

## Dithiocarbamates have a common toxic effect on zebrafish body axis formation

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### 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<sub>2</sub>), 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*) was perturbed well after cessation of treatment with pyrrolidine-DTC (PDTC), dimethyl-DTC (DMDTC), NaM, MITC, and CS<sub>2</sub>. 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<sub>2</sub> 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 triciaine (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.

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**Keywords:** Copper chelation; Development; Isothiocyanates; Mixture; *collagen 2a1*; *No tail*

### 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 methylisothiocyanate (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 as NaM (McGuire, 2003). ITCs are also under extensive study as cancer chemopreventive agents for some forms of cancer (Callaway et al., 2004). Due to these important uses, it is essential to determine if dithiocarbamates 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

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eligibility decision (RED) as mandated by the FQPA 1996 and NRDC Consent Decree. Furthermore, DTCs such as thiram (DTC disulfide), macozeb, and maneb have undergone recent voluntary cancellations of some of their agricultural uses (U.S. EPA, 2005a, 2005c). 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  $\mu\text{g/l}$  (U.S. EPA, 2004a, 2004b). 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 and Sedlak, 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 U.S. 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, 1992d, 1992e; 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 1). This strongly suggests a conserved developmental toxicity that is completely unstudied at the molecular level.

Table 1  
Developmental toxicity from dithiocarbamate exposure is conserved across several species

| Author (Year)                 | Species        | Developmental toxicity                           |
|-------------------------------|----------------|--|
| Zavanella et al. (1984)       | Newt           | Abnormal and malformed limb regeneration         |
| Birch and Prahlad (1986)      | <i>Xenopus</i> | Malformed notochord                              |
| Ghate (1985)                  | <i>Xenopus</i> | Malformed notochord                              |
| Van Leeuwen et al. (1986a,b)  | 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, skeletal anomalies, cranio-facial defects |
| Rath et al. (2004)            | Chick          | Tibia hyperplasia                                |
| Hodge (1993)                  | Rabbit         | Skeletal anomalies, cleft palate, meningocele    |
| Tinston (1993)                | Rat            | Skeletal anomalies, hydrocephaly, anophthalmia   |
| Matthiaschik (1973)           | Mice           | Skeletal anomalies, cleft palate, micrognathia   |
| Roll (1971)                   | Mice           | Skeletal anomalies, cleft palate, micrognathia   |

Humans are certainly exposed to DTCs through occupational settings and food residues (Cole, 1998; Caldas et al., 2004; Panganiban et al., 2004). However, despite anecdotal reports of adverse developmental outcomes in humans, developmental toxicity in mammalian studies requires 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., 2006; Corsini et al., 2006; Pruett et al., 2006; Valentine et al., 2006). The thiol containing DTCs will interact with sulfhydryl groups forming thiol protein adducts and disrupt cellular antioxidant levels (Nobel et al., 1997; Chung et al., 2000; Tonkin et al., 2000; Cheng and Trombetta, 2004). 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) (Heikkila et al., 1976; Fitsanakis et al., 2002; Furuta et al., 2002; 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., 2006).

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., 2006). 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 that 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 1 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<sub>2</sub> have the potential to elicit a common toxic effect on zebrafish notochord development. These studies also revealed that neocuproine, a phenanthroline copper chelator, was effective at inducing a similar distortion. PDTc, DMDTC, NaM, MITC, and CS<sub>2</sub> 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 h light/10 h dark photoperiod (Westerfield, 1995). Fish were maintained in reverse osmosis water supplemented with a commercially available salt solution (0.6% Instant Ocean) and were 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  $\mu$ S 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). 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 h 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 is 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 min 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  $\times$  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 et al. (2006). 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, 5 $\times$  SSC, and 0.1% Tween for 1 h and then hybridized overnight at 70 °C with digoxigenin-labeled antisense probe in 50% formamide, 5 $\times$  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 2 $\times$  SSC, 0.2 $\times$  SSC, and 0.1 $\times$  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 MgCl<sub>2</sub>, 100 mM NaCl, and 0.1% Tween-20. The collagen 2a1, no tail and myoD antisense RNA probes have been described (Yan et al., 1995; Weinberg et al., 1996).

**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).

With the exception of CS<sub>2</sub>, PDTC, nabam, and sulfuraphane, this range was sufficient to determine approximate LC<sub>50</sub>s and the induction of notochord distortions (Table 3). Further study of CS<sub>2</sub> determined a lethal threshold between 31 and 62 ppm (400–800  $\mu$ M), 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. 1 for representative pictures of distortions). DTC disulfides and ferbam were significantly more potent than the other molecules (Table 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<sub>2</sub> and MITC were selected for more detailed studies. Refined notochord dose–responses with a narrowed concentration range for CS<sub>2</sub>, PDTC, and DMDTC were determined to complement what was already known for NaM and MITC (Fig. 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<sub>2</sub> had the broadest range, but the threshold for the notochord distortion was rather dramatic beginning at 103  $\mu$ M (Table 3, Fig. 1).

