



## Sulfhydryl systems are a critical factor in the zebrafish developmental toxicity of the dithiocarbamate sodium metam (NaM)

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### ABSTRACT

Dithiocarbamates (DTCs) are sulfhydryls (thiol)-containing compounds, often associated with metals, and have both antioxidant and pro-oxidant abilities depending on the compound, experimental system and condition. In this study we investigated whether cell death plays a role in the manifestation of DTC-induced notochord distortions in the developing zebrafish and if thiol-containing compounds or antioxidants could modify this developmental toxicity. Sodium metam (NaM) induced notochord distortions could not be protected with the antioxidants ascorbic acid, trolox (synthetic vitamin E) or lipoic acid. However, NaM-induced distortions could be protected with co-exposure to glutathione or *N*-Acetyl Cysteine. Staggering the NaM and glutathione exposures in consecutive 10 h developmental windows also resulted in protection. There were no discernable changes in TUNEL positive cells, a marker of apoptotic cells, at 24 h post-fertilization (hpf) in NaM, dimethyl-dithiocarbamate, carbon disulfide, or neocuproine exposed embryos. Live NaM-exposed embryos incubated with acridine orange, a general stain for cell death, for 1 h beginning at 11, 18 and 24 hpf showed clusters of stained nuclei near the somitogenic front but not in the cells making up the notochord. Overall, induction of apoptotic pathways and widespread cell death are not involved in the manifestation of the adverse developmental outcomes following NaM exposure. However, cellular thiol status or critical sulfhydryl moieties are important considerations in the mechanisms of DTC developmental toxicity.

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### 1. Introduction

Dithiocarbamates (DTCs) are sulfhydryl (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 sulfhydryl 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 perturbing the developmental targets. At environmentally relevant levels all DTCs and key degradation products induce a common toxic effect resulting in a distorted zebrafish notochord (Tilton et al., 2006). The most sensitive window of exposure occurs during somitogenesis and has been linked to transcriptional changes in the development of the periph-

eral nervous and muscular systems (Haendel et al., 2004; Teraoka et al., 2005; Tilton and Tanguay, 2008). While little is known about the molecular mechanisms of this toxicity, alterations of metal or sulfhydryl systems could result in death of cells critical for proper development, likely through one of the several oxidative stress pathways.

In the presence of copper, DTCs can redox cycle to form DTC-disulfides which will in turn oxidize pools of GSH thereby altering the oxidative state of cells without the production of reactive oxygen species (ROS) (Nobel et al., 1995, 1997; Burkitt et al., 1998). DTCs and DTC disulfides have been shown to directly oxidize catalytic thiols, inhibiting pro-apoptotic enzymes, such as caspase-3, and form cysteine-adducts (Valentine et al., 1995; Nobel et al., 1997; Tonkin et al., 2003). Additionally, several studies demonstrated the ability of thiols to diminish DTC-induced toxicity in a variety of model systems suggesting the importance of cellular thiol levels (Fitsanakis et al., 2002; Furuta et al., 2002; Thompson et al., 2002; Chen and Liao, 2003; Cheng and Trombetta, 2004). Enzymes important for the glutathione system are expressed and active in the developing zebrafish embryo as early as gastrulation (Thisse et al., 2001; Wiegand et al., 2001; Best et al., 2002). However, the impact of

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thiol containing toxicants such as DTCs on developing vertebrate systems has not been studied.

DTCs increase the activity of glutathione peroxidase, superoxide dismutase and alter glutathione ratios differently than reactive oxygen species generated by paraquat (Barlow et al., 2005). DTCs may also alter cell signaling pathways important for processes of cell proliferation and apoptosis differently than what is known to occur with increased cellular levels of metals (Chung et al., 2000). *N*-Acetyl cysteine (NAC), a synthetic precursor to glutathione, was found to activate and antagonize the pyrrolidine-DTC inhibition of the transcription factor, NFκB, which is a key component of apoptotic and proliferation pathways (Fernandez et al., 1999).

There is also ample evidence to suspect a possible role for copper and genes involved with copper homeostasis in some, if not all, DTC reported toxicities (Fitsanakis et al., 2002; Furuta et al., 2002; Mendelsohn et al., 2006; Valentine et al., 2006). We previously demonstrated the identical notochord distortions induced by sodium metam (NaM) and its major degradation product, methylisothiocyanate (MITC), were not protected by exogenous copper while those same distortions induced by the copper chelator neocuproine and dimethyldithiocarbamate (DMDTC) can be protected with the addition of copper (Tilton et al., 2006). Given the complex links between DTCs, copper, reactive oxygen species generation and apoptotic pathways, we investigated whether developmental exposure to NaM leads to inappropriate cell death in areas necessary for proper notochord development and if thiol-containing compounds or antioxidants could modify these NaM induced notochord distortions.

