nmol GSH per mg total protein and were shown to improve the development of mouse embryos after chemical insult (Gardiner *et al.* 1998). In addition both zebrafish and rat embryos express GCL genes as early as gastrulation (Hansen *et al.* 2004; Thisse *et al.* 2001). GCL overexpression in drosophila increased longevity with global and neuronal specific drivers illustrating the protective effect of glutathione to vulnerable biological targets (Orr *et al.* 2005). For these same reasons glutathione homeostasis is a potential target for chemical insult.

In this study, the major degradation product, MITC, was unresponsive to GSH likely reflecting its inability to form disulfide with glutathione or its high affinity for the target. Both GSH and copper likely hinder DTCs or their degradation products from interacting with the target in a manner dependent on the affinity of DTCs with these two

modifying factors. For example, DMDTC has previously shown to have opposite characteristics to NaM in its responsiveness to GSH and copper (Chapter 2). However, it is likely that DMDTC is stabilized by copper preventing its degradation (Weissmahr 2000). In these studies, DMDTC-induced distortions were unprotected but greatly diminished at the higher concentrations of GSH indicating a possible threshold of protection by GSH. In our earlier studies, DMDTC was found to be approximately three times more potent than NaM at causing a notochord distortion (Chapter 2). Some studies have shown an inversely proportional relationship between isothiocyanate (ITC) structural complexity and their ability to inhibit generation of superoxide via NADPH oxidase (Miyoshi *et al.* 2004). In those studies, both MITC and the benzyl ITC dithiocarbamte derivative had the lowest potential to inhibit superoxide generation. Additionally, evidence supports that the structural complexity of DTCs influences their ability to form covalent bonds with sulfhydryl groups (Scozzafava *et al.* 2001). Perhaps DMDTC has a higher affinity for the target relative to glutathione. Further studies are needed to extend our understanding of the DTC structure-activity relationships in the whole animal, particularly in target tissues of the developing embryo.

From these data it would appear that excessive or widespread creation of ROS is not likely the mechanism by which DTCs elicit their developmental effects. In our previous study ,the response to DTC insult appeared to involve the induction of electrophile responsive genes through the antioxidant response element resulting in increases in glutathione-S-transferase expression (Chapter 3). The DTC, maneb, increased the activity of glutathione peroxidase and altered glutathione ratios differently than ROS generated by paraquat (Barlow *et al.* 2005). Glutathione transferase and

glutathione peroxidase activities have been measured throughout zebrafish development (Wiegand *et al.* 2001; Wiegand *et al.* 2000). We would predict that DTCs are interacting with biological thiols of their molecular targets in the differentiating somite.

DTCs have been shown to transport and increase levels of intracellurlar redox active metals *in vitro* resulting in ROS-mediated oxidation of P53 cysteine thiols and catechols (Fitsanakis *et al.* 2002; Furuta *et al.* 2002). Moreover, CS_2 was shown to increase the susceptibility of plasma LDL to Cu^{2+} oxidation, among other measures of oxidative stress (Chen and Liao 2003; Wronska-Nofer *et al.* 2002). This indicates there is still a possible role for copper as a significant modifying factor in the mechanism of developmental toxicity for some DTCs.

Previous studies have reported the inability of the dithiocarbamate thiram to increase the number of apoptotic cells during development (Teraoka *et al.* 2005). We confirmed these observations using the same TUNEL method with several DTCs (Fig 4S-1). We then evaluated cell death in general using acridine orange staining in the living animal and found that there was no obvious increase in the number of cells dying in response to NaM exposure throughout somitogenesis. There were perhaps cell specific staining occurring in the spinal interneurons or more likely Rohon-Beard cells based on their proximity to the dorsal surface. Upon further investigation, it is possible cell death of these cells may play a role in the impairment neuromuscular development and communication resulting distortions of the notochord. Taken together, it is clear that large events of cell death do not lead to DTC-mediated distorted notochord and perturbed muscle development in the developing zebrafish.

Glutathione depletion from DTC disulfide formation, competition with thiol adduct formation, and/or regulation of redox sensitive thiols likely increases the susceptibility of developing vertebrates. Further study will be needed to understand how DTCs perturb the molecular mechanisms involved in the maintenance of thiol status under exposure to toxicants such as DTCs.

Supplemental Data

TUNEL experiments using zebrafish embryos exposed to several DTCs and degradation products to determine the role of unscheduled apoptosis in DTC developmental toxicity.

Purpose: In order to confirm the results of Teraoka *et al.* (2005) and investigate whether other members of the DTC class induce unwarranted apoptosis we performed experiments with CS2, NCu, DMDTC, MITC, and NaM using the TUNEL method.

Materials and Methods. Embryos were exposed to the minimal concentration required to cause notochord distortions in embryos using methods described elsewhere. A positive control of 130 uM ethanol was used to Embryos were fixed in 4 % paraformaldhyde overinight at 4 on a gentle rocker. Embryos were then rinsed three times in PBSTx and incubated in 1ug/mL proteinase K at 37 for 30 minutes. Embryos were then rinsed in PBSTx and re-fixed for 20 minutes. Following several rinses over 20 minutes embryos were equilibrated in TTase buffer, CoCl2, and water for 15 minutes. The reaction mixture containing FL-dUTP and TTase enzyme was allowed to incubate on

the embryos for 60 minutes on ice, followed by 60 minutes at 37. Samples were then rinsed with PBSTx and mounted for viewing as described elsewhere.

Results and Findings. There is a clear lack of apoptotic cells in exposed embryos (Fig 4S-1a, b). This confirms the report of Teraoka *et al.* (2005). The ethanol positive control failed to give a significant enough response over the course of three experiments to provide a dynamic range in apoptotic cell number to conclude that the compounds tested in this study were not inducing apoptosis at undetectable levels which could be biologically significant. In the case of DMDTC and NCu there could even be suppression of apoptosis by DTCs. In place of these studies a much more sensitive assay albeit less specific, acridine orange, was used in Chapter 4.