In order to investigate the role of metals and the DTC metal chelating properties in this developmental toxicity, a series of

Table 2  
Chemicals under study

| Common names                               | Structure | Chemical name                                   | Uses  |
|--|-----------|---|---|
| <i>Dithiocarbamates</i>                    |           |   |   |
| Metam                                      |           | Sodium monomethyl dithiocarbamate               | Fumigant, herbicide, insecticide                  |
| Metam disulfide                            |           | Monomethyl dithiocarbamate disulfide            | Metam environmental degradation product           |
| DMDTC                                      |           | Dimethyldithiocarbamate, 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   |
| <i>Isothiocyanates</i>                     |           |   |   |
| MITC                                       |           | Methyl isothiocyanate                           | Pesticide, degradation product                    |
| AITC                                       |           | Allyl isothiocyanate                            | Mustard oil, pesticide                            |
| Sulforaphane                               |           | 4-Methylsulfinylbutyl isothiocyanate            | Found in cruciferous vegetables                   |
| ANIT                                       |           | Alpha-1-naphthylisothiocyanate                  | Experimental compound                             |
| <i>Dithiocarbamate degradation product</i> |           |   |   |
| Carbon disulfide                           |           | Carbon disulfide                                | Chemical precursor, alkyl dtc degradation product |

Table 3  
Dithiocarbamates and not isothiocyanates cause notochord distortions

| Compound                                   | Notochord distortion | Notochord NOEL ppb ( $\mu\text{M}$ ) | $\text{LC}_{50}$ ppb ( $\mu\text{M}$ ) |
|--|----------------------|--------------------------------------|--|
| <i>Dithiocarbamates</i>                    |                      |                                      |  |
| Metam <sup>a</sup>                         | 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)                          |
| <i>Isothiocyanates</i>                     |                      |                                      |  |
| MITC <sup>a</sup>                          | Yes                  | 16 (0.1)                             | 137 (1.87)                             |
| Allyl isothiocyanate                       | No                   | n/a                                  | 40–400 (0.4–4.0)                       |
| Sulfuraphane                               | No                   | n/a                                  | >40 K (>220)                           |
| ANIT                                       | No                   | n/a                                  | 400–4000 (2.1–21.6)                    |
| <i>Dithiocarbamate degradation product</i> |                      |                                      |  |
| Carbon disulfide                           | Yes                  | 3930 (52)                            | 31–62 K (400–800)                      |

<sup>a</sup> All other exposures were from 4 to 24 h post-fertilization. Twenty 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  $\text{LC}_{50}$  in test embryos unless otherwise noted.

metal chelators were tested under similar conditions for their ability to produce notochord distortions (Table 4). Only the membrane permeable copper chelator neocuproine (NCu) (2,9-dimethyl,1,10-phenanthroline) had an adverse effect on notochord development (Fig. 2). NCu elicited the same notochord distortion beginning at concentrations of 38  $\mu\text{M}$  which is 100 times less potent than the DTCs tested (Fig. 2). This leads us to investigate notochord- and muscle-specific transcriptional markers for five representative molecules (NaM, MITC,  $\text{CS}_2$ , 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 time point evaluated (36 hpf) which is 12 h after the cessation of the exposure. In control embryos, collagen 2a1 expression is dramatically decreased by 24 hpf (Fig. 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. 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. 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. 4a). Significant lethality appeared between 20 and 200  $\mu\text{M}$  (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. 4b). Moreover, there was a significant decrease in mortality which would be expected from the 200  $\mu\text{M}$  (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. 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  $\mu\text{M}$  copper were normal ( $6\% \pm 4$ ), however, this was not statistically significant and the copper lethality remained unchanged (Fig. 5a). There was no protection of NaM,  $\text{CS}_2$ , and MITC-induced notochord distortions with copper co-treatment (Fig. 5b). There was an apparent increase in the mortality expected from 20 to 200  $\mu\text{M}$   $\text{CuSO}_4$  alone in both  $\text{CS}_2$  and MITC co-treatments; however, this was not statistically significant (Fig. 5b). In addition, the apparent copper protection of  $\text{CS}_2$  induced distortions was also not statistically significant from the  $\text{CS}_2$  controls.

The gene expression of *collagen 2a1* and *no tail* was evaluated in animals treated with DMDTC, NCu, and NaM in combination with copper or tricaine (Figs. 6a, 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 et al. (2006) 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 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.

