The Role of Methylmercury on Thioredoxin Reductase Activity in Breast Cancer Cells

By

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Abstract

Metalloestrogens are small ionic metals and metalloids that activate the estrogen receptor (ER) in a manner similar to estrogen. When metalloestrogens bind to the ER, there is an increase in the expression of estrogen-related genes and proliferation of estrogen-dependent breast cancer. Mercury has been shown to cause proliferation of estrogen-dependent breast cancer cells through its role as a metalloestrogen. Mercury, in the form of methylmercury (MeHg), induces oxidative stress via overproduction of reactive oxygen species (ROS), and inhibits cellular antioxidant defense mechanisms. Specifically, MeHg inhibits the enzyme thioredoxin reductase (TrxR), which is a crucial enzyme involved in the scavenging of ROS and plays a role in many signaling pathways. In this study, a method for measuring TrxR activity in cell culture and tissues was developed. Furthermore, a method for measuring ROS levels in zebrafish tissue was perfected and used to determine ROS concentration in the tissues of tumor and non-tumor bearing fish. Although the method for measuring TrxR activity in cells and tissue remains to be perfected, ROS levels measured in the gastrointestinal (GI) system of tumor-bearing fish were significantly greater than those in non-tumor bearing (control) fish. This result was consistent with the observation of tumor formation in the GI system. Further studies using methylmercury must be performed in order to decipher its role in tumor formation and ROS production in vivo.
### Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Name</th>
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<tbody>
<tr>
<td>BSA</td>
<td>Bovine Serum Albumin</td>
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<tr>
<td>Cys</td>
<td>Cysteine</td>
</tr>
<tr>
<td>DCFH-DA</td>
<td>2', 7'-dichlorofluorescin diacetate</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco’s Modified Eagle Medium</td>
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<tr>
<td>ER</td>
<td>Estrogen receptor</td>
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<tr>
<td>FAD</td>
<td>Flavin adenine dinucleotide</td>
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<tr>
<td>FBS</td>
<td>Fetal Bovine Serum</td>
</tr>
<tr>
<td>Gly</td>
<td>Glycine</td>
</tr>
<tr>
<td>H$_2$O$_2$</td>
<td>Hydrogen peroxide</td>
</tr>
<tr>
<td>Hank’s Balanced Salt Solution</td>
<td>HBSS</td>
</tr>
<tr>
<td>HER2</td>
<td>Human epidermal growth factor receptor 2</td>
</tr>
<tr>
<td>Hg$^0$</td>
<td>Elemental mercury</td>
</tr>
<tr>
<td>Hg$^{2+}$</td>
<td>Mercuric cation</td>
</tr>
<tr>
<td>HgCl$_2$</td>
<td>Mercuric chloride</td>
</tr>
<tr>
<td>MCF-7</td>
<td>Michigan Cancer Foundation-7</td>
</tr>
<tr>
<td>MeHg</td>
<td>Methylmercury</td>
</tr>
<tr>
<td>O$_2^•$</td>
<td>Superoxide anion</td>
</tr>
<tr>
<td>•OH</td>
<td>Hydroxyl radical</td>
</tr>
<tr>
<td>PR</td>
<td>Progesterone receptor</td>
</tr>
<tr>
<td>Pro</td>
<td>Proline</td>
</tr>
<tr>
<td>RNR</td>
<td>Ribonucleotide reductase</td>
</tr>
<tr>
<td>ROS</td>
<td>Reactive oxygen species</td>
</tr>
<tr>
<td>SOD</td>
<td>Superoxide dismutase</td>
</tr>
<tr>
<td>Trx</td>
<td>Thioredoxin</td>
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<tr>
<td>TrxR</td>
<td>Thioredoxin reductase</td>
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Chapter I—Introduction

1.1 Breast Cancer

In the United States, cancer is responsible for 1 in every 4 deaths, and is the second most common cause of death among both men and women (ACS, 2015). Cancer cells have three major characteristics that distinguish them from non-cancerous cells: resistance to apoptosis, unlimited ability to grow, and ability to break away from the site of origin and metastasize to other areas in the body (Frank, 2013). What typically puts an individual at risk for developing a form of cancer are harmful interactions with the environment, most notably carcinogens that mutate the individual’s DNA. Mutations in the DNA due to carcinogens may promote the transcription of proteins that promote cellular growth. Furthermore, mutations to tumor-suppressing genes that function to stop cellular growth also may result in the development of cancer (Frank, 2013).

Although all forms of cancer share a common feature, the ability for cancerous cells to grow uncontrollably, each individual form of cancer has certain characteristics that set it apart from the rest. The tissue type and location in the body where the cancer originates defines the type of cancer with which an individual is afflicted. In adults, epithelial cells are most prone to turning cancerous due to their high rate of division and firsthand exposure to the environment (Frank, 2013). Each person affected by the same type of cancer possesses unique molecular properties. These unique differences among patients and cancer types are termed prognostic factors, and are used by oncologists to help aid in patient diagnosis and method of treatment (Frank, 2013).
Breast cancer is the second most common form of cancer for women in the United States (U.S. Cancer Statistics, 2015). In 2015, it was estimated that 60,000 cases of breast cancer in situ were diagnosed, and 40,000 women would die of invasive breast cancer (Cancer Facts and Figures, 2015). In breast cancer, the cells that turn cancerous are those located in various areas that make up the breast (Figure-1). The specific location in the breast where the cancer arises determines the classification. For example, although cancer arising from cells that make up ducts is most common, other forms of breast cancer include lobular, inflammatory, and tubular (Frank, 2013).
Figure-1: Female breast anatomy. Breast cancer originates from cancerous epithelial cells that make up sections of the breast (URMC, 2016).