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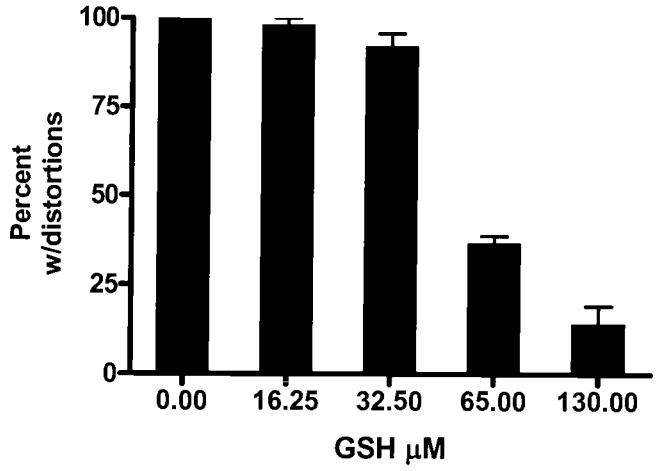


Figure 4-1. Glutathione protects metam sodium induced notochord distortions.

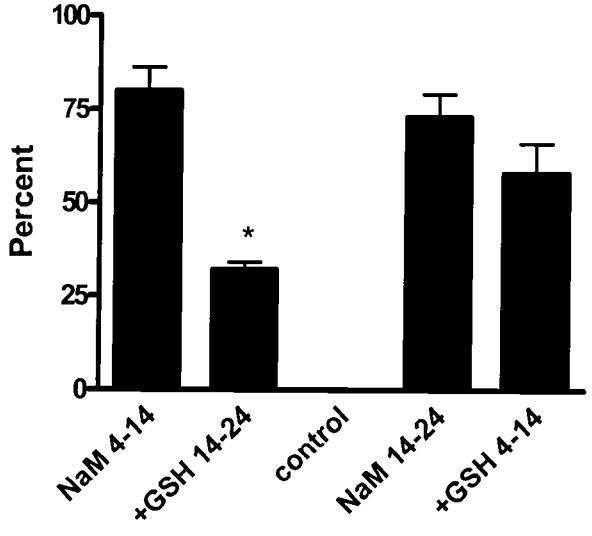


Figure 4-2. The effects of metam sodium in narrowed windows of developmental exposure before and after equivalent exposure to glutathione.

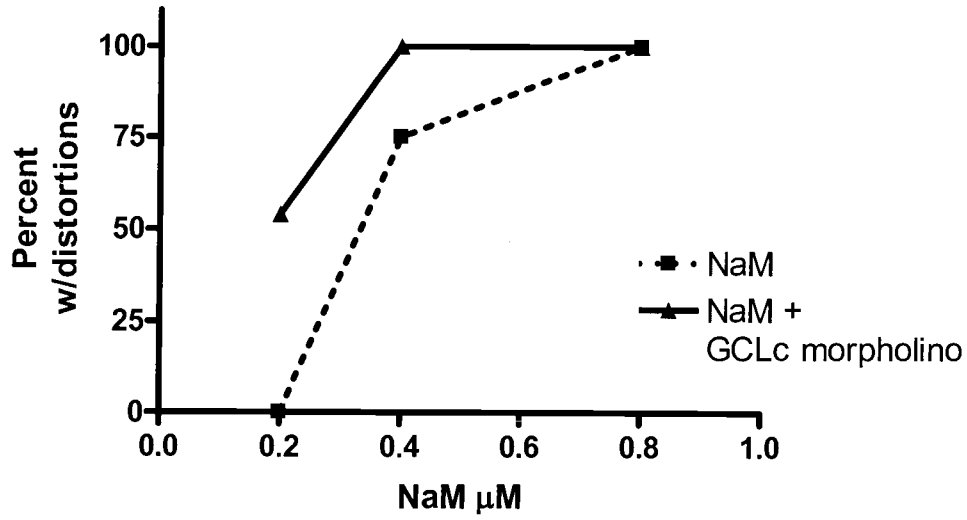
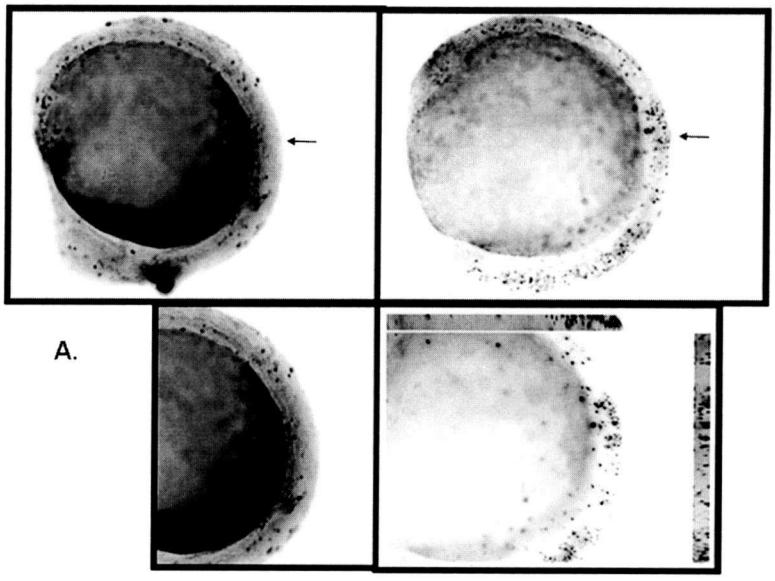
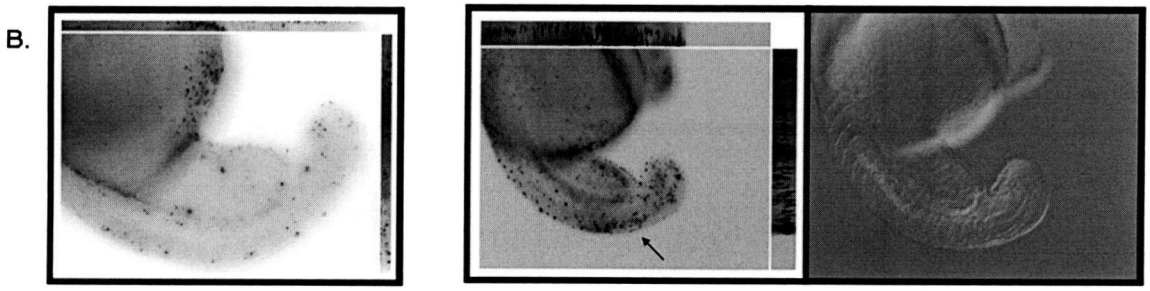