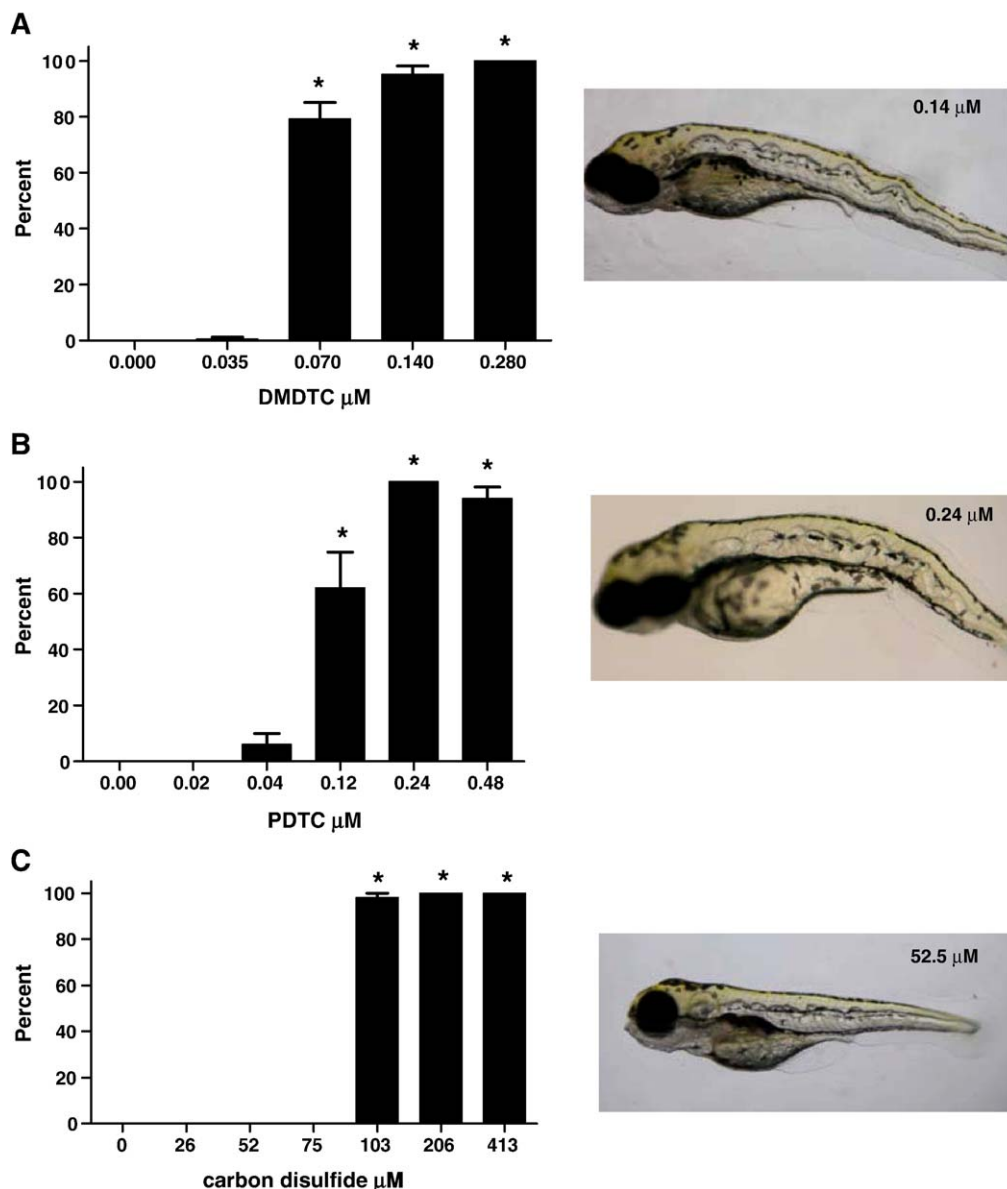


Fig. 1. The percentage of embryos exhibiting notochord distortions with treatment to DMDTC, PDTC, and  $\text{CS}_2$ . (A) DMDTC, concentrations greater than  $0.12 \mu\text{M}$  were statistically different from controls ( $P < 0.001$ ). (B) PDTC, concentrations greater than  $0.24 \mu\text{M}$  were statistically different from controls ( $P < 0.001$ ). (C)  $\text{CS}_2$ , concentrations greater than  $103 \mu\text{M}$  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.

