Abstract

Metallothionein (MT) is a low-molecular weight protein with many physiological functions, including contributing to zinc homeostasis and serving as an antioxidant. MT can reduce the efficacy of chemotherapy drugs by inducing drug resistance in cancerous cells. On the other hand, it can also offer protection to normal cells against side effects of cancer treatment (i.e. radiation therapy). Clearly, optimum levels of MT in a tissue-specific manner are important for the ultimate positive outcomes of cancer treatment. Zinc is a potent inducer of MT in various tissues (i.e. peripheral blood cells) and has been linked to resistance to chemotherapy drugs. However, it presently is not known whether zinc supplementation influences the resistance of breast cancer cells to chemotherapy drugs and offers protection to normal cells from chemotherapy drug-induced cytotoxicity. I hypothesized that peripheral blood cells, but not breast cancer cells, will be responsive to zinc supplementation leading to an upregulation of MT synthesis. This increased MT synthesis will then protect peripheral blood cells from chemotherapy drug-induced cytotoxicity by preventing oxidative stress. The overall objective of my thesis research project was to examine the potential role of MT in minimizing the cytotoxic effects of chemotherapy drugs on peripheral blood cells.

Human blood mononuclear THP-1 cells and breast cancer MBA-MB-231 cells were cultured in DMEM for 3 days followed by zinc supplementation using zinc sulfite at 0, 25, 50 or 100 µM for 24 hrs. Zinc supplementation resulted in a dose-dependent increase in total cellular zinc concentration and the abundance of the labile intracellular pool of zinc in THP-1 cells, but not in MDA-MB-231 cells. Similarly, zinc supplementation at 50 and 100 µM also significantly increased the abundance of MT1x and MT2a mRNA in THP-1 cells, while zinc supplementation at 100 µM increased the abundance of MT3a mRNA. In contrast, the abundance of MT1x,
MT2a and MT3a mRNAs was not affected by zinc supplementations in MDA-MB-231. When THP-1 cells were exposed to 2µM etoposide, a chemotherapy drug, for 24 hrs, zinc supplementation at 50 and 100 µM reduced etoposide-induced apoptosis by 15 and 33%, respectively with an inverse correlation between zinc supplementation and etoposide-induced apoptosis ($r^2 = 0.99$). Furthermore, zinc supplementation also significantly reduced caspase-3 and -9 activities and etoposide-induced DNA oxidative damage. The antioxidant properties of MT likely reduced the oxidative stress of etoposide resulting in the inhibition of apoptosis. In human donor peripheral cells, zinc supplementation at 50 and 100 µM caused a significant elevation in CFU and a 25% increase in the IC$_{50}$ compared to the control. Overall, these results suggested that zinc plays a potentially pivotal role in against chemotherapy (i.e. etoposide)-induced cytotoxicity in normal cells.
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Co-Authorship Statement

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INTRODUCTION

Resistance to apoptosis (programmed cell death) is a hallmark of cancerous cells. Most chemotherapeutic drugs exert their anti-cancer activities through the induction of apoptosis by stimulating apoptotic pathways, including initiating DNA-damage or impairing DNA repair. The goal of chemotherapy is to effectively and selectively eliminate cancerous cells, whilst having marginal effects on non-cancerous cells. Cancerous cells are resistant to apoptosis due to mutations in apoptotic regulatory proteins, presence of drug-efflux pumps, and detoxification mechanisms, all of which render the ineffectiveness of chemotherapeutic drugs (Lowe & Lin, 2000; Hickman et al., 2002). On the other hand, chemotherapy drugs cause cytotoxicity to normal cells, which often limit the dosage of chemotherapy drugs thus reducing efficacy of the chemotherapy treatment.

A number of chemotherapy drugs have been shown to have detrimental side effects on gastrointestinal and hematopoietic tissues. Hematopoietic side effects can lead to cytopenia (reduction in the number of blood cells) causing anemia and/or reduced immune function (Du et al., 1990). Due to these complications and resultant hematopoietic side effects arising from chemotherapy drugs and other anti-cancer treatments (i.e. radiation therapy), dosage reductions and/or delays in drug administration may be required in order to maintain patient quality of life (Ferretti et al., 1990). This disrupts the ability of chemotherapy drugs to effectively inhibit the growth of cancerous cells and cancer progression, providing an opportunity for the cancerous cells to further grow and metastasize. Therefore, effective strategies designed to reduce chemotherapy drug-induced cytotoxicity can enhance the effectiveness of chemotherapy and save lives. The co-administration of antioxidants with chemotherapy has been a valued strategy to strengthen crucial cells such as peripheral blood cells to prevent on the onset of hematopoietic
complications during chemotherapy. However, its use in a clinical setting has been limited due to the ineffectiveness and inconsistency of certain antioxidants. There has been a growing support to further profile antioxidants to determine the type of antioxidant and the administration conditions needed to prevent chemotherapy drug-induced cytotoxicity.
CHAPTER 1
Literature Review

1.1 Drug-induced apoptosis

Chemotherapy drugs exert its anti-cancer activity by primarily activating apoptotic pathways to effectively inhibit tumor growth and survival. There are two pathways by which chemotherapy drugs initiate apoptosis; the extrinsic (death receptor) pathway or the intrinsic (mitochondrial) pathway (Kerr et al., 1972). Both pathways employ cysteine aspartyl-specific proteases (caspase) that cleave substrates such as nuclear lamins and poly(ADP)-ribose polymerase that subsequently initiate the execution and degradation of the cell. The extrinsic apoptotic pathway involves death receptors, including Fas, TNFR, DR3, DR4, and DR5 located on the plasma membrane. When these receptors are activated by ligands (i.e., Fas-associating protein with death domain), they trigger the recruitment of intracellular adapter proteins (Taylor et al., 2008). These proteins then activate caspase-8 and 10, the initiator caspase proteins, which, in turn, activate the conversion of procaspase-3 to caspase-3, the executioner caspase, resulting in apoptosis (Salvesen, 2002).

The intrinsic apoptotic pathway involves the release of cytochrome c from the mitochondria to cytosol. Cytochrome c diffuses out of the mitochondria into the cytosol through the mitochondrial pore channels. The opening of these channels is influenced by the ratio of pro-apoptotic (i.e. Bax) and anti-apoptotic (i.e. Bcl-2) proteins, whereby a greater ratio of pro-apoptotic to anti-apoptotic proteins will initiate cytochrome c release from the mitochondria (Appendix 1; Taylor et al., 2008). Upon entering cytosol, cytochrome c activates caspase-9, an initiator caspase protein, which then activates caspase-3, resulting in apoptosis (Salvesen, 2002).
Pro-apoptotic proteins act as a surveillance system, which initiate apoptosis when DNA damage is severe or unrepairable. This surveillance system serves as a defensive mechanism to prevent harmful gene mutations from being integrated into the genome and being passed on to progeny, thereby preventing malignant transformation. At the center of this apoptotic regulation is p53, an essential transcription factor that is activated by DNA damage. Under mild level of activation, p53 can halt cellular proliferation by the upregulation of p21 that triggers G1-S cell cycle arrest, allowing DNA repair to occur (Maddika et al., 2007). However, when DNA damage is more severe, there is a high of activation of p53 that results in the transcription of pro-apoptotic genes, PUMA and NOXA (Maddika et al., 2007). This p53 activation leads to the translocation of Bax, a pro-apoptotic protein, from the nucleus to the mitochondria, which allows for the opening of the mitochondrial pore channels that will ultimately induce apoptosis. However, mutations of these apoptotic genes such as p53 in many cancerous cells prevent appropriate activation of apoptosis and result in the need for a greater stimulus to initiate apoptosis (Chan et al., 2000). This is an important consideration, as cancerous cells typically require a higher dosage of chemotherapy drugs to achieve cell death as compared to non-cancerous cells. Therefore, clinically effective dosages of chemotherapy drugs often cause severe side effects to normal cells.