## 2. Materials and methods

### 2.1. 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®) at a pH and conductivity range of 6.8–7.0 and 450–520 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.

### 2.2. Embryo exposures

Embryos showing proper development were selected for exposures and placed in 20 mL water and Teflon sealed clear glass vials when they reached 4 h post-fertilization (hpf). Solvent and no treatment controls were present with every experiment and

no exposure was used in further analysis if mortality was greater than 20% in either control. Typically no mortality was observed. Unless embryos were sampled at an earlier developmental time points, exposures were from 4 to 24 hpf in order to capture the major developmental milestones targeted by DTCs. For a number of compounds used in these studies, NaM (1.0 μM), MITC (1.0 μM), PDTC (0.24 μM), neocuproine (38 μM) and dimethyl DTC (DMDTC) (0.14 μM), the concentrations used were experimentally determined to produce notochord distortions in 100% of the embryos under the aforementioned conditions (Tilton et al., 2006). Experiments were PDTC, neocuproine, and dimethyl DTC were used, working stocks were made up in DMSO prior to the experiment and used to spike the exposure vials with the test compound (DMSO 0.05%, v/v). The working stocks of the remaining compounds were made up in fish water or as described elsewhere (Tilton et al., 2006). Exposures to generate control and exposed embryos for TUNEL and acridine orange assays were conducted as described (*N* = 3, 20 embryos per vial) and further details are found below.

To investigate the ability of thiols and antioxidants to modify the NaM induced toxicity we first determined whether exposure to these compounds alone could induce the distorted notochord phenotype (Table 1). Exposures were conducted with a range of at least four concentrations, nominal 4, 40, 400 ppb and 4 ppm, and development was evaluated at 24 h post-fertilization (*N* = 2 per treatment, 20 embryos per vial). If a clear concentration mortality relationship was not observed within this range further experiments were conducted to determine the sub-lethal exposure concentrations used in experiments with NaM co-exposures. Working stocks of these thiols and antioxidants were made up in exposure water immediately prior to the addition of embryos to minimize pre-exposure degradation or oxidation. Co-exposures with 1.0 μM NaM (*N* = 2 per treatment, 20 embryos per vial) and at least two experimentally determined sublethal concentrations of the thiol and antioxidants were used to determine whether they would alter the DTC-induced distorted notochord phenotype. Exposure to diethyl maleate and butylsulfonimine, two chemicals known to deplete reduced glutathione levels in zebrafish embryos, were also prepared for co-exposure to 0.2, 0.4, 0.8 μM NaM (*N* = 2 per treatment, 20 embryos per vial) to evaluate whether these compounds created embryos more sensitive to the effects of NaM.

Follow-up studies of the glutathione protection were generated from embryos co-exposed to 1.0 μM NaM and either 16, 33, 65 or 130 μM GSH (*N* = 5, 10 animals per treatment) and evaluated at 24 hpf for notochord distortions. For NaM experiments during restricted windows, embryos were removed from the first exposure solution 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 (*N* = 5, 10 animals per treatment). To test whether glutathione could protect the embryos from the effects of other DTCs we performed a co-exposure with each of these individual compounds with reduced glutathione from 4 to 24 h post-fertilization, (*N* = 2

**Table 1**  
Chemicals with antioxidant or thiol properties screened as modifiers of sodium metam (NaM) induced notochord distortions in the zebrafish embryo

Chemical under study	Characteristics	Lethality threshold	Protection from NaM induced notochord distortions
GSH	Reduced glutathione	150 μM	Y
<i>N</i> -Acetyl cysteine	Synthetic glutathione precursor, thiol containing	50–250 μM	Y (25 μM)
Diethyl maleate (DEM)	Depletes GSH levels	23–46 μM	N
Butylsulfonimine (BSO)	Inhibition of glutathione synthesis	14–28 μM	N
Trolox	Synthetic vitamin E, antioxidant, no thiols	1.3 mM	N
Ascorbic acid	Vitamin c precursor, antioxidant, no thiols	180–360 μM	N
Lipoic acid	Antioxidant multi-functional, thiol containing	12 μM	N

'Lethality threshold' refers to the concentration at which mortality was observed in a greater number than controls (>1–2 embryos) within a 20 h exposure period, 4–24 h post-fertilization, (*N* = 2 per treatment, 20 embryos per vial). The ability of these compounds to diminish the distorted notochord phenotype was tested from 4 to 24 hpf by co-exposing sodium metam (1.0 μM) and several concentrations of these chemicals beneath the lethality threshold within a 20 h exposure period, 4–24 h post-fertilization (*N* = 2 per treatment, 20 embryos per vial). The two glutathione modulators, DEM and BSO, were also tested at 0.2, 0.4, 0.8 μM NaM.