DTC disulfides and ferbam, which contains multiple DTC moieties, are much more potent relative to the other molecules tested (Table 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., 2006; 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-catalyzed decomposition to  $\text{CS}_2$  and, based on our observations, appears to associate strongly with the embryo unlike the other molecules under study. Overall, the ethylene-bis-

Table 4  
Metals and chelators tested for notochord distortions

| Abbreviations                         | Chemical names  | Chelator type                  | Lethality threshold |
|---------------------------------------|---|--------------------------------|---------------------|
| <i>Membrane permeable chelators</i>   |   |                                |                     |
| DFOM                                  | Desferrioxamine mesylate                                | Fe, Cu, and Zn divalent metals | >2.4 mM             |
| NCu                                   | Neocuproine   | Cu (II)                        | >38 $\mu$ M         |
| TPEN                                  | <i>NNNN</i> -tetrakis-(2-pyridylmethyl ethylenediamine) | Divalent metals                | >1.9 mM             |
| <i>Membrane impermeable chelators</i> |   |                                |                     |
| BPDS                                  | Bathophenanthroline disulfonic acid                     | Fe (II)                        | 12 to 23 mM         |
| BCPS                                  | Bathocuproine disulfonic acid, disodium salt            | Cu (II)                        | >8.8 mM             |
| <i>Non-specific chelators</i>         |   |                                |                     |
| EDTA                                  | Ethylenediaminetetraacetic acid                         | Multi-valent metals            | >1 mM               |
| <i>Metals</i>                         |   |                                |                     |
| 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 h post-fertilization with nominal concentrations between 4 ppb and 4 ppm. Fifteen to twenty 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.

dithiocarbamates (EBDCs) appear to be the least potent of the DTCs tested (Table 2). This may be due to the fact that EBDCs form many sub-class-specific degradation products, none of which is 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 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 (Zhang et al., 1996; Zhang, 2000; Callaway et al., 2004). 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 acts independently to cause similar notochord distortions.

CS<sub>2</sub> 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<sub>2</sub> is the causative agent. CS<sub>2</sub> 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<sub>2</sub> formation at the target. If poor absorption and/or distribution of CS<sub>2</sub> occurred, this would result in the observation of lower potency of CS<sub>2</sub> reported in this study. DTC-generated CS<sub>2</sub> is a well-established neurotoxicant and both DTC-generated CS<sub>2</sub> and diethyl-DTC will independently form similar cysteine adducts (Valentine et al., 1995; Tonkin et al., 2003). 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<sub>2</sub> infers that copper plays a role in the developmental