Necrosis, another form of cell death, is associated with strenuous events such as hypoxia, inflammation and toxic chemicals, and is not a preferred mode of action of chemotherapy drugs. Necrotic death has detrimental effects on the human body as it can stimulate the release of degradation enzymes from lysosomes causing damage to the surrounding tissues. In contrast, apoptotic death is the favored form of cell death since apoptotic cells are phagocytosed by the
immune system, preventing the accumulation of dead cells, consequently allowing replenishment with healthy new cells.

DNA damage is a primary initiator of the intrinsic pathway and is often the mode of action of many chemotherapeutic drugs. These drugs can cause DNA damage by several primary mechanisms. Some of the most effective chemotherapeutic drugs are alkylating drugs (induce integration of N-methylated bases), DNA cross-linking drugs (induce inter- and intra-DNA strand linkages), and topoisomerase inhibitors (prevent DNA from uncoiling during replication) (Roos & Kaina, 2006). Many chemotherapy drugs also have a secondary mode of action, which involves the generation of free radicals that can damage many cellular components to induce apoptosis (Roos & Kaina, 2006).

Chemotherapy drugs can also induce apoptosis indirectly through promoting the generation of free radicals. Free radicals can be defined as atoms or molecules that have an unpaired electron and are chemically highly reactive to biological molecules (Hussain et al., 2003). Free radicals are commonly classified into two categories: (i) reactive oxygen species that consist of hydroxyl radical (\(\cdot OH\)) and superoxide (\(\cdot O_2\)), and (ii) reactive nitrogen species consisting of nitric oxide (\(\cdot NO\)), peroxynitrite (\(\cdot ONOO\)) and nitrous anhydride (\(\cdot N_2O_3\)) (Hussain et al., 2003). These free radicals are formed by a number both enzymatic (i.e. monooxygenase and xanthine oxidase) and non-enzymatic reactions (i.e. Fenton and Haber-Weiss reactions) (Conklin, 2004). Furthermore, cytotoxic drugs can cause an inflammatory response leading to the recruitment of mast cells and leukocytes. These cells release free radicals that can damage neighboring epithelial and stromal cells (Hussain et al., 2003). To some degree, all cytotoxic drugs can potentially induce apoptosis through generating free radical metabolites.
Once generated, free radicals target cellular components such as DNA, proteins and lipids that can cause the induction of apoptosis. For example, damage to DNA repair proteins allows the accumulation of modified (i.e. oxidized) nucleotides that consequently halt cellular proliferation and promote apoptosis (Witkiewicz-Kucharczyk & Bal, 2006). It has been shown that, in many DNA-repair proteins, structures directly involved in the protein-DNA interactions (i.e. zinc-finger motifs) are particularly susceptible to free radicals-induced damage. Damage to these structures compromises functions of these proteins (Witkiewicz-Kucharczyk & Bal, 2006). In addition, free radicals can also cause lipid peroxidation and the formation of reactive by-products, such as malondialdehyde and 4-hydroxynoneal, which induce DNA damage (Hussain et al., 2003). Finally, free radicals can directly induce several types of DNA damage such as strand breakage, base oxidation, and DNA-protein cross-linkage (Roos & Kaina, 2006). One of the most common modified nucleotides from free radical exposure is 8-oxo-7,8-dihydroguanine (8-oxoG), which is a known biomarker for oxidative stress (Wu et al., 2004).

1.2. Chemotherapy-induced oxidative stress and antioxidant supplementation

Protection of hematopoietic progenitor cells from chemotherapy drug-induced cytotoxic exposure is essential to protect patients from side effects during chemotherapy. Bone marrow contains hematopoietic stems cells from which all blood cells are derived, including myeloid (monocytes and macrophages, neutrophils, basophils, eosinophils, erythrocytes, megakaryocytes/platelets, and dendritic cells) and lymphoid lineages (T-cells, B-cells, and NK-cells). The suppression of bone marrow or blood cell lineage activities causes deficiencies in blood cells that can lead to life-threatening diseases. For example, deficiency in leukocytes can
lead to the development of opportunistic bacterial and viral infections, and deficiency in red blood cells causes anemia and reduced oxygen transport (McGuire, 1998).

Newer experimental approaches to address chemotherapy drug-induced bone marrow toxicity during chemotherapy have been developed. Hematopoietic stem cell transplants (HSCT) have been used as a common strategy during chemotherapy to reconstitute normal bone marrow function and eliminate bone marrow infiltrative processes that can lead to immunodeficiency disorders. Bone marrow cell transplantation allows cancer patients to receive higher doses of chemotherapy drug than patients can usually tolerate, allowing for enhanced treatment efficacy (McGuire, 1998). However, HSCT can have major complications and side effects such as infection (i.e. sepsis), veno-occlusive disease, and graft-versus-host disease (Wadleigh et al., 2003; Le Blanc et al., 2004). Another approach is to use an isolated infusion technique to deliver chemotherapy drugs directly to the tumorigenic tissue (i.e. liver or lung), whereby a relatively higher dose of chemotherapy drug is delivered locally to prevent excessive damage to the systemic system (i.e. the immune system; Thompson et al., 1998). However, this approach does not eradicate micrometastases, metastatic cells in bloodstream are known for their role in tumor recurrence, as the chemotherapeutic effects of the drugs is limited to the tumor site and not to the systematic system.

A widely debated, non-invasive strategy in chemotherapy is the co-administration of antioxidants, whose principle action is to strengthen the cellular defense of bone marrow cells against the chemotherapy drug-induced cytotoxicity. Cancer patients typically have depleted serum levels of magnesium, selenium, and zinc, minerals, which are cofactor for enzymes that exhibit antioxidant properties. Levels of these minerals in the circulation are often depleted further during chemotherapy (Ma & Jiang, 1993; Shenberg et al, 1995; Federico et al., 2001).
Furthermore, patients undergoing chemotherapy have a reduction in endogenous plasma antioxidant levels, which can weaken cellular free radical defense system and hence increase systemic cytotoxic response to chemotherapy. Patients who underwent high-dose chemotherapy often had a reduction in antioxidants in general circulation such as vitamin C, vitamin E and β-carotene (Clemens et al., 1990, Meister, 1991).