**Table 2**  
Reduced glutathione co-exposure with dithiocarbamates and related compounds

Chemical under study	Characteristics	Concentration tested	GSH protection?	GSH concentration threshold
Dimethyl dithiocarbamate	Alkyl dithiocarbamate	0.14 $\mu$ M	Y	65 and 130 $\mu$ M
Pyrollidine dithiocarbamate	Will not form carbon disulfide	0.24 $\mu$ M	N	–
Methylisothiocyanate	NaM degradate	1.0 $\mu$ M	N	–
Carbon disulfide	Common dithiocarbamate degradate	103 $\mu$ M	N	–

A 20 h co-exposure period, 4–24 h post-fertilization ( $N=2$  per treatment, 20 embryos per vial). Chemicals under study were tested at the minimum concentration necessary to induce 100% notochord distortions. Positive controls for each compound expressed the distorted notochord phenotype in all embryos. Reduced glutathione was tested from 33, 65, and 130  $\mu$ M.

per treatment, 20 embryos per vial) (Table 2). As previously stated these compounds were tested at the minimum concentration necessary to induce 100% notochord distortions. Reduced glutathione was tested at 33, 65, and 130  $\mu$ M.

### 2.3. Tunel

To observe apoptotic cell death, terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick end labeling (TUNEL) analysis was performed in whole mount zebrafish embryos at 24 hpf. Embryos were exposed to the minimal concentration required to cause notochord distortions in embryos from 4 to 24 hpf as described above. Embryos were then fixed in 4% paraformaldehyde overnight at 4 °C on a gentle rocker. Groups of five embryos from the same treatment were placed in individual eppendorf tubes and rinsed three times in PBSTx and incubated in 1 ug/mL proteinase K at 37 °C for 30 min. Embryos were then rinsed in PBSTx and re-fixed for 20 min. Following several rinses over 20 min embryos were equilibrated in TTase buffer,  $\text{CoCl}_2$ , and water for 15 min. The reaction mixture containing FL-dUTP and TTase enzyme was allowed to incubate on the embryos for 60 min on ice, followed by 60 min at 37 °C. Samples were then rinsed with PBSTx and mounted for viewing. Z-stacks were acquired at 10  $\mu$ m increments throughout the depth of the tissue, between 8 and 12 stacks deep. Z-stacks were acquired using Axiovision software, AxioCam HR (Zeiss) mounted to a Zeiss Axiovert 200 M motorized inverted microscope.

### 2.4. Acridine orange

To observe both necrotic and apoptotic cell death, live embryos were evaluated using acridine orange (AO) staining. Following exposure to the desired developmental period embryos were dechorionated and 5 animals per treatment were placed in 2 mL eppendorf tubes. They were then incubated at 5  $\mu$ g/mL acridine orange for 1 h. AO is 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. The experiment was repeated for all the developmental time points. Z-stacks and single plane images were captured as described and representative pictures are shown. The animals were then euthanized with a lethal dose of tricane (MS-222).