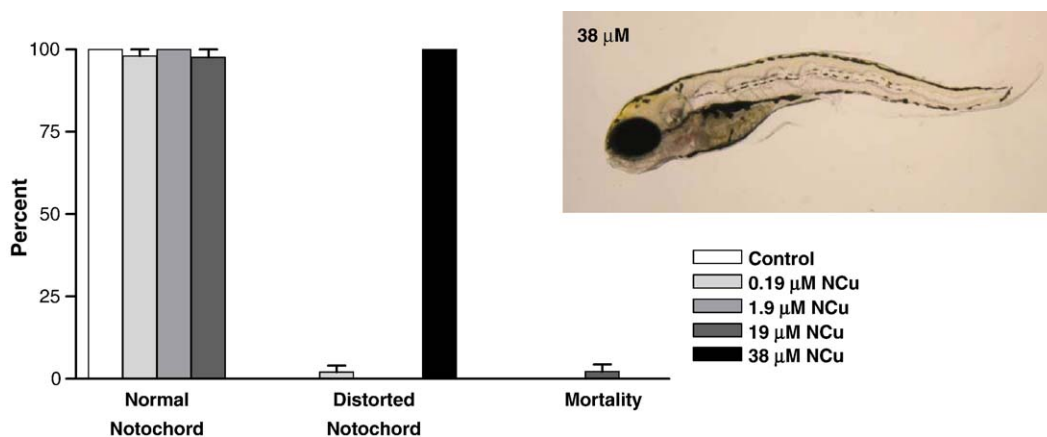
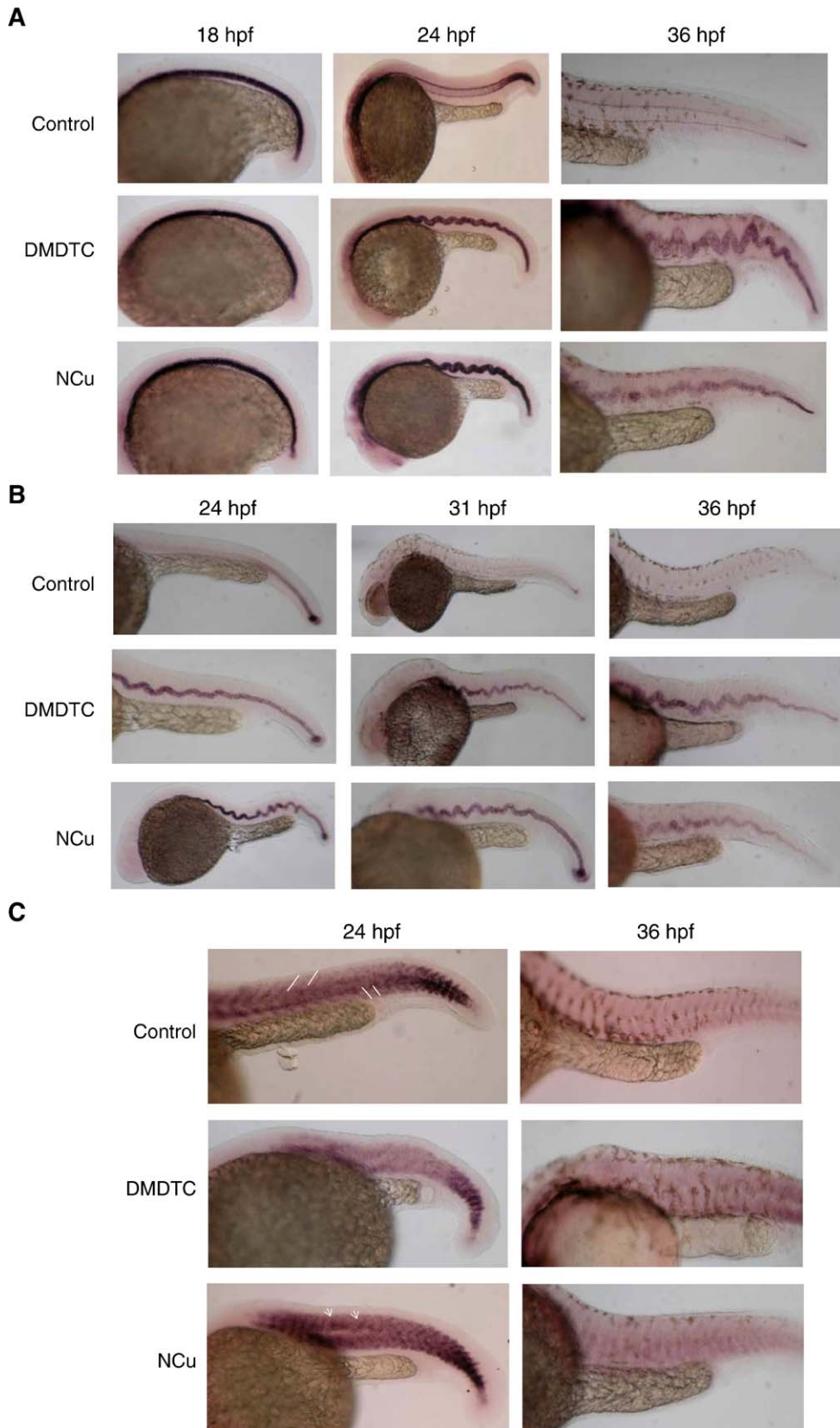


Fig. 2. Concentration-dependent responses to the membrane permeable copper chelator neocuproine. *N* = 5 per nominal concentration with 10 animals per vial.

toxicity (Fig. 2, Table 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 un-

available to the target (Fig. 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