Figure 4-3. Suppression of glutamyl cysteine ligase increases the susceptibility of zebrafish embryos to 1.0uM metam sodium from 4 to 24 hours post fertilization.

Figure 4-4. Acradine orange staining in 14, 19, and 24 hour post fertilization control and treated zebrafish embryos. **(A)** Control embryos left column top 8.5x and bottom is 12.5x versus treated embryos in left column. Arrow shows the approximate location of the first somite. **(B)** 19.5 hpf control embryo left. Treated embryo showing acradine orange staining and the approximate location of the solitogenic front, arrow. DIC image of same treated animal to the right showing progression of somitogenesis. **(C)** Control and treated embryos at 24 hpf (left, right).


A.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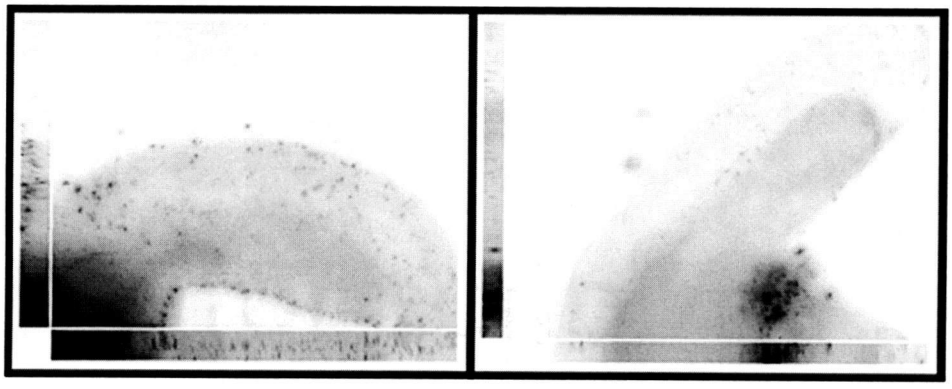

C.

Table 4-1: Antioxidants and glutathione modulators which do not alter NaM induced distortions

Chemical under study	**function**	**Lethality threshold**
Diethyl maleate (DEM)	depletes reduced glutathione levels	23-46 μM
butylsufoximine (BSO)	inhibition of a rate limiting enzyme	14-28 μM
Trolox synthetic vitamin E	antioxidant	1.3 mM
Ascorbic acid vitamin C precursor	antioxidant	180 to 360 μM
Lipoic Acid	antioxidant	12 μM

Figure 4S-1. Whole mount TUNEL images of 24 hour post fertilization control and treated zebrafish embryos. **(A)** Inverted z-stacks of 24 hpf zebrafish embryos (12-20 slices at 10um) exposed to ethanol as a positive control and no treatment negative control. Apoptotic cells appear black. **(B)** The presence of TUNEL positive cells in NCu, CS2, DMDTC, NaM and MITC.