Many factors put an individual at risk for developing breast cancer. Factors an individual can control, known as modifiable factors, include weight gain after the age of 18, the use of menopausal hormonal therapy, heavy smoking, and the use of oral contraceptives. Non-modifiable factors include breast tissue density, family history of breast cancer, and type 2 diabetes (Cancer Facts and Figures, 2015). What makes one form of breast cancer more or less fatal than another are prognostic factors such as the ability for the cancer to metastasize, and the presence/absence of receptors located on the cells (Frank, 2013).
The three main forms of breast cancer are hormone receptor positive, human epidermal growth factor receptor 2 (HER2) positive, and triple negative. These forms account for 65%, 25%, and 10% of breast cancer diagnoses, respectively. Hormone-receptor positive breast cancer is when the patient has estrogen and/or progesterone receptors located in their breast tissue, but the HER2 receptor is absent. The presence of either the estrogen or progesterone receptor (ER, PR) is an important prognostic factor, for it allows the patient to be treated with hormonal therapies that target the ER or PR. These hormonal therapies often prove to be very effective (Frank, 2013). HER2 positive breast cancer is defined as the presence of the HER2 receptor located on the surface of breast cancer cells. The ER and PR may be either present or absent in these patients. HER2’s normal function is to control the growth and division of healthy, non-cancerous breast cells. In HER2 positive breast cancer patients, the HER2 protein is overexpressed, which in turn leads to uncontrolled cell growth (Breastcancer.org, 2015). Treating patients with HER2 positive breast cancer involves the use of anti-HER2 therapies, such as the drug Herceptin. Triple negative breast cancer (the rarest form of breast cancer) involves the absence of HER2, ER, and PR. For this reason, neither hormonal nor anti-HER2 therapies are effective means of treatment. Breast cancer patients with triple negative breast cancer often resort to chemotherapy as their primary means of treatment (Frank, 2013).
1.2 Estrogen and the Estrogen Receptor

In order for breast cancer to thrive, cancerous cells depend on hormones created by the body (Frank, 2013). These hormones are synthesized by endocrine glands and are circulated throughout the bloodstream. In patients with breast cancer, the hormone of interest is 17β-estradiol, a steroid hormone more commonly known as estrogen (Yu et al., 2010). Before women undergo menopause, the ovaries are the main source of estrogen. The main functions of estrogen in premenopausal women are the development of secondary sex characteristics, maintenance of cognitive function, regulation of bone mass, and regulation of cell-cycle regulatory genes (Nelson et al., 2001; Yu et al., 2010). When the ovaries stop producing estrogen after menopause, extragonadal sites, such as adipose tissue in the breast, are the main sources of estrogen (Simpson, 2003). Estrogen produced at extragonadal sites exerts its effects locally, rather than at distant tissues. In women with breast cancer, the concentration of estrogen in breast tumors is at least 20 times greater than that in circulation (Simpson, 2003). Therefore, the estrogen that is responsible for breast cancer development is primarily that which is produced in the breast itself.

Estrogen exerts its proliferative effect on breast epithelial cells through its interaction with the estrogen receptor (ER) located in the cytoplasm. The ER is a dimer composed of either two ERα subunits, or two ERβ subunits. ERα facilitates mitosis, while ERβ facilitates antimitogenic processes (Byrne et al., 2013). Once the two subunits bind estrogen, the complex moves into the nucleus, where it is able to target genes that regulate the cell cycle (Figure-2; Yu et al., 2010). ER
positive breast cancer occurs when either estrogen or estrogen-like molecules over-actively bind to the ER, thus causing excessive stimulation of the intercellular signaling pathways associated with cell division (Yu et al., 2010).

![Figure-2: Mechanism of estrogen activity through association with estrogen receptors. Estrogen crosses the cell membrane and enters the cytoplasm. Once in the cytoplasm, estrogen binds to the estrogen receptors to form a dimer. This complex then moves into the nucleus, where it binds to the promoter region of DNA to promote transcription (Yu et al., 2010).](image)

1.3 Metalloestrogens

Metalloestrogens are small ionic metals and metalloids that activate the ER in a manner similar to estrogen (Byrne et al., 2013). When metalloestrogens bind to the ER, there is an increase in the expression of estrogen-related genes and
proliferation of estrogen-dependent breast cancer (Byrne et al., 2013).

Metalloestrogens are found in food, water, air, and occupational settings, which makes them an extremely relevant topic of study.

Some metals are essential to organismal metabolism and enzymatic function since they are commonly located at the active site of enzymes. For example, iron plays a significant role in human respiration due to its presence in hemoglobin molecules. Chromium, copper, and nickel are examples of metals that are essential to human health when present at low concentrations. Non-essential metals such as lead, mercury, and cadmium are toxic in that they either mimic or block the function of essential metals. Many non-essential metals also possess carcinogenic properties, most notably cadmium (Byrne et al., 2013). Cadmium has been extensively studied as a metalloestrogen. As humans age, it accumulates in the body due to its slow rate of excretion and long biological half life (Yu et al., 2010). Many epidemiological studies have linked cadmium to breast cancer, for it has been shown to cause proliferation and increased expression of estrogen related genes in estrogen-dependent breast cancer cells (Byrne et al., 2013).

Cadmium binds to the ligand-binding domain (LBD) of ERα by mimicking the role of calcium. This suggests that in order for metals to bind to ERα, a +2 charge is typically required. This class of metalloestrogens is called bivalent cations (Byrne et al., 2013). Mercury is another bivalent metal that, although less well studied than cadmium, has been shown to cause proliferation of estrogen-dependent breast cancer cells through the ER (Byrne et al., 2013).
1.4 Methylmercury

Mercury is a non-essential metal that has the ability to induce toxic effects when ingested. Humans are subjected to mercury exposure through air, cigarette smoke, occupational exposure, and, most notably, dietary means. It is estimated that the daily intake of mercury is between 0.25 and 0.28 µg/day (Byrne et al., 2013).

Mercury enters the environment through both natural and anthropogenic sources. For example, mercury is naturally emitted into the air through volcanic eruptions and forest fires, but anthropogenically through mining and combustion of fossil fuels (Morel et al., 1998; Ceccatelli et al., 2010). The Environmental Protection Agency estimates that between 5,000 and 8,000 metric tons of mercury are emitted annually through both natural and anthropogenic sources (EPA, 2016). Anthropogenic sources account for approximately 30% of annual mercury emission, whereas natural sources account for 10%. The remaining 60% of mercury emission is due to “re-emission” of previously emitted mercury that has built up in soils and oceans over time (UNEP, 2013). Whether it be through natural or anthropogenic causes, when mercury is released into the atmosphere it enters the global mercury cycle (Figure-3; Utah DEQ, 2016).