### 2.5. Statistics

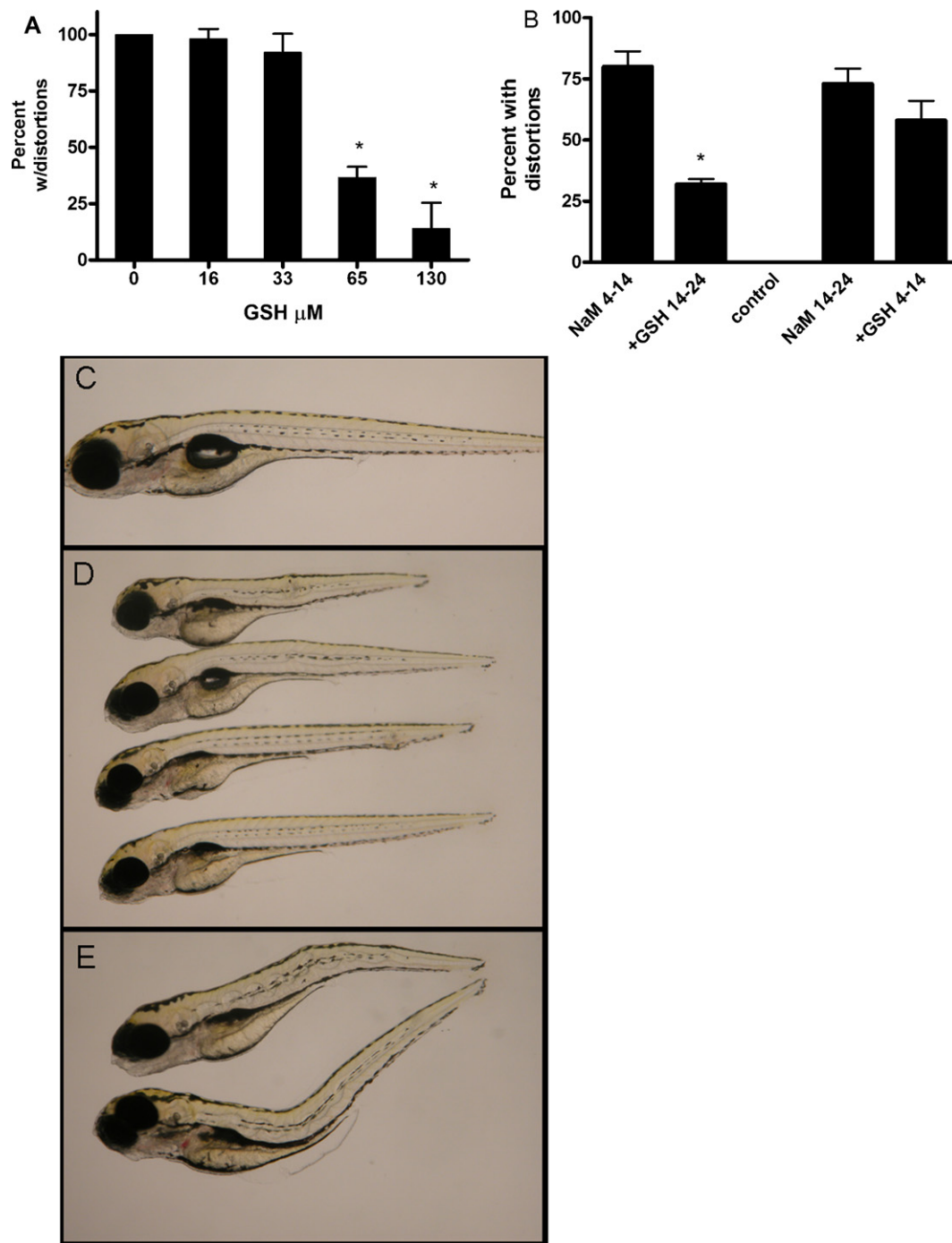
Data are illustrated as the mean with standard deviation using GraphPad Prism v4.0 for Windows (GraphPad Inc.) where appropriate. ANOVA statistical analysis was performed to test significance of the effect. Where treatment effects were shown to be significant ( $p < 0.0001$ ) the Tukey Test for pairwise multiple comparison was applied.

## 3. Results

Several known antioxidants and pharmacological agents were studied to determine if the developmental toxicity caused by 1.0  $\mu$ M NaM could be altered by these compounds. The chemicals were initially tested individually in a range of concentrations to determine sub-lethal concentrations and to confirm that the test chemicals were penetrating the embryo (Table 1). Furthermore, none of test chemicals induced a distorted notochord on their own at any concentration tested. Each chemical was then tested at several sub-lethal concentrations in combination with 1.0  $\mu$ M NaM to determine their potential to modify NaM induced notochord distortions. Reduced glutathione and *N*-acetyl cysteine, each thiol-containing compounds, were capable of protecting the embryos from expressing this phenotype. Ascorbic acid and trolox (a synthetic vitamin E analog), are known to buffer cells against the creation of reactive oxygen species. Lipoic acid has ROS buffering capabilities and is known to induce glutathione systems in mammals. There was no statistically significant protection of NaM-induced distortions by any of these compounds (Table 1). To pharmacologically diminish the pools of glutathione in the zebrafish diethyl maleate (DEM), a GSH depletor, and butylsulfonimine (BSO), inhibitor of glutathione synthesis, were evaluated in co-exposures across the established dose–response curve of NaM (0.2, 0.4, 0.8, 1.0  $\mu$ M) (Table 1). There was no detectable change in the NaM induced dose–response curve with the addition of these compounds.