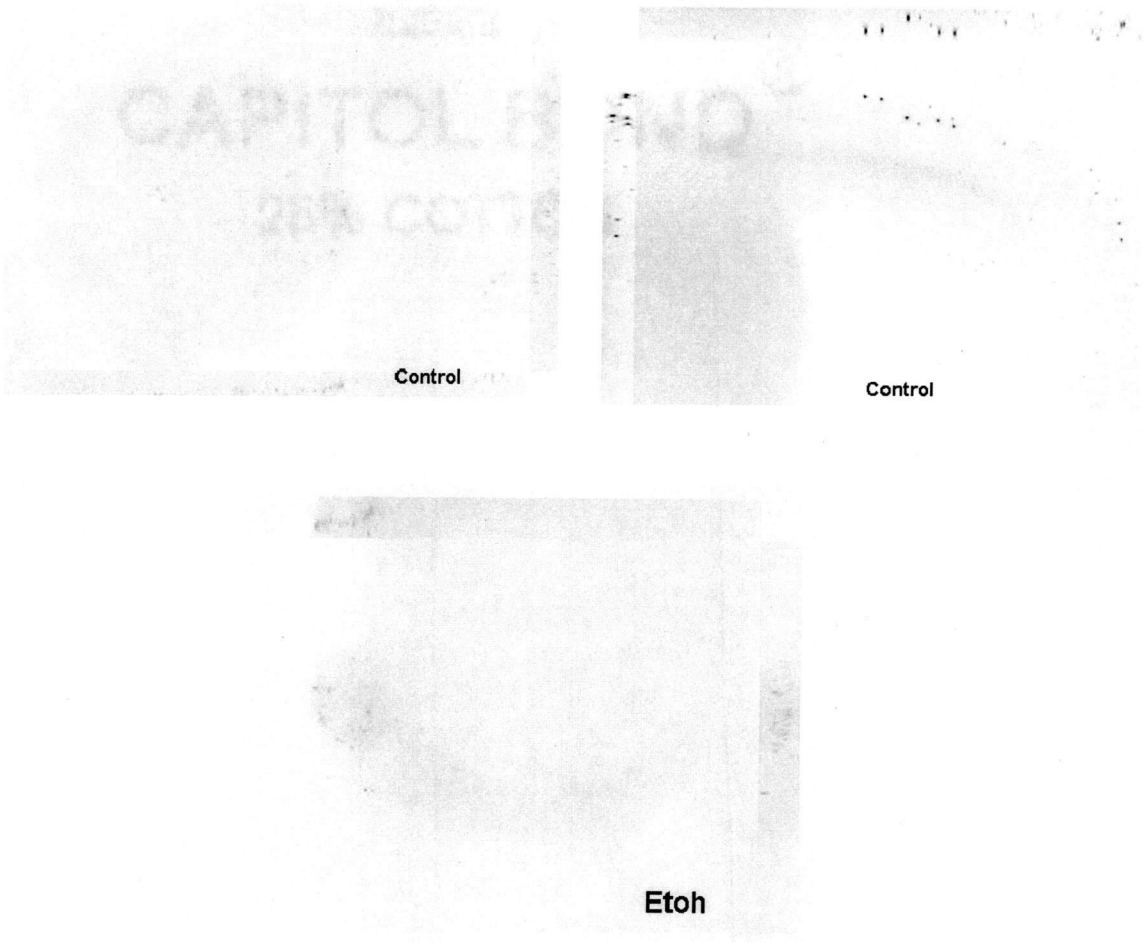


Figure 4S-1A.

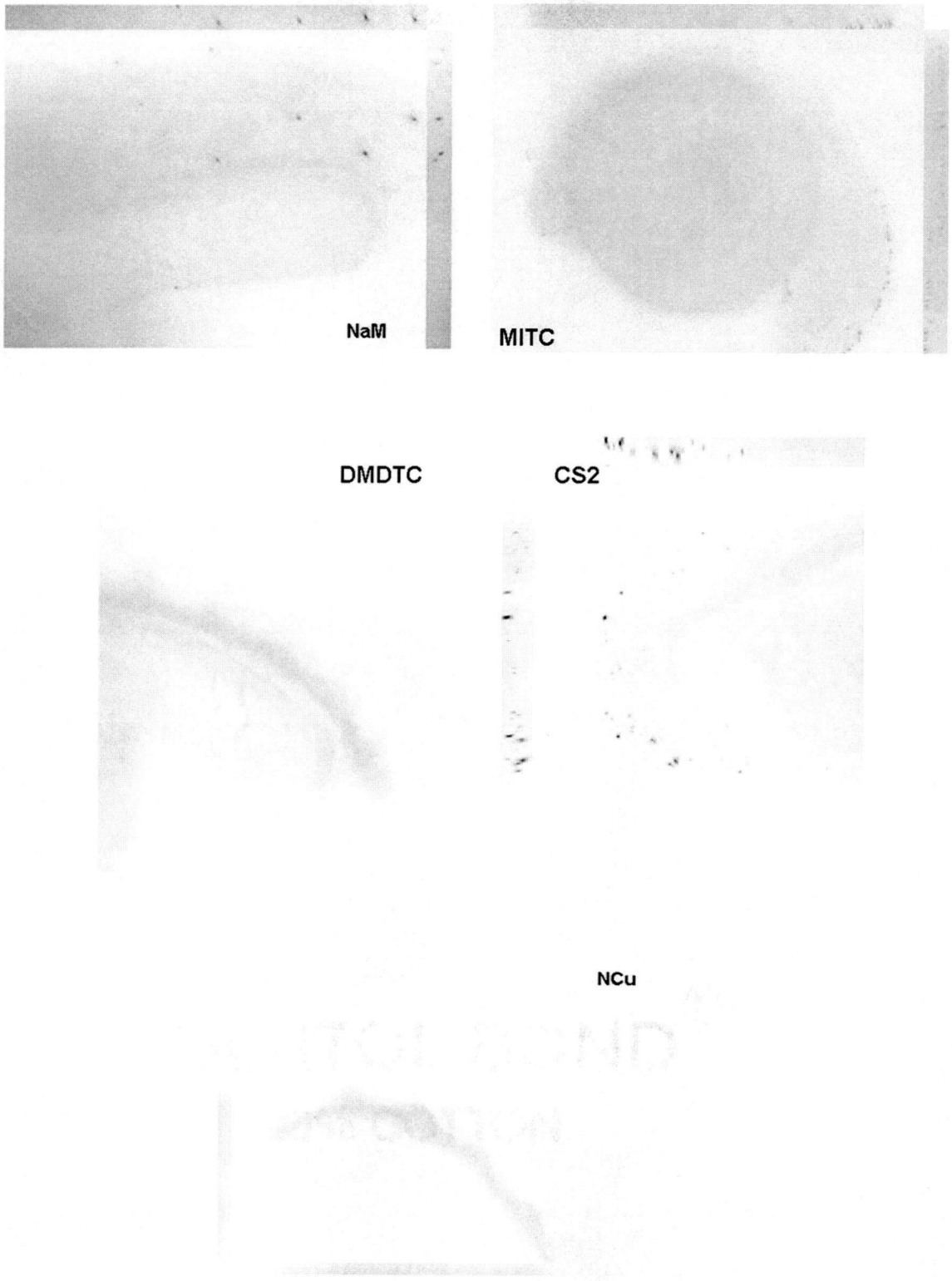


Figure 4S-1B.