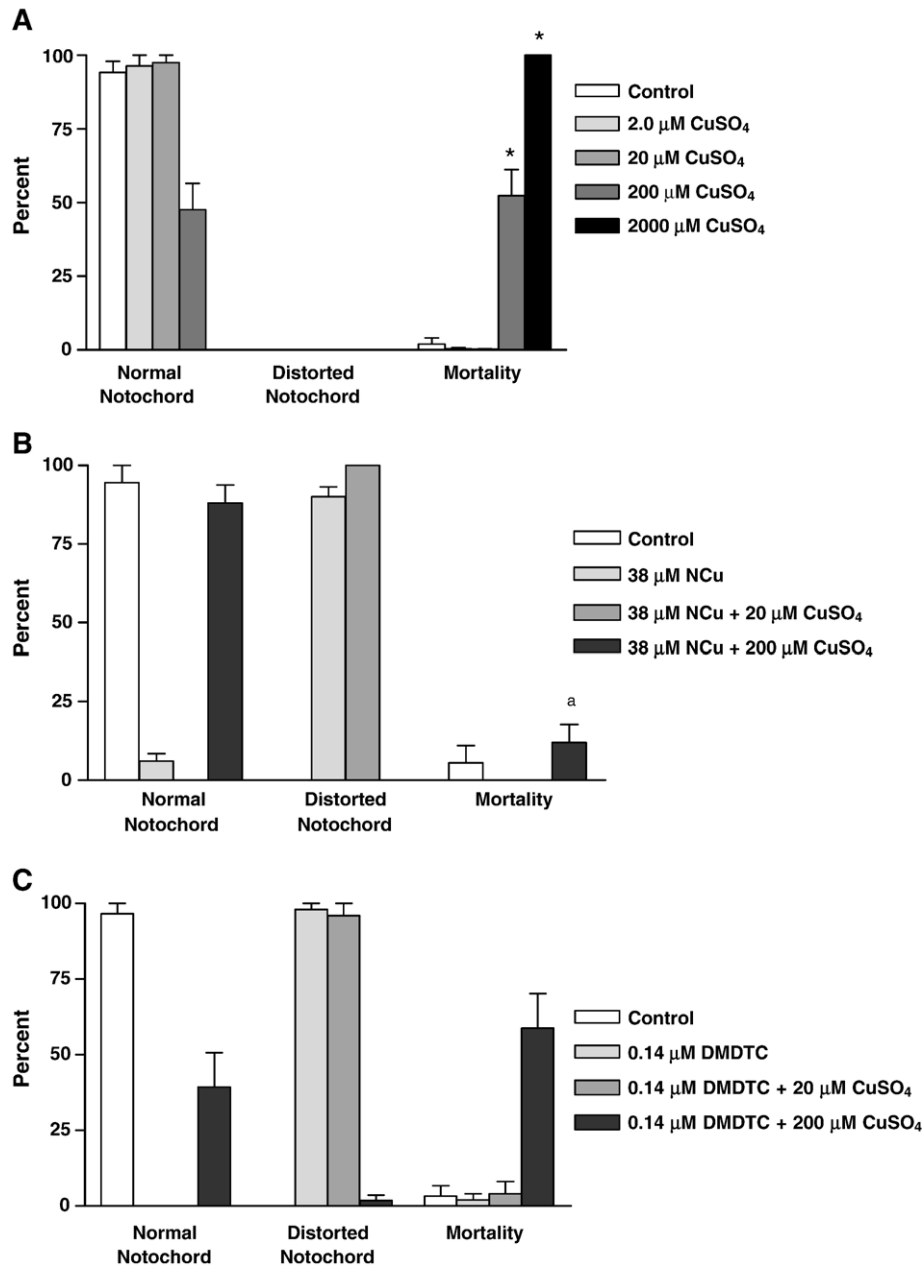


Fig. 4. Protection of notochord distortion with copper in the developing embryo. (A) Copper sulfate dose–response. (\*) Statistically significant increase in mortality from controls ( $P < 0.001$ ) by Kruskal–Wallis one way analysis of variance on ranks with pairwise comparisons performed using Tukey 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.

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-choliner-

gic nerves was diminished upon NCu copper chelation. Furthermore, DEDTC also did not modulate the NCu effect in co-exposure (Gocmen et al., 2000; De Man et al., 2001; Gocmen et al., 2005). Considering DTC, CS<sub>2</sub> or MITC formation would

Fig. 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): 0.38 μM 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): 0.38 μM 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): 0.38 μM neocuproine treated embryos at 24 and 36 hpf. Arrows point to areas of intense *myoD* expression near the distorted notochord.