Additional studies revealed there was a concentration-dependent decrease in the number of embryos exhibiting NaM-induced notochord distortions when co-exposed to increasing concentrations of reduced glutathione (Fig. 1A). The severity of the notochord distortion was diminished in animals exposed to 33  $\mu$ M GSH and 1.0  $\mu$ M NaM, while the presence of the distorted notochord was unchanged (Fig. 1A and D). A statistically significant decrease in the appearance of a distorted notochord phenotype was observed after co-exposure to 65  $\mu$ M GSH and 1.0  $\mu$ M NaM, (Fig. 1A, C, and D). By reducing the exposure period from 20 h to two consecutive 10 h 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. This study was performed to test whether there was simply a thiol conjugation occurring between NaM and GSH in the test solution, thereby reducing DTC bioavailability. Animals exposed to NaM from 4 to 14 hpf followed by 130  $\mu$ M GSH from 14 to 24 hpf had significantly fewer notochord distortions than those that were exposed to only NaM from 4 to 14 hpf (Fig. 1B). In animals that were pre-exposed to GSH the severity of the distortion was noticeably diminished, however there was no difference in the number of embryos exhibiting notochord distortions,

To evaluate whether this glutathione protection was unique to NaM, we investigated the potential for GSH to protect against the induction of distorted notochords by other DTCs and related compounds. GSH exposure, over the same concentration range

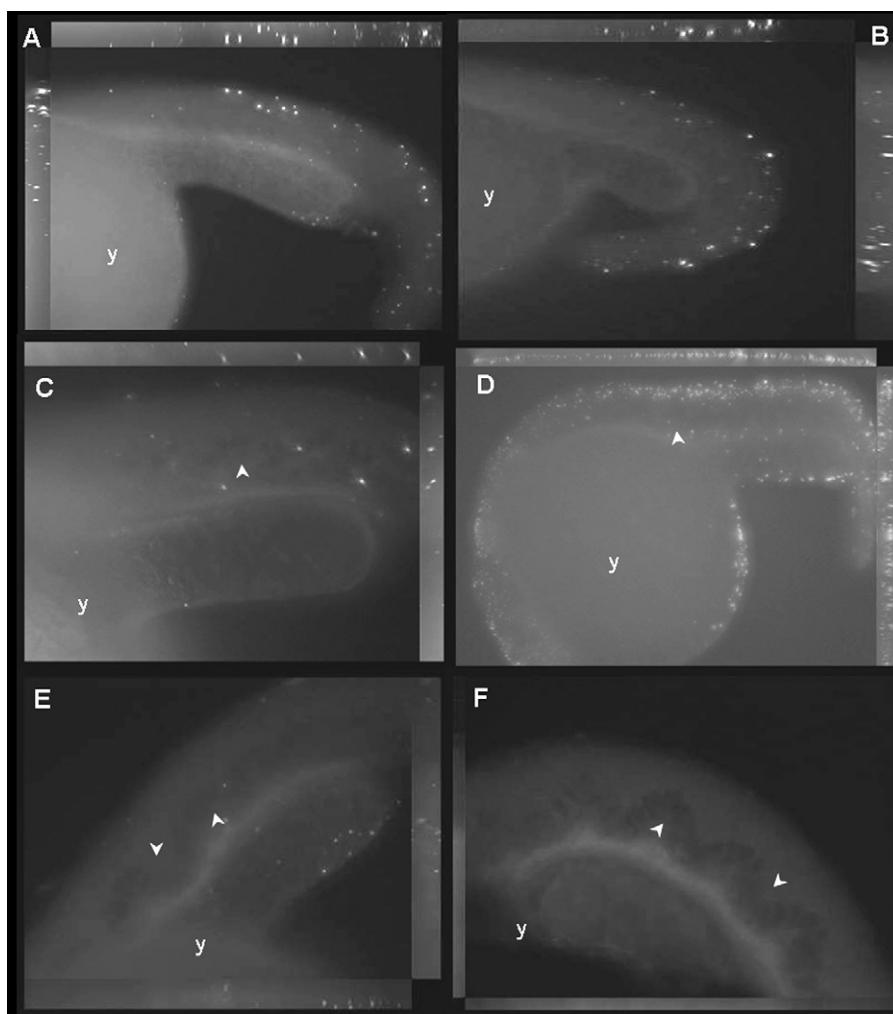


**Fig. 1.** (A) Percent of zebrafish embryos exhibiting a notochord distortion in the presence of 1  $\mu\text{M}$  sodium metam (NaM) and increasing concentrations of reduced glutathione (GSH) co-exposures from 4 to 24 h post-fertilization (hpf). \*Statistically significant from 1  $\mu\text{M}$ +0  $\mu\text{M}$  GSH positive controls,  $p < 0.001$ , Tukey. (B) Percent of embryos with a sodium metam (NaM) induced notochord distortion exposed in 10 h windows of development alone (NaM 4–14 and NaM 14–24) or with reduced glutathione preceding or following NaM exposure (NaM 4–14+GSH 14–24 and GSH 4–14+NaM 14–24). \*Statistically significant from NaM only positive controls,  $p < 0.001$ , Tukey. (C) Representative image of GSH and no treatment control embryo at 5 days post-fertilization. (D) Representative images of 5 day post-fertilization embryos exposed to NaM and GSH from 4 to 24 hpf exhibiting diminished notochord distortions, top three, or complete protection bottom. (E) Representative images of NaM-induced notochord distortions following a 4–24 hpf exposure.