Chapter 5: Conclusion, Ramifications, Future Directions

DTC developmental toxicity is well established, but poorly understood. Dithiocarbamates according to the U.S. EPA have a mechanism of action involving, "the inhibition of metal-dependent and sulfhydryl enzyme systems in fungi, bacteria, plants, as well as mammals." We hypothesized that by using the zebrafish development model we could better inform the mechanism of action of DTCs and for the first time establish a molecular understanding of DTC developmental toxicity in vertebrates. We have established that all types of dithiocarbamate pesticides and some degradation products have the potential to elicit a common toxic effect on development resulting in a distorted notochord and a significant impact to the body axis. We provide evidence to support the hypothesis that metal chelation is not the primary mechanism of action by which DTCs impact the developing vertebrate. By manipulating the exposure window of zebrafish we hypothesized that somitogenesis was the targeted developmental process. We tested this by using the Affymetrix microarray to observe gene expression induced by the N-methyl dithiocarbamate, metam sodium (NaM). Throughout this process it is clear that genes related muscle development are perturbed. These gene signatures are consistent with the morphological changes observed in larval and adult animals and that somitogenesis is the developmental target. Novel findings include the targeting of many redox sensitive targets and a possible role for the TGFβ signaling pathway. Thiol status is a critical modifying factor in DTC developmental toxicity but this toxicity does not result from dramatic cell death. It is possible this outcome is reached by DTCs and its primary degradation products through several pathways. Taken together we can hypothesize that the development mechanism of action of DTCs involves the depletion, oxidation, or

adduct formation of critical thiols in the somites of developing vertebrates. It is likely copper plays some role but it is not the target. The proximate toxicant is likely a DTC mixture of parent and degradation products acting to alter the redox thiol state of the animal.

By toxicants targeting somitogenesis a large period of development is susceptible to toxicant exposure. This likely explains some of the varied responses to tissues shown to be impaired by DTC developmental exposures because the timing, as well as the dose, determines the systems perturbed. Furthermore, because somites develop into diverse systems such as the muscular-skeletal system, the number of possible targets and the ways they can be perturbed is significantly greater. This is supported by studies demonstrating related ramifications to the notochord and somite derived tissue from toxicants such as cadmium, fipronil, and malathion. All of these studies were performed using the zebrafish model. Currently endpoint specific regulatory requirements related to EDSTAC, STAB-EU and TSCA require screening of large number of compounds for a variety of endpoints. Zebrafish are uniquely poised to fill these needs as well as the recommendations put forth by NRC 2000 on Developmental Toxicology and Risk Assessment..

The studies in this dissertation are a part of a larger approach to utilize toxicant gene signatures to identify novel targets of toxicants. Our understanding of DTC developmental toxicity is hampered by our inability to monitor the complex products from DTC degradation. One approach would be to compare the gene signatures from a variety of DTCs in a manner not unlike the experimental design in Chapter 2. Another approach is to utilize the products of large scale mutagenesis screens in zebrafish to

identify single point mutation mutants with phenotypes of interest. One such mutant is leviathion Fig 5-1. It phenocopies DTC developmental toxicity at the morphological level as well as in the way it responds to modifying factors such as tricaine (Fig 5-2). At the molecular level we have preliminary evidence to suggest that the neurotoxicity of DTC developmental exposure is phenocopied in leviathon (Fig 5-3). Taking at least two novel compounds, 7f11, and neocuproine, in addition to the diversity of DTC chemistries and the mutant leviathon it should be plausible to generate molecular signatures that are shared between these small molecules considering their common toxic effect. This will provide a broad understanding of the factors which determine susceptibility to DTCs and those biochemistries that result in a similar developmental toxicity.

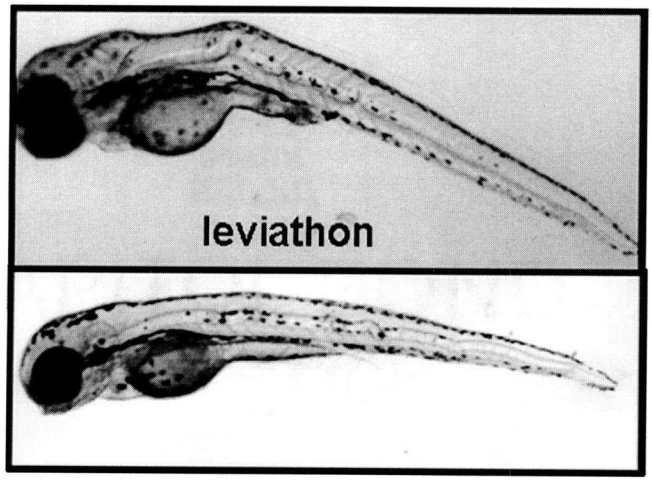


Figure 5-1. The mutant leviathan phenocopies the morphology of DTC developmental toxicity.

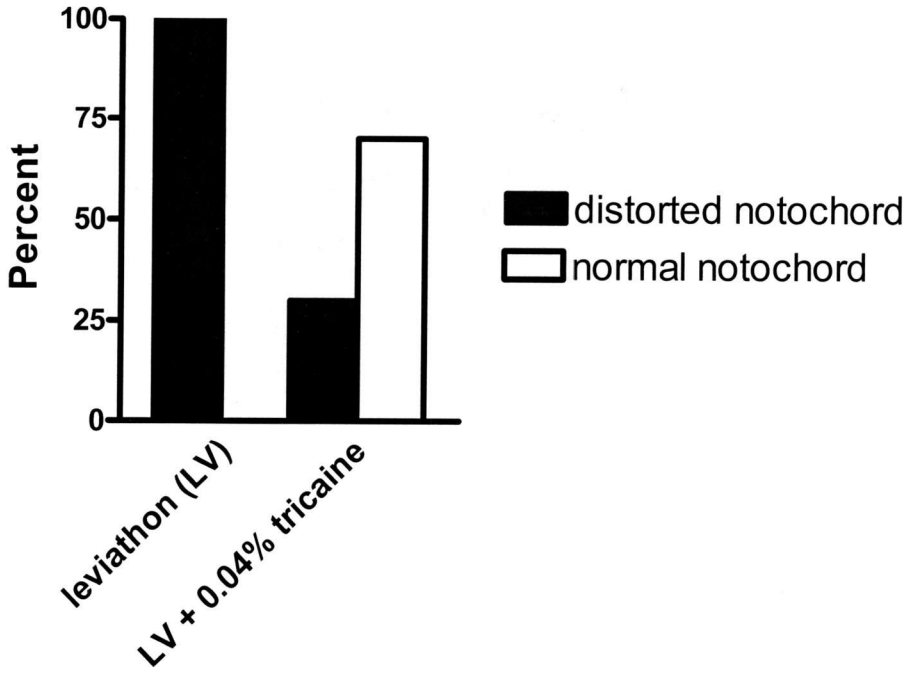


Figure 5-2. The mutant leviathon phenocopies the responses of DTC developmental toxicity.