Although the chief sources of mercury emission come from industrialized regions on land, mercury can remain in the atmosphere for up to a year before returning back down to land or sea. This allows for mercury to be distributed over vast areas of the globe (Morel et al., 1998). In the atmosphere, elemental mercury (Hg\(^0\)) is slowly oxidized to the mercuric cation (Hg\(^{2+}\)). Once in the ionic form,
$\text{Hg}^{2+}$ is able to dissolve and return to Earth’s surface via precipitation (Morel et al., 1998).

When $\text{Hg}^{2+}$ enters an aquatic environment such as lakes, rivers, and oceans, the majority of mercury is reduced back to $\text{Hg}^0$ and volatizes back into the atmosphere, leaving only a small fraction of mercury in the $\text{Hg}^{2+}$ state. The fraction that does remain in the ionic state is able to settle into the sediment, where it is taken up by sulfate-reducing and other bacteria (Morel et al., 1998). Mercury enters bacteria chiefly through diffusion across the cell membrane by binding free chloride ions in the water. The mercuric chloride complex ($\text{HgCl}_2$) is fairly lipid soluble and uncharged, making it relatively easy to diffuse into the bacterium. Once in the cytoplasm, some species of bacteria transform mercury into the organic form methylmercury ($\text{MeHg}$) via enzyme-mediated pathways; however, the exact mechanism and location of mercury within the cell during methylation remains a topic of study (Morel et al., 1998; Benoit et al., 2003). When $\text{MeHg}$ is in the water, it has the potential to bioaccumulate in aquatic organisms such as plankton and fish. $\text{MeHg}$ is biomagnified through the aquatic food chain, and is ultimately introduced to humans through the consumption of large predatory fish such as shark, tuna, swordfish, and mackerel (FDA, 2014).
Figure 3: The global mercury cycle as it occurs in bodies of water. Mercury is deposited into aquatic systems by both natural and anthropogenic sources. $\text{Hg}^0$ is oxidized to ionic mercury $\text{Hg}^{2+}$, which is then converted into MeHg by sulfate-reducing bacteria in the sediment of bodies of water. Once in organic form, mercury is able to bioaccumulate and biomagnify up the food chain (Utah DEQ, 2016).

Due to its ability to biomagnify up the aquatic food chain and thus enter humans, MeHg poisoning has become a major concern to public health. Massive health disasters have occurred primarily in populations where fishing is the main source of food, i.e. Japan, Canada, and New Zealand (Ceccatelli et al., 2010). When humans ingest MeHg-contaminated food, the absorption of MeHg takes place primarily in the small intestine, and is excreted through feces (Clarkson et al., 2007). During digestion, MeHg binds to free thiol (R-SH) and selenol (R-SeH) groups, giving it the ability to enter the bloodstream/tissues through amino acid transporters. For example, when MeHg attaches to the thiol residue on cysteine, it forms a molecule that closely resembles the amino acid methionine.
This variation is subtle enough for MeHg to enter cells through methionine transporters (Clarkson et al., 2007). The ability for MeHg to form complexes with thiols and selenols is what allows it to be distributed throughout various tissues in the body, including passage through the blood-brain barrier (Ceccatelli et al., 2010).

When MeHg is introduced to the brain, it causes many neurological impairments that include numbness of the extremities, imbalance, deafness, and abnormal eye movement. The first autopsy on a person who suffered occupational MeHg exposure revealed morphological changes in the brain and a decrease of cells in the cerebellum. Severe poisoning of MeHg has led to death (Kaur et al., 2006; Ceccatelli et al., 2010). Despite the main symptoms of mercury poisoning being neurological in origin, mercury has been shown to cause proliferation of estrogen-dependent breast cancer cells through its role as a metalloestrogen (Martin et al., 2003; Byrne et al., 2013; Gaudet et al., in review). MeHg toxicity has been linked to disruption of calcium homeostasis, induction of oxidative stress via overproduction of reactive oxygen species (ROS), and a reduction in cellular antioxidant defense (Ceccatelli et al., 2010). Specifically, MeHg inhibits the enzyme thioredoxin reductase (TrxR), which is a crucial enzyme involved in the scavenging of ROS and plays a role in many signaling pathways (Carvalho et al., 2008).
1.5 The Thioredoxin System

The thioredoxin system, composed of thioredoxin (Trx), thioredoxin reductase (TrxR), and NADPH, is one of the most important intracellular defense mechanisms against ROS. It is no surprise, therefore, that the thioredoxin system is conserved across many domains of life. In more complex organisms, components of the thioredoxin system have multiple roles (Arner and Holmgren, 2000). In mammals, the thioredoxin system is responsible for reducing protein disulfides by the addition of hydrogen atoms, which is accomplished by TrxR transferring electrons from NADPH to Trx. When Trx is in the reduced form, its purpose is to act as a disulfide reductase (Holmgren et al., 2010). Reduced thioredoxin has many other cellular functions, many of which involve cellular growth, DNA binding, and antioxidant defense (Figure-4; Mustacich and Powis, 2000).
Figure-4: The thioredoxin system is composed of NADPH, TrxR, and Trx. Reduced Trx is involved in many cellular pathways involving cell growth, DNA synthesis/repair, gene transcription, and antioxidant defense (Mustacich and Powis, 2000).

Trx is a 10-12 kDa protein that is capable of undergoing reversible oxidation/reduction at the two cysteine residues present at the active site (Mustacich and Powis, 2000). The active site of Trx contains four amino acids that are generally conserved across organisms: Cys-Gly-Pro-Cys (Holmgren et al., 2010). The 3D structure of Trx contains a common domain termed the “thioredoxin fold” which is composed of five β strands surrounded by four α helices (Arner and Holmgren, 2000). As stated previously, Trx functions to reduce disulfides, and is therefore a key contributor to redox regulation of protein folding and signaling. Since many systems require redox mechanisms that are thiol-dependent, Trx has a long evolutionary history with DNA and defense
against oxidative damage (Holmgren et al., 2010). Trx influences DNA replication, repair, and the transcription of genes through its interaction with transcription factors and enzymes such as ribonucleotide reductase (RNR). Trx reduces RNR, which is involved in DNA synthesis and repair. NF-κB and AP1 are both transcription factors that require reduction via Trx in order to bind DNA upstream of genes involved in the inflammatory response (Arner and Holmgren, 2000). Trx is also involved in the inhibition of apoptotic pathways through its interaction with apoptosis signaling kinase 1 (ASK1) (Arner and Holmgren, 2000). When levels of Trx are upregulated or overexpressed, it aids in protection against oxidative damage. It does this by donating electrons to thioredoxin peroxidases or peroxiredoxins that reduce H$_2$O$_2$. Through the reduction of H$_2$O$_2$, oxidative damage and the initiation of apoptosis are prevented (Arner and Holmgren, 2000; Holmgren et al., 2010). It is important to note that although Trx targets many different proteins, oxidized Trx can only be reduced by TrxR. Therefore, any alteration to TrxR activity would alter the activity/function of Trx (Mustacich and Powis, 2000).