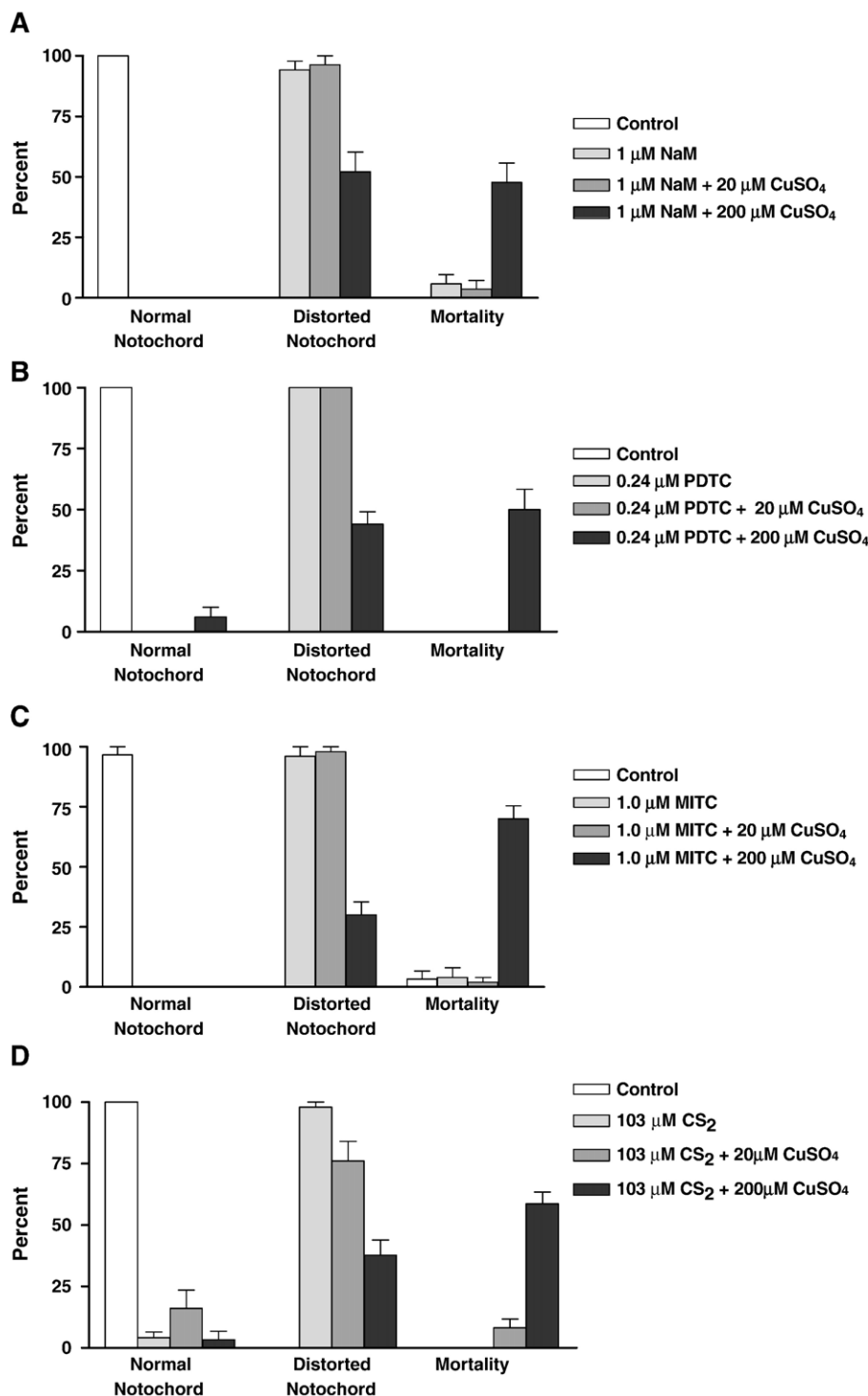


Fig. 5. Lack of notochord distortion from copper co-exposure. (A) NaM, top graph; PDTC, bottom graph. (B) MITC, top graph; CS<sub>2</sub>, bottom graph.  $N = 5$  per nominal concentration with 10 animals per vial.

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's group 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., 2006).

A classical explanation for the DTC-mediated toxicity is the interaction of DTCs with metal containing enzymes such as dopamine beta-hydroxylase and Cu/Zn superoxide dismutase (Heikkila et al., 1976; Simonian et al., 1992). If metal containing