Administration of antioxidants designed to replenish depleted antioxidant levels is thought to enhance the antioxidant defense system against the reactive cytotoxic drugs and increase the ability of the cells to withstand the cytotoxicity of chemotherapy drugs. A recent systematic review identified sixteen randomized-controlled clinical trials that studied the concurrent use of antioxidants and chemotherapy (Lawenda et al., 2008). The results are inconclusive with respect to the safety and efficacy of antioxidant interventions. A majority of these studies had insufficient power to evaluate endpoints (i.e. survival rates) and no conclusion could be drawn from these studies. Another systemic review of thirty four randomized-controlled clinical trials found that twenty four studies reported evidence of decreased toxicity, nine studies reported no difference and one study reported an increased toxicity with the antioxidant intervention compared to the placebo group (Block et al., 2008). Similar to Lawenda and coworkers’ study (2008), lower statistical power and weak experimental design used to evaluate the end points compromised the significance of these findings.

The common antioxidants used in these studies include both dietary (i.e. ascorbate, β-carotene, and α-tocopherol) and non-dietary (i.e. glutathione, methionine). The assessment of antioxidant behaviors during chemotherapy is difficult due to the variability of antioxidant efficacy between cancerous cells compared to normal cells. The general criteria for antioxidants used in chemotherapy treatments include: (i) non-interference with chemotherapeutic drugs; (ii)
enhancement of the cytotoxic effects of the drugs in cancerous cells; (iii) protection of normal cells against chemotherapy toxicities; and, (iv) increased patient survival (Lawenda et al., 2008). Our knowledge in this area is severely limited. Further research is greatly needed to profile antioxidants by antioxidant effect (i.e. free-radical scavenging), cellular actions (i.e. cell signaling, gene expression), differential bioavailability, distribution, metabolism, and potency, to address potential usages in a chemotherapy setting (Lawenda et al., 2008).

In addition to free radical scavenging properties, some antioxidants have a unique ability to directly quench chemotherapy drugs and prevent cytotoxic effects on the human body. Cysteine-rich proteins have a strong reducing capacity and nucleophilic strength (tendency to donate electrons) due to the sulfhydryl group (-SH) of cysteine. This nucleophilic property is beneficial for sequestering electrophilic compounds (tendency to accept electrons), which are primarily alkylating and platinum-based drugs used in chemotherapy. Glutathione (GSH), N-acetyl cysteine (NAC) and metallothionein (MT) are cysteine-rich compounds that have strong antioxidant capacity, and as such are potentially valuable as additive agents to chemotherapeutic regimes due to the above described mechanisms.

Due to the potential effectiveness of MT as a co-administered antioxidant in chemotherapy, the remainder of the literature review will focus specifically on the mechanism and potential benefits of the addition of MT to cytotoxic drugs. I will discuss in-depth the specific properties of MT that make it an ideal candidate for antioxidant support during chemotherapy. Also, I will discuss the administration of zinc, a potent inducer of MT, and the role it has in drug tolerance.
1.3 Metallothionein

1.3.1 Structure

MT has gained much attention in molecular research due to its diverse biological functions. MT plays an important role in copper and zinc homeostasis and exhibits protective roles against radiation and oxidative stress. It is also involved in the detoxification of heavy metals such as cadmium and is implicated in biochemical functions such as cellular proliferation and apoptosis (Davis & Cousins, 2000).

MT is a cysteine-rich, low molecular weight (6-7 kDa) protein that can bind up to seven zinc ions in two domains: the α and β domains, to form a stable molecule (Figure 1). The apo-protein part of the molecule is known as thionein (T_R). Sulfur has a unique metal binding affinity for Zn^{2+}, Fe^{2+}, Cd^{2+}, and Cu^{2+}, and is able to accommodate multiple bonds; however, Zn^{2+} is one of the predominant metals bound to MT under physiological conditions (Braun et al., 1992). The 28 intramolecular zinc-sulfur bonds are responsible for the formation of α and β domains in MT as zinc ions can bridge the multiple cysteine ligands into clusters (α domain: Zn_4Cys_{11}; β domain: Zn_3Cys_9; Figure 1). This clustered conformation is also implicated in the zinc-transferring properties inherent in both MT domains (Jiang et al., 2000).

MT has four isoforms, all of which contain 20 cysteine residues, yet differ in their total amino acid composition (Bell & Vallee, 2009). The difference in amino acid composition may account for functional diversity among the MT isoforms. MT-1 and -2 are expressed in all organs, whereas MT-3 and -4 are only expressed in certain tissues or diseased states. The expression of MT-1 and -2 is regulated at the transcription level and their expressions are responsive to metals (i.e. zinc), glucocorticoids, cytokines and a variety of chemical signals. The
**Figure 1.1: Metallothionein structure** (A) Cysteine amino acid structure. The side chain residue of cysteine is sulfhydryl group (-SH). (B) MT structure. MT is composed on 20 cysteine residues, in which, the sulfur groups are bound to zinc ions to form the α and β domains (Bell & Vallee, 2009).
genes of these isoforms contain metal responsive element (MRE) and glucocorticoid responsive element (GRE) in their promoter regions and are the principle regulatory site of MT transcription. MRE, in turn, is regulated by metal-responsive transcription factor-1 (MTF-1) that is activated by the presence of zinc ions. MTF-1 contains six zinc-finger motifs (the DNA binding domain); of these six potential zinc-binding sites, four to five are bound to zinc under normal physiological conditions (Davis & Cousins, 2000). The remaining zinc-binding sites are usually unoccupied. Under the conditions where zinc concentration exceeds the normal physiological zinc concentrations, the excess zinc ion(s) occupy the vacant zinc-finger site. The binding of zinc to these sites in the MTF-1 enables the formation of the proper structural conformation of the motif and the subsequent translocation from the cytosol to the nucleus. Once entering the nucleus, MTF-1 binds to the MRE regions on the MT gene to initial transcription (Heuchel et al., 1994). MT-3 and -4 lack the MRE regions and are therefore relatively unresponsive to heavy metals.