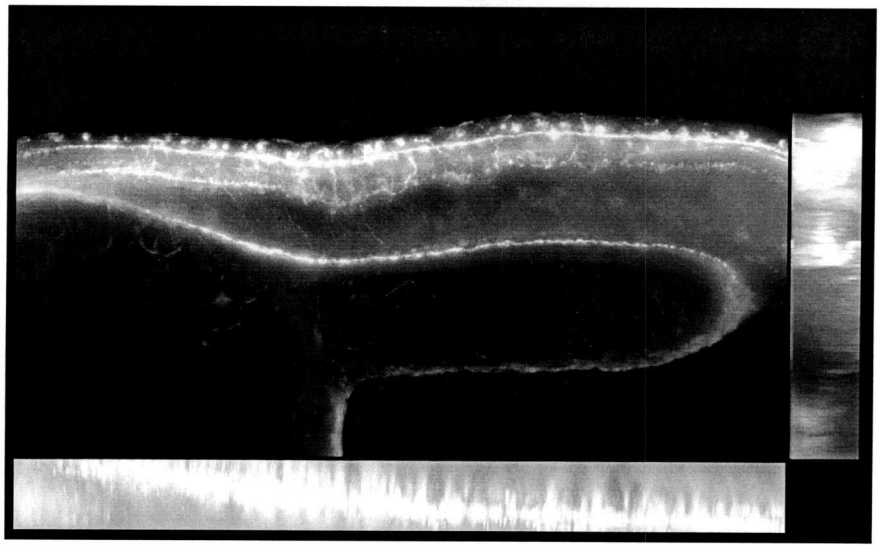
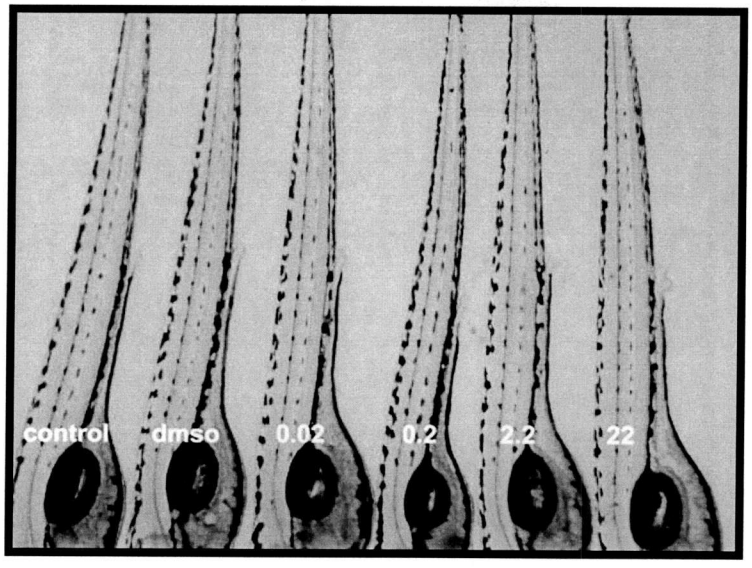
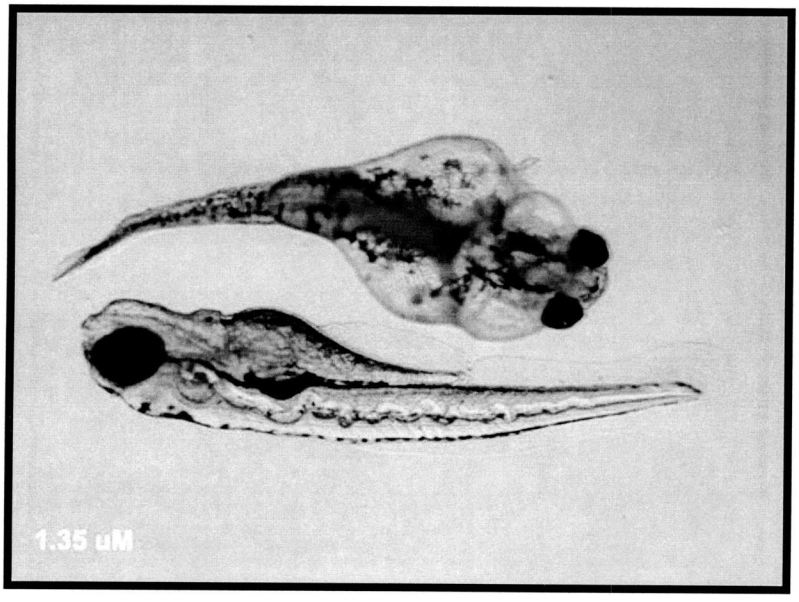


Figure 5-3. Alpha acetylated tubulin in leviathon embryos show similar developmental disturbances of peripheral neurons.