1.6 Thioredoxin Reductase

TrxR originates from the flavoprotein family of enzymes, and is a homodimeric selenoenzyme that ranges between 112-130 kDa (Arner and Holmgren, 2000; Mustacich and Powis, 2000). The enzyme contains a flavin adenine dinucleotide (FAD) prosthetic group, a NADPH binding region, and a
redox active site that is responsible for reducing substrate (Mustacich and Powis, 2000).

The active site is composed of cysteine and selenocysteine amino acids, which are necessary for catalytic activity (Arner and Holmgren, 2000). TrxR is able to reduce many different substrates, and not all functions are linked to Trx. For example, TrxR directly reduces hydroperoxides and is responsible for the recycling of ascorbate (vitamin C). Ascorbate is an antioxidant that protects cells against oxidative stress. Since humans are not capable of synthesizing their own ascorbate, they rely heavily on the thioredoxin system (Holmgren et al., 2010).

1.7 Methylmercury and Thioredoxin Reductase

It is known that MeHg has a high affinity for thiol and selenol groups. Thus, MeHg interacts with TrxR by covalently attaching to the selenocysteine residue located at the active site of TrxR (Carvalho et al., 2008; Wagner et al., 2010). This covalent attachment inhibits the active site of TrxR, which renders it non-functional. Without the ability for TrxR to reduce Trx, the defensive mechanisms against oxidative stress are compromised (see section 1.9). It has been reported that the inhibition of TrxR with MeHg leads to a decrease in activity in a concentration dependent basis, producing antiproliferative effects (Carvalho et al., 2008).
1.8 Thioredoxin Reductase and Cancer

Since TrxR is the only known enzyme to convert oxidized Trx to reduced Trx, much research has focused on the reaction of TrxR on drugs designed to treat inflammation and cancer. In fact, plasma levels of Trx and TrxR are effective markers for diseases like HIV and cancer, as most cancer cells exhibit high levels of both Trx and TrxR (Holmgren et al., 2010). For this reason, TrxR has become a fairly new target for anti-cancer therapies by changing the function of TrxR from inhibiting cell death, to promoting cell death (Powis et al., 1998).

Due to TrxR’s ability to counteract ROS produced by carcinogens such as MeHg, TrxR is beneficial in preventing cancer; however, it also may play a role in the promotion of cancer due to its growth-promoting and antiapoptotic functions (Saitoh et. al., 1998). Little is known regarding the activity of TrxR in MeHg-induced toxicity/proliferation of breast cancer, so determining the role of TrxR in MeHg-induced breast cancer is a crucial first step in developing therapeutics for this type of cancer.

1.9 ROS and Cancer

Reactive oxygen species (ROS) are radicals, ions, or molecules that possess unpaired electrons in their outermost electron shells. Due to this property, ROS are extremely reactive and are involved in many cellular signaling and metabolic functions (Liou and Storz, 2010; Manda et al., 2009). ROS are separated into two groups: free oxygen radicals and non-radical ROS. Examples of free oxygen radicals that are well studied in cancer include the superoxide
anion (O$_2^-$) and the hydroxyl radical (•OH). Superoxide is typically produced as a by-product of the respiratory chain, whereas the hydroxyl radical is produced when hydrogen peroxide (H$_2$O$_2$) reacts with iron (the Fenton reaction) (Manda et al., 2009). The non-radical form of ROS that is most studied in cancer is hydrogen peroxide, as it is involved in signaling pathways and freely diffuses in and out of tissues. Furthermore, hydrogen peroxide is necessary for the generation of the hydroxyl radical, as it acts as a reactant in the Fenton reaction (Manda et al., 2009).

In non-phagocytic cells, ROS are produced mainly in the mitochondria. The superoxide anion is generated as a by-product of the respiratory chain at complexes I and III. Once generated, superoxide can leak through the outer mitochondrial membrane and into the cytoplasm, where it is converted to H$_2$O$_2$ by the antioxidant enzyme superoxide dismutase (SOD) (Liou and Storz, 2010).

ROS play a key role in cellular redox signaling, for they are involved in the signaling of mitogen-activated protein kinases (MAPKs), protein phosphatases, receptor tyrosine kinases, and transcription factors (Liou and Storz, 2010; Manda et al., 2009). Although ROS are necessary for essential cellular functions, levels of ROS must be kept in check by antioxidant defense mechanisms.

SOD is the enzyme involved in scavenging the superoxide anion and converting it to H$_2$O$_2$. SOD is typically referred to as the first line of defense in protecting cells against ROS (Kinnula and Crapo, 2003). Since H$_2$O$_2$ is also regarded as a type of ROS, it too is able to trigger signaling pathways and cause
cellular damage. It is therefore essential for the cell to be able to eliminate excess levels of $\text{H}_2\text{O}_2$. To do this, catalase converts $\text{H}_2\text{O}_2$ to water and oxygen. As discussed previously, the thioredoxin system also functions to eliminate ROS from cells. When antioxidant defense mechanisms are unable to keep up with the amount of ROS being produced, a condition called oxidative stress results (Manda et al., 2009).

Oxidative stress is enhanced in cancerous cells, which leads to tumor promotion and a decrease in apoptotic signaling (Nogueira and Hay, 2013). ROS further functions in cancer cells to promote cell-cell adhesion and energy metabolism (Liou and Storz, 2010). Increased metastasis is a factor regulated by increased ROS. In mice injected with the human breast cancer cell line MCF-7, higher levels of endogenous ROS were seen in cells that metastasized (Liou and Storz, 2010).