shown in Fig. 1, did not protect the embryos from the MITC, DTC degradate carbon disulfide, or pyrrolidine dithiocarbamate induced notochord distortions (Table 2). At 65 and 130  $\mu\text{M}$  GSH the severity of the distortions induced by dimethyl dithiocarbamate were clearly diminished but not abolished.

Using the TUNEL assay on fixed embryos, we determined whether NaM and related compounds induce apoptotic cells death

at 24 hpf. There are no appreciable differences in apoptotic cells at 24 hpf between the predictable light constellation of TUNEL positive cells of the control compared to carbon disulfide, neocuproine, NaM, and DMDTC exposed embryos (Fig. 2A–C and E, F). In these exposed animals there were fewer than 10 apoptotic cells identified in the notochord region and typically <50 cells throughout the embryo. This was similar to controls. MITC exposed embryos



**Fig. 2.** Whole mount apoptotic cell labeling (TUNEL) of 24 h post-fertilization (hpf) control and 4–24 hpf exposed zebrafish embryos. Apoptotic cells appear white. (A) Representative Z-stack image (12–20 slices at  $10\ \mu\text{m}$ ) of untreated control zebrafish embryo. Y: yolk sac. (B) Representative Z-stack image of  $103\ \mu\text{M}$  carbon disulfide exposed zebrafish embryo. Y: yolk sac. (C) Representative Z-stack image of  $1\ \mu\text{M}$  sodium metam (NaM) zebrafish embryo. Y: yolk sac. Arrow head pointing to distorted notochord. (D) Representative Z-stack image of  $1\ \mu\text{M}$  methylisothiocyanate (MITC) zebrafish embryo. Y: yolk sac. Arrow head pointing to distorted notochord. (E) Representative Z-stack image of  $0.14\ \mu\text{M}$  dimethyldithiocarbamate (DMDTC) zebrafish embryo. Y: yolk sac. Arrow heads pointing to distorted notochord. (F) Representative Z-stack image of  $38\ \mu\text{M}$  neocuproine (NCu) zebrafish embryo. Y: yolk sac. Arrow head pointing to distorted notochord.

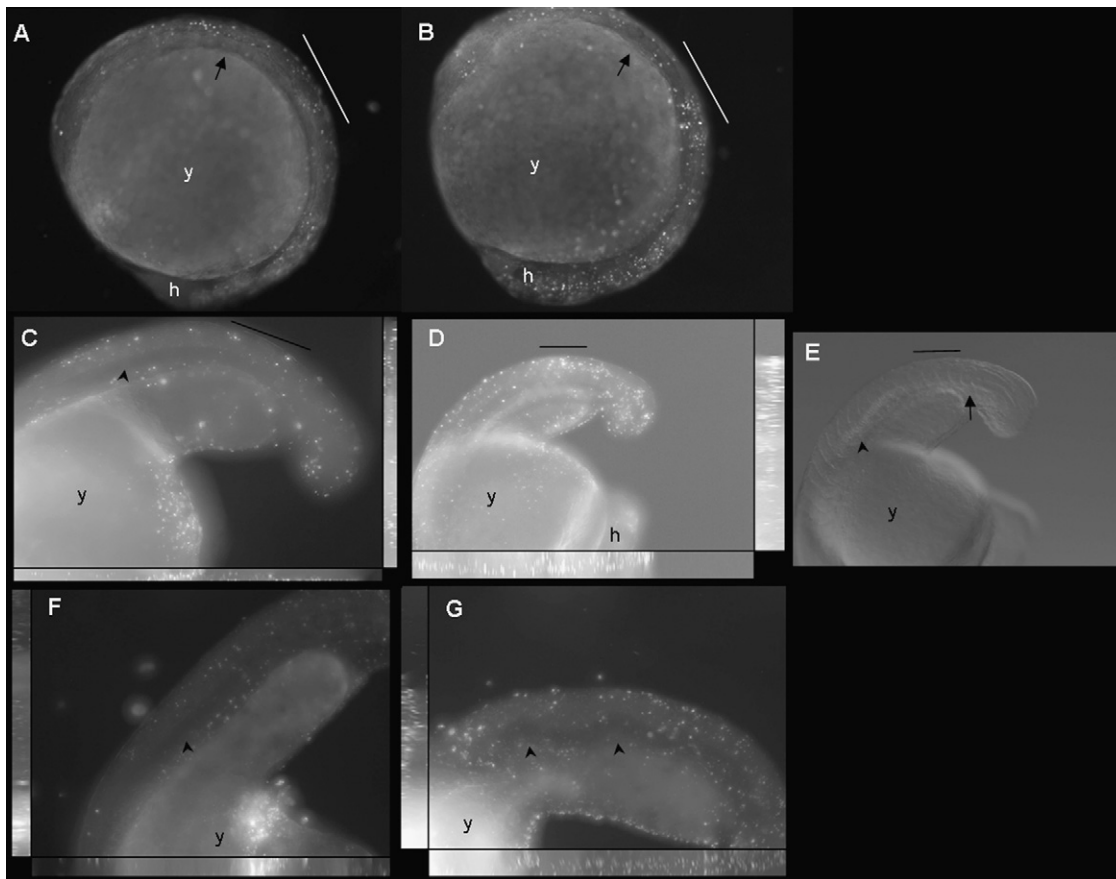
consistently showed numerous apoptotic cells with more than 100 cells observed throughout the embryo (Fig. 2D). This included cells within the brain and peripheral nervous system and the cells making up the notochord or notochord sheath, suggesting more widespread effects by this compound. In the case of DMDTC and neocuproine, it would be tempting to speculate that a suppression of apoptosis occurred due to the few, if any, TUNEL positive cells observed (Fig. 2E and F). However the small number of cells exhibited by controls makes this type of observation difficult.