Appendix 2-A

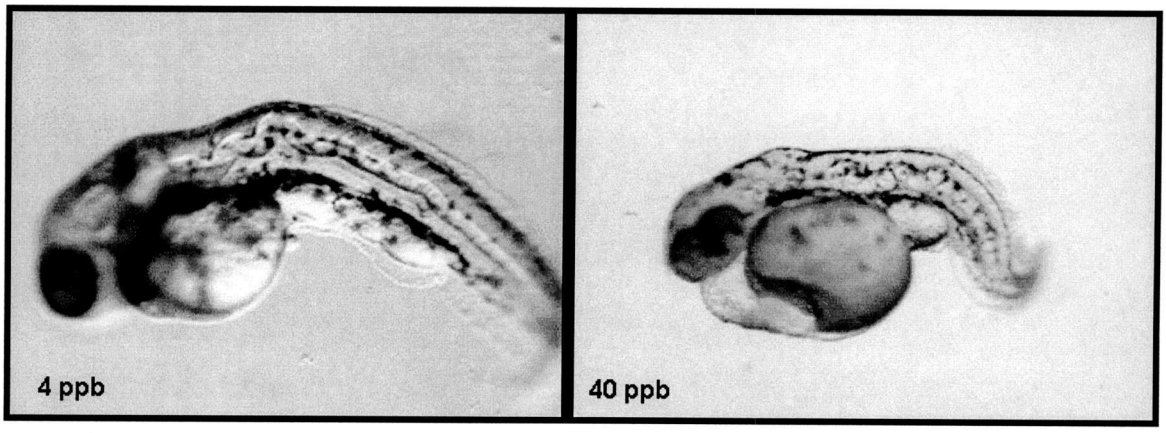


sulfurophane

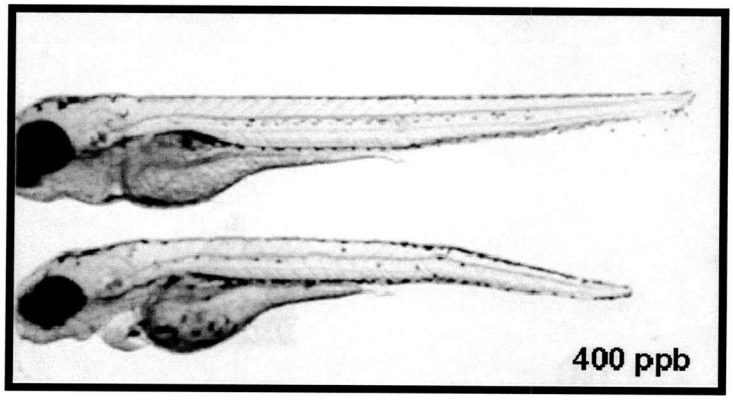


disulfiram

Figure A. Sulfurophane and disulfiram have uses in experimental and clinical medicine. No toxic threshold was identified with sulfurophane (> 20 ppm) and animals exposed to 0.02 to 22 ppm showed no signs of notochord distortions (heads pointed down). One half of the animals at 1.35 uM disulfiram exhibited yolk and/or cardiac edema in addition to a distorted notochord. This was only seen with disulfiram which is prescribed to alcoholics to inhibit aldehyde dehydrogenase.



ferbam



Metam sodium disulfide

Figure B. Representative pictures of two alkyl dithiocarbamates. Ferbam which is three dimethyl dithiocarbamate moiety's associated to iron. A potential aquatic degradation product of metam sodium was created by Dr. Rodney Thompson, Beckman laboratory, and was found to induce notochord distortions.