The mechanism behind the increased levels of ROS in cancerous cells can be explained via the Warburg Effect. Since cancer cells divide rapidly, they are in constant need of nutrients and energy in the form of ATP. However, the tumor environment is typically one with low levels of oxygen, therefore aerobic respiration is not ideal. To ensure they are getting enough energy, cancer cells switch their main source of ATP from aerobic respiration to glycolysis; this is the Warburg Effect (Liou and Storz, 2010). The Warburg Effect contributes to oxidative stress because this alteration in cell metabolism enhances levels of superoxide production in the mitochondria (Kinnula and Crapo, 2003).
1.10 Hypothesis

Prior studies regarding the role of MeHg on TrxR activity (Carvalho et al., 2008; Wagner et al., 2010), as well as studies done on the effects of metalloestrogens on cancer cells (Martin et al., 2003; Ceccatelli et al., 2010; Byrne et al., 2013; Gaudet et al., in review), have shown that MeHg has many complex effects on cancer cells. One study found that treatment of MCF-7 cancer cells with 1nM MeHg leads to cellular proliferation (Gaudet et al., in review). In contrast, inhibiting TrxR with MeHg leads to a decrease in TrxR activity, causing antiproliferative effects (Carvalho et al., 2008). Furthermore, inhibiting TrxR with MeHg causes an increase in ROS production in cancer cells, which can lead to either tumor promotion or suppression (Nogueira and Hay, 2013). Identifying the role of MeHg on TrxR activity and ROS production is therefore crucial in understanding MeHg’s tumor suppression/promotion properties.

Previous studies by Christensen and Gaudet have shown that at intermediate concentrations (10nM, 100nM), ER positive MCF-7 breast cancer cells did not proliferate compared to untreated cells. I hypothesize that the inhibition of TrxR may counteract the metalloestrogenic effects of MeHg in breast cancer cells treated with intermediate MeHg concentrations. I further hypothesize that the production of ROS stimulated by higher MeHg concentrations contributes to cell death. To test these hypotheses, reliable methods for determining TrxR activity and ROS concentration must be established. Thus, the goal of this study is to develop such methods for use in cell culture and tissues.
Chapter II—Materials and Methods

2.1 Experimental Design

The goal of this study was to establish methods to elucidate the role of TrxR in MeHg-induced breast cancer. To do this, ER positive MCF-7 breast cancer cells were used. Cells were cultured under a MeHg concentration gradient using the protocol outlined by Christensen and Gaudet (Gaudet et al., in review). Proliferation assays were conducted to confirm the trend seen by Christensen and Gaudet and to provide a platform for this study. A kinetic assay employing selenocysteine was used to measure TrxR activity (Cunniff et al., 2013). In addition, a fluorometric ROS assay (LaBel et al., 1992; Moussavi Nik, 2014) was done to measure ROS concentration in tumor-bearing zebrafish with xenografts of human MCF-7 breast cancer cells.

2.2 Cell Culture

MCF-7 human invasive ductal breast carcinoma cells were gifted from the Filardo Lab at Brown University (Providence, RI). These cells express both the ER and PR. Cells were grown in flasks containing phenol red-free Dulbecco’s Modified Eagle Medium (DMEM)/F-12 supplemented with 5% fetal bovine serum (FBS) and 2µM sodium selenite (ThermoFisher). Cells were maintained in a humidified incubator at 37°C with 5% CO₂. Cells were grown until the flask reached 70% confluence, when they were then split into new flasks.
2.3 Preparation of Cell Lysate

Multiple methods were used to lyse MCF-7 cells (Figure-7). A method based on Cunniff et al (2013) was deemed most appropriate. Cells were washed 1x with cold phosphate-buffered saline (PBS) and lysed in culture flask by adding NP-40 lysis buffer (50mM Tris [pH 7.5], 150mM NaCl, 1% TritonX-100, and 1% NP-40) and Halt™ protease inhibitors (ThermoFisher). Cell lysate was scraped from culture flask on ice and transferred to 1.5 ml microcentrifuge tubes (Cunniff et al., 2013). Lysates were then centrifuged for 10 minutes at 14,000 rpm at 4°C. Supernatant was transferred into clean 1.5 ml microcentrifuge tubes in 200µl aliquots and stored in -80°C to save for Bradford, ROS, and kinetic enzyme assays.

2.4 Determining Protein Concentration

Before running the kinetic enzyme assay for TrxR, protein concentration of cell lysate samples was determined using the Bradford method (Bradford, 1976). A standard curve was developed each day using bovine serum albumin (BSA) standards ranging from 25.2 to 125.9 µg/ml. Protein concentrations were determined by the absorbance at 595nm on a Agilent UV/VIS spectrometer.

2.5 Selenocysteine Assay for Thioredoxin Reductase

A kinetic enzyme assay for TrxR (Cunniff et al., 2013) was used to determine its specific activity. In this assay, TrxR oxidizes NADPH to NADP⁺ and H⁺. TrxR then transfers H⁺ and electrons to selenocysteine, thus reducing it to
–SeH (Figure-5). Since NADPH is the electron source for this reaction, measuring the decrease of absorbance for NADPH at 340nm is used to determine TrxR activity. This assay is superior to prior methods because selenocysteine can only be reduced by TrxR; thus, a single assay of cell lysate can be used to quantify the activity of TrxR (Cunniff et al., 2013).

To carry out this reaction, reagents were added to a microcuvette (UV) in the following order: 940µl Tris buffer (50mM and 1mM EDTA [pH 7.5]), 20µl NADPH, and 20µl cell lysate. After gentle mixing, 20µl selenocysteine (40mM) was added to the cuvette and absorbance measured at 340nm for 2 minutes. A detailed protocol for this assay is located in Appendix I, and sample assay curves are shown in Appendix II.