In addition, we evaluated the cellular uptake of acridine orange, a general indicator of cell death, in the living embryo at 14, 18 and 24 hpf in control and NaM-exposed embryos during the sensitive developmental period of somitogenesis. As observed using TUNEL at 24 hpf, there was no dramatic increase in cell death in exposed animals relative to controls at 24 hpf (data not shown) or at any treatment (Fig. 3A–D). There was, however, an interesting clustering pattern of AO labeled cells occurring at the location of the somitogenic front at 14 and 18 hpf. This corresponded with the period of somitogenesis occurring during the 1 h AO treatment. These AO positive cells appeared throughout the somites and were not restricted to the notochord.

#### 4. Discussion

From the data presented here, the thiol status of the developing vertebrate is a critical factor in the sensitivity to sodium metam (NaM). This may suggest an alteration of reduced and oxidized glutathione pools in the embryo or a chemical interaction (e.g. disulfide bond) between GSH and NaM. DTCs have been shown to impair catalytic thiols, pro-apoptotic enzymes, and form cysteine adducts without the production of reactive oxygen species (Valentine et al., 1995; Nobel et al., 1997; Tonkin et al., 2003). Further, the DTC maneb was shown to alter glutathione ratios differently than ROS generated by paraquat (Barlow et al., 2005), and several studies have demonstrated the ability of thiols to diminish DTC-induced toxicity in a variety of model systems (Fitsanakis et al., 2002; Furuta et al., 2002; Thompson et al., 2002; Chen and Liao, 2003; Cheng and Trombetta, 2004).

DMDTC has previously been shown to be more potent relative to NaM at inducing notochord distortions (Tilton et al., 2006). While complete glutathione protection was found to be unique to NaM compared the other DTCs (Table 2), DMDTC-induced distortions were diminished in severity at the high concentrations of