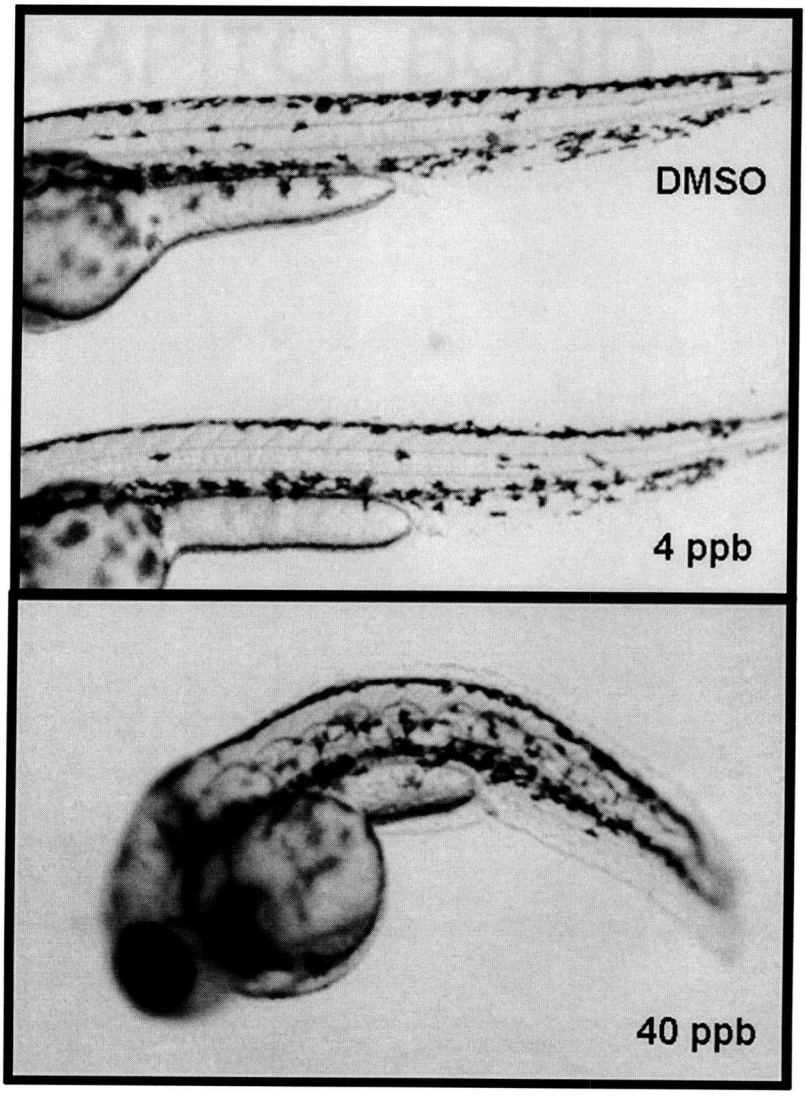
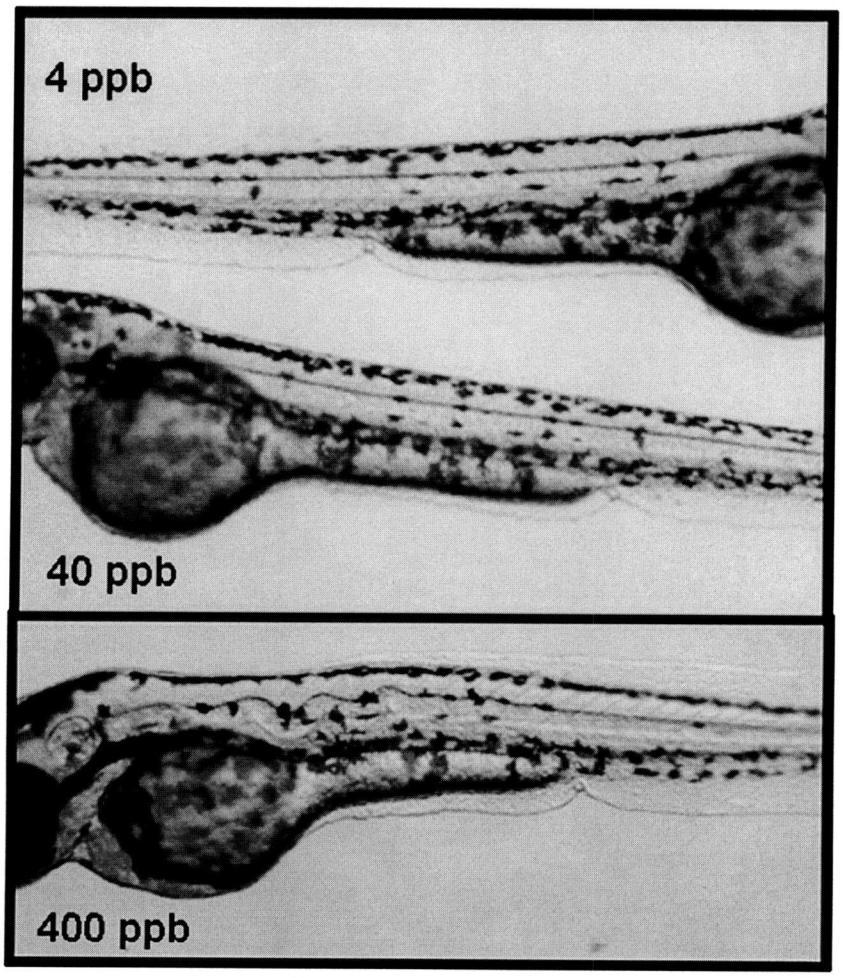
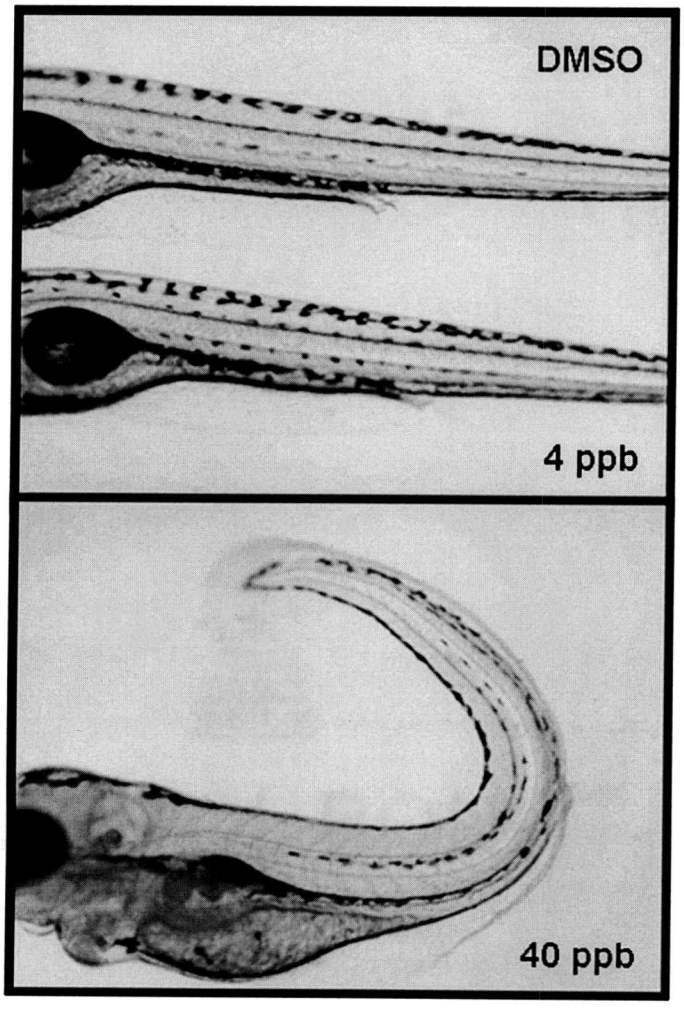


Figure C. Pyrollidine DTC is a widely used experimental DTC.



maneb

Figure D. Much higher concentrations of EBDCs such as maneb are required to cause a distorted notochord.



AITC

Figure E. Allyl ITC was not with out effects to the body axis. At 40 ppb a consistent curling of the tail was observed in less than 40% of the embryos tested.