![Figure-5: The reaction carried out during the selenocysteine assay. Selenocysteine is reduced using H⁺ and electrons supplied by reduced TrxR (shown as TR$_{\text{red}}$). The ultimate source of electrons is from NADPH (Cunniff et al., 2013).](image)

2.6 ROS Assay with DCFH-DA

A fluorescent assay using 2’, 7’-dichlorofluorescin diacetate (DCFH-DA) was used to measure ROS levels in zebrafish tissue (LaBel et al., 1992; Moussavi Nik, 2014). In this assay, DCFH-DA is added to homogenized tissue. DCFH-DA crosses cellular membranes, and is converted to non-fluorescent 2’, 7’-...
dichlorofluorescin (DCFH) by intracellular esterases (LaBel et al., 1992). In the presence of ROS, DCFH is oxidized to 2’, 7’-dichlorofluorescein (DCF), which is fluorescent. The mechanism behind this reaction is shown in Figure-6. (LaBel et al., 1992)

In order to calibrate the assay, it was first run using H$_2$O$_2$ standards. In this case, the DCFH-DA was hydrolyzed with base to convert it to DCFH, and then the DCFH was reacted with H$_2$O$_2$ as described below. A linear relationship was observed between peroxide concentration and DCF fluorescence intensity (Appendix V). This relationship was used to convert measured fluorescence intensity to equivalent H$_2$O$_2$ concentration in tissue for each fish assay. All ROS measurements in fish were normalized to the masses of tissue used for the assay.

For assays involving zebrafish, brain and gastrointestinal (GI) tissue were dissected from sacrificed wild type and tumor-bearing fish. Tumor-bearing fish were created using xenografts of human MCF-7 breast cancer cells. Tissue was weighed, and then homogenized in 1 ml of ice-cold Tris-HCl buffer (100mM Tris-HCl [pH 7.4], 0.25M sucrose). Tissue homogenate was then transferred to a 1.5 ml microcentrifuge tube and centrifuged for 30 minutes at 14,000 rpm at 4°C. Supernatant was then transferred to a clean 1.5 ml microcentrifuge tube and kept on ice until needed.

In the ROS assay, tissue supernatant (20 µl for GI tissue, 40 µl for brain tissue) was added to Tris-HCl buffer (1.58 ml for GI tissue assays, 1.56 ml for brain tissue assays) along with 400 µl of 500 µM DCFH-DA (2 ml total volume). The reaction was carried out in the dark for 30 minutes at 25°C. Reagent and
tissue blanks were also prepared. Fluorescence was measured at an excitation wavelength of 488nm and an emission wavelength of 525nm using a PerkinElmer LS 55 fluorescence spectrometer. Appendix IV shows a sample spectrum of DCF fluorescence from an assay of zebrafish brain and gastrointestinal tract samples. Despite the fact that the tumor cells were tagged with a fluorescent marker, there was no interference from background fluorescence, as indicated by low fluorescent values for tissue blanks.
Figure-6: The mechanism for the reaction carried out during the ROS assay.

DCFH-DA is a non-fluorescent compound that crosses cellular membranes via diffusion. Once inside the animal cell, DCFH-DA is deacetylated by esterases. After deacetylation occurs, ROS inside the cell oxidize the compound into DCF, which is fluorescent (LaBel et al., 1992).
Chapter III—Results and Discussion

3.1 Goals and Objectives

One goal of this study was to develop a kinetic assay that could be used to measure the activity of TrxR in human breast cancer cells treated with MeHg. In addition, a fluorometric ROS assay was developed to measure ROS levels in tumor-bearing zebrafish with xenografts of human MCF-7 breast cancer cells. Methods reported in the literature (Cunniff et al., 2013; LaBel et al., 1992; Moussavi Nik, 2014) were adapted and expanded to suit the needs of this study.

3.2 Selenocysteine Assay for Thioredoxin Reductase

To determine the effect of MeHg on TrxR activity in MCF-7 breast cancer cells, a kinetic enzyme assay using selenocysteine was used (Cunniff et al., 2013). Cells were grown in flasks containing phenol red-free Dulbecco’s Modified Eagle Medium (DMEM)/F-12 supplemented with 5% fetal bovine serum (FBS) and 2µM sodium selenite (ThermoFisher). When cells reached 70% confluence, they were lysed according to the procedure outlined in section 2.3. Cell lysate was then used to measure activity of TrxR (see section 2.5).

After establishing that the selenocysteine assay successfully measured the activity of purified TrxR (Appendix I, IIa), the method development centered around collecting and lysing MCF-7 breast cancer cells. Figure-7 summarizes the various methods used to obtain cell lysates.
Mammalian cells, having no cell wall, should in theory be relatively simple to lyse. First attempts to lyse cells involved the use of TritonX-100, which makes cell membranes more permeable. Cells were lifted off the culture flask using trypsin, then spun down and resuspended in 0.5 ml Hank’s Balanced Salt Solution (HBSS) and 10µl of 10% TritonX-100. Cells were then centrifuged for 30 minutes at 14,000 rpm at 4°C. Supernatant was collected and used in the selenocysteine assay. TrxR activity could not be measured using this lysis method. The next method used to lyse cells involved freezing and thawing in a -80°C freezer. Since mammalian cell membranes are rather fragile, multiple cycles of freezing and thawing should in theory be harsh enough to lyse cells open. Once again, cells were lifted off the culture flask with trypsin, spun down, and resuspended in HBSS. 200µl aliquots were transferred to microcentrifuge tubes and placed in a -80°C freezer until needed. Before use in assays, these samples were centrifuged for 30 minutes at 14,000 rpm at 4°C. Supernatant was collected and used in the selenocysteine assay. TrxR activity could not be measured using this lysis method. The next few methods used to lyse cells again focused on mechanical means to disrupt cell membranes. Cells were lifted off the culture flask with trypsin, spun down, and resuspended in HBSS. Aliquots of 0.5 ml were transferred to microcentrifuge tubes. Cells were then subjected to either an electric tissue homogenizer or sonication. Cells were then centrifuged for 30 minutes at 14,000 rpm at 4°C. Supernatant was collected and used in the selenocysteine assay. None of these methods produced successful assays to measure TrxR activity.
The next lysis methods attempted involved removing trypsin from the cell-collection procedure. Trypsin, being a protease, functions to hydrolyze proteins. To ensure that trypsin was not destroying TrxR, cells were instead manually scraped off the culture flask in the presence of lysis buffer and proteases (see section 2.3). When this method was unsuccessful, cell medium was supplemented with selenium in the form of sodium selenite (2µM). Supplementing MCF-7 cells with sodium selenite has been shown to increase TrxR activity 37-fold when compared to media with no added selenium. Furthermore, this increase in TrxR activity was accompanied by a 3-fold increase in TrxR protein level (Gallegos et al., 1997). The cells lysis method outlined in section 2.3 was once again employed; however, even with supplemental selenium, TrxR activity could not be measured.