**Fig. 3.** Acridine orange staining in living 14, 19, and 24 h post-fertilization (hpf) control and treated zebrafish embryos. (A) Representative single plane image of a control embryos following at 15.5 hpf following a 1 h incubation in AO. Arrow shows the forming somites. The bar shows the approximate area of somitogenesis during the 1 h AO staining. Y: yolk, h: head. (B) Representative single plane image of a 15.5 hpf embryo exposed to 1  $\mu\text{M}$  sodium metam from 4 hpf and incubated in AO for 1 h beginning at 14 hpf. Arrow shows the forming somites. The bar shows the approximate area of somitogenesis during the 1 h AO staining. Y: yolk, h: head. (C) Representative Z-stack image (12–20 slices at 10  $\mu\text{m}$ ) of a control embryo at 21 hpf following a 1 h incubation in AO beginning at 19 hpf. Arrow head shows the distorting notochord. The bar shows the approximate area of somitogenesis during the 1 h AO staining. Y: yolk. (D) Representative Z-stack image (12–20 slices at 10  $\mu\text{m}$ ) of an embryo at 21 hpf exposed to 1  $\mu\text{M}$  sodium metam from 4 hpf and incubated in AO for 1 h beginning at 19 hpf showing a cluster of AO positive cells at the somitogenic front. The bar shows the approximate area of somitogenesis during the 1 h AO staining. Y: yolk, h: head. (E) DIC image of the same embryo shown in (D). Arrow head shows the distorting notochord. The arrow points to the somitogenic front at 21 hpf. The bar shows the approximate area of somitogenesis during the 1 h AO staining. Y: yolk. (F) Representative Z-stack image (12–20 slices at 10  $\mu\text{m}$ ) of a control embryo at 25 hpf following a 1 h incubation in AO beginning at 24 hpf. Arrow head points to the normal notochord. Y: yolk. (G) Representative Z-stack image (12–20 slices at 10  $\mu\text{m}$ ) of an embryo at 25 hpf exposed to 1  $\mu\text{M}$  sodium metam from 4 hpf and incubated in AO for 1 h beginning at 24 hpf. Arrow head points to the distorted notochord. Y: yolk.

GSH. This may suggest potency differences among these related compounds. Other studies have reported that the structural complexity of DTCs influences their ability to form covalent bonds with sulfhydryl groups (Scozzafava et al., 2001). These data clearly illuminate the need to extend our understanding of the complex DTC structure–activity relationships in the whole animal, particularly in target tissues of the developing embryo. In using the glutathione modulators butylsulfonamide or diethyl maleate we observed no notochord distorts induced by these compounds alone. This suggests that DTCs do not simply create a glutathione deficient environment. These pharmacological agents also did not alter or enhance the NaM induced notochord distortion. Previous studies in zebrafish embryos have demonstrated these compounds are effective at making the embryo more sensitive to toxicant induced oxidative stress (Usenko et al., 2008). Therefore, the ability of glutathione to protect the developing zebrafish from NaM exposure may stem from the protection of biochemically important sulfhydryls targeted during somitogenesis by NaM, rather than combating reactive oxygen species generation.

The DTC thiram, which also induces a distorted notochord, was reported to not cause an increase in the number of apoptotic cells

(Teraoka et al., 2006). We confirmed these observations using the same TUNEL method with several other DTCs, the copper specific chelator, neocuproine, and the common degradation product, carbon disulfide, all of which induce distorted notochords. The NaM degradation product, MITC, however showed more widespread TUNEL labeling of apoptotic cells following exposure, distinguishing itself from the other compounds tested. There is some evidence for 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 dithiocarbamate derivative had the lowest potential to inhibit superoxide generation. MITC was unresponsive to GSH in this study possibly reflecting its inability to form a disulfide with glutathione or its high affinity for the target.

The localized cell death, as measured by acridine orange, may be reflective of the mis-regulation of neuronal and muscular genes in the somites (Tilton and Tanguay, 2008). Investigating the specific cell types undergoing apoptosis will be required to understand their role more clearly. Widespread cell death caused by, for example, metal catalyzed reactive oxygen species generation does not underlie the developmental toxicity of NaM. Given that all the compounds

tested induce notochord distortions and only MITC shows some induction of apoptosis, the induction of apoptotic pathways cannot play a significant role in NaM developmental toxicity. Rather, it may be of interest to investigate whether DTCs or DTC-disulfides suppress normal apoptotic processes during development (Nobel et al., 1995, 1997; Burkitt et al., 1998).

While DTC developmental toxicity is conserved across all vertebrates, aquatic organisms are considered to be particularly sensitive. The embryos overall thiol status likely plays a significant role in the increased margin of safety observed for DTC developmental toxicity in higher vertebrates. For example, in 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). While zebrafish have  $\mu\text{M}$  quantities of glutathione (Pullela et al., 2006), egg-laying aquatic organisms such as fish have a finite input from the parents relying solely on their own biochemical processes to repair and defend against contaminant insult early in development. Furthermore, conditions which could impair thiol status such as genetic factors, nutritional deficiencies, and exposure to other thiol modifying contaminants should not be overlooked in the risk assessment of DTCs during development.

From these studies, NaM-induced notochord distortions are reversible with thiols and unresponsive to antioxidants. Furthermore, the lack of induction of apoptosis and a diminutive, albeit localized, clustering of cell death observed by acridine orange staining support the that critical sulfhydryls or cellular thiol status, rather than reactive oxygen species generation, plays a role in the mechanism of DTC developmental toxicity. Clearly further exploration of the role of thiols and metal homeostasis in the development of DTC developmental toxicity is warranted.

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