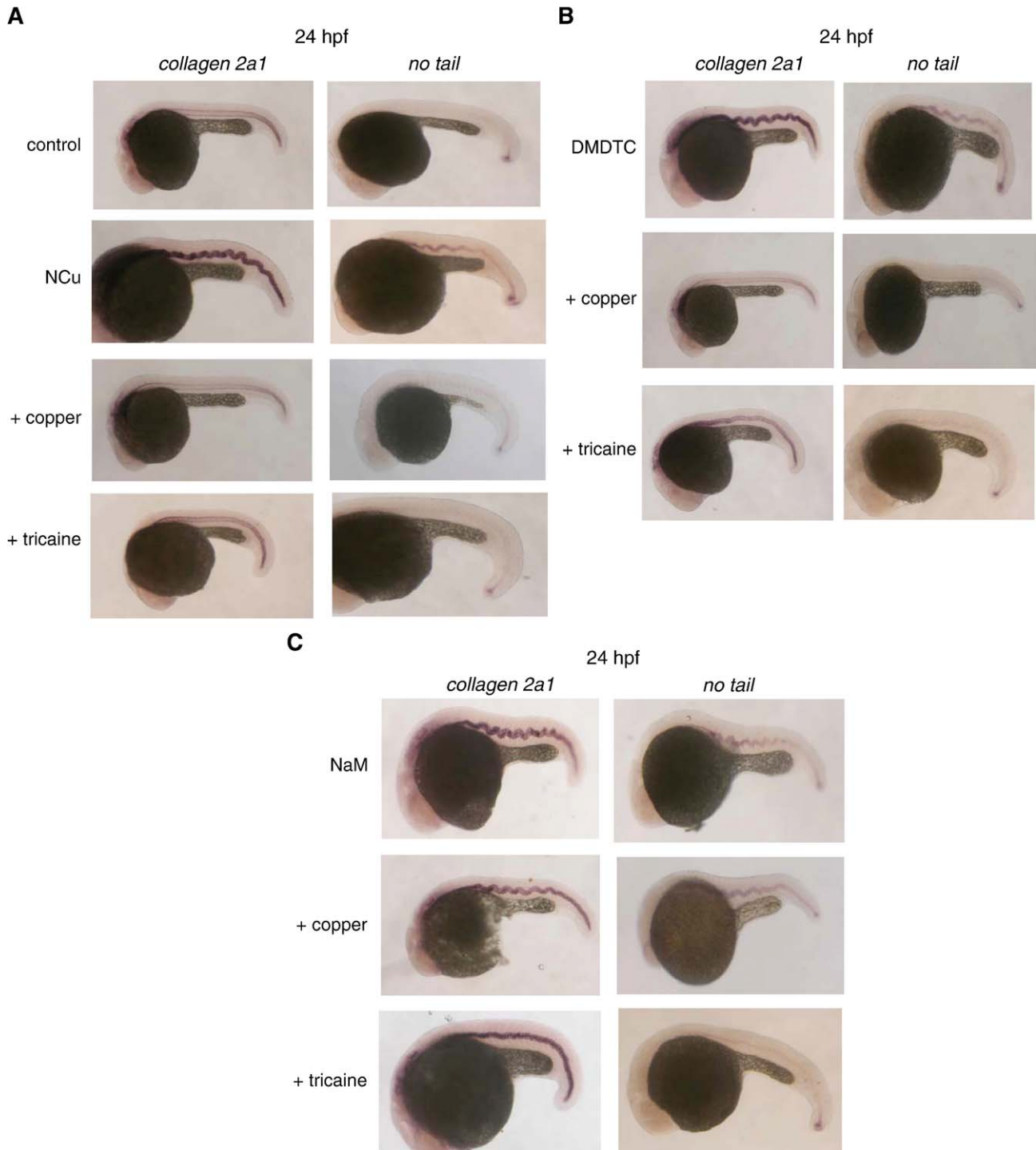


Fig. 6. Whole animal in situ hybridization of zebrafish embryos. (A) Top panel (left to right): no treatment *collagen 2a1* and *no tail* controls embryos at 24 hpf (representative of copper, tricaine, and carrier controls). Bottom panel (first row): 24 hpf *collagen 2a1* and *no tail* 0.38  $\mu$ M neocuproine treated embryos. (Second row) 24 hpf 0.38  $\mu$ M neocuproine + 0.04% tricaine (at 17 hpf) treated embryos. (Third row) 24 hpf 0.38  $\mu$ M neocuproine + 200  $\mu$ M copper sulfate treated embryos. (B) (First row) 24 hpf *collagen 2a1* and *no tail* 1.0  $\mu$ M metam treated embryos. (Second row) 24 hpf 1.0  $\mu$ M metam + 0.04% tricaine (at 17 hpf) treated embryos. (Third row) 24 hpf 1.0  $\mu$ M metam +200  $\mu$ M copper sulfate treated embryos. (C) (First row) 24 hpf *collagen 2a1* and *no tail* 0.12  $\mu$ M DMDTC treated embryos. (Second row) 24 hpf 0.12  $\mu$ M DMDTC + 0.04% tricaine (at 17 hpf) treated embryos. (Third row) 24 hpf 0.12  $\mu$ M DMDTC + 200  $\mu$ M copper sulfate treated embryos.

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<sub>2</sub>, NaM, and MITC clearly interacted with the target despite the presence of excess copper, this mechanism of toxicity cannot be a direct explanation for 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. 4). Undoubtedly, DTCs have metal chelating and transport abilities and CS<sub>2</sub> is highly reactive with biological metals (Oskarsson, 1987; Danielsson et al., 1990; IRIS, 1992a; 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 and Sedlak, 2000). Moreover, PDTC, NaM, MITC, and CS<sub>2</sub> 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<sub>2</sub>, and NCu lend support to the hypothesis that these molecules are acting on the embryo in a similar manner (Figs. 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., 2006). 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 et al. (2006). In the detailed analysis of gene expression presented in our study, none of the six molecules was 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 cannot 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* was examined in animals treated with NCu, DMDTC, and NaM in combination with copper or tricaine (Figs. 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 (Figs. 6a and b). Consistent with the morphological data reported in this study, *collagen 2a1* and *no tail* expression are not responsive to copper and NaM co-treatment (Figs. 5 and 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., 2006). 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 (Figs. 6a and b). Disruption of collagen is consistent with previous reports investigating the histopathology of DTCs in fish (Birch and Prahlad, 1986; Van Leeuwen et al., 1986a). Collagen is clearly important for the proper formation and stability of the notochord among other developmental processes, 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<sub>2</sub> 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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