The upregulation of MT genes results in the increased synthesis of \( T_R \). \( T_R \) is structurally unstable due to the susceptibility to undergo proteolysis; however, upon binding to zinc ion (\( \text{Zn}^{2+} \)) to form MT, it is resistant to proteolysis and thus stable (Feldman et al., 1978). The precise mechanism for the stability differences is not known; however, it is postulated that the metal (i.e. \( \text{Zn}^{2+} \)) binding to \( T_R \) stabilizes the MT against the degradation from soluble proteases (Feldman et al., 1978). Importantly, since “free” zinc can potentially induce cytotoxicity, the zinc-sensing MTF-1 triggered increase in \( T_R \) synthesis prevents zinc-induced cytotoxicity by lowering cellular zinc ion levels. Thus, \( T_R \) is important to cellular zinc homeostasis.
1.3.2 Redox activity

The sulfur residue of the cysteine has important redox activities in cysteine-rich proteins such as GSH and MT. Sulfur compounds have multiple oxidation states that correspond to numerous redox forms, where thiol (\(-\text{SH}\)) and disulfide (\(-\text{SS}-\)) groups are the common forms of sulfur in biological tissues. Under oxidative conditions, the number of disulfide forms increases (Feng et al., 2006). Due to the multiple transition states of sulfur, there are three primary forms of MT: (i) metal-bound MT (\(-\text{S-Zn}\)), (ii) apo-MT (thionein, \(T_{R}; -\text{SH}\)), and oxidized MT (thionin, \(T_{O}; -\text{SS}-\)) (Bell & Vallee, 2009). The strong free radical scavenging properties of MT is presumably due to the close proximity among the 20 cysteine residues in MT allowing for the interchangeability between thiol and disulfide forms. Furthermore, the sulfur residues of the cysteine ligands can be oxidized by numerous types of free radicals including hydrogen peroxide, superoxide, hypochlorous acid and nitric oxide (Fliss & Menard, 1992; Kroncke et al., 1994). Cells transfected with MT-1 had a 6-fold increase in resistance to tert-butyl hydroperoxide (a reactive oxygen species), and 4-fold increase in resistance to S-nitroso-N-acetylpenicillamine (a reactive nitrogen species) -induced cytotoxicity (Schwarz et al., 1994; Schwarz et al., 1995).

Available evidence has shown that MT has a higher antioxidant capacity than GSH, a well-recognized cysteine-rich antioxidant. MT is up to 800-fold more potent compared to GSH in sequestering hydroxyl radicals in isolated rats livers (Abel & Ruiter, 1989) and 10-fold more effective than GSH in prevention of lipid peroxidation induced by adriamycin, a chemotherapy drug, in liver microsomes from male Wistar strain rats (Miura et al., 1997). It is not clear as why MT is a more potent antioxidant than GSH; however, the stronger free radical scavenging
properties of MT could be attributed to its structure, specifically the α- and β-domains. The zinc-sulfur residues are capable of donating electrons to free radicals, which, in turn, is converted to stable free radical metabolite while the zinc-sulfur residues in MT form disulfide bonds. The exact mechanism of the disulfide bond formation is presently unknown. It has been found that the intra-, but not the inter-molecular disulfide bonds occur in the α- and β-domains of MT (Romero-Isart & Vasak, 2002). In contrast, GSH forms inter-molecular disulfide bonds with other GSH molecule under oxidative stress (Brennan et al., 2006). The intra-molecular disulfide bonds likely have an enhanced efficiency in disulfide bond formation due to the close proximity of the cysteine residues as opposed to the less efficient inter-molecular disulfide bond formation.

The transitional states of MT have important implications under oxidative conditions. Oxidative stress causes a decrease in $T_R/T_O$ ratio with a subtle decrease in the amount of MT, which likely arises from the oxidation of either MT or $T_R$, both free radical scavenger forms of MT, causing the formation of disulfide bonds and accumulation of $T_O$ (Krezel et al., 2007). The formation of disulfide bond also causes the release of zinc ions from the zinc-sulfur residues. Human ovarian cells transfected with ras-oncogene, a gene involved in oxidative stress, results in an elevated of labile intracellular zinc levels and a lower $T_R/T_O$ ratio compared to the non-transfected cells (Krezel et al., 2007). Furthermore, oxidative redox potential in different cellular states of human colon cancer cells has shown to be correlated with a decrease in $T_R$ and an increase in $T_O$ (Krezel & Maret, 2006).

The redox cycle (MT/$T_R/T_O$) involves the thiol/disulfide exchange with certain redox compounds such as GSH/GSSG and selenocystamine. GSH is capable to reduce $T_O$ back to $T_R$ while it is oxidized to GSSG; however, GSH is not a very efficient reducing agent even at high concentrations. The catalytic properties of selenium-compounds (i.e. selenocystamine) can
improve the redox cycle efficiency of GSH/GSSH and MT (Chen & Maret, 2001).

Theoretically, the GSH/GSSG redox couple also works in the reverse direction as GSSG can oxidize $T_R$ to $T_O$; however, this reverse conversion is physiologically not common due the high ratio (100:1) of GSH/GSSG (Kang, 2006).

**1.3.3 Zinc transfer**

MT facilitates the transfer of zinc ions to other zinc-requiring proteins, initiating their activity and thus influencing various cellular processes. Zinc-requiring proteins include zinc metalloenzymes (i.e. alkaline phosphatase and mitochondrial aconitase), and zinc-finger transcription factors (i.e. TFIIIA and SP1) (Maret, 2004; Feng et al., 2005; Krezel et al., 2007). On the other hand, the transfer of zinc from MT to zinc donor proteins can also inhibit the activity of some enzymes including caspase-3 and fructose 1,6-diphosphatase (Perry et al., 1997). The transfer of zinc ions requires a direct interaction between MT and the corresponding zinc acceptor, as the absence of MT in cells prevents the transfer of zinc ions to zinc-donor proteins (Maret, 2004). However, the precise mechanism for zinc transfer from MT to zinc acceptors is puzzling due to the high-binding affinity of MT for zinc ions. In theory, zinc transfer is expected to occur from a protein with lower zinc binding constant to one with higher zinc binding constant. The binding constant of zinc to MT is 1,000 times greater than other known zinc-requiring proteins, indicating that MT should not transfer zinc ions to other proteins (Jacob et al., 1998). It seems that certain agents and cellular states can facilitate the transfer of zinc from MT to its acceptor by the modification of the thiol (sulfur-residue) groups in MT. For example, the oxidation of the thiol groups of MT result in the formation of disulfide bonds and
the consequent release of zinc ions that then are transferred to other zinc-requiring proteins (Jacob et al., 1998). There has been other evidence to support that MT acts as a zinc chaperone to deliver zinc to zinc-requiring processes on demand. Specifically, MT has been found to translocate to the mitochondria and the nucleus, with subsequent effects on cellular respiration (Ye et al., 2001), gene regulation (Cherian & Apostolova, 2000) and cell proliferation (Apostolova & Cherian, 2000).

1.3.4 Involvement of metallothionein in drug resistance

Elevated MT levels have been observed in a wide variety of cancers, including breast, prostate, ovary and lung cancer, and an elevated MT level appears to be a marker of poor prognosis in breast carcinomas (Vazquez-Ramirez et al., 2000; Zhang et al., 2000). Furthermore, elevated MT expression has been associated with the resistance of tamoxifen, which is the first line chemotherapy drug for estrogen-positive breast cancer, in patients with primary invasive ductal breast cancers (Surowiak et al., 2003). This line of evidence suggests a possible association between MT and drug resistance. Furthermore, subcellular MT distribution also appears to be altered in breast cancer patients with a three-fold increase in nuclear MT level and two-fold increase in cytosolic MT level (Florianczyk & Grzybowska, 2000). The cytosolic MT is thought to quench free radicals to prevent oxidative stress, whereas the nuclear MT is thought to traffic zinc from cytosol to the nucleus to regulate cell proliferation and cell cycle (Apostolova & Cherian, 2000; Cherian & Apostolova, 2000).

MT can directly decrease the effectiveness of drugs by binding to the molecules and quenching free radicals to prevent these highly reactive compounds to reach their final drug
target sites (i.e. the nucleus) (Doz et al., 1993). MT has a high binding affinity to certain classes of drugs due to the sulfhydryl-rich residues. MT acts as a neutrophile that can neutralize electrophilic chemotherapy drugs including alkylating and platinum (Pt)-based drugs. Electrophilic compounds can replace the zinc and bind to the thiol residue to form a MT-drug complex, resulting in the retention and neutralization of the electrophilic compound in the MT molecule. Mass spectrophotometry analysis indicates that Pt compounds are sequestered by MT-2 to form numerous MT-Pt derivatives, which cause the displacement and release of zinc from MT in exchange for Pt ion (Knipp et al., 2007). Evidence suggests that hepatocellular carcinomas patients who have higher levels of MT do not response to platinum-based chemotherapy drugs such as cisplatin and carboplatin (Endo et al., 2004). There may be other potential chemotherapy drugs that have yet to be investigated in regards to their binding affinity for MT.

Along with the interaction between MT and reactive compounds, MT can also quench free radicals generated by chemotherapy drugs that subsequently induce apoptosis. Free radicals, by definition, have an “unpaired” electron and are, therefore, chemically reactive to cellular components in cells. Zinc/thiolate sites in MT can be oxidized by free radicals as the sulfhydryl residues donate their electrons to the free radical, in order for the free radical to obtain “paired” electron configuration. As a result, the free radical metabolite becomes chemically stable and thus non-reactive. The oxidation of the zinc/thiolate causes the release of zinc ions into the cytosol as discussed earlier. The release of the zinc ion from MT, either as a result of the direct binding of reactive compounds to MT or the oxidation by free radicals, can cause significant fluctuations in the labile intracellular zinc levels. The labile intracellular zinc levels in a cell are normally tightly controlled, typically within a range from 0.1-0.5 nM, depending on the type and
state of the cell (Atar et al., 1995; Ayaz & Turan, 2006). However, the oxidation of MT can cause a raise in intracellular zinc levels. Oxidative treatment in epithelial colon mucosa cells causes a 3-5 nM increase in the labile intracellular zinc levels compared to the control levels (Cima et al., 2006). The increase in the labile intracellular zinc levels can be sensed by MTF-1, which then binds to these MREs in the promoter regions of MT gene to causes an elevation in transcription activity. Increase in MT synthesis, in turn, represents a higher zinc buffering capacity, which leads to normalization of the labile intracellular zinc levels. Besides MT, several other genes have also been identified to contain MREs (Table 1). Since stressful situations such as electric stimulation, diabetes, and oxidative stress result in fluctuation in intracellular zinc levels (Atar et al., 1995; Ayaz & Turan, 2006; Cima et al., 2006), it should not be surprising that MREs are typically found on stress-response genes (Table 1.1).

It has also been speculated that stress-response proteins such as p53 and NF-κB are influenced by fluctuations in intracellular zinc levels and MT. p53 is a zinc-finger transcription factor that has prominent roles in the regulation of apoptosis, cell cycle, cell proliferation and angiogenesis (Maddika et al., 2007). p53 is mutated in a diverse types of cancer including colon, lung and breast and is associated with drug resistance by preventing the initiation of apoptosis (Chan et al., 2000). The zinc-finger domain of p53 undergoes conformation changes under zinc-deficient conditions preventing p53 to bind to target DNA domains to regulate transcription level of various genes including apoptotic genes (Fan & Cherian, 2002). The distorted conformation of the zinc-finger motif behaves similar to the mutated p53 form found in tumors. However, under high zinc-supplementation conditions, it is postulated that elevated transcription levels of TR can compete for the zinc ions in zinc finger motif to regulate the folding of zinc metalloproteins such as p53 (Notta & Koroparnick, 2006). This is supported by the strong
Table 1.1: MRE sequences found in stress-response genes

<table>
<thead>
<tr>
<th>Gene</th>
<th>Function</th>
<th>Number of MRE</th>
<th>Binding of MTF-1</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>MT-1/2</td>
<td>Zinc storage/homeostasis</td>
<td>6</td>
<td>++++</td>
<td>Heuchel et al., 1994</td>
</tr>
<tr>
<td>γ-GSH</td>
<td>Glutathione synthesis</td>
<td>1</td>
<td>+/+/-</td>
<td>Gunes et al., 1998</td>
</tr>
<tr>
<td>γ-GST</td>
<td>Glutathione metabolism</td>
<td>3</td>
<td>n/a</td>
<td>Gunes et al., 1998</td>
</tr>
<tr>
<td>AFP</td>
<td>Free radical scavenger</td>
<td>5</td>
<td>+++</td>
<td>Lichtlen et al., 2001</td>
</tr>
<tr>
<td>Tear lipocalin</td>
<td>Inhibits lipid peroxidation</td>
<td>5</td>
<td>+++</td>
<td>Lichtlen et al., 2001</td>
</tr>
<tr>
<td>C/EBPα</td>
<td>Inflammatory response</td>
<td>5</td>
<td>+++</td>
<td>Lichtlen et al., 2001</td>
</tr>
</tbody>
</table>

*a* γ-GSH = γ-glutamyl-cysteine synthetase; γ-GST = γ-glutamyltranspeptidase;  
*b*Binding affinity of MRE regions on target genes to MTF-1

NF-κB is transcription a factor that regulates genes involved in cellular immunity response, growth, and apoptosis. NF-κB functions as a pro- or anti-apoptotic regulator depending on the nature of apoptotic stimulus; however, it is generally considered to be more anti-apoptotic under oxidative conditions (Kaltschmidt et al., 2000). Overexpression of NF-κB in tumor cells induces drug resistance to chemotherapy drugs (Karin et al., 2002). The anti-apoptotic function of NF-κB is mediated through regulating the transcription of anti-apoptotic genes such as TRAF-1 and -2, Bcl-2, XIAP, and cIAP-1 and -2 (Wang et al., 1999). NF-κB has two main subunits: the p50 and p65, which form a heterodimer that is localized to the nucleus to transcribe target genes. However, under normal physiological states, the nuclear localization signal on the p50/p65 heterodimer is inhibited by IκB, which retains p50/p65 in its inactive state in the cytosol. IκB is degraded by a signal-induced IκB kinase that phosphorylates IκB to undergo ubiquitination, thus allowing p50/p65 to translocate to the nucleus to activate its target genes. Several studies have found that MT influences NF-κB to protect against apoptosis (Fanzo et al., 2002; Notta & Koroparnick, 2006; Uzzo et al., 2006). MT-1 and -2 knockout fibroblastic cells have a reduced level of p65 subunit with no effect in p50 subunit or IκB levels, resulting in a >50% reduction in NF-κB DNA binding affinity to target oligonucleotides. This enhances the sensitivity of the cells to tert-butylhydro-peroxide (a free radical generator) -induced apoptosis compared to the MT-wildtype cells. Furthermore, zinc supplementation and, presumably, the upregulation of MT in human aortic endothelial cells has been reported to increase the expression of Bcl-2, an anti-apoptotic mitochondrial protein, which is activated by the presence of NF-κB (Fanzo et al., 2002). However, there is also evidence to support that zinc and MT can
negatively influence NF-κB as zinc inhibits NF-κB DNA binding activity in a dose-dependent manner (Kim et al., 2003, Uzzo et al., 2006). Further, zinc supplementation increases IkB activity, which subsequently prevents the nuclear localization of NF-κB (Uzzo et al., 2006). The use of different cell types and cellular states between these studies would suggest the possible reason for the discrepancy in these findings.

1.3.5 Metallothionein and chemotherapy drug-induced cytotoxicity

There are numerous reports suggesting MT has a protective role against chemotherapy drug-induced cytotoxicity in normal cells (Roosen et al., 1994; Gogu & Agrawal, 1996; Satoh et al., 2000); however, the mode of action and tissue-specific MT regulation in response to chemotherapy is far from clear. An ideal chemotherapy strategy would be to have minimum cytotoxic effects to normal cells through enhancing protective capacity of normal cells, while imposing maximum cytotoxicity on cancerous cells. The relationship between the cytotoxic effects of a chemotherapy drug on normal cells and cancerous cells can be described by a therapeutic index, which is defined as the ratio of the amount of a therapeutic agent that is able to cause a therapeutic effect to the amount that causes cytotoxic side effects (Becker, 2007).

Possible strategies aimed at improving therapeutic index would be: (i) to reduce MT levels in cancerous cells to increase the ability of chemotherapy drug to cause apoptosis or (ii) to induce MT synthesis by nontoxic metals (i.e. zinc) in normal cells to increase resistance to apoptosis during cytotoxic exposure (Cherian et al., 2003). Chemotherapy drugs are frequently toxic to highly dividing cells, such as cancerous cells, bone marrow, intestinal epithelium, and hair follicles due to the high turnover rate in these cell populations (Cherian et al., 2003). In contrast,
resting tissues such as skeletal muscle are less susceptible to chemotherapy drug-induced cytotoxicity. The cytotoxicity of chemotherapy drugs to peripheral blood cells can cause detrimental effects to the body and lower the limit of the tolerable dose of chemotherapy drugs. Thus, increase in MT level in peripheral blood cells can improve the survival of these cells so that a higher dosage of chemotherapy drugs can be used. Since various metals induce MT synthesis, it is critical to identify an MT inducer that is selective for peripheral blood cells and not to tumor cells. It is well established that zinc is a potent inducer of MT synthesis. Thus, zinc supplementation might be able to selectively upregulate MT in peripheral blood cells while having little response in tumor cells. The selective upregulation of MT in peripheral blood cells would protect against the cytotoxicity of chemotherapy drugs on peripheral blood cells, while not influencing the effect of chemotherapy drugs on the tumor site. This would prevent complications such as delays and/or reduction in drug dosage in chemotherapy administration.

1.4 Zinc

1.4.1 Zinc homeostasis

Zinc is an essential trace element to humans and plays an important role in a wide range of physiological functions, including regulating cell signaling, second messenger metabolism (i.e., kinase activity) and phosphatase activity, etc. (Kambe et al., 2004). Thus, it should not be surprising that intracellular zinc levels are under tight homeostatic regulation, which requires the interplay among ZIPs, a family of transporters involved in zinc influx, ZnTs, a family of transporters involved in zinc efflux, and zinc-binding proteins such as MT (Cousins et al., 2003). The expression of zinc transporters is altered in certain disease states such as breast cancer
resulting in the alteration in intracellular zinc levels to promote tumor malignancy (Franklin et al., 2005). The levels of zinc in serum and malignant tissues are altered in some types of carcinomas. Zinc levels in serum and carcinomas of the liver, gallbladder, digestive tract and prostate tend to be reduced (Chakravarty et al., 1986; Gupta et al., 2005; Costello & Franklin, 2006), whereas in breast carcinomas, zinc levels in serum and malignant tissues are decreased and elevated zinc level, respectively (Schwartz et al., 1974; Margalioth et al., 1983). It is postulated that the changes in serum and malignant tissue zinc levels are due to alterations in the expression of zinc transporter genes such as ZIPs. For example, in prostate cancer, ZIP1 expression is suppressed leading to a lower intracellular zinc levels (Franklin et al., 2005; Costello & Franklin, 2006), whereas ZIP10 has been shown to contribute to the metastasis of breast cancer to the lymph node (Kagara et al., 2007).

While the basal expression of zinc transporters in carcinomas is important to understand possible role of zinc transporters on tumor malignancy, it is not certain how these types of cells respond to zinc supplementation. There is little known about the responsiveness of cancerous tissue to varying zinc status; however, zinc supplementation and its regulation of MT levels have been studied extensively in peripheral blood cells, particularly, blood monocytes and mononuclear cells. Zinc supplementation (15 mg/day) in young males for 10 days caused a rapid upregulation of MT synthesis in blood monocytes and mononuclear cells, but erythrocytes were less responsive (Cao & Cousins et al., 2000). Global gene screening of zinc transporters in THP-1 cells, a blood monocyte cell line that responds similarly to human mononuclear cells, indicates that only ZnT1 and MT was significantly upregulated (3-fold and 13-fold, respectively), whereas the other seven zinc transporters expressed in THP-1 cells were not affected by zinc supplementation (Cousins et al., 2003). This may suggest that THP-1 cells have a weak zinc
homeostasis mechanism due to the lack of responsiveness of other zinc transporters and that the regulation of zinc transporters are unable to solely maintain labile intracellular zinc levels. It is likely that the increase in labile intracellular zinc levels caused by zinc supplementation, resulted in the dramatic upregulation of MT levels to bind to labile intracellular zinc levels in an attempt to maintain physiological labile intracellular zinc levels.

Alterations in extracellular zinc levels caused from zinc supplementation or zinc deficiency conditions can regulate the transcriptional activation and post-transcriptional modifications of zinc transporters to modulate intracellular zinc levels. The expressions of ZnT transporters, involved in the control of zinc efflux, are mainly regulated at the transcription level by MTF-1. ZnT transporters contain MRE sequences and the increase in intracellular zinc levels caused MTF-1 to activate the transcriptional of ZnT transporters genes. Similarly, under high intracellular zinc levels, MTF-1 can also promote MT synthesis through binding to the MREs in MT genes. This to accommodates the increased need to control the level of labile intracellular zinc to prevent zinc-induced cytotoxicity.

The regulation of ZIP transporter gene expression is also not well understood, although available evidence suggests that extracellular levels of zinc influences cellular metabolism of ZIP transporters. Increased extracellular zinc level through zinc supplementation promotes ZIP4 endocytosis, resulting in a decreased number of ZIP4 in the plasma membrane (Kim et al., 2004). Since ZIP transporters, such as ZIP1, ZIP2 and ZIP4, are mainly involved in zinc influx across the plasma membrane, a reduction in the abundance of ZIP transporters from the plasma membrane would reduce the influx of zinc ions caused from the increase in extracellular zinc levels. Peripheral blood cells lack the basal expression of ZIP4 gene, which may be one of the factors causing the accumulation of zinc under high intracellular zinc levels (Aydemir et al.,
Expression and regulation of zinc transporter genes in cancerous cells are essentially unknown. Since cancerous cells are known for their higher rate of proliferation and zinc is essential for cell proliferation and growth, it is possible that the expression of zinc transporter genes are altered to ensure sufficiently high level of intracellular zinc in order to sustain their higher rate of cell proliferation and growth.

1.4.2 Zinc, antioxidant properties in cancer

Serum zinc level in patients with malignant tumors such as head-and-neck, prostate and hepatocellular carcinomas are lower than normal (Brys et al., 1997; Liaw et al., 1997; Doerr et al., 1998; Guven et al., 1999). Chronic zinc deficiency causes the elevation of lipid peroxidation that can increase the susceptibility of cells to injury induced by oxidative stress (Stefanidou et al., 2006). It is postulated that low serum zinc level in cancer patients may contribute to a compromised antioxidant defense in peripheral blood cells since zinc has a diverse function in the catalytic properties of over 300 enzymes and a structural component of many proteins (Ames, 1998; Mocchegiani et al., 2000; Prasad & Kucuk, 2002). Based on the current evidence regarding the role of zinc in antioxidant function, there are two primary antioxidant mechanism that zinc influences: (i) the activity of copper-zinc superoxide dimutase (CuZnSOD) and (ii) the maintenance of MT levels.

Patients with breast carcinomas have reduced activities of CuZnSOD with an elevation in lipid peroxidation in peripheral blood cells (Kasapovic et al., 2008). Since, zinc is a structural component of CuZnSOD, low serum zinc levels are thought to reduce the activity of CuZnSOD (Tapiero & Tew, 2003). It has been shown that patients with hepatocellular carcinomas had a
reduction in zinc serum levels that was correlated to a reduction in CuZnSOD activities (Guven et al., 1999). However, there are some report inconsistencies regarding the connection between zinc and CuZnSOD, as zinc deficiency has been shown to slightly elevated CuZnSOD (Ho & Ames, 2002; Ho et al., 2003). It is suggested that zinc deficiency causes oxidative stress that results in a response to upregulate antioxidant enzymes to combat the accumulation of free radicals (Ho & Ames, 2002).

The reduction in MT levels caused by low serum zinc levels in peripheral blood cells has also been found to influence the antioxidant capacity of these cells (Cao & Cousins, 2000). Since MT has a predominant role in quenching free radicals, the decline of MT levels can increase the susceptibility to oxidative stress resulting in compromised function of peripheral blood cells in cancer patients. However, zinc supplementations in patients with head and neck cancers who are in mild zinc deficiency do not replenish erythrocytes antioxidant levels, including CuZnSOD, catalase, and glutathione peroxidase, compared to the placebo group (Ertekin et al., 2004a). This zinc supplementation group, conversely, exhibited improved recovery from radiation in a follow-up study (Ertekin et al., 2004b). This evidence suggests that zinc supplementation is unable to strengthen the depleted antioxidant enzymes, but could potentially compensate for the lack of antioxidant capacity by regulating other cellular defenses mechanisms, presumably MT levels.

1.4.3 Zinc, metallothionein and drug resistance

Zinc can induce MT synthesis in the heart, liver, bone marrow, and bone marrow progenitor cells in the blood, as well as in cancerous cells. Bone marrow progenitor cells such as
Peripheral blood mononuclear cells (PBMC) are considered the most responsive to zinc-induced MT synthesis (Cao & Cousins, 2000; Sullivan et al., 1998; Doz et al., 1993). However, the responsiveness of MT synthesis to zinc supplementation and consequently drug resistance appeared to vary from tissue to tissue. There is an apparent association between the responsiveness of tissue-specific upregulation of MT synthesis to MT inducers with prevention of chemotherapy drug-induced cytotoxicity. Satoh et al. (2000) profiled three types of metal MT inducers (i.e. zinc, copper, and bismuth) and analyzed the tissues responsive to the MT inducers using mice xenografted in colon carcinoma cells. These researchers then investigated if there are alterations in the efficacy (i.e. reductions in tumor size) and side-effects (i.e. bone marrow toxicity and cardiotoxicity) of adriamycin, a chemotherapy drug, in relation to tissue-specific regulation of MT levels. Zinc supplementation results in a significant elevation of MT synthesis in the bone marrow, heart, kidney, liver and even the cancerous cells (Satoh et al., 2000). Zinc pretreatment, followed by adriamycin administration, resulted in a complete reduction in adriamycin-induced cardiotoxicity and bone marrow toxicity; however, it has no effect on tumor size (Satoh et al., 2000). Interestingly, bismuth administration exerts an ideal MT tissue distribution, in which, MT synthesis is significantly elevated in the bone marrow, heart, kidney and liver, but remains unaffected in the tumor cells. This tissue specific induction in MT synthesis is associated with a decrease in adriamycin-induced cardiotoxicity and bone marrow toxicity, which are similar to the effects observed with zinc supplementation, but causes a complete reduction in tumor size compared to the control. These observations showed that those tissues that are responsive to MT inducers are resistant to adriamycin-induced cytotoxicity. In addition, the type of MT inducer is also crucial for selective upregulation in MT synthesis in normal cells compared to cancerous cells. The responsiveness of different types of cancerous
cells to zinc-induced MT synthesis is presently unclear; however, it is reasonable to speculate that the responsiveness to zinc administration is likely differ among different types of cancerous cells since zinc homeostasis apparently varies with tissues and type of carcinomas (Margalioth et al., 1983; Costello & Franklin, 2006). Further research is needed to profile the zinc responsive of MT other types of carcinomas.

There is some evidence to support that zinc administration offers a differential chemoprotection between normal cells and cancerous cells. Roosen et al. (1994) pretreated glioma cells (a brain tumour cells) and peripheral blood cells with zinc sulfate, followed by treatment of carmustine, a drug used in the treatment of brain cancers. Zinc pretreatment reduces the carmustine-induced cytotoxicity in peripheral blood cells, while it has no effect on the drug tolerance in glioma cells (Roosen et al., 1994). Based on the current literature, it appears that zinc pretreatment has a protective effect on hematopoietic progenitor cells from cytotoxic drugs or radiation (Roosen et al., 1994; Gogu & Agrawal, 1996; Satoh et al., 2000; Ertekin et al., 2004c); however, it is still debatable whether zinc supplementation causes drug resistance in cancerous cells.

Recently, zinc supplementation has been introduced in clinical settings to reduce the cytotoxicity of aggressive therapies such as radiotherapy. Oral zinc sulfate supplementation (150 mg/day) has been shown to prevent radiation-induced oropharyngeal mucositis in patients with head-and-neck cancers (Ertekin et al., 2004b). Conventional radiotherapy, especially high-dose radiotherapy, can cause oral mucositis, a radiation-induced inflammation and ulceration of the oral epithelial cells. Oral mucositis can lead to a reduction in food intake and increase susceptibility to infections that can eventually limit the dose and/or delays administration of anti-cancer treatment (i.e. radiotherapy or chemotherapy), which can compromise the efficacy of the
treatment. Patients administered zinc sulfate showed a significant reduction in the degree of oral mucositis and delay in onset of oral mucositis compared to a placebo group (Ertekin et al., 2004b). Although the impact of zinc on cancerous growth was not measured, those patients treated with zinc sulfate were able to tolerate a higher radiotherapy dose compared to the placebo group, potentially leading to a more effective treatment against head-and-neck cancers. Others report similar findings, with no exacerbation of chemotherapy-induced side effects (i.e. weight loss and low white blood cell count) in response to zinc supplementation (Lin et al., 2006; Lin et al., 2007). Lin et al. (2007) performed a three-year follow-up on patients with head-and-neck carcinoma and found that the zinc supplementation group has a better three-year local-free survival rate. Researchers also found that patients in the experimental group with stages III-IV disease has a much better three-year survival rates when they received concurrent chemoradiotherapy. These studies are indicative of the clinical implications of zinc supplementation during radiotherapy and/or chemotherapy, which can be effective in protecting cells with high turnover rates.

In summary, MT is a low-molecular weight protein with many known physiological functions, including contributing to zinc homeostasis and as an antioxidant. MT can reduce the efficacy of chemotherapy drugs by inducing drug resistance in cancerous cells. On the other hand, it can also offer protection to normal cells against side effects of cancer treatment (i.e. radiation therapy). Clearly, optimum levels of MT in a tissue-specific manner are important to the ultimate outcomes of cancer treatment. Zinc is a potent inducer of MT in a various tissues and has been shown to lead to drug resistance to chemotherapy drugs. However, it presently is not known whether zinc supplementation influences the resistance of breast cancer cells to chemotherapy drugs and offers protection to normal cells from drug-induced cytotoxicity.
1.5. Hypothesis

The hypothesis for my thesis research project is that peripheral blood cells, but not breast cancer cells, will be responsive to zinc supplementation leading to an upregulation of MT synthesis. This increased MT synthesis will then protect peripheral blood cells from chemotherapy drug-induced cytotoxicity by preventing oxidative stress.

1.6. Overall Objective and Specific Aims

The overall objective of my thesis research project was to examine the potential role of MT in minimizing the cytotoxic effects of chemotherapy drugs on peripheral blood cells. The specific aims were:

1) To establish the responsiveness of blood mononuclear cell line (THP-1) and breast cancer cell line (MBA-MB-231) to zinc supplementation;

2) To determine whether zinc supplementation increased drug tolerance in THP-1 cells to etoposide, a chemotherapy drug, and to explore the possible cellular defense mechanisms involved; and

3) To explore the effect of zinc supplementation to influence the etoposide-induced cytotoxicity in human donor peripheral cells.
1.7. References


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CHAPTER 2
Protection of Zinc against Etoposide-induced Hematopoietic Toxicity

2.1 Introduction

Protecting hematopoietic progenitor cells from chemotherapy drug-induced cytotoxicity is essential to minimize the side effects of chemotherapy in cancer patients. Among the strategies used to minimize the side effects of chemotherapy, hematopoietic stem cell transplantation (HSCT) has been used to re-establish normal bone marrow function, thereby preventing immunodeficiency disorders (McGuire, 1998). HSCT allows patients to receive a higher dose of chemotherapy than chemotherapy without HSCT, thus increasing the efficacy of chemotherapy. However, HSCT has limited effectiveness against solid cancers and has major side effects such as infection (i.e., sepsis), veno-occlusive disease, and graft-versus-host disease (Wadleigh et al., 2003; Le Blanc et al., 2004).

Elevated oxidative stress is a common mechanism whereby chemotherapeutic drugs induce cytotoxicity in normal cells such as bone marrow cells. Concurrent administration of antioxidants with chemotherapy drugs has been used as a strategy to strengthen the cellular anti-oxidative stress defense system in bone marrow cells (Block et al., 2008). However, some antioxidants (i.e. β-carotene) also protect bone cancerous cells against chemotherapy drugs resulting in a lower effectiveness of chemotherapy, limiting the usefulness of this adjunct therapy.

Chemotherapy drugs typically have primary and secondary modes of action to induce apoptosis. The primary mode of the action is the specific stimulus that elicits apoptosis through

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1 A version of this chapter will be submitted for publication. Purtzki, M. J. and Xu, Z. Protection of Zinc against Etoposide-induced Hematopoietic Toxicity.
triggering a specific apoptotic mechanism such as topoisomerase inhibition and microtubules assembly inhibition. The secondary mode of action is the production of free radicals through the metabolism of chemotherapy drugs to its free radical intermediate metabolites (Conklin, 2004; Appendix 1).

Metallothionein (MT), a low molecular weight, cysteine-rich protein, is a powerful antioxidant that can quench free radicals due to the presence of many sulfhydryl-rich residues located in the protein (Krezel et al., 2007). MT can also directly bind to drug molecules, which can prevent them from reaching its final cellular target sites (e.g., the nucleus), thereby, limiting the effectiveness of the drug (Doz et al., 1993). Furthermore, MT can indirectly reduce chemotherapy drug–induced cytotoxicity by increasing intracellular zinc availability, which in turn can alter the activity of proteins regulated by zinc. MT has a high zinc-binding capacity with seven moles of zinc ions per mole of MT. Oxidation of zinc-thiol sites in MT causes the release of zinc ions from MT into the cytosol, resulting in an elevation of intracellular levels of free zinc ions. Higher levels of intracellular free zinc ions can lead to the activation of other zinc-dependent enzymes such as protein kinase C, phosphoinositol-3 kinase and tyrosine-specific kinase (LaRochelle et al., 2001) that could lead to reduce chemotherapy drug-induced cytotoxicity.

MT is constitutively synthesized in a wide range of tissues and its synthesis can be induced by a number of factors, including zinc. The zinc-induced synthesis of MT has been shown to occur in a tissue–specific manner, and provide a basis for prevention of chemotherapy-induced cytotoxicities. Zinc administration has been shown to induce MT synthesis in bone marrow (Futamachi et al., 1998), skin fibroblast (Leccia et al., 1999), endothelial (Meerarani et
al., 2000) and neuronal (Milton et al., 2001) cells to prevent chemotherapy drug-induced cytotoxicity in these cells. However, zinc administration is thought to provide little protection in certain tumor cells against chemotherapy drug-induced cytotoxicity due to the lack of zinc uptake in these cells. This supports the notion that zinc administration has little effect on drug-resistance in tumor cells. This apparent selective MT-mediated protection against chemotherapy drug-induced cytotoxicity in normal, vital cells such as peripherals blood cells makes zinc a potentially important agent in reducing chemotherapy drug-induced cytotoxicity. However, our knowledge of the relationship between zinc administration and MT synthesis, and chemotherapy has not been