ABSTRACT

Disruption of Copper Homeostasis by Copper Chelating Agents in Embryonic Zebrafish

Christina R. Hagan

Director: Dr. Erika Abel

Disruption of copper homeostasis has been well-documented in cancers and neurodegenerative diseases. Recent studies highlight the potential efficacy of metal chelating compounds in the treatment of altered copper phenotypes. Disulfiram (DSF) is a dithiocarbamate that chelates copper, and DSF treatment has been shown to limit growth of cancer cells. These findings stimulated a search for other thione-containing copper chelating agents with capacity to alter abnormal cellular physiology. Thiomaltol (Htma) and dithiomaltol (Httma) are thiol derivatives of 3-hydroxy-2-methyl-4-pyrone (maltol) with high affinity for copper. In the current study, the efficacy of these compounds in eliciting copper-related phenotypes was compared to that of DSF using the embryonic zebrafish model. Embryonic viability and morphology following DSF, Htma and Httma was assessed in the presence and absence of copper supplementation. Treatment with DSF, Htma and Httma induced mortality or malformations characteristic of copper deficiency in a concentration-dependent manner (LC50 = 41.0, 23.0, 6.4 μM, Htma, Httma and DSF, respectively). Unexpectedly, co-treatment with 2.0 μM copper sulfate ($CuSO_4$) exacerbated the effects of malformation-inducing levels of Htma, but not DSF; embryos co-treated with $CuSO_4$ and nonlethal concentrations of Htma exhibited 100% mortality within 24 hours. Additionally, follow-up studies revealed that addition of $CuSO_4$ to the culture water 15 minutes after treatment with high-dose DSF (but not Htma) could temporarily 'rescue' the embryos from lethality. The differing responses to copper supplementation of Htma versus DSF-treated embryos suggest unique interactions within the copper homeostatic pathway beyond simple direct chelation. Future studies will address the potential intracellular targets of Htma versus DSF.

APPROVED BY DIRECTOR OF HONORS THESIS:

_____

Dr. Erika Abel, Department of Biology

APPROVED BY THE HONORS PROGRAM:

_____

Dr. Elizabeth Corey, Director

DATE: _____

DISRUPTION OF COPPER HOMEOSTASIS BY COPPER CHELATING AGENTS IN EMBRYONIC ZEBRAFISH

A Thesis Submitted to the Faculty of

Baylor University

In Partial Fulfillment of the Requirements for the

Honors Program

By

Christina Hagan

Waco, Texas

May 2016

TABLE OF CONTENTS

DEDICATION

I would first like to express my appreciation to my biochemistry professor, thesis advisor, and lab director Dr. Erika Abel for spurring me to work in the zebrafish laboratory and investing in me greatly throughout this process. Thank you to my other lab director Dr. Crystal Usenko for helping me to develop an appreciation for research with the model organism *Danio Rerio* as well as gathering "lots of data" and Dr. Patrick Farmer for his insights and collaboration.

I am especially grateful for my roommates Suzanne Beecher and sister Bethany Hagan for supporting me throughout my laboratory work, including late nights in lab, and the thesis writing process. A special thank you to my mother who taught me "to whom much is given, much is required" and to my father for his constant support, many fun brainstorming sessions and instilling a passion for science in me. Finally, I couldn't be more thankful for Aaron Hopkins, the best lab partner and friend I could have hoped for.

CHAPTER ONE

Introduction

The Essentiality of Copper

Copper is an essential micronutrient for all living organisms [1]. Copper is found in the cuprous (Cu(I)) and cupric (Cu(II)) forms *in vivo* and readily converts between these redox states. The redox activity of copper explains its utility as a catalytic cofactor for a variety of enzymes and the potential of the free ion to initiate unwanted reactions intracellularly. Free copper has the capacity to displace metal cofactors from binding sites, such as Zn(II) ions of zinc finger domains in transcription factors, as well as prompt formation of reactive hydroxyl radicals with high potential for inducing cellular damage [2]. Therefore, the movement of copper within the cell is necessarily mediated by transmembrane proteins such as CTR1 and ATP7A, regulators of influx and efflux respectively, and intracellular chaperones, such as ATOX1 that specifically delivers copper to the Golgi. In states of dietary copper deficiency or dysfunction of copper cellular import, these chaperones inadequately supply copper to cuproenzymes, manifesting in a characteristic phenotype of severe neurological and connective tissue defects and often lethality. Underlying copper homeostasis is a complex system of interconnecting pathways, where copper interacts specifically with a number of cuproenzymes and other copper binding proteins to perform its essential functions.

Copper Homeostasis

In humans, copper homeostasis is necessary for proper functioning at both the biochemical and organismal level. The recommended dietary allowance of copper for adults is 0.013 mg Cu/kg per day however, as little as 0.017 mg Cu/kg may induce toxicity [3]. The duodenum is the primary site of copper absorption, while some absorption also takes place in the stomach and ileum [4]. Serum albumin protein and ceruloplasmin are the primary plasma copper transporters, delivering copper to tissues for cellular import. Ceruloplasmin is limited to copper loading in the liver while albumin binds copper *in situ*; albumin copper is the major component of the plasma copper exchangeable pool. The albumin-copper binding complex has a short half-life while ceruloplasmin binds copper less transiently with the ability to recognize cell membrane receptors and release copper at specific tissue sites [5]. The teratogenesis that results from maternal copper deficiency illustrates the essentiality of adequate copper intake and distribution through the plasma to normal physiological development. Studies in a variety of species reveal a similar phenotype of brain defects, including collapse of cerebral hemispheres and hypomyelination, and significant deformities of connective tissue in fetuses experiencing copper deficiency during prenatal development. Even copper deficient mothers who do not manifest with phenotypic characteristics of copper deficiency may give birth to severely affected fetuses [6].

Consuming adequate levels of dietary copper is only one factor in maintaining copper homeostasis. Adequate cellular import and distribution of copper must also be maintained. The transmembrane protein Ctr1 primarily mediates copper import (Figure 1). The chaperone proteins COX17, ATOX1 and CCS serve to transport imported copper

to specific cuproenzymes in the mitochondria, the Golgi and the cytoplasm. Copper binding is necessary to the function of these biologically essential cuproenzymes.

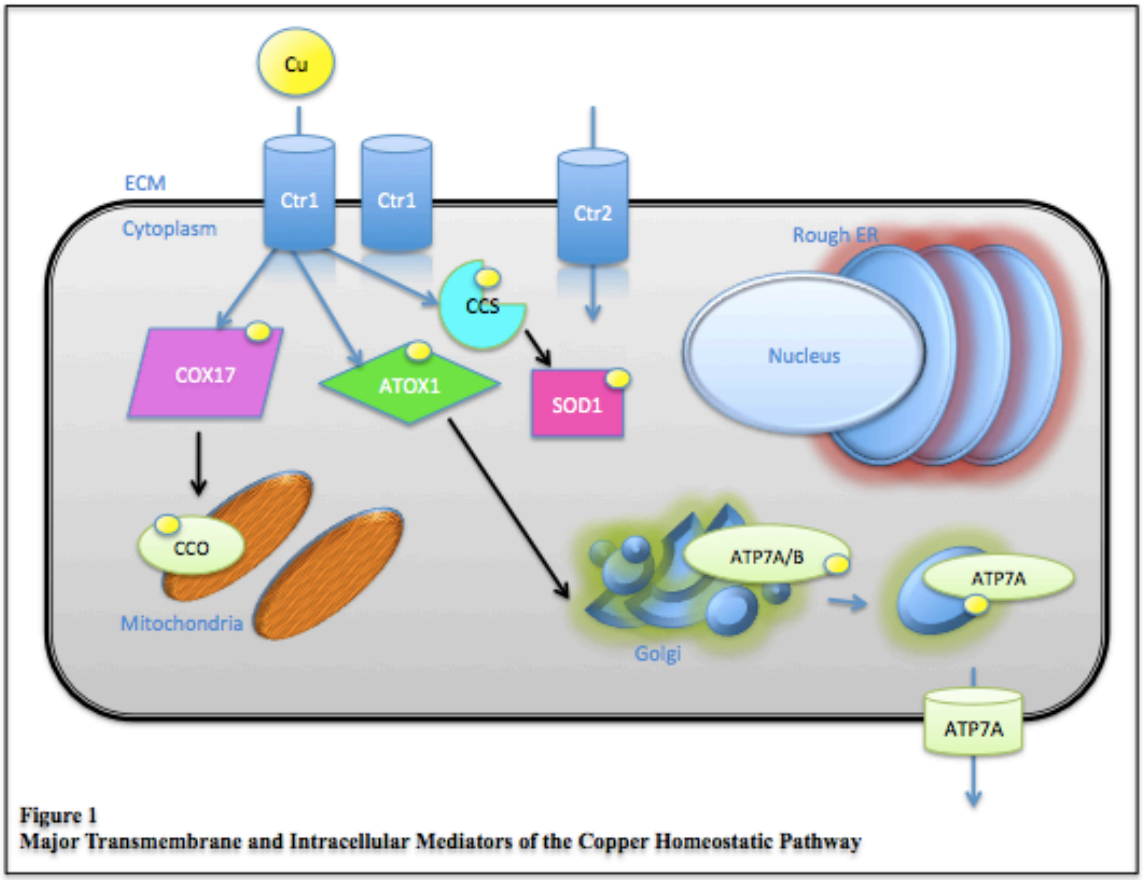


Figure 1
Major Transmembrane and Intracellular Mediators of the Copper Homeostatic Pathway

Copper Influx: CTR1

The cellular influx of copper from plasma is primarily mediated by the integral membrane transport protein CTR1. The human CTR family has two members, CTR1 and CTR2, which are products of the *SLC31A1* and *SLC31A2* genes respectively. CTR1, localized in the plasma membrane, possesses a high affinity for copper while low copper-affinity CTR2 is localized primarily in intracellular vesicles but may also reside in the plasma membrane. CTR1 and CTR2 are structurally similar. CTR1 is a homotrimer with three 190-residue monomer units encircling a central pore. The N-terminal domain is

located extracellularly and contains motifs abundant in histidine and methionine residues proposed to promote Cu(I) aggregation. The CTR1 N-terminal domain is followed by three transmembrane domains, an intracellular loop that links the first and second transmembrane domains and a C-terminal cytoplasmic tail that likely functions to open and close the central pore. CTR1 lacks an ATPase domain and is energy-independent [7]. Given that little free copper exists *in vivo*, it is unlikely that an appreciable transmembrane copper gradient exists to drive CTR1 activity. CTR1 is probably driven by the gradient of other ions, such as potassium. The mechanism by which CTR1 binds Cu(I) and transfers it to intracellular chaperones has yet to be elucidated. However, inhibition of CTR1 alters chaperone activity in cells, indicating the integral role of CTR1 in the cellular influx of copper [8].

Overexpression of human CTR1 in cultured cells results in cytoplasmic copper accumulation while high expression of CTR2 has negligible effects on copper metabolism. The critical function of CTR1 as the main avenue for copper influx was determined in a murine model with targeted *Ctr1* mutagenesis. The homozygous $CTR1^{-/-}$ embryos showed mortality early in development while the heterozygotes manifested copper deficiency specifically in the brain. Embryos with the $CTR1^{-/-}$ null mutation failed to develop any observable mesoderm or allantois, the result of either lysyl oxidase inhibition or blockage of mesodermal migration during embryogenesis [9]. Especially high expression of CTR1 in the choroid plexus and low measured copper levels in the brain determined by atomic absorption spectrophotometry in homozygotes highlight the dependency of the brain on CTR1 for copper import.

The embryonic zebrafish model of human development has also been utilized to examine the role of CTR1. *zCTR1* sequencing revealed significant homology between the zebrafish transporter and mammalian *Ctr1*. Mutant z*Ctr1* zebrafish embryos showed early mortality while knockout of z*Ctr1* using morpholino oligonucleotides (MOs) reflected the phenotype of the heterozygote *Ctr1* mutant mice [9]. These embryos developed normally until organogenesis, when malformations in the head region, especially a decrease in eye size, were observed. Staining with acridine orange supported that effects on the CNS, head and eyes resulted from cellular death [10]. The outcomes in zebrafish and mice $Ctr1^{-/-}$ mutants support the critical role of CTR1 in copper import and maintenance of copper homeostasis.

Chaperones and Cuproenzymes

There are wide arrays of copper binding proteins and metallochaperones within the cell including CCS (Copper chaperone for superoxide dismutase), COX 17 (Cytochrome c oxidase assembly protein 17) and ATOX1 (Antioxidant copper chaperone 1) [11]. The role of copper chaperones is to deliver copper to copper-requiring cytoplasmic enzymes or subcellular compartments for incorporation into cuproenzymes and to prevent toxic accumulation of intracellular copper. To this end, CCS delivers copper to superoxide dismutase 1 (SOD1). SOD1 is a homodimer where copper binding is integral to the stability of each monomer [12]. As a cytoplasmic cuproenzyme that facilitates the conversion of harmful intracellular reactive oxygen species (ROS) to molecular oxygen and hydrogen peroxide, SOD1 performs a process known as "dismutation" [13]. ROS species have been implicated in a wide number of diseases and

SOD proteins are important players in defense against intracellular antioxidant formation [14]. CCS copper delivery is specific to SOD, and the interaction between these proteins is well-characterized [15].

The chaperone COX17 delivers copper to Cytochrome C Oxidase (CCO) located in the inner mitochondrial membrane. The cuproenzyme CCO catalyzes the final step of the electron transport chain, transferring electrons from cytochrome c to molecular oxygen. In copper deficient phenotypes, the activity of CCO is decreased which results in mitochondrial dysfunction. For example, Rossi et al. analyzed CCO in copper-deficient rats. Isolated rat heart mitochondria showed decreased CCO activity. Further analysis revealed that copper deficiency resulted in impairment of heme incorporation into CCO. Because of this not only was CCO synthesis impaired, but also iron homeostasis was affected [16]. Interactions between the iron and copper homeostatic pathways are tissue specific, highly complex, and beyond the scope of this study [17]. However, the Rossi et al. study highlights the severe multiplicity of effects that result from copper deficiency-induced CCO dysfunction.

Finally, ATOX1 delivers copper to the cuproenzymes ATPase7A and ATPase7B. These P-type ATPases are primarily found in the trans Golgi network (TGN) and are important in the metalation of copper-requiring proteins in the secretory pathway [11]. Additionally, ATP7A translocates to the plasma membrane to catalyze copper efflux from cells in certain instances, localizing to the cell membrane to facilitate copper export in situations of elevated copper [11]. ATP7B is highly active in the liver, incorporating copper into apo-ceruloplasmin for eventual excretion to blood and interacting with COMM (copper metabolism gene MURR1) domain-containing protein 1 to facilitate the

biliary excretion of copper. When copper is elevated, ATP7B localizes in the apical membrane and functions as a copper exporter. The role of ATP7B in cells other than hepatocytes is thought to be insignificant and not well characterized [18].