It was hypothesized that perhaps the lysis buffer or the cell extracts were interfering with the assay. To test this hypothesis, 20µl of purified TrxR was added to the assay along with larger volumes of cell lysate (40µl, 60µl, 80µl, 100µl). Tests indicated that cell lysate and cell lysis buffer samples spiked with purified TrxR resulted in similar rates compared to TrxR activity in assay buffer (Figure-8). Typical kinetic curves over the course of the assay are shown in Appendix IIb. These results indicated that nothing in the assay mixture was directly interfering with measuring TrxR activity.

We were able to successfully lyse the cells using the method outlined by Cunniff et al., 2013 (section 2.3). Visualization of cells under a microscope after treatment with lysis buffer and protease inhibitors indicated that cells were indeed
lysed. Despite a successful way to lyse cells, measuring TrxR activity in cell lysate was not successful (Appendix IIIb). The initial reaction rates (absorbance/second) were comparable to what we obtained in blanks. It is important to note that before each assay, total protein content was determined via the Bradford method (Bradford, 1976). Total protein levels in cell lysates were comparable to those reported in previous studies (Cunniff et al., 2013).

Despite adding increasing volumes of cell lysate to the assay and performing various methods using MCF-7 cancer cells (Figure-7), measuring detectable TrxR activity was not successful. We shifted to trying the selenocysteine assay in zebrafish tissue to determine if more tissue was needed for the assay to be successful. Zebrafish tissue was collected from sacrificed fish and homogenized in 1 ml of ice-cold PBS. Tissue was then centrifuged for 20 minutes at 14,000 rpm at 4°C. Supernatant was transferred into clean 1.5 ml microcentrifuge tubes in 200µl aliquots and stored in -80°C to save for Bradford, ROS, and kinetic enzyme assays. The selenocysteine assay was then performed according to the procedure described in section 2.5. Despite the use of zebrafish tissue, the assay again proved to be unsuccessful in detecting TrxR activity (Appendix IIIa). Once again, initial reaction rates (absorbance/second) were comparable to blanks.

Further troubleshooting is required in order to obtain successful results from the selenocysteine assay. Obtaining successful measurements of TrxR activity in MCF-7 breast cancer cells treated with MeHg will provide important information regarding the proliferative/toxic effects of MeHg. Note that the assay
was not performed in cells previously treated with MeHg, for it had to be established in untreated cells before moving on to cells treated with MeHg. Since MCF-7 cells depend on estrogen to thrive, efforts to stimulate cell growth with estrogen may result in increased TrxR activity that can be measured using the selenocysteine assay.

Efforts to measure other enzymes involved in cellular processes (catalase, glutathione reductase, and glutathione peroxidase) were also unsuccessful. It was therefore concluded that measuring the activity of these enzyme was below the detection limit of the method, for it was highly unlikely that the cells did not produce any of these vital enzymes. To increase the sensitivity of the assay, steps can be done to purify TrxR from cell lysates. This would involve the use of dialysis. Purifying or purchasing a higher-grade selenocysteine is another step that could be done to increase assay sensitivity.
Figure 7: Flow chart depicting methods used to measure TrxR activity in MCF-7 breast cancer cells. A master stock of MCF-7 cells was split into 4 flasks. Once cells reached 70% confluence, the following lysis techniques were performed, ultimately ending with the selenocysteine assay for TrxR activity.
To determine ROS levels in tumor-bearing zebrafish with xenografts of human MCF-7 breast cancer cells, a fluorescent assay with DCFH-DA was used. ROS levels were measured in the GI and brain tissue of nine tumor and nine non-tumor bearing (control) zebrafish. Fluorescent values were measured at an emission wavelength of 525nm, and converted to H$_2$O$_2$ concentration using a standard curve (see Appendix V). The concentrations were then normalized to mass of tissue and expressed as mmol H$_2$O$_2$/mg tissue (Appendix VI). Student’s t-tests were used to compare means between tumor-bearing and control fish. H$_2$O$_2$ levels in the GI tissue of tumor-bearing (treated) zebrafish were significantly (p≤0.01) greater than those in control zebrafish (Figure-9). The average level of H$_2$O$_2$ in the GI tissue of control fish was 1.5 ± 0.8 mmol H$_2$O$_2$/mg tissue. In
tumor-bearing fish, the average level of $\text{H}_2\text{O}_2$ was $2.4 \pm 0.5 \text{ mmol H}_2\text{O}_2/\text{mg GI tissue}$. These results suggest that the presence of tumors in the GI system is correlated with elevated levels of ROS; however, the same correlation was not observed in brain tissue (Figure-10). In brain tissue, the average level of $\text{H}_2\text{O}_2$ in control fish was $2.4 \pm 0.9 \text{ mmol H}_2\text{O}_2/\text{mg tissue}$. In tumor-bearing fish, the average level of $\text{H}_2\text{O}_2$ was $2.78 \pm 1.02 \text{ mmol H}_2\text{O}_2/\text{mg brain tissue}$.

The increased level of ROS in the GI tissue of tumor-bearing zebrafish is most likely the result of observed tumor formation. Since MCF-7 cells were injected into the body cavity, it was expected to see tumor growth in organs around the site of injection. Tumors were not observed in the brains of zebrafish with MCF-7 xenografts (S. Morrow, personal communication, 2016). Brain tissue was included in this study because it provided another tissue type for comparison. Furthermore, the brain is also a common site of metastasis in breast cancer (ACS, 2015). The lack of tumor metastases to the brain is most likely due to the poor metastatic potential associated with MCF-7 breast cancer cells (Lacroix and Leclercq, 2004). The absence of tumors in the brains of our tumor-bearing fish is consistent with levels of ROS that are not significantly different from control fish.
Figure 9: Average levels of H$_2$O$_2$ in the GI tissue of both tumor and non-tumor bearing zebrafish. H$_2$O$_2$ levels were significantly greater in tumor-bearing fish (p≤0.01). The asterisk denotes significance at 99% confidence (p=0.01, n=9).

It has been shown that treating cancerous cells with hydrogen peroxide prior to injection into mice increased metastasis, even in the weakly-invasive MCF-7 cell line. (Liou and Storz, 2010). This is further evidence that endogenous levels of ROS may contribute to cancer metastasis. To further investigate the role of ROS in tumors, breast cancer cells with a higher metastatic potential should be used. Alternatively, supplemental estrogen or an estrogen-mimicking compound could be given to test organisms. Using either a cell line with a higher metastatic potential or supplementing with estrogen will ensure the presence of more tumors. Further studies using methylmercury should be performed in order to decipher its role in tumor formation and ROS production in vivo.
Figure-10: Average levels of H₂O₂ in the brain tissue of both tumor and non-tumor bearing zebrafish. There was no significant difference in H₂O₂ level between tumor and non-tumor bearing fish (p=0.45, n=9).
Chapter IV—Conclusions

It was hypothesized that the inhibition of TrxR may counteract the metalloestrogenic effects of MeHg in breast cancer cells treated with intermediate MeHg concentrations, and that the production of ROS stimulated by higher MeHg concentrations contributes to cell death. Although measuring TrxR activity in MCF-7 cancer cells and in zebrafish tissue was not successful, methods for measuring TrxR activity and ROS levels were established. The results of this study indicate that ROS levels in tumor-bearing fish, specifically in organs around the site of injection, are increased when compared to non-tumor bearing fish.

The selenocysteine method for determining TrxR activity had never been performed in our lab prior to this study, and will now be the common method used. Additional experiments must be done to perfect the assay to assure success in cell culture and in tissues. It was concluded that no components of the lysis buffer or assay mixture interfered with measuring TrxR activity, so future experiments should focus on adding larger volumes of cell lysate to the assay. Furthermore, protein purification and Western blotting should be done to ensure that the cells do in fact possess the TrxR protein in their cytoplasm. Although it is highly unlikely that the cells do not possess this crucial enzyme, biochemical techniques should be performed to verify the presence of TrxR. Past studies have been able to successfully measure TrxR activity in MCF-7 cancer cells (Gallegos et al., 1997), which indicates that this cell line can be used to measure activity of this enzyme.
The ROS assay using DCFH-DA was perfected and will now serve as the standard for measuring ROS in tissue; however, extending this method to cell culture should be attempted. Results from the ROS assay indicated significantly higher levels of ROS in the GI system of tumor-bearing fish when compared to control fish. This suggests that the presence of tumors in these organs is associated with the generation of ROS. Levels of ROS in the brains of tumor-bearing fish were not significantly different when compared to control fish. This is most likely the result of tumors not metastasizing to the brain after being injected into the body cavity.

The methods developed in this study can be used to better understand the role of MeHg in ER positive breast cancer, specifically how it interacts with TrxR and its role in producing ROS. For future studies, injecting fish with a cell line that has a high metastatic potential would ensure metastasis occurs. Furthermore, introducing estrogen or an estrogen-mimicking compound (such as MeHg) should be given to the organism to ensure better tumor formation and metastasis. Studies using MeHg must be performed in order to decipher its role in tumor formation and ROS production in vivo.
References


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Appendix

Appendix I

Thioredoxin Reductase Assay Using Selenocysteine

1. Turn on UV/VIS spectrometer and software program and wait 15 min to allow the machine to warm up.

2. Set the program to the “KINETICS” method.

3. Click “setup” and set wavelength to 340nm and measurement time to 120s.

4. Blank the machine with 1 ml of buffer in a 1.5 ml cuvette.

5. Click “Time-based measurement” and change the filename.

6. Each assay contains the following solutions, which are to be added in the following order:
   a. 940µl Tris buffer (50mM and 1mM EDTA [pH 7.5])
   b. 20µl NADPH
   c. 20µl cell lysate. Mix well after this addition
   d. 20µl selenocysteine (40mM). Mix gently for about 5 seconds, then place in the holder and click “Start”

7. After each run is complete, record the rate and print the plot

NOTE:

- Before running assays containing cell lysate, run blanks containing buffer, NADPH, and selenocysteine.
Appendix II

a) A time trace from a selenocysteine assay using purified TrxR. The decrease of NADPH was measured over the course of 2 minutes, with the following assay mixture:
   a. 940µl Tris buffer (50mM and 1mM EDTA [pH 7.5])
   b. 20µl NADPH
   c. 20µl pure TrxR.
   d. 20µl selenocysteine (40mM).

\[ \text{Rate (AU/s): } 5.20 \times 10^{-4} \]

b) A time trace from a selenocysteine assay of cell lysate spiked with purified TrxR. As expected, NADPH absorbance decreased over the course of the assay. From this plot, it can be concluded that nothing in the assay was interfering with measuring TrxR activity. The assay mixture contained:
   a. 920µl Tris buffer (50mM and 1mM EDTA [pH 7.5])
   b. 20µl NADPH
   c. 20µl cell lysate
   d. 20µl pure TrxR.
   e. 20µl selenocysteine (40mM).

\[ \text{Rate (AU/s): } 5.25 \times 10^{-4} \]
Appendix III

a) A time trace from a selenocysteine assay of zebrafish tissue extract. The decrease of NADPH was measured over the course of 2 minutes. The assay mixture contained:
   a. 940µl Tris buffer (50mM and 1mM EDTA [pH 7.5])
   b. 20µl NADPH
   c. 20µl zebrafish tissue extract.
   d. 20µl selenocysteine (40mM).

Rate (AU/s): 2.78x10^{-5}

b) A time trace from a selenocysteine assay of MCF-7 cell lysate. The decrease of NADPH was measured over the course of 2 minutes. The assay mixture contained:
   a. 940µl Tris buffer (50mM and 1mM EDTA [pH 7.5])
   b. 20µl NADPH
   c. 20µl cell lysate extract.
   d. 20µl selenocysteine (40mM).

Rate (AU/s): 1.60x10^{-4}
Appendix IV

Spectrum measuring DCF fluorescence in zebrafish tissue. The peak is shown at 525nm on the x-axis.

Key:

Red line: GI tissue
Green line: Brain tissue
Blue line: Blank (no tissue)
Turquoise: GI tissue blank (no DCFH-DA)
Pink: Brain tissue blank (no DCFH-DA)
Appendix V

Standard curve used to determine H$_2$O$_2$ concentration in ROS assays.

![ROS Assay Standard Curve](image)

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Appendix VI

Measured H$_2$O$_2$ levels, expressed as mmol H$_2$O$_2$/mg tissue, in GI and brain tissue of both tumor-bearing (treated) and non-tumor bearing (control) zebrafish.

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