ATP7A is expressed throughout human body tissues and is important for the trafficking of copper into the Golgi in most cells excluding hepatocytes [18]. In mammalian models as well as zebrafish model organisms, ATP7A expression is developmentally regulated. ATP7A expression peaks during the postnatal period prior to synaptogenesis, but later concentrates in distinct cerebellar regions [19]. ATP7A provides copper to enzymes processed through the secretory pathway, including tyrosinase, peptidyl α-amidating monooxygenase, lysyl oxidase and enzymes in the catecholamine synthetic pathway [11]. Tyrosinase is synthesized by melanocytes and is an essential mediator of melanin formation. The enzyme is incorporated into

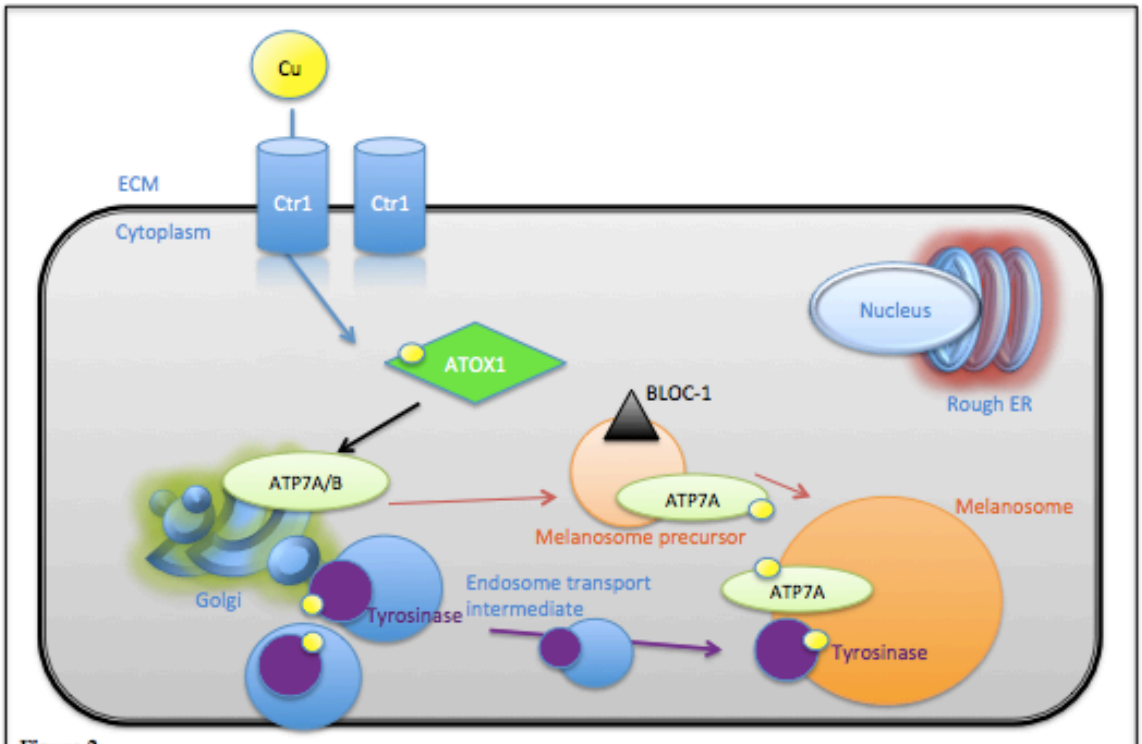


Figure 2:
Tyrosinase is specific to pigment producing cells. Inefficient copper loading of Tyrosinase in the Golgi results in reloading of copper into Tyrosinase upon the enzyme's incorporation into the mature melanosome. Introduction of copper to melanosomes is facilitated by ATP7A, which is trafficked from the Golgi to melanosomes under the direction of BLOC-1.

melanosomes, where its luminal-facing catalytic domain has two essential copper-binding sites [20]. Of special interest, tyrosinase loads limited copper in the TGN, instead becoming copper-bound in melanosomes (Figure 2). Copper specifically gains entrance to melanosomes via ATP7A, which localizes to melanosomes in a process dependent on the biogenesis of lysosome-related organelles complex-1 (BLOC-1). This intersection between ATP7A copper transport pathways and the function of tyrosinase in melanosomes explains ATP7A-deficient phenotypes including decrease or total lack of pigmentation [21]. Additionally, peptidyl α-amidating monooxygenase requires copper to function in the hydroxylation of specific glycine residues on neuropeptides, a step necessary for their bioactivation [22]. Lysyl oxidases are extracellular cuproenzymes that link collagen and elastin. These enzymes are critical to the formation of the axial skeleton during embryogenesis as well as preservation of connective tissue integrity throughout life. Finally, the conversion of dopamine to norepinephrine, an essential process to CNS functioning, is accomplished by Dopamine-beta-hydroxylase (DBH) [23]. ATP7A expression is especially high in cells comprising the blood brain barrier, evidence of its involvement in copper delivery to the brain [18].

Copper Efflux

ATP7A also functions in copper cellular efflux, especially in conditions of elevated copper. ATP7A primarily localizes in the trans Golgi network (TGN), although some ATP7A is constitutively trafficked to and from the plasma membrane. This trafficking mechanism has been extensively studied. While clathrin-coated vesicles and associated adaptor proteins AP-1and AP-2 relay copper to the TGN following

endocytosis, Ras-related proteins in the brain (Rabs) are responsible for movement of ATP7A from the Golgi to the plasma membrane. Rabs are GTPases that, in alternating between GTP-bound and unbound states, mediate vesicle formation, movement and fusion in post-Golgi and endosomal trafficking [24]. When intracellular copper levels rise, the steady state distribution of ATP7A shifts toward the plasma membrane. When cellular copper decreases to normal ranges, ATP7A may return to the TGN [25].

As a member of the P-type ATPase family, ATP7A consists of an actuator domain, nucleotide-binding domain and phosphorylation domain [18]. In addition are eight transmembrane domains within the protein and six metal-binding domains (MBD) at the N-terminus. While copper binding at the N-terminal MBDs has no affect on the copper transporting ability of ATP7A, it is essential that copper be bound to at least three of the MBDs, specifically MBDs 4-6, for ATP7A trafficking to occur [26]. Further, the catalytic cycle of P-type ATPases includes formation of an acyl-phosphate catalytic intermediate, and studies show that trafficking of ATP7A is dependent on formation of this intermediate structure (Figure 3). Petris et al. reported that all trafficking was prevented upon introduction of mutations in ATP7A that blocked formation of the phosphorylated intermediate. However, mutations that induced permanent phosphorylation of the ATPase caused constitutive protein trafficking from the TGN to the cell membrane [27].

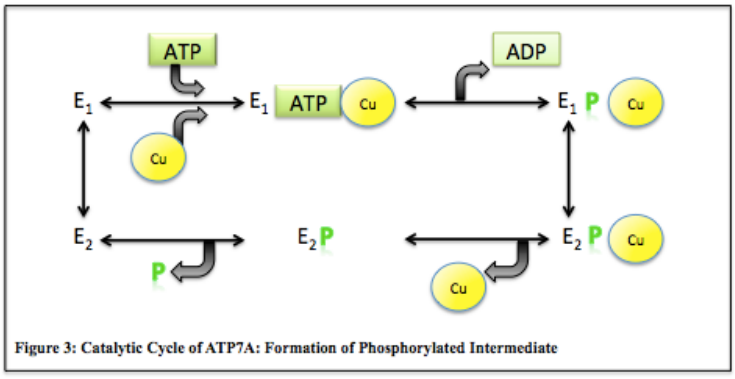


Figure 3: Catalytic Cycle of ATP7A: Formation of Phosphorylated Intermediate

Omeprazole is an over-the-counter medication for patients with gastroesophageal reflux disease. Omeprazole has been shown to prevent melanogenesis in human epidermal melanocytes and murine melanoma cells through inhibition of catalysis and trafficking of ATP7A. In a recent study, effects on pigmentation were not observed until 48-72 hours following exposure to omeprazole, which is consistent with the turnover rate for tyrosinase. Omeprazole can participate in disulfide bond formation, suggesting that it inhibits trafficking through directly binding to ATP7A and blocking formation of the acyl-phosphate intermediate [28]. The sensitivity and responsiveness of ATP7A to intracellular copper concentrations and the widespread activity of the secretory enzymes dependent on ATP7A underscore its importance as a copper-mediating protein. Each of these findings highlights the critical role of intracellular copper trafficking in the maintenance of cellular architecture, pigmentation and physiology.

In summary, a dynamic interplay of cuproenzymes and other metalloproteins function to maintain copper homeostasis. Normal physiology in organisms is dependent on maintaining the integrity of these copper-transporting pathways.

Copper in Disease

Disruption of copper homeostasis has been well-documented in numerous cancers and neurodegenerative diseases. The phenotypic manifestations of copper-altered states are explained by the defective functioning of copper-dependent proteins and the body's response mechanisms. Without binding of metal cofactors, apoenzymes demonstrate little to no activity and characteristic symptoms of copper deficiency result. However, copper excess is also detrimental to cells when copper levels exceed that which the cell is

equipped to handle.

Birth Defects

Altered copper homeostasis has been associated with a variety of congenital abnormalities. For example, an association between myelomeningocele and the SOD1 protein has been reported. The myelomeningocele phenotype is more common in SOD1 mutants given the loss of protection against ROS formation [29]. Lysyl oxidase is also important during embryogenesis and inhibition of its expression or activity results in lack of integrity of collagenous structures throughout the body. Knockout of lysyl oxidase-like 3 in mice resulted in spinal cord deformity, cleft palate and a shortened mandible [30].

Loss of lysyl oxidase-like 3 in zebrafish has been shown to impact notochord formation. In zebrafish, the notochord is the first organ to complete development. It develops from a derivative of the mesoderm, the chordamesoderm, whose inner layer of cells differentiates rapidly, developing large, fluid-filled vacuoles and whose overlying epithelium matures to secrete an external notochord sheath. By 24 hours post fertilization, the notochord occupies nearly the full volume of the embryo. Lysyl oxidase is highly critical to the vacuolation, matrix synthesis and sheath formation that characterize notochord formation [31]. Gasner et. al showed that morpholino-induced knockouts of lysyl oxidase genes expressed during notochord formation sensitized developing zebrafish embryos to copper deficiency. In such states, undulating deformities of the notochord were observed [32]. This emphasizes the importance of gene-nutrient interactions, including the micronutrient copper, during embryogenesis.

Cancer

High tumor levels of copper have been documented in numerous malignancies, including breast, lung, stomach and colorectal cancers as well as squamous cell carcinoma, acute lymphocytic leukemia and others. Increased copper concentrations in solid tumors are positively correlated with cancer stage. It is proposed that oxidative stress in cancer cells results from increased cellular metabolism, inflammation and mitochondrial dysfunction. The cellular ROS defense mechanisms are often insufficient to deal with these significant levels of stress. Beyond this, copper levels are also increased in cancer cells, as the metal is a stimulator of angiogenesis [33]. An illustration of altered copper homeostasis in malignancy is seen in the context of melanoma. Melanoma cells exhibit a weakened ability to deal with oxidative stress, resulting in high intracellular ROS concentrations. Melanoma cells also exhibit increased uptake of copper relative to melanocytes. It was shown that addition of exogenous copper in the presence of metal chelators to melanoma cells promoted further oxidative production and apoptosis [34]. The abnormal copper metabolism and high levels of ROS in melanoma cells have become of special interest to researchers seeking to specifically target cells of this serious cancer type.

Neurodegenerative Diseases

Additionally, copper homeostasis is altered in a variety of neurodegenerative diseases. Some forms of amyotrophic lateral sclerosis (ALS) are associated with increased expression of mutant SOD1 protein and its chaperone CCS. Elevated concentrations of high copper-affinity CCS heighten copper demand and cause

competition between it and other cuproenzymes, especially cytochrome c oxidase. The potential for copper supplementation to reduce competition in this context has become a recent focus for researchers in developing ALS therapies [35].

Pathogenesis associated with mutant ATP7A reveals a distinct phenotype, most often the result of Occipital Horn Syndrome and the more severe Menkes disease, an x-linked recessive disorder that results in non-functional ATP7A. Affected individuals have widespread symptoms, including neurodegeneration and weakening of connective tissues, and mortality is often observed before age three [36]. A Menkes model in zebrafish has been developed, where morpholino-induced knockout of ATP7A shows analogous outcomes of nervous system and connective tissue deformities in developing embryos. Malformations include lack of pigment, expanded hindbrain ventricles and a wavy notochord. Further, ATP7A knockouts were greatly sensitized to copper deficient states. In general, as whole body copper levels decreased, tissues with relatively high expression of ATP7A including the ventricles and notochord preferentially accumulated copper. This model may be useful in exploring not only Menkes disease, but also other birth defects including congenital scoliosis and syndromes involving gene-copper interactions during development [37].

The recognition of the role of copper in disease continues to increase. The morbidity and mortality associated with these copper-related diseases including myelomeningocele, melanoma and Menkes diseases are significant and has prompted a search for copper homeostasis-targeting therapies.

Copper: A Therapeutic Target

Initial efforts to treat copper deficiency involved oral and intravenous administration of copper. Acquired copper deficiency, such as that following gastric bypass surgery or long-term jejunostomy feedings, is treated with oral and parenteral copper histidine supplements [38]. These supplements restore normal serum copper but incompletely resolve nervous system deficits [39]. The limitations of copper histidine are also recognized in attempts to facilitate copper transport across the blood brain barrier. Doses of copper histidine high enough to induce kidney toxicity are required for copper to traverse the highly selective barrier between the blood and the brain [40]. Therefore, oral copper supplementation is not effective in many instances and other methods for manipulating copper homeostasis are being explored, including identifying specific copper-binding protein inhibitors. Further, excess copper, as detected in cancerous tissues, may be equally destructive. Alternate mechanisms for restoring homeostasis in situational copper excess are also of interest.

Lung cancer screen 1 (LCS-1) is a non-copper chelating SOD1 inhibitor. LCS-1 slows progression of specific strains of lung cell adenocarcinoma where SOD1 is overexpressed. Development of LCS-1 analogs with greater affinity is underway [41]. Omeprazole, mentioned above, is a proton pump inhibitor that blocks ATP4A in gastric parietal cells. It is also shown to affect tyrosinase by inhibiting ATP7A and is currently being studied as a candidate topical treatment for inhibiting pigment formation [28].

While supplementation and direct enzyme inhibition have proved effective in certain contexts, another copper manipulation strategy involves the use of metal chelating agents. A chelator is a molecule that binds a metal at multiple attachment sites. Metal

chelation has been traditionally used as a therapy for metal poisoning however, another therapeutic application for these compounds in cancer treatment has been proposed. D-penicillamine (D-pen) is an alpha-amino acid metabolite of penicillin and a highly hydrophilic copper chelator that is efficacious in inducing copper excretion from the body. In chelating copper, D-pen reduces C(II) to C(I), with formation of hydrogen peroxide accompanying this reaction. Copper and hydrogen peroxide potentiate formation of ROS species, a result of Fenton chemistry (Figure 4). Already high levels of ROS characterize cancer, a hypothesized by-product of increased metabolism in malignant cells and mitochondrial mutations. Cancer cells are also marked by relatively low levels of antioxidant molecules including SOD1 and glutathione reductase as well as irregularly high copper concentrations. Glutathione may be oxidized to its disulfide form in the presence of copper, also leading to generation of hydrogen peroxide. Co-treatment with D-pen and copper sulfate induced cytotoxicity in human breast cancer and leukemia cells, while independently these doses of copper sulfate and D-pen did not generate cytotoxic effects [42]. By augmenting the already high ROS in cancer cells, D-pen and copper promoted apoptosis of malignant cells. However, the major limitation of D-pen treatment is its high hydrophilicity. Attempts to increase the lipophilicity of D-pen have included use of a gelatin-D-pen conjugate [43]; however, many researchers turned their attention to copper chelating compounds with greater membrane permeability.

The toxicity of H_20_2 is largely based on its conversion to the hydroxyl radical by interactions with transition metals such as copper through Fenton chemistry.

$$H_20_2 + Cu^{+} \rightarrow Cu^{2+} + OH^{-} + OH^{\bullet}$$

Figure 4: The Fenton Reaction

Copper Ionophores

Ionophores are a subclass of metal chelators that not only bind metal ions, but also shuttle the bound ions across cell membranes. The hydrophobicity of these compounds facilitates the unique metal transport mechanism. A very recent study identified the ionophore diacetyl-bis(4-methyl thiosemicarbazonato)copper(II) (Cu^{II}(ATSM)) as a potential candidate for treatment of mutant SOD related amyotrophic lateral sclerosis (ALS). (Cu^{II}(ATSM)) is a member of the copper-chelating family bis(thiosemicarbazones) with methyl substituents that result in a relatively low reduction potential of the (Cu^{II}(ATSM)) complex. The presence of these substituents makes copper less likely to dissociate from (Cu^{II}(ATSM)) in the intracellular environment, illustrating that the action of ionophores is dependent on the reduction potential of their metal-coordinated complex [40]. In highly reducing, or hypoxic, environments however, (Cu^{II}(ATSM)) will release its coordinated copper ion. In the context of a mouse model of ALS, (Cu^{II}(ATSM)) is effective at delivering copper to the central nervous system, which proved transformational to the longevity of mice with the disease. Treatment with (Cu^{II}(ATSM)) prolonged lifespan of SOD/CCS double-knockout mice by an average of 1.5 years [35].

Another ionophore, disulfiram (DSF), is a sulfur-based metal chelating compound that was first recognized for its inhibition of the enzyme aldehyde dehydrogenase. DSF is an FDA-approved drug currently prescribed under the name Antabuse to alcohol dependent individuals. DSF interferes with ethanol metabolism to elicit adverse reactions to alcohol consumption as a deterrent approach [44]. It does so through direct inhibition of aldehyde dehydrogenase (ALDH), a mediator in the retinoic acid pathway. DSF also

strongly chelates copper, although this chelating ability is technically a function of its metabolite diethyldithiocarbamate (deDTC). Both DSF and deDTC are part of a larger family of compounds known as dithiocarbamates (DTC) [45]. The DTC family is compromised of molecules that form highly stable interactions with various metals [46]. deDTC is a monoanionic, bidentate species that complexes with Cu(II) to form the ionophoric complex $Cu(deDTC)_2$ [47]. In 2004, Cen et al. reported that disulfiram was able to facilitate intracellular copper uptake by melanoma cell lines in various stages. The extracellular conversion of DSF to deDTC and subsequent metal binding only occurred in the presence of copper, not independently or in the presence of other metals including iron. Interestingly, melanoma cells appeared particularly sensitive to this treatment relative to other cells [34].

More recently, Allensworth et al. expounded on this mechanism in the context of inflammatory breast cancer. Experiments utilized two lines of breast cancer tumor cells, SUM149 and rSUM149, which differ in their expression levels of the copper transporter Ctr1. DSF was equally effective at chelating copper and transporting it into the SUM149 and rSUM149 cells, indicating a Ctr1-independent mechanism for copper cell entry with DSF [48]. The metabolite of DSF, deDTC in complex with copper has also been shown to inhibit tyrosinase, to inhibit proteasome activity and to influence the activity of NFκB. [49].

Disulfiram also shows promise in increasing the bioavailability of copper as a treatment for various diseases. A case study in 2005 of a patient with melanoma in the buccal region showed that topical treatment with DSF and copper eradicated the malignant growth [50]. In a recent case study involving three patients with Menkes

disease and Occipital horn syndrome, oral Disulfiram along with parenteral administration of copper-histidine improved outcomes in one of the patients [51]. Ongoing clinical trials and studies in murine models are currently underway. The multimodal compound DSF and its metabolites are promising to the pharmaceutical field and are being extensively studied in many contexts.

The Maltol Derivatives

As mentioned previously, the efficacy of metal chelators with thiol derivatives, such as those in the dithiocarbamate family, spurred researchers to examine other chelators with properties similar to DSF. Wanting to capitalize on the copper chelating properties of DSF while minimizing other potential interactions, such as the inhibition of aldehyde dehydrogenase enzymes, the Farmer Research Group turned to thiol derivatives of the common food additive maltol [47].

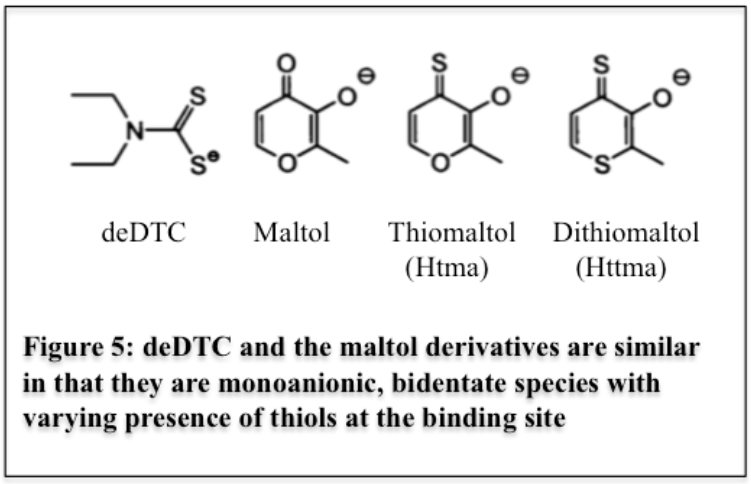


Figure 5: deDTC and the maltol derivatives are similar in that they are monoanionic, bidentate species with varying presence of thiols at the binding site

Maltol is a heterocyclic compound with both alpha hydroxyl and ketone moieties. Binding is observed between these substituents and metal ions, although high copper concentrations are necessary [47]. Thiomaltol (Htma) is a derivate of maltol with a thione substituent in place of the ketone group (Figure 5). Htma was first synthesized in 1969 but interest in its biological activity did not develop until recently. In contrast to

maltol, Htma binds copper at significantly lower concentrations of copper, indicating the influential role of thione groups in copper chelation by Htma and another maltol derivative, dithiomaltol (Httma). Httma was first synthesized by members of the Farmer group in 2004 via a novel one-pot synthesis heterocyclic exchange reaction. In Httma, the heterocyclic ring oxygen is replaced with a sulfur atom. A unique property of Httma is that when complexed with zinc, the $Zn(Httma)_2$ complex is intrinsically fluorescent. Similar to deDTC, the maltol derivatives are monoanionic, bidentate species with a varying number of thione groups present at the binding site [47].

Embryonic Zebrafish Model

In the current study, the copper chelating agents DSF and the maltol derivatives were assessed using the embryonic zebrafish model. Zebrafish (*Danio rerio*) are a valuable model organism with utilities in the fields of genetics, developmental biology, and pharmaceutical toxicology. Zebrafish genome analyses reveal that while *Danio rerio* gene products are distinct from their human orthologues, particular domains of zebrafish proteins that serve as sites of drug-protein interactions are nearly 100% equivalent with the analogous regions of human protein domains [52]. Therefore assessment of pharmaceuticals in zebrafish provides great insight into the actions of potential therapeutic compounds in humans.

The embryonic zebrafish model has many advantages. Set-up and maintenance of a zebrafish aquatic housing system is both space and cost-effective relative to that of other model organisms. Zebrafish produce a large number of offspring in a single spawning period. Developing zebrafish readily uptake compounds from their

environment through skin and gills. The embryonic chorion can be removed manually or enzymatically via pronase digestion for treatment at early developmental time points. Finally, the translucent nature of zebrafish embryos and their well-characterized stages of development allow for easy visualization and assessment of phenotypic abnormalities during embryogenesis.

The initial focus of the present study was to assess the abilities of DSF and the maltol derivatives to block pigmentation in embryonic zebrafish. The embryonic zebrafish model has proven a useful tool for assessing the activity of tyrosinase during melanogenesis and its organism-wide effects on pigmentation [53]. It was hypothesized that DSF and the maltol derivatives Htma and Httma would inhibit tyrosinase due to their manipulation of copper homeostasis via metal chelation. However, at pigment-blocking treatment concentrations of DSF and the maltol derivatives, significant malformations and mortality were observed.

The focus of this study shifted to comparing the toxicities of DSF and Htma in zebrafish and to assessing the outcomes of copper supplementation on the effects of these compounds. It was hypothesized that DSF would be more toxic relative to the maltol derivatives due to its additional function in inhibiting ALDH and that copper supplementation should influence the effects of DSF and Htma. It was expected that characteristic manifestations of copper deficiency would be observed following treatment with DSF and Htma, but that this phenotype would be at least partially ameliorated upon copper supplementation. It was also thought that high levels of supplemental copper might induce embryonic cell death due to formation of intracellular ROS, directly correlated with the level of copper co-incubated with DSF or Htma prior to exposure.

The following experiments showed dramatic phenotypic manifestations of copper deficiency and distinct outcomes between DSF and Htma when supplemented with copper, suggesting potentially unique interactions for these compounds within the copper homeostatic pathway.

CHAPTER TWO

Methods and Procedures

Experimental Animals

Adult zebrafish were raised as a breeding stock in the Abel and Usenko Zebrafish Toxicology Laboratory at Baylor University (Waco, TX, USA). Wildtype zebrafish (Tropical 5D) were obtained from Oregon State University (Corvallis, OR, USA). Adult zebrafish were maintained in polycarbonate tanks in a circulating housing system at 28°C with a 14 h light: 10 h dark photoperiod and were fed flake food fed twice daily. Fish water consisted of distilled water with 0.26% Instant Ocean Aquarium Salt™ to maintain a pH of 7.0 and a conductivity of 400 uSem/cm. All fish were treated according to IACUC protocols.

Spawning took place between select tanks twice weekly, signaled by the laboratory lights turning on in the morning. Two hours later, embryos were collected into plastic petri dishes and at 6 hours post fertilization (hpf) were dechorionated with Pronase. At 24 hpf, dechorionated embryos were visually assessed. Those that appeared phenotypically normal and in the correct developmental stage (Pharyngula, 24 hpt) were transferred with a glass pipette into 96-well microplates, one fish per well, for treatment in a minimum of 200 µL of exposure solution in fish water (Figure 6).

Exposure Methods

Exposure solutions were prepared by diluting 10 mM stock solutions of DSF, Htma, Httma, and maltol [in DMSO (Dimethyl Sulfoxide)] or 200 mM $CuSO_4$ (in water)

to achieve the desired concentration with ≤ 0.5% vehicle in the final treatment. For example, 4 mL of fish water were pipetted into 15 mL conical vials, and 4 µL of 10 mM stock solution were added to each to generate a 4 µM exposure solution. Water was subsequently removed from each well containing a single embryo in the microplate and 200 µL of treatment solution was applied. Embryos were scored for viability (presence of heartbeat) and assessed for notochord malformations, pericardial edema and yolk sac edema at 24, 48 and 120 hpt. The developing embryos were examined under a dissecting microscope (Fisher Scientific Stereomaster) and images were gathered and stored using a Nikon SMZ 1500 photo-microscope and NIS-Elements D image manager.

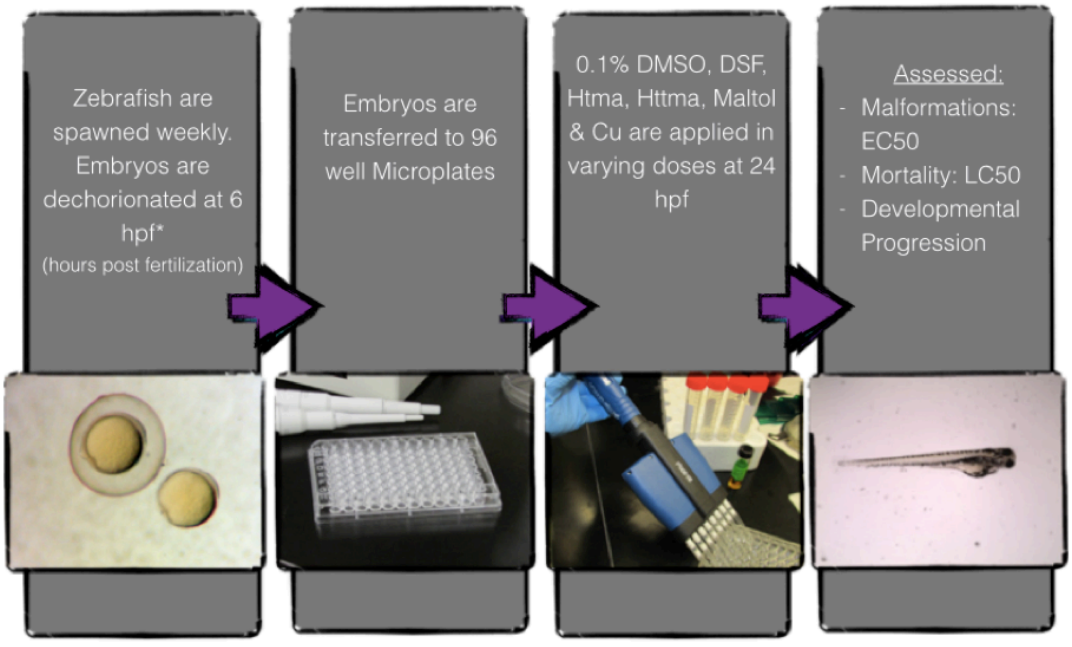


Figure 6: Treatment Method

Toxicity Dose Response Analysis

Dechorionated embryos were exposed, as outlined above, to the following concentrations of DSF: 0.625 μM, 0.125 μM, 2.5 μM, 5 μM, 10 μM, 25 μM and 50 μM. 0.1% DMSO in fish water served as the control. In separate studies, embryos were exposed to equivalent concentrations of Htma, Httma and maltol. For each living embryo, the presence of pericardial edema, yolk sac edema and notochord abnormalities was determined and accordingly charted. This assessment was repeated at 48 and 120 hpt. For example, embryos that exhibited malformations but lacked a heartbeat at 24 hpt were counted in the mortality group at that time point and at all subsequent assessment time points.

Pigmentation Assay

A pigmentation assay was performed on all Htma-treated embryos still viable at 120 hpt. These zebrafish embryos were euthanized and their bodies collected, centrifuged and frozen at 120 hpt. Frozen samples were later digested with 100 microL of 1.0M NaOH for 5 min. Samples were cooled at room temperature and mildly vortexed to produce a pigment homogenate that was loaded into individual wells of a 96 well plate. Using a plate reader spectrophotometer (DTX 880 Multimode Detector, Beckman Coulter), UV absorbance at 405 nm was measured immediately as previously described [54]. Values were normalized by the number of fish bodies collected.

Htma or DSF and Copper Co-Treatment

Copper test solutions were made by dissolving appropriate masses of $CuSO_4 \bullet 5H_2O$ in fish water (distilled water; Instant Ocean Aquarium Salt™) to make 0.2, 2.0 and 20.0 µM copper sulfate stock solutions. Of important note, Instant Ocean Aquarium Salt™ mix contains 0.00115 ppm (0.18 microM) copper sulfate. 2.5 µM Htma or DSF were co-incubated in 15 mL conical vials with fish water and the respective doses of copper sulfate test solutions (Figure 7). Exposures were performed with these combinatorial solutions as described in the Exposure Methods section above, with 0.1% DMSO, Htma-only or DSF-only and copper-only treatments serving as negative and positive controls respectively.

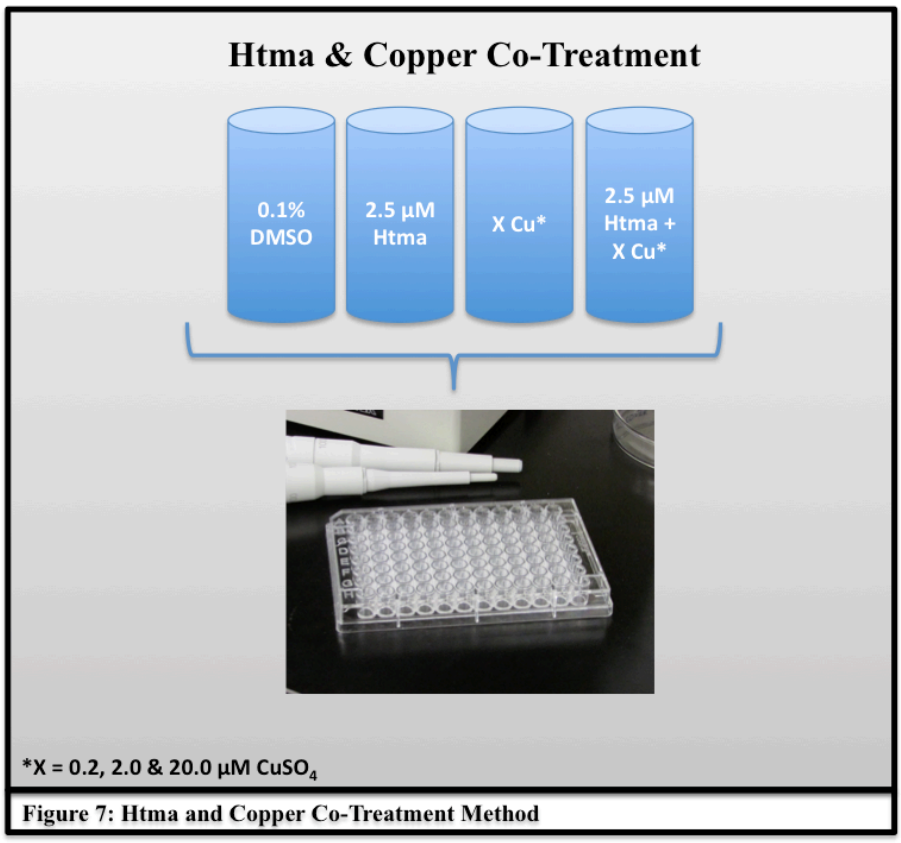


Figure 7: Htma and Copper Co-Treatment Method

Copper Supplementation Post-Treatment

Embryos dechorionated at 6 hpf were treated with 25 µM Htma at 24 hpf. 15 minutes following this exposure, 20 µM $CuSO_4$ was added to the existing exposure solution of half of the Htma-treated embryos in the microplate, with the Htma-treated embryos without copper addition serving as a positive control. This procedure was repeated for embryos treated with 25 µM DSF (Figure 8).

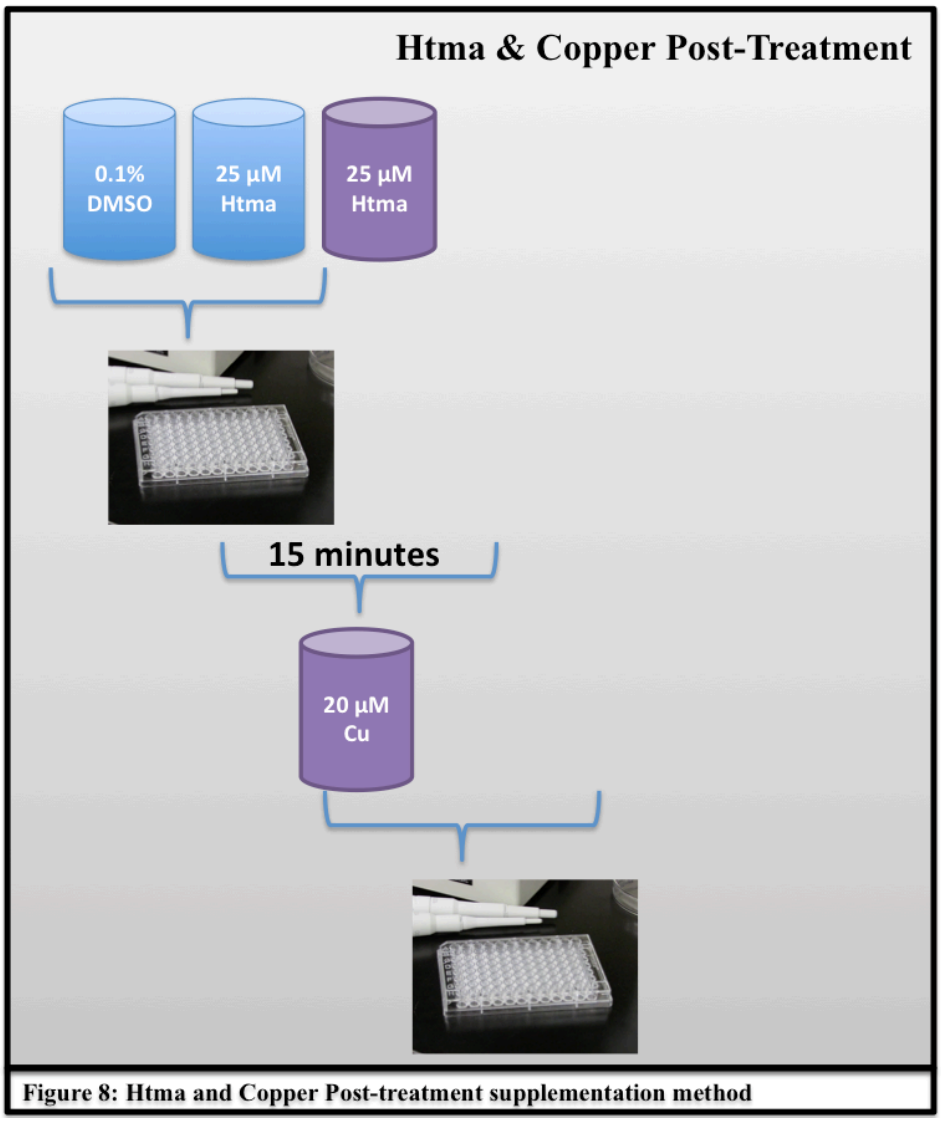


Figure 8: Htma and Copper Post-treatment supplementation method

CHAPTER THREE

Results

3.1 Toxicity Dose-Response – DSF, Htma, Httma & Maltol at 120 hpt

The embryonic zebrafish model was utilized to assess the relative toxicities of DSF, maltol and the maltol derivatives in an *in vivo* model. Treatment of 24 hpf embryos with DSF proved acutely toxic to developing zebrafish, inducing lethality in a concentration-dependent manner (Figure 9A). Mortality following exposure to DSF, even in the low micromolar range, was observed by 120 hours post treatment (hpt). Exposure to 10 μM DSF led to lethality in greater than 75% of embryos in the respective treatment arm (Fisher's exact test, $p < 0.05$). By 120 hpt no embryos exposed to 50 μM DSF were alive. These results are in line with previously published reports demonstrating potent toxicity of DSF in embryonic zebrafish [56].

By comparison, no toxicity was observed following exposure to the non-thione-containing, weak copper chelating agent, maltol, at equivalent doses. However, when the oxygen atom of the ketone group and heterocyclic ring oxygen of maltol are substituted with sulfur to generate potent copper chelation capacity, the associated toxicity in embryonic zebrafish is greatly enhanced. Htma and Httma induced mortality in a concentration-dependent manner. Notably, however, these compounds induced toxicity at higher average doses relative to DSF. That the mortality induced by Htma and Httma is nearly equivalent indicates that the second sulfur atom of Httma likely does not play a significant role in the compound's induced toxicity. The second sulfur atom substitution in Httma does not replace the alpha hydroxyl group of Htma which serves as a metal

attachment site, but instead substitutes for the heterocyclic ring oxygen (Figure 5) [47]. DSF had the lowest calculated LC50 (DSF LC50 = 6.4 µM) indicating the greatest potency in inducing mortality as compared to the other compounds assessed (Figure 9B).

A

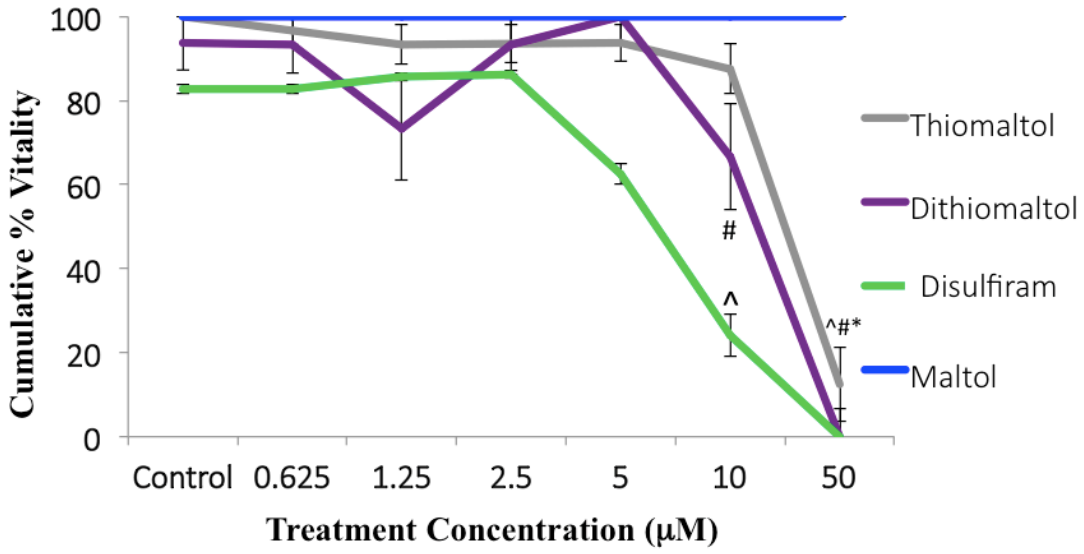


Figure 9: DSF, Htma, and Httma induce mortality in embryonic zebrafish in the micromolar range. **(A)** Mortality increases in a concentration dependent manner following treatment with DSF, Htma, and Httma as compared to vehicle treated controls. Mortality was assessed at 120 hours post treatment. $p<0.05$, Fisher's exact test; Htma (#), Httma (*), DSF (^), N=16 larvae/treatment group
(B) DSF has the lowest LC50 indicating the highest acute toxicity in developing zebrafish. EC50 values indicate DSF induced malformations at a lower average dose as compared to Httma and Htma. Maltol did not induce malformations or mortality.

B

Compound	LC50	EC50
DSF	6.4 µM	0.14 µM
Maltol	NA	NA
Htma	41.0 µM	1.39 µM
Httma	23.0 µM	1.91 µM

3.2 120 hour Time-Course: DSF & Htma-Induced Mortality

To determine the time of onset of toxicity following treatment with DSF or Htma and to gain insight into their toxicity-inducing mechanisms, mortality following treatment

with DSF or Htma at various concentrations was assessed at 24, 48 and 120 hpt (Figures 10A & 10B). DSF-induced lethality was observed as early as 24 hpt at all treatment concentrations. 25 µM DSF resulted in death of all embryos assessed within 24 hours of exposure, while 50 µM Htma did not induce total lethality at any time point assessed. Htma was equivalently or less lethal than DSF at every time point, and the significant mortality associated with 50 µM Htma was not observed until 120 hpt. These observations highlight the increased potency of DSF relative to Htma, which may result from DSF's ability to interact with other molecules essential to key developmental pathways, including inhibition of the enzyme aldehyde dehydrogenase, in addition to chelating copper.

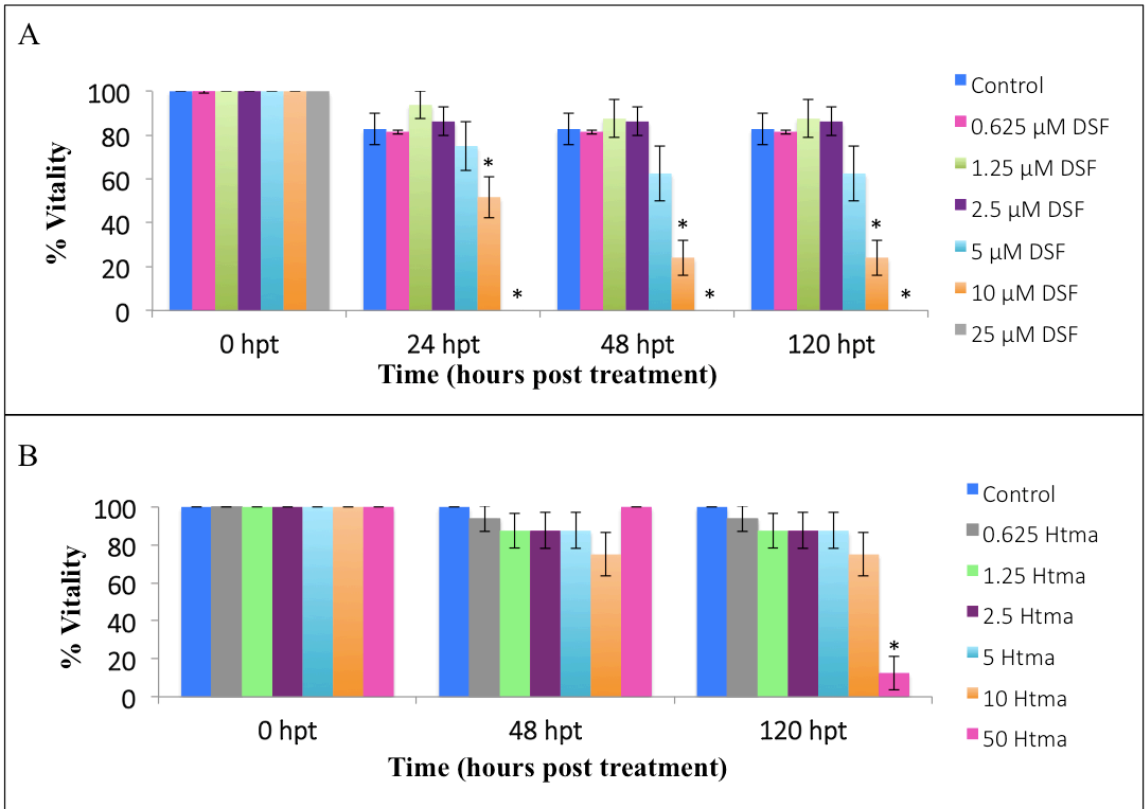


Figure 10: Vitality of DSF- or Htma-treated embryos over a 120 hour time course. (A) Vitality in zebrafish embryos following treatment with DSF decreases in a concentration-dependent manner over a 120 hour time course. High dose DSF results in complete mortality within the first 24 hours following treatment. **(B)** The mortality induced by Htma follows the same trend as DSF, however, it is less potent at nearly all time points. High dose Htma does not generate significant mortality until 120 hpt. $p<0.05$, Fisher's exact test, (*) indicates statistical significance, N=16 larvae/treatment group

3.3 Pigmentation at 120 hr following Htma treatment

An interesting observation was noted following treatment with non-lethal doses of Htma. Larvae that had been exposed to Htma showed an obvious heartbeat and circulation, but a clear appearance and malformations were also evident (Figure 11).

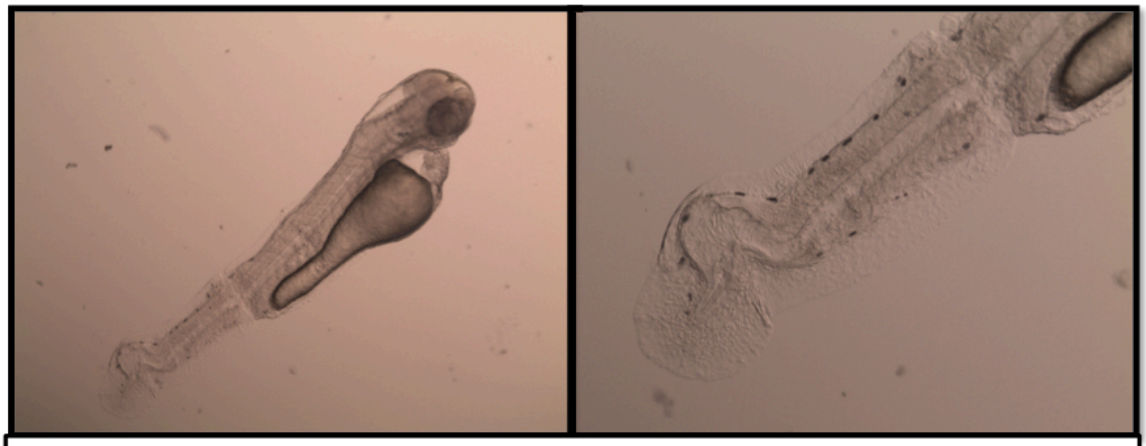


Figure 11: Notochord malformations and reduction in pigmentation following Htma exposure in embryonic zebrafish. Treatment with 25 μM Htma resulted in an undulating notochord in the caudal-most portion of the zebrafish embryo body axis. The embryo appeared clear, exhibiting an obvious lack of pigmentation.

To validate visual observations, larvae were euthanized at 120 hpt, homogenized and UV absorbance at 405 nm was assessed. Only living fish, differentiated by the presence of a beating heart, were included in the assessment. The observed decrease in pigmentation was confirmed with absorbance analysis (Figure 12). Similar results were observed following treatment with DSF. These findings could indicate that Htma and DSF indirectly block the activity of the copper-requiring enzyme tyrosinase via intracellular copper chelation. As tyrosinase is an essential mediator in the melanin synthesis pathway, a dysfunctional form of the enzyme would lead to a reduction in pigmentation equivalent to that shown in Figure 11. However, the observed decrease in pigmentation in these experiments paralleled the toxicity induced by Htma (Figure 12). Pigmentation in developing zebrafish embryos is first observed in the rostral region at approximately

24 hours post fertilization and melanogenesis occurs progressively down the embryo body axis [53]. Given that Htma exposures were performed at 24 hpf, the toxicity induced by Htma or DSF could be interfering with developmental processes including melanogenesis, rather than acting specifically on tyrosinase.

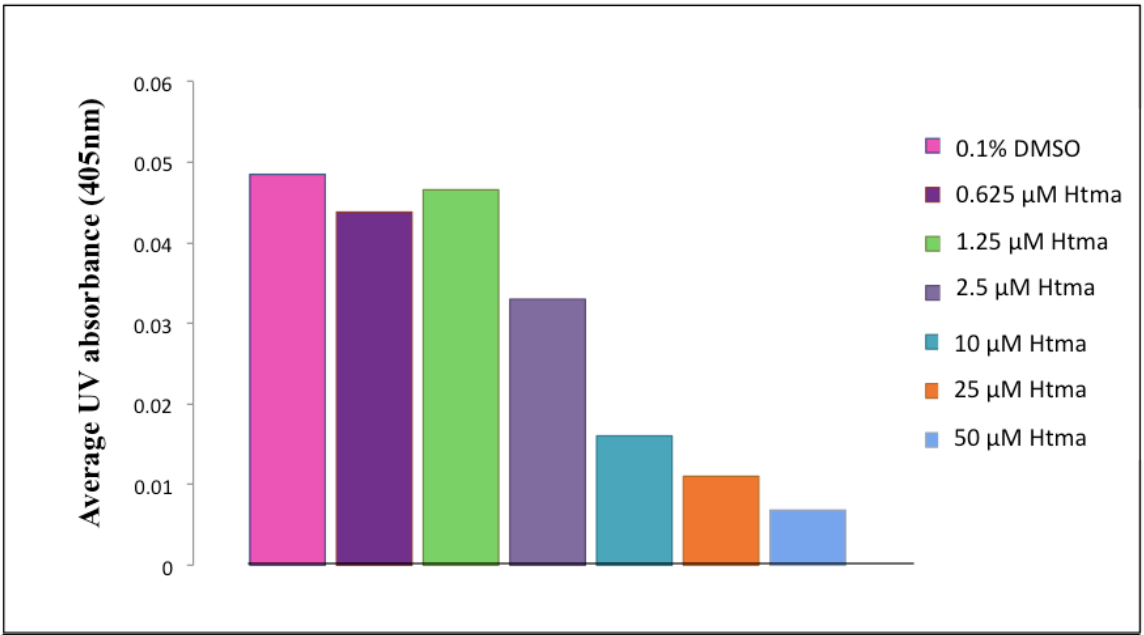
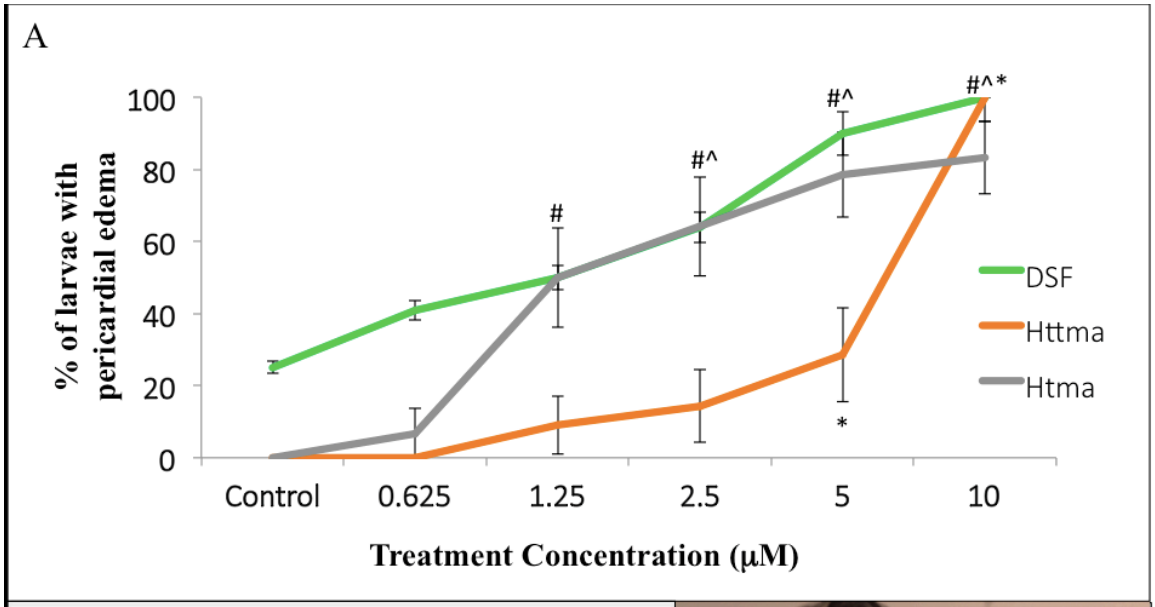


Figure 12: Pigmentation of Htma-treated embryos at 120 hours post treatment.
Exposure to Htma reduces pigmentation in developing zebrafish embryos. Pigmentation correlates negatively with the Htma concentration of the treatment solutions. The average absorbance at 405 nm was normalized by the fish count in each treatment arm.

3.4 Pericardial Edema observed at 120 hpt with test agents

In addition to effects on pigmentation, numerous physical malformations were induced in zebrafish embryos treated with DSF, Htma and Httma. Each agent induced a concentration-dependent increase in the incidence of pericardial edema (PE) over controls. The incidence of PE in the treated larvae approached 100% for all tested compounds in the mid-micromolar range when examined at 120 hpt (Figure 13). Httma had a less pronounced effect on the incidence of PE than either Htma or DSF at lower

treatment concentrations (EC50 values: 0.14 µM DSF, 1.39 µM Htma, 1.91 µM Httma). The comparatively higher EC50 value for Httma indicates that the double thione-containing derivative of maltol has the lowest PE-inducing potency compared to Htma and DSF.



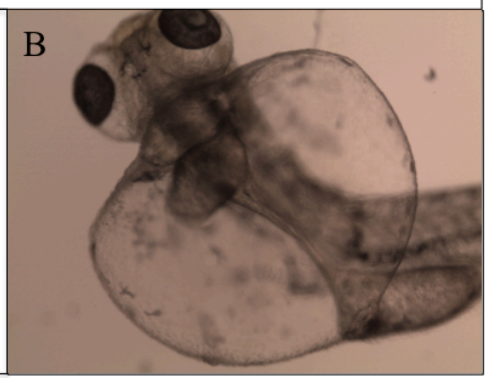


Figure 13: DSF, Htma and Httma induce pericardial edema in developing zebrafish.
(A) DSF, Httma, and Htma induce pericardial edema (PE), with the incidence of PE approaching 100% for all tested compounds in the low micromolar range. (B) Severe PE induced by 25 µM Htma at 120 hpt. $p<0.05$, Fisher's exact test, Significance: Htma #, Httma *, DSF ^, N=16 larvae/treatment group

3.5 Notochord Malformations observed at 120 hpt with test agents

In addition to pericardial edema, dramatic notochord malformations were observed following treatment with DSF and the maltol derivatives. 100% of the embryos treated with sublethal doses of DSF, Htma and Httma exhibited notochord abnormalities

at 120 hpt (Figure 14). Again, DSF proved more potent than the maltol derivatives, inducing notochord deformities at the lowest relative treatment concentrations.

The malformations induced by DSF and the maltol derivatives included large lesions in both the zebrafish body and tail region (Figure 15). These lesions appeared as disruptions of the notochord, characterized by localized expansion and disorganization of notochordal sheath components. Rarely, undulations in the caudal portion of the zebrafish body axis were observed (Figure 11). The notochord malformations induced by the maltol derivatives and DSF were phenotypically similar. While PE is a general indicator of toxicity, the notochord malformations observed here mimic those of a well-established model of copper deficiency in embryonic zebrafish [55], indicating that the toxicity induced by these compounds is copper-specific.

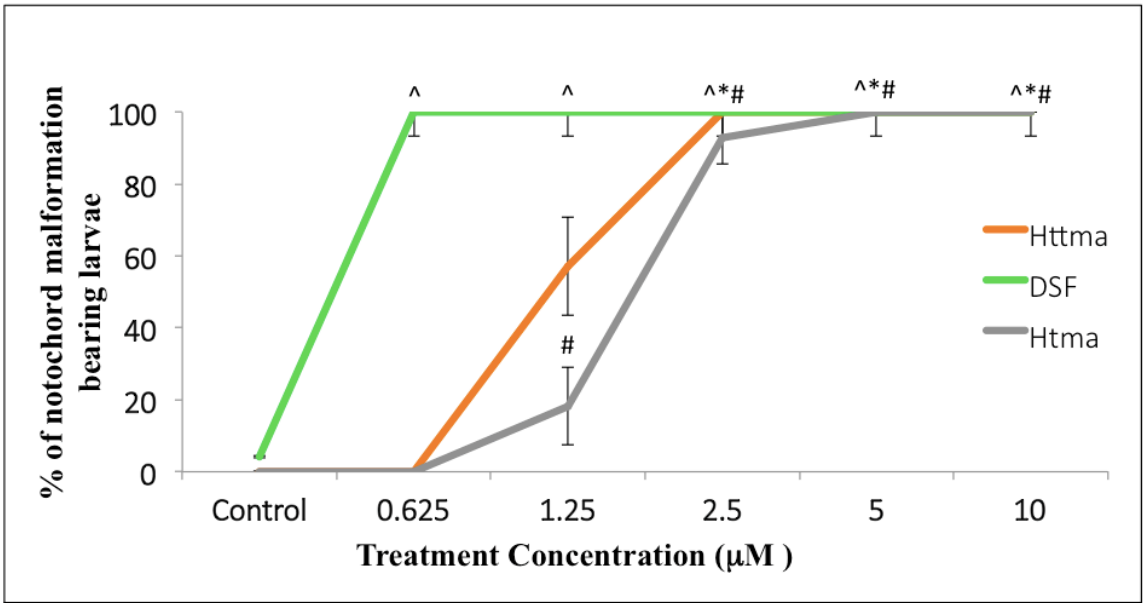
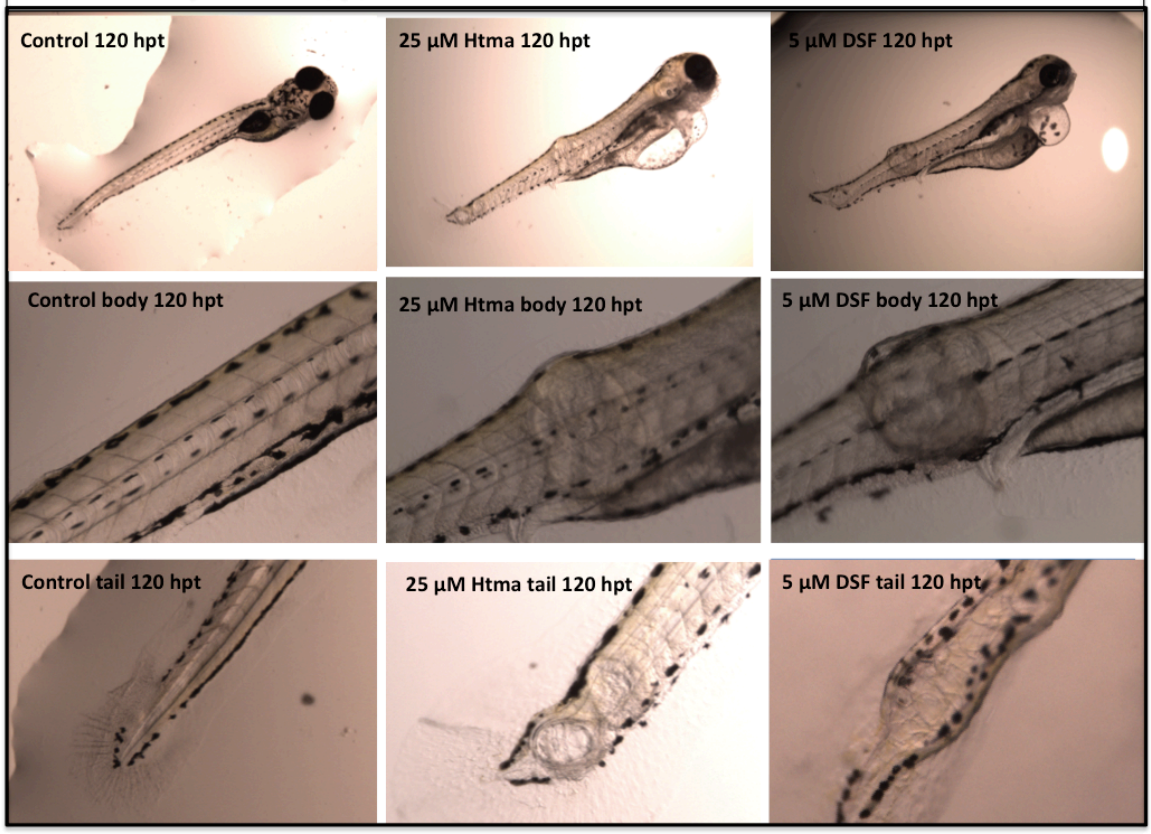


Figure 14: DSF, Htma and Httma induce structural malformations of the notochord in zebrafish during development. Dramatic notochord malformations were observed in 100% of embryos treated with DSF, Htma, and Httma at high concentrations. DSF induced malformations in the sub-micromolar range. $p<0.05$, Fisher's exact test, Significance: Htma #, Httma *, DSF ^ N=16 larvae/treatment group

Figure 15: DSF & Htma induce notochord malformations characteristic of copper deficiency. Lesions are evident on the zebrafish body axis and tail following treatment with Htma or DSF. 0.1% DMSO served as control. Photographs were taken at 120 hours post treatment (hpt).



3.6 Appearance of DSF- and Htma- Induced Notochord Malformations over 120 hour time course

To gain insight into the mechanism by which DSF and Htma induce these notochordal lesions, the onset of appearance of notochord malformations following DSF and Htma exposure was investigated more fully. DSF treatment resulted in notochord deformities in embryonic zebrafish, even in the low micromolar range, by 24 hpt (Figure 16). At this time point, treatment with 1.25 µM DSF resulted in the lowest average incidence of notochord malformations, with 33.33% of embryos in the respective treatment arm presenting with notochordal deformities. By 48 hpt, 100% of the zebrafish

treated at this low concentration were malformed. 5 µM and 10 µM DSF induced notochord malformations in 100% of the embryos by 24 hpt. That low doses of DSF induced such significant structural malformations in a short period supports that DSF exposure likely affects, either directly or indirectly, an important mediator in notochord formation and integrity.

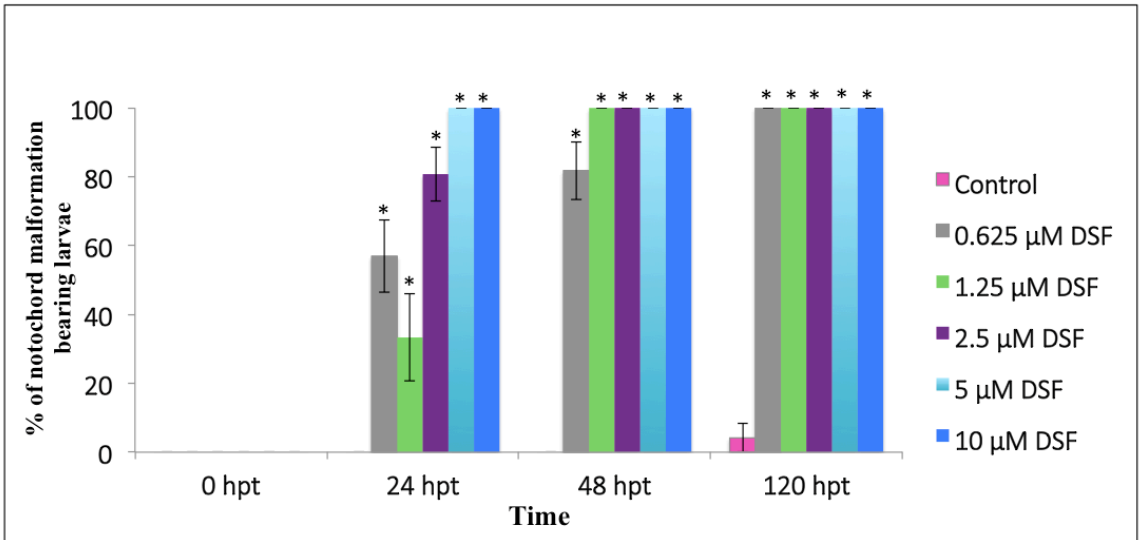


Figure 16: 120 Hour Assessment of DSF-induced notochord malformations in embryonic zebrafish. DSF induces notochord malformations in the low micromolar range in embryonic zebrafish by 24 hpt. 100% of embryos treated with 5 µM DSF reveal deformities of the notochord in the first 24 hours following treatment. $p<0.05$, Fisher's exact test, (*) indicates statistical significance, N=16 larvae/treatment group

Interestingly, treatment with Htma also resulted in embryonic notochord deformities that were almost identical in morphology to those induced by DSF. All embryos exposed to 2.5 µM and higher concentrations of Htma exhibited malformations of the notochord by 24 hpt (Figure 17). 1.25 µM Htma treatment showed significant notochord perturbation by 120 hpt. It is notable that while Htma was slower in causing lethality in developing embryos relative to DSF (Figure 10), 2.5 µM Htma showed a more dramatic impact in inducing malformations of the notochord by 24 hpt compared to the equivalent dose (2.5 µM) of DSF. While the phenotypic similarity of the lesions induced by Htma and DSF support a similar malformation-inducing mechanism for these

compounds, the distinct time points and concentrations at which notochord deformities appeared must be considered.

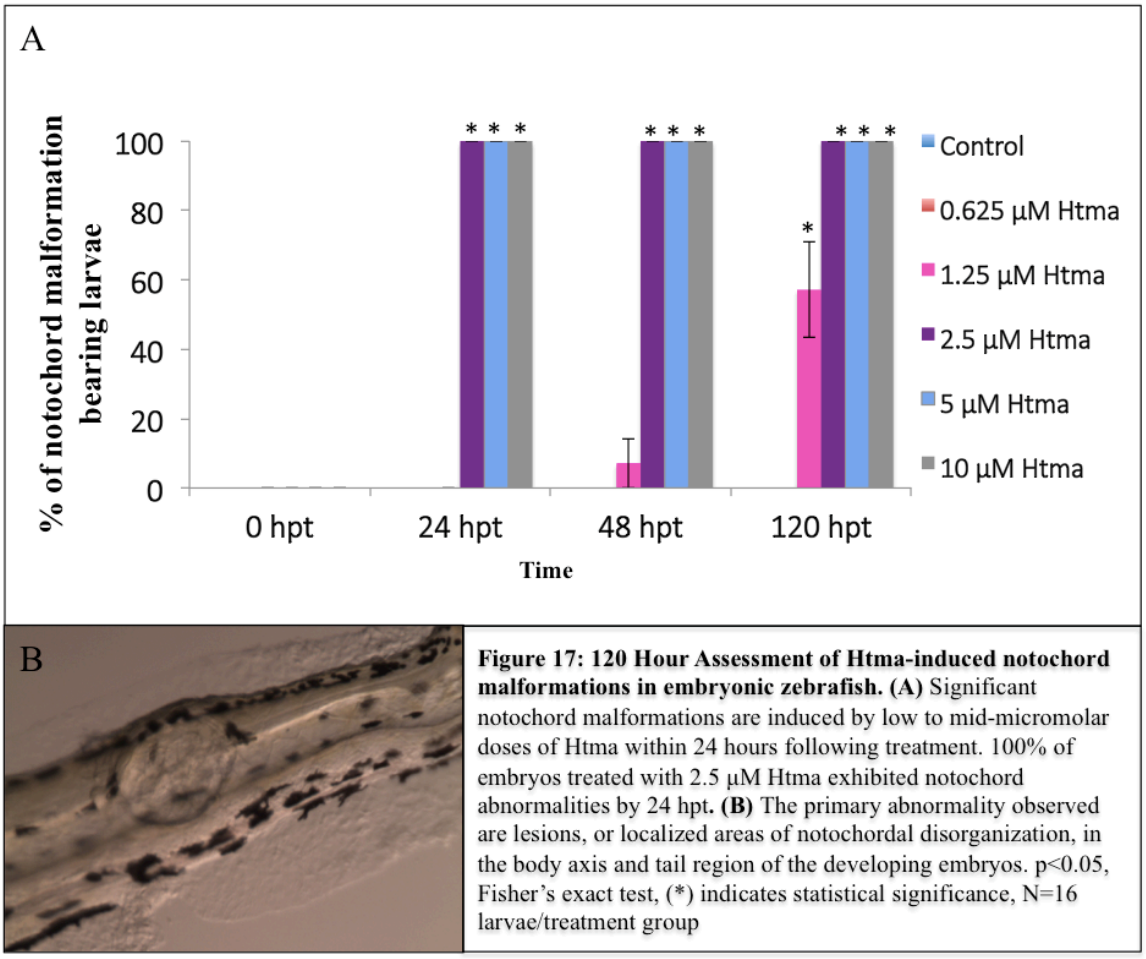


Figure 17: 120 Hour Assessment of Htma-induced notochord malformations in embryonic zebrafish. (A) Significant notochord malformations are induced by low to mid-micromolar doses of Htma within 24 hours following treatment. 100% of embryos treated with 2.5 µM Htma exhibited notochord abnormalities by 24 hpt. **(B)** The primary abnormality observed are lesions, or localized areas of notochordal disorganization, in the body axis and tail region of the developing embryos. $p<0.05$, Fisher's exact test, (*) indicates statistical significance, N=16 larvae/treatment group

3.7 Htma and Copper Sulfate Co-Treatment

Outcomes following co-treatment with a copper chelating agent in combination with copper supplementation have proven distinct from that of the copper chelator alone in a variety of contexts, including treatment of melanoma cells in humans and amyotrophic lateral sclerosis in a murine model [34, 35]. For this reason, the impact of co-incubation of non-lethal concentrations of copper sulfate and Htma on embryos was explored. The results of this experiment varied depending on the concentration of copper

utilized (Figure 18). Co-exposure to either 0.2 or 2.0 µM $CuSO_4$ and 2.5 µM Htma showed a surprising and highly significant potentiative effect, resulting in complete lethality of zebrafish embryos in this treatment arm within 24 hours of exposure. Treatment with 2.5 µM Htma alone induced no increase in mortality over controls. Interestingly, co-supplementation with high dose copper sulfate (20.0 µM) and Htma did not induce a significant increase in the incidence of lethality over controls at any time point assessed. These results were unexpected and prompted a comparison of these outcomes with those following combinatorial treatments of DSF and copper sulfate.

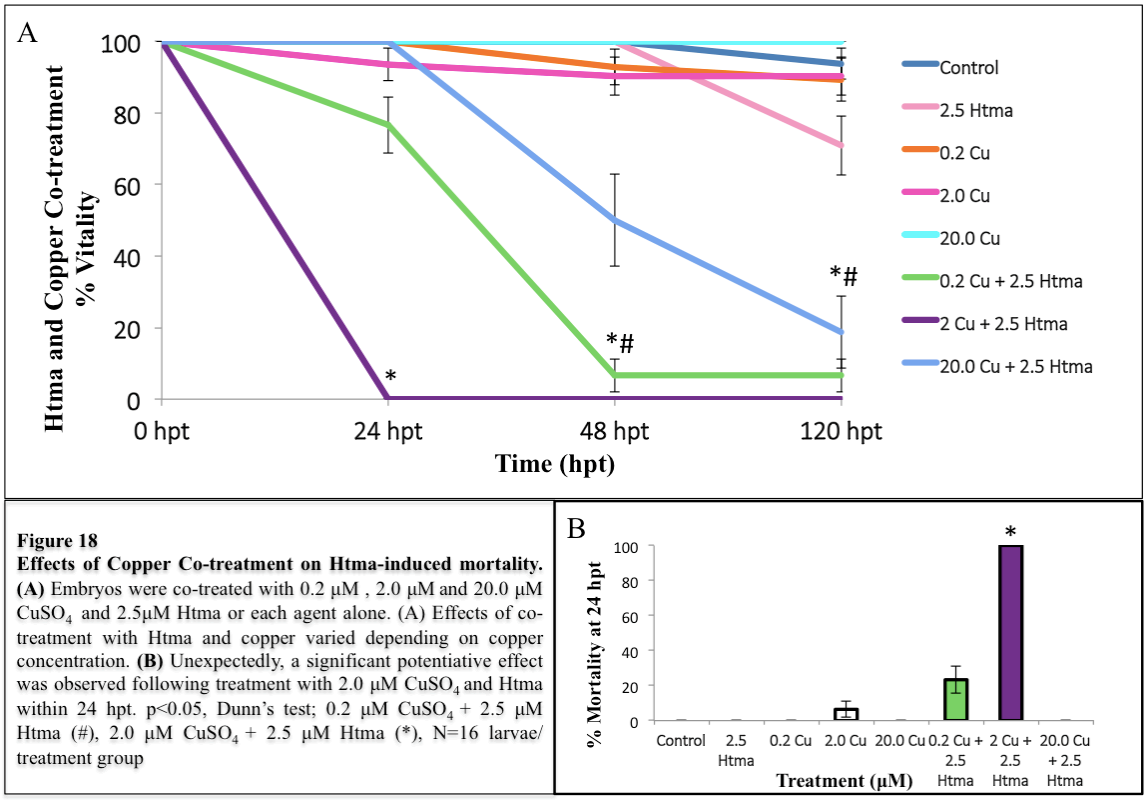


Figure 18
Effects of Copper Co-treatment on Htma-induced mortality. **(A)** Embryos were co-treated with 0.2 µM , 2.0 µM and 20.0 µM $CuSO_4$ and 2.5µM Htma or each agent alone. (A) Effects of co-treatment with Htma and copper varied depending on copper concentration. **(B)** Unexpectedly, a significant potentiative effect was observed following treatment with 2.0 µM $CuSO_4$ and Htma within 24 hpt. $p<0.05$, Dunn's test; 0.2 µM $CuSO_4$ + 2.5 µM Htma (#), 2.0 µM $CuSO_4$ + 2.5 µM Htma (*), N=16 larvae/ treatment group

3.8 DSF and Copper Sulfate Co-Treatment

Interestingly, exposure to DSF co-incubated with $CuSO_4$ produced more subtle results than that of Htma and $CuSO_4$ in combination (Figure 19). Mortality was first seen at 48 hpt, only following treatment with 2.5 µM DSF in combination with the highest dose of $CuSO_4$ (20.0 µM). Lethality induced by co-treatment with mid-dose copper sulfate (2.0 µM $CuSO_4$) and DSF was not observed until 120 hpt. Analysis of mortality at 120 hpt revealed that copper supplementation with DSF caused some potentiation of DSF-induced toxicity, however this effect was far less potent and less accelerated than that of Htma in combination with copper. Further, the mortality induced by DSF and copper sulfate combinatorial solutions in this study was not great enough to be statistically significant.

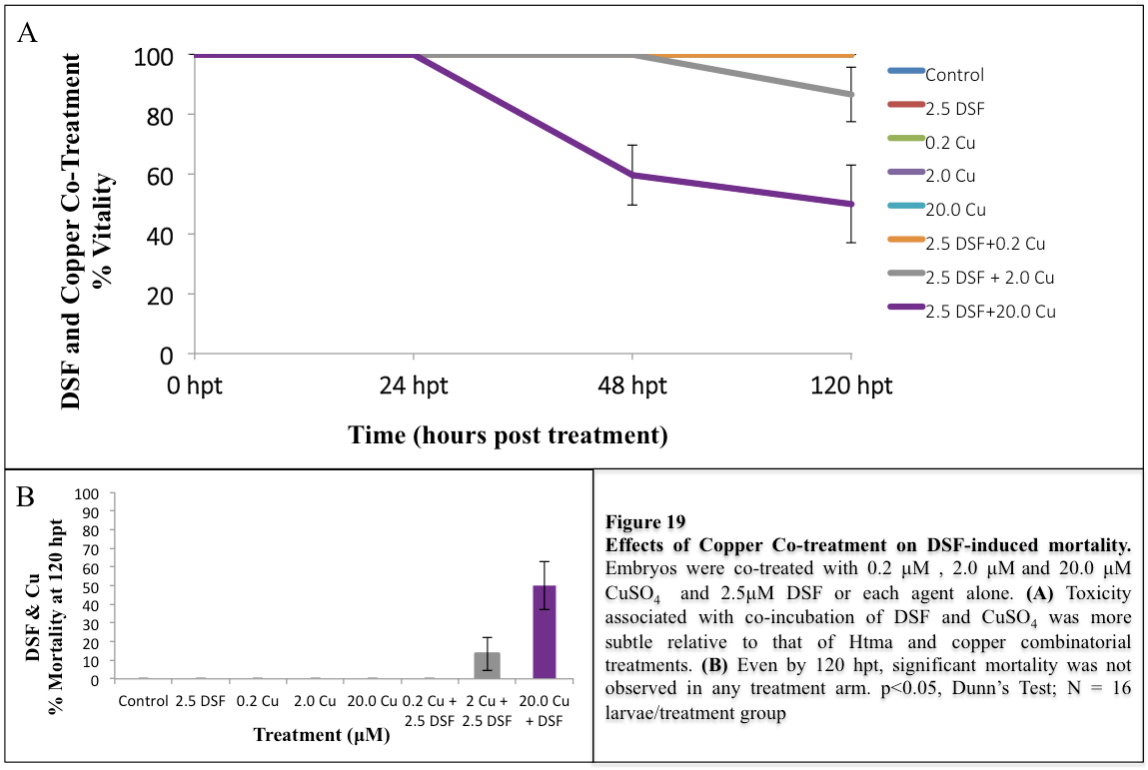


Figure 19
Effects of Copper Co-treatment on DSF-induced mortality. Embryos were co-treated with 0.2 µM , 2.0 µM and 20.0 µM $CuSO_4$ and 2.5µM DSF or each agent alone. **(A)** Toxicity associated with co-incubation of DSF and $CuSO_4$ was more subtle relative to that of Htma and copper combinatorial treatments. **(B)** Even by 120 hpt, significant mortality was not observed in any treatment arm. $p<0.05$, Dunn's Test; N = 16 larvae/treatment group

3.9 DSF and Copper Supplementation Post-Treatment

In response to the unexpected results of the Htma and copper co-treatment studies and because Htma or DSF in complex with copper is highly hydrophobic, an experiment was designed to examine the role of solubility in the observed outcomes. The possibility exists that high concentrations of copper in solution with Htma or DSF limits the bioavailability of either the chelating agent or the chelator-Cu complex. Embryos were first incubated with a high dose of the copper chelating agent (Htma or DSF), alone, with the aim of allowing sufficient time for the agents to enter cells before introduction of $CuSO_4$ could interfere with absorption. 15 minutes following this treatment, $CuSO_4$ was added to the microtiter wells of the treated embryos and lethality was assessed at 24 and 48 hpt. The effects of 20.0 μM $CuSO_4$ addition with high dose 25 μM DSF revealed a protective role for copper (Figure 20). At 24 hpt, 25 μM DSF induced mortality in 93% of the embryos in the respective treatment arm. However, copper sulfate prevented DSF-induced lethality at 24 hpt. This copper-induced "rescue" was transient though as 100% lethality was observed in the DSF and post-treatment copper supplemented embryos by 48 hpt. Whether copper addition temporarily ameliorates effects of copper deficiency within the cell or whether it complexes with DSF and precipitates in the fish water, transiently preventing DSF from cellular entry, is not clear. However, that DSF-induced toxicity was observed in both treatment arms indicates that DSF did eventually gain entry to cells. Future studies should allow DSF a longer incubation period prior to copper supplementation to aid in further elucidating this mechanism.

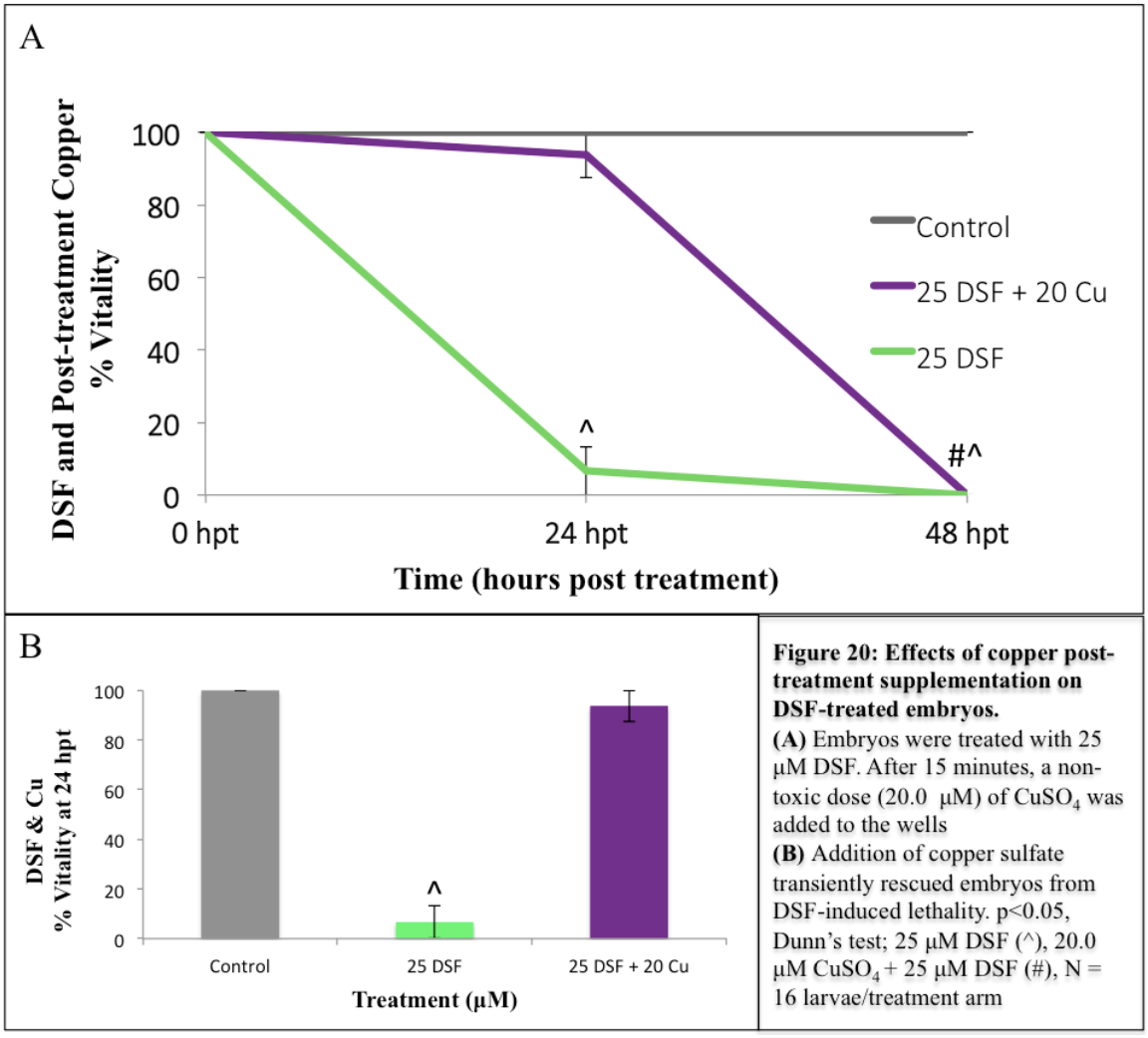


Figure 20: Effects of copper post-treatment supplementation on DSF-treated embryos.
(A) Embryos were treated with 25 µM DSF. After 15 minutes, a non-toxic dose (20.0 µM) of $CuSO_4$ was added to the wells
(B) Addition of copper sulfate transiently rescued embryos from DSF-induced lethality. $p<0.05$, Dunn's test; 25 µM DSF (^), 20.0 µM $CuSO_4$ + 25 µM DSF (#), N = 16 larvae/treatment arm

3.10 Htma and Copper Supplementation Post-Treatment

Post-treatment $CuSO_4$ addition to 25 µM Htma-treated embryos revealed a distinct pattern from that of DSF and copper post-treatment supplementation. 25 µM Htma is not lethal to developing embryos by 48 hpt, in accordance with the initial dose-response studies (Figure 9). Addition of 20.0 µM $CuSO_4$ 15 minutes following treatment resulted in significant and nearly complete (93%) mortality by 24 hpt, mimicking the potentiation effect seen in the Htma and copper co-treatment studies (Figure 21). 100% lethality was observed by 48 hpt in this treatment group.

These results shed light on the solubility question presented earlier. The impact of solubility on the observed outcomes was first proposed when co-exposure to high dose copper sulfate (20.0 μM $CuSO_4$) and 2.5 μM Htma revealed a "protective effect" at 24 hpt (Figure 18). It was thought that such a high dose of copper might facilitate precipitation of Htma. In the copper post-treatment studies described here however, 20.0 μM $CuSO_4$ addition following treatment with a ten fold higher dose of Htma (25 μM Htma) resulted in a highly significant potentiation effect, inducing nearly complete mortality by 24 hpt. It seems that if solubility were truly the issue, the relatively higher concentrations of ions in this copper supplementation post-treatment experiment would facilitate precipitation of the Htma-copper complex. However, this was not the case, leaving room for proposal of alternative hypotheses regarding the mechanism of Htma-induced toxicity.

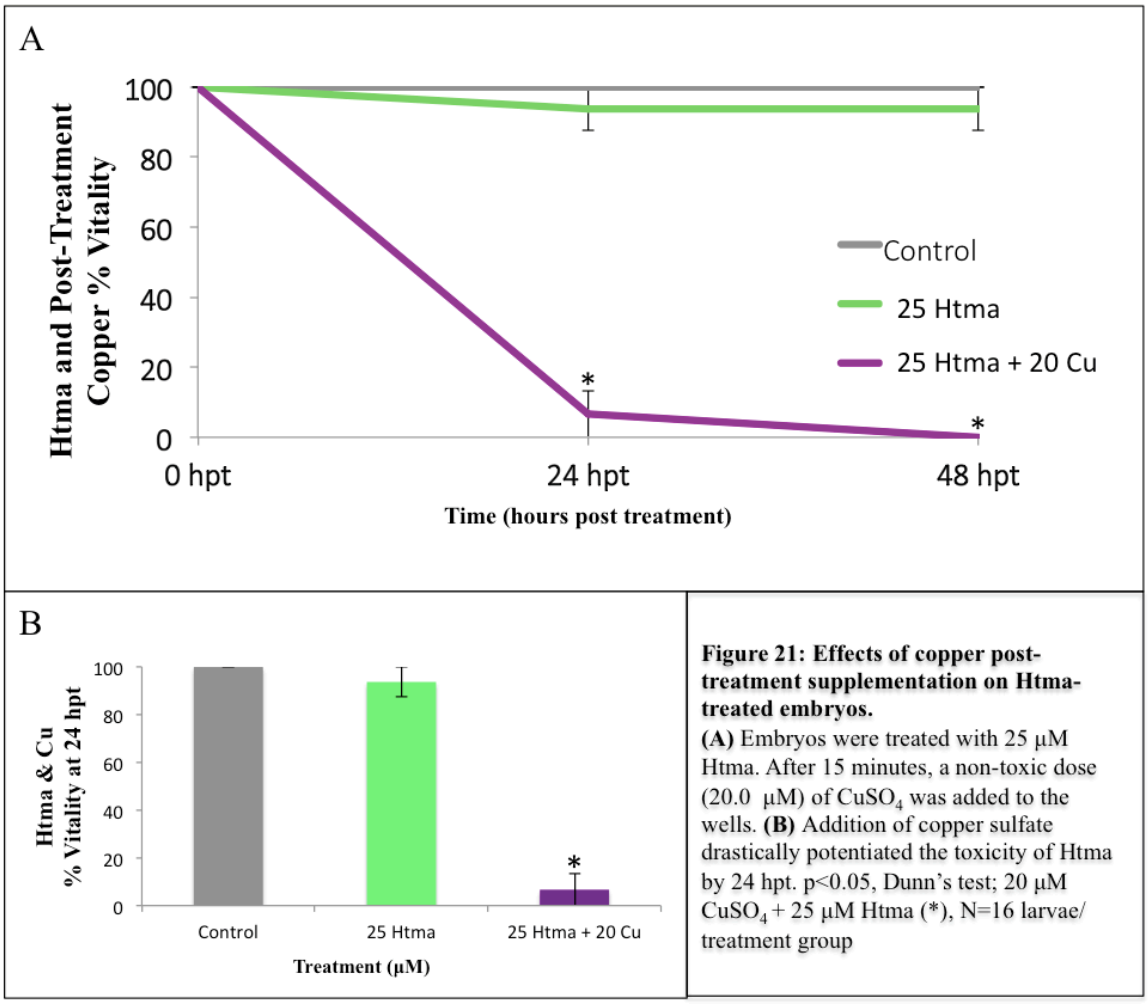


Figure 21: Effects of copper post-treatment supplementation on Htma-treated embryos.
(A) Embryos were treated with 25 µM Htma. After 15 minutes, a non-toxic dose (20.0 µM) of $CuSO_4$ was added to the wells. **(B)** Addition of copper sulfate drastically potentiated the toxicity of Htma by 24 hpt. $p<0.05$, Dunn's test; 20 µM $CuSO_4$ + 25 µM Htma (*), N=16 larvae/ treatment group

CHAPTER FOUR

Discussion

In the current study, the embryonic zebrafish model was utilized to explore the effects of the copper-chelating agents DSF, maltol and maltol derivatives, Htma and Httma, *in vivo*. As anticipated, DSF proved the most acutely toxic of the compounds assessed, inducing both malformations and mortality in the low micromolar range (Figure 9A). Htma and Httma also proved toxic, but at higher treatment concentrations. Further, the toxicity of the maltol derivatives was observed at later time points than that of DSF (Figures 4A, 10A&B). The toxicity induced by these copper chelating agents is supported by the embryonic yolk sac, which contains an internal reservoir of copper [45]. The increased potency of DSF may be due to its dual role in both copper chelation and inhibition of the enzyme aldehyde dehydrogenase, an essential mediator in the retinoic acid pathway. The absence of malformations or mortality following treatment with maltol supports the role of the sulfur containing moieties of Htma and Httma in their induced toxicity.

The malformations observed following treatment with DSF, Htma and Httma include pericardial edema (PE), lack of pigmentation and deformities of the notochord. PE is a common endpoint of various mechanisms that alter cardiac output. Diverse stressors and mutants that significantly disrupt cardiac output have been shown to generate a singular phenotype of PE and eventual heart failure [55]. Therefore the pericardial edema induced by DSF and the maltol derivatives at mid-range micromolar

doses here is a strong indicator of toxicity, although it does not give direct insight to the mechanism of toxicity (Figure 13).

Previous chemical and genetic modification studies have shown that notochord defects in zebrafish embryos represent a sensitive model for disrupted copper homeostasis with notochord defects as the primary phenotype [56]. The effects of copper deficiency in the developing zebrafish notochord have been well-characterized. In 2006, Tilton et al. described a common "wavy" or undulating notochord phenotype induced by a variety of dithiocarbamate (DTC) compounds including DSF [56]. DTC-induced notochord malformations were shown in a later study to first appear at 18 hpf, the time at which muscular contractions in the body axis commence during development [57].

Gasner et al. demonstrated an essential role for the cuproenzyme lysyl oxidase in establishing the integrity of the notochord sheath, reporting that the effects of the lysyl oxidase inhibitor β-aminopropionitrile in embryos mimicked the effects of notochord perturbation resulting from DTC-induced copper deficiency. They also showed that partial knockdown of two lox genes, loxl1 and loxl5, and the lysyl oxidase substrate col2a sensitized embryos to copper deficient states [32]. In 2010, van Boxtel et al. reported that DTCs directly inhibit the activity of lysyl oxidase. Although the mechanism has not been definitively elucidated, it is proposed that DTCs may inhibit catalysis via binding copper within the enzyme or that lysyl oxidase activity is highly sensitive to intracellular copper concentrations, thus exhibiting altered activity in the presence of chelators [58].

The notochord malformations induced by DSF and Htma in the present study did not reveal an obvious wavy notochord phenotype (Figure 15). However, undulation in

the tail region was recognized in a small fraction of exposed embryos (Figure 11) [59]. The primary abnormalities observed include impressive lesions in the middle and posterior embryo body axis as well as in the vicinity of the tail and caudal fin. Under a dissecting microscope, these lesions appeared as swellings or proliferations of notochordal components, with expansion limited by the notochord sheath. A publication by Haendel et al. on treatment with the DTC sodium metam informed these observations. In Haendel's study, exposure of embryos to sodium metam from 4-14 hpf resulted in undulating notochords in greater than 85% of the treated embryos. None of the zebrafish exposed to sodium metam in the time frame 14-24 hpf developed a wavy notochord. However, these embryos showed a phenotype identical to the DSF and Htma-treated fish in our study, characterized by localized expansions of the notochord on varied regions of the body axis and tail [60]. A meticulous analysis of the malformations observed in the present study supported the sensitivity of the notochord to treatment at specific time points during differentiation and the varied phenotypes associated with these exposures. Overall, the malformations induced by DSF and the maltol derivatives support copper chelation as the underlying mechanism of the observed notochord phenotype and that the toxicity generated is copper-specific.

In addition to PE and notochord deformities, lack of pigmentation was observed in embryos following treatment with Htma (Figure 11). Transcription of the tyrosinase gene first occurs at 16.5 hpf in the retinal pigment epithelium and pigmentation in the eye may be observed as early as ~24 hpf. Following this, activation of tyrosinase expression and subsequent pigmentation is visualized progressively down the zebrafish body axis toward the caudal region [53]. While the results here show that pigmentation is indeed

reduced following treatment with Htma, this concentration-dependent pigment reduction correlates with the presence of PE and notochord abnormalities. Given that exposures were performed at 24 hpf, whether Htma is specifically targeting tyrosinase and/or is inducing toxicity that affects other aspects of melanogenesis cannot be ascertained. Experiments performed at a later time point, following completion of melanogenesis, may shed new light on this mechanism.

Given that in many contexts the treatment efficacy of copper and DSF in combination is greater than that of treatment with either compound alone, including treatment of melanoma, breast cancer, and potentially Menkes disease, copper supplementation with Htma and DSF was examined in the current study. The co-treatment copper supplementation studies were designed to confirm the effectiveness of Htma or DSF in facilitating copper cellular entry and intracellular copper accumulation that would directly correlate with the concentration of copper sulfate co-incubated with it prior to treatment. Given the copper deficient phenotypes associated with Htma and DSF, it was thought that addition of copper sulfate should aid in overcoming the effects of copper deficiency resulting from intracellular copper chelation by Htma or DSF. However it was also believed that high intracellular levels of copper, induced by Htma and DSF-facilitated copper cell entry, may induce embryonic lethality. While co-treatment with $CuSO_4$ did cause effects distinct from treatment with Htma alone, outcomes varied inconsistently with the supplemented $CuSO_4$ concentration. The magnitude and accelerated effect of intermediate-dose 2.0 μM $CuSO_4$ addition, which caused complete lethality in the zebrafish by 24 hpt, was startling (Figure 18). Given this effect, the similar but less dramatic potentiation of low-dose 0.2 μM $CuSO_4$ with 2.5 μM

Htma was reasonably expected. However, the "protection" induced by high-dose 20.0 μM $CuSO_4$ and Htma requires an explanation, especially as DSF showed a significantly less potent and delayed potentiation effect, with 20.0 μM $CuSO_4$ and DSF co-treatment causing the greatest toxicity (Figure 19). This "protective" effect of 20.0 μM $CuSO_4$ and Htma could be related to solubility, as the Htma complex is highly hydrophobic. Addition of high concentrations of ions to solution may cause Htma to precipitate, manifesting phenotypically as a decrease in potency.

However, an alternate hypothesis to explain this phenomenon from a biological standpoint may also be proposed. This effect could result from Htma specifically targeting a dynamic mediator of copper homeostasis. For example, the P-type ATPase copper transporter ATP7A is localized primarily in the Golgi, where it supplies copper to cuproenzymes of the secretory pathway, but a small fraction of the transporter is also localized in the plasma membrane to facilitate copper export. In situations of high intracellular copper, the equilibrium of ATP7A between the Golgi and the plasma membrane shifts, and a large fraction of ATP7A localizes in the plasma membrane to expand the available copper cellular exit routes. An increase in ATP7A activity is also associated with high intracellular copper concentrations [19].

Htma inhibition of ATP7A could help to explain the unexpected outcomes of co-incubating varied concentrations of copper sulfate with Htma prior to exposure. The complete mortality induced by low dose (0.2 μM) $CuSO_4$ by 48 hpt could be the result of Htma binding ATP7A and blocking copper efflux from the cell. ROS generation from increased intracellular copper levels and defective functioning of cuproenzymes in the secretory pathway due to ATP7A inhibition would contribute to the observed mortality at

48 hpt. The significant lethality observed in embryos within 24 hours of exposure to Htma and intermediate dose (2.0 μM) $CuSO_4$ can be similarly explained by direct inhibition of ATP7A by Htma. Higher concentrations of supplemented copper in this treatment arm would lead to greater intracellular accumulation of copper, explaining why mortality was observed at an earlier time point here. Inhibition of ATP7A by Htma can also clarify the protective effect of higher concentrations of copper (20.0 μM $CuSO_4$) with 2.5 μM Htma. 20.0 μM $CuSO_4$ would increase the activity and localization of ATP7A to the plasma membrane. This high concentration of copper relative to Htma could make interactions between Htma and the target less likely, manifesting as a "protective effect". The significance of the unexpected results of copper and Htma co-treatment is reiterated upon examination of DSF and copper co-supplementation, where significant mortality was not observed at any time point. Only a subtle potentiation effect was observed at 48 hpt, with 2.5 μM DSF and the highest dose of copper sulfate (20.0 μM $CuSO_4$) showing an expected greater toxicity relative to DSF co-incubated with an intermediate dose of copper (2.0 μM $CuSO_4$).

The interesting results of the copper co-treatment experiments prompted a further examination of whether the observed outcomes might actually result from reduced solubility of Htma or DSF in the presence of copper. To address this question, DSF or Htma-treated embryos were given exogenous copper 15 minutes following treatment with 25 μM $CuSO_4$, with the aim of giving Htma or DSF sufficient opportunity to enter cells and induce toxicity before addition of copper. As expected from the prior dose-response studies, 25 μM Htma did not induce significant lethality by 48 hpt (Figure 21). However, the dramatic potentiation effect of Htma and copper was again observed in this

experiment, despite the higher concentration of Htma and the delayed addition of copper sulfate. At 24 hpt, treatment with high dose Htma and high dose copper resulted in complete lethality in the treatment arm, supporting the hypothesis that the potentiation observed may be more than a simple solubility issue. Interestingly, the complete mortality induced by 25 μM DSF by 24 hpt was transiently rescued by addition of high dose copper sulfate (Figure 20).

That manifestations of copper deficiency caused by DSF may be temporarily overcome in the presence of copper supports that interactions between DSF and cuproenzymes are likely mediated by the copper ion rather than by direct DSF inhibition. The results here support that while DSF induces effects via a copper chelation-dependent mechanism, an alternative target, such as interaction with a copper homeostatic mediator, may exist for Htma. However, despite these proposed distinct mechanisms, equivalent malformations are observed in the notochord following treatment with these compounds.

As described above, notochord abnormalities result from dysfunctional lysyl oxidase and phenotypic manifestations of copper deficiency vary with the time point at which embryos are exposed to copper deficient states during development [60]. The effectiveness of copper addition in the reversal of notochord malformations and other copper deficiency-induced abnormalities will vary depending on factors such as the age of the developing embryo, the mechanism by which copper deficiency is induced and its severity, and the time point(s) at which exogenous copper is given. Although the ameliorating effect observed in this study with DSF is transient, treatment at any early enough time point so as to prevent significant defects during embryogenesis as well as

renewal exposures of copper may be able to more completely reverse DSF-induced malformations.

Htma-exposed embryos showed equivalent manifestations of copper deficiency in the notochord despite exhibiting distinct interactions with supplemented copper. If Htma inhibits ATP7A, copper loading into the secretory enzyme lysyl oxidase is blocked, resulting in the same abnormal notochord phenotype as that induced by DSF copper chelation. However, exogenous copper addition would not reverse or ameliorate notochordal effects in Htma-exposed embryos, as dysfunctional ATP7A could not transport copper into the Golgi despite high levels of intracellular copper.

However, other factors may be influencing the observed outcomes in this study, and future studies should further examine the role of exogenous copper addition in reversing copper deficient phenotypes in embryonic zebrafish to shed light on the mechanism of DSF and Htma-induced toxicity. Because inclusion of copper sulfate in the treatment media of Htma- exposed embryos may block uptake of Htma or vise versa, pre-incubation with Htma or DSF should be increased by several hours. Embryos should be washed prior to exogenous copper sulfate addition to eliminate the possibility of chelation by any Htma or DSF that remains in the fish water. Experiments where copper sulfate and DSF or Htma exposures are performed in reverse order should also be conducted. Embryonic exposure to copper sulfate several hours preceding addition of Htma or DSF will help to confirm whether the chelating compounds are truly facilitating copper cellular entry or potentially blocking copper efflux.

Future studies will also examine the role of copper transport in Htma- vs. DSF – induced effects on zebrafish development and viability by measuring the intracellular

accumulation of copper following treatment with Htma and DSF. Copper accumulation within zebrafish lysates collected at 120 hpt must be characterized by Inductively Coupled plasma-mass spectrometry to determine the relative copper whole-body concentrations of control and treated embryos. Copper measurement of the lysates will confirm that copper uptake has occurred and that Htma and DSF enhanced this uptake. Further, examination of the subcellular distribution of copper following treatment with Htma or DSF will help to direct which copper transporters may be involved in the observed effects and which proteins should be specifically targeted, such as ATP7A, for assessment of copper transporter knockdown on Htma- or DSF- induced toxicity.

In conclusion, DSF and the maltol derivatives proved to be potent copper chelators in embryonic zebrafish, inducing toxicity and malformations characteristic of copper deficiency in exposed embryos. As anticipated, copper supplementation influenced the potency of these compounds. However, the dramatic response of Htma to copper supplementation suggests a distinct mechanism for Htma in the copper homeostatic pathway beyond copper chelation. Future studies should address the potential intracellular targets of Htma versus DSF.

REFERENCES

1. Health http://copperalliance.org.uk/copper-and-society/health (accessed Mar 13, 2016).

2. Bertinato, J.; L'Abbé, M. R. Maintaining Copper Homeostasis: Regulation of Copper-Trafficking Proteins in Response to Copper Deficiency or Overload. *J. Nutr. Biochem.***2004**, *15* (6), 316–322.

3. Toxicological Profile for Copper: Relevance to Public Health http://www.lenntech.com/recommended-daily-intake.htm (accessed Mar 13, 2016).

4. Griffith, D. P.; Liff,D.; Ziegler, T. R.; Esper, G. J.; Winton, E. F. Acquired Copper Deficiency: A Potentially Serious and Preventable Complication Following Gastric Bypass Surgery. *Obesity (Silver Spring)* **2009**, *17* (4), 827–831.

5. O'Dell, B. L.; Sunde, R. A. *Handbook of Nutritionally Essential Mineral Elements*; CRC Press, 1997.

6. (Keen, C. L.; Uriu-Hare, J. Y.; Hawk, S. N.; Jankowski, M. A.; Daston, G. P.; Kwik-Uribe, C. L.; Rucker, R. B. Effect of Copper Deficiency on Prenatal Development and Pregnancy Outcome. *Am J Clin Nutr* **1998**, *67* (5), 1003S – 1011S.

7. Tsigelny, I. F.; Sharikov,Y.; Greenberg, J. P.; Miller, M. A.; Kouznetsova, V. L.; Larson, C. A.; Howell, S. B. An All-Atom Model of the Structure of Human Copper Transporter 1. *Cell Biochem Biophys* **2012**, *63* (3), 223–234.

8. Kim, H.; Wu, X.; Lee, J. SLC31 (CTR) Family of Copper Transporters in Health and Disease. *Mol Aspects Med* **2013**, *34* (2-3), 561–570.

9. Kuo, Y.-M.; Zhou, B.; Cosco, D.; Gitschier, J. The Copper Transporter CTR1 Provides an Essential Function in Mammalian Embryonic Development. *PNAS* **2001**, *98* (12), 6836–6841.

10. Mackenzie, N. C.; Brito, M.; Reyes, A. E.; Allende, M. L. Cloning, Expression Pattern and Essentiality of the High-Affinity Copper Transporter 1 (ctr1) Gene in Zebrafish. *Gene* **2004**, *328*, 113–120.

11. Zhao, L.; Xia, Z.; Wang, F. Zebrafish in the Sea of Mineral (iron, Zinc, and Copper) Metabolism. *Front. Pharmacol* **2014**, *5*, 33.

12. Hilton, J. B.; White, A. R.; Crouch, P. J. Metal-Deficient SOD1 in Amyotrophic Lateral Sclerosis. *J Mol Med (Berl)* **2015**, *93* (5), 481–487.

13. Superoxide Dismutase: Boosting the Body's Primary Antioxidant Defense - Life Extension http://www.lifeextension.com/magazine/2006/6/report_sod/page-01 (accessed Feb 9, 2016).

14. Zelko, I. N.; Mariani, T. J.; Folz, R. J. Superoxide Dismutase Multigene Family: A Comparison of the CuZn-SOD (SOD1), Mn-SOD (SOD2), and EC-SOD (SOD3) Gene Structures, Evolution, and Expression. *Free Radical Biology and Medicine* **2002**, *33* (3), 337–349.

15. Furukawa, y.; O'halloran, T. V. Posttranslational Modifications in Cu,Zn-Superoxide Dismutase and Mutations Associated with Amyotrophic Lateral Sclerosis. *Antioxid Redox Signal* **2006**, *8* (5-6), 847–867.

16. Rossi, L.; Lippe, G.; Marchese, E.; Martino, A. D.; Mavelli, I.; Rotilio, G.; Ciriolo, M. R. Decrease of Cytochrome c Oxidase Protein in Heart Mitochondria of Copper-Deficient Rats. *Biometals* **1998**, *11* (3), 207–212.

17. Collins, J. F.; Prohaska, J. R.; Knutson, M. D. Metabolic Crossroads of Iron and Copper. *Nutr Rev* **2010**, *68* (3), 133–147.

18. Kodama, H.; Fujisawa, C.; Bhadhprasit, W. Inherited Copper Transport Disorders: Biochemical Mechanisms, Diagnosis, and Treatment. *Curr. Drug Metab.* **2012**, *13* (3), 237–250.

19. Telianidis, J.; Hung, Y. H.; Materia, S.; Fontaine, S. L. Role of the P-Type ATPases, ATP7A and ATP7B in Brain Copper Homeostasis. *Front Aging Neurosci* **2013**, *5*.

20. Petris, M. J.; Strausak, D.; Mercer, J. F. B. The Menkes Copper Transporter Is Required for the Activation of Tyrosinase. *Hum. Mol. Genet.* **2000**, *9* (19), 2845–2851.

21. Setty, S. R. G.; Tenza, D.; Sviderskaya, E. V.; Bennett, D. C.; Raposo, G.; Marks, M. S. Cell-Specific ATP7A Transport Sustains Copper-Dependent Tyrosinase Activity in Melanosomes. *Nature* **2008**, *454* (7208), 1142–1146.

22. Meskini, R. E.; Culotta, V. C.; Mains, R. E.; Eipper, B. A. Supplying Copper to the Cuproenzyme Peptidylglycine A-Amidating Monooxygenase. *J. Biol. Chem.* **2003**, *278* (14), 12278–12284.

23. Kaler, S. G.; Holmes, C. S. Catecholamine Metabolites Affected by the Copper-Dependent Enzyme Dopamine-Beta-Hydroxylase Provide Sensitive Biomarkers for Early Diagnosis of Menkes Disease and Viral-Mediated ATP7A Gene Therapy. *Adv. Pharmacol.* **2013**, *68*, 223–233.

24. Holloway, Z. G.; Velayos-Baeza, A.; Howell, G. J.; Levecque, C.; Ponnambalam, S.; Sztul, E.; Monaco, A. P. Trafficking of the Menkes Copper Transporter ATP7A Is Regulated by Clathrin-, AP-2–, AP-1–, and Rab22-Dependent Steps. *Mol. Biol. Cell* **2013**, *24* (11), 1735–1748.

25. Petris, M. J.; Mercer, J. F.; Culvenor, J. G.; Lockhart, P.; Gleeson, P. A.; Camakaris, J. Ligand-Regulated Transport of the Menkes Copper P-Type ATPase Efflux Pump from the Golgi Apparatus to the Plasma Membrane: A Novel Mechanism of Regulated Trafficking. *EMBO J.* **1996**, *15* (22), 6084–6095.

26. Voskoboinik, I.; Strausak, D.; Greenough, M.; Brooks, H.; Petris, M.; Smith, S.; Mercer, J. F.; Camakaris, J. Functional Analysis of the N-Terminal CXXC Metal-Binding Motifs in the Human Menkes Copper-Transporting P-Type ATPase Expressed in Cultured Mammalian Cells. *J. Biol. Chem.* **1999**, *274* (31), 22008–22012.

27. Petris, M. J.; Voskoboinik, I.; Cater, M.; Smith, K.; Kim, B.-E.; Llanos, R. M.; Strausak, D.; Camakaris, J.; Mercer, J. F. B. Copper-Regulated Trafficking of the Menkes Disease Copper ATPase Is Associated with Formation of a Phosphorylated Catalytic Intermediate. *J. Biol. Chem.* **2002**, *277* (48), 46736–46742.

28. Matsui, M. S.; Petris, M. J.; Niki, Y.; Karaman-Jurukovska, N.; Muizzuddin, N.; Ichihashi, M.; Yarosh, D. B. Omeprazole, a Gastric Proton Pump Inhibitor, Inhibits Melanogenesis by Blocking ATP7A Trafficking. *Journal of Investigative Dermatology* **2015**, *135* (3), 834–841.

29. Kase, B. A.; Northrup, H.; Morrison, A. C.; Davidson, C. M.; Goiffon, A. M.; Fletcher, J. M.; Ostermaier, K. K.; Tyerman, G. H.; Au, K. S. Association of Copper-Zinc Superoxide Dismutase (SOD1) and Manganese Superoxide Dismutase (SOD2) Genes with Non-Syndromic Myelomeningocele. *Birth Defects Res A Clin Mol Teratol* **2012**, *94* (10), 762–769.

30. Zhang, J.; Yang, R.; Liu, Z.; Hou, C.; Zong, W.; Zhang, A.; Sun, X.; Gao, J. Loss of Lysyl Oxidase-like 3 Causes Cleft Palate and Spinal Deformity in Mice. *Hum Mol Genet* **2015**, *24* (21), 6174–6185.

31. Stemple, D. L. Structure and Function of the Notochord: An Essential Organ for Chordate Development. *Development* **2005**, *132* (11), 2503–2512.

32. Gansner, J. M.; Mendelsohn, B. A.; Hultman, K. A.; Johnson, S. L.; Gitlin, J. D. Essential Role of Lysyl Oxidases in Notochord Development. *Dev Biol* **2007**, *307* (2), 202–213.

33. Gupte, A.; Mumper, R. J. Elevated Copper and Oxidative Stress in Cancer Cells as a Target for Cancer Treatment. *Cancer Treat. Rev.* **2009**, *35* (1), 32–46.

34. Cen, D.; Brayton, D.; Shahandeh, B.; Meyskens, F. L.; Farmer, P. J. Disulfiram Facilitates Intracellular Cu Uptake and Induces Apoptosis in Human Melanoma Cells. *J. Med. Chem.* **2004**, *47* (27), 6914–6920.

35. Williams, J. R.; Trias, E.; Beilby, P. R.; Lopez, N. I.; Labut, E. M.; Samuel Bradford, C.; Roberts, B. R.; McAllum, E. J.; Crouch, P. J.; Rhoads, T. W.; et al. Copper Delivery to the CNS by CuATSM Effectively Treats Motor Neuron Disease in SODG93A Mice Co-Expressing the Copper-Chaperone-for-SOD. *Neurobiology of Disease*.

36. Tümer, Z.; Møller, L. B. Menkes Disease. *Eur J Hum Genet* **2010**, *18* (5), 511–518.

37. Mendelsohn, B. A.; Yin, C.; Johnson, S. L.; Wilm, T. P.; Solnica-Krezel, L.; Gitlin, J. D. Atp7a Determines a Hierarchy of Copper Metabolism Essential for Notochord Development. *Cell Metab.* **2006**, *4* (2), 155–162.

38. Jayakumar, S.; Micallef-Eynaud, P. D.; Lyon, T. D. B.; Cramb, R.; Jilaihawi, A. N.; Prakash, D. Acquired Copper Deficiency Following Prolonged Jejunostomy Feeds. *Ann. Clin. Biochem.* **2005**, *42* (Pt 3), 227–231.

39. Griffith, D. P.; Liff, D.; Ziegler, T. R.; Esper, G. J.; Winton, E. F. Acquired Copper Deficiency: A Potentially Serious and Preventable Complication Following Gastric Bypass Surgery. *Obesity (Silver Spring)* **2009**, *17* (4), 827–831.

40. Helsel, M. E.; Franz, K. J. Pharmacological Activity of Metal Binding Agents That Alter Copper Bioavailability. *Dalton Trans.* **2015**, *44* (19), 8760–8770.

41. Somwar, R.; Erdjument-Bromage, H.; Larsson, E.; Shum, D.; Lockwood, W. W.; Yang, G.; Sander, C.; Ouerfelli, O.; Tempst, P. J.; Djaballah, H.; et al. Superoxide Dismutase 1 (SOD1) Is a Target for a Small Molecule Identified in a Screen for Inhibitors of the Growth of Lung Adenocarcinoma Cell Lines. *Proc Natl Acad Sci U S A* **2011**, *108* (39), 16375–16380.

42. Gupte, A.; Mumper, R. J. Copper Chelation by D-Penicillamine Generates Reactive Oxygen Species That Are Cytotoxic to Human Leukemia and Breast Cancer Cells. *Free Radical Biology and Medicine* **2007**, *43* (9), 1271–1278.

43. Gupte, a. Targeting the metal chelator d-penicillamine to exploit the elevated copper and oxidative stress associated with cancer. *University of Kentucky Doctoral Dissertations* **2008**.

44. Antabuse (disulfiram) dosing, indications, interactions, adverse effects, and more. http://reference.medscape.com/drug/disulfiram-343199#10 (accessed Mar 13, 2016).

45. Pike, M. G.; Mays, D. C.; Macomber, D. W.; Lipsky, J. J. Metabolism of a Disulfiram Metabolite, S-MethylN,N-Diethyldithiocarbamate, by Flavin Monooxygenase in Human Renal Microsomes. *Drug Metab Dispos* **2001**, *29* (2), 127–132.

46. Kanchi, S.; Singh, P.; Bisetty, K. Dithiocarbamates as Hazardous Remediation Agent: A Critical Review on Progress in Environmental Chemistry for Inorganic Species Studies of 20th Century. *Arabian Journal of Chemistry* **2014**, *7* (1), 11–25.

47. Brayton, D. F. Targeting Melanoma via Metal Based Drugs: Dithiocarbamates, Disulfiram Copper Specificity, and Thiomaltol Ligands. Ph.D., University of California, Irvine: United States -- California, 2006.

48. Allensworth, J. L.; Evans, M. K.; Bertucci, F.; Aldrich, A. J.; Festa, R. A.; Finetti, P.; Ueno, N. T.; Safi, R.; McDonnell, D. P.; Thiele, D. J.; et al. Disulfiram (DSF) Acts as a Copper Ionophore to Induce Copper-Dependent Oxidative Stress and Mediate Anti-Tumor Efficacy in Inflammatory Breast Cancer. *Molecular Oncology* **2015**, *9* (6), 1155–1168.

49. Degradation of NF-κB, p53 and other regulatory redox-sensitive proteins by thiol-conjugating and -nitrosylating drugs in human tumor cells. - PubMed - NCBI http://www.ncbi.nlm.nih.gov/pubmed/23354308 (accessed Mar 13, 2016).

50. Farmer, P. J. Baylor University, Waco, TX. Personal communication, 2016.

51. Ogawa, E.; Kodama, H. Effects of Disulfiram Treatment in Patients with Menkes Disease and Occipital Horn Syndrome. *Journal of Trace Elements in Medicine and Biology* **2012**, *26* (2-3), 102–104.

52. Langheinrich, U. Zebrafish: A New Model on the Pharmaceutical Catwalk. *Bioessays* **2003**, *25* (9), 904–912.

53. Camp, E.; Lardelli, M. Tyrosinase Gene Expression in Zebrafish Embryos. *Dev. Genes Evol.* **2001**, *211* (3), 150–153.

54. Lee, T. H.; Seo, J. O.; Baek, S.-H.; Kim, S. Y. Inhibitory Effects of Resveratrol on Melanin Synthesis in Ultraviolet B-Induced Pigmentation in Guinea Pig Skin. *Biomol Ther (Seoul)* **2014**, *22* (1), 35–40.

55. Chen, J. Impaired Cardiovascular Function Caused by Different Stressors Elicits a Common Pathological and Transcriptional Response in Zebrafish Embryos. *Zebrafish* **2013**, *10* (3), 389–400.

56. Tilton, F.; La Du, J. K.; Vue, M.; Alzarban, N.; Tanguay, R. L. Dithiocarbamates Have a Common Toxic Effect on Zebrafish Body Axis Formation. *Toxicology and Applied Pharmacology* **2006**, *216* (1), 55–68.

57. Teraoka, H.; Urakawa, S.; Nanba, S.; Nagai, Y.; Dong, W.; Imagawa, T.; Tanguay, R. L.; Svoboda, K.; Handley-Goldstone, H. M.; Stegeman, J. J.; et al. Muscular Contractions in the Zebrafish Embryo Are Necessary to Reveal Thiuram-Induced Notochord Distortions. *Toxicology and Applied Pharmacology* **2006**, *212* (1), 24–34.

58. Van Boxtel, A. L.; Kamstra, J. H.; Fluitsma, D. M.; Legler, J. Dithiocarbamates Are Teratogenic to Developing Zebrafish through Inhibition of Lysyl Oxidase Activity. *Toxicology and Applied Pharmacology* **2010**, *244* (2), 156–161.

59. Anderson, C.; Bartlett, S. J.; Gansner, J. M.; Wilson, D.; He, L.; Gitlin, J. D.; Kelsh, R. N.; Dowden, J. Chemical Genetics Suggests a Critical Role for Lysyl Oxidase in Zebrafish Notochord Morphogenesis. *Mol. BioSyst.* **2007**, *3* (1), 51–59.

60. Haendel, M. A.; Tilton, F.; Bailey, G. S.; Tanguay, R. L. Developmental Toxicity of the Dithiocarbamate Pesticide Sodium Metam in Zebrafish. *Toxicol. Sci.* **2004**, *81* (2), 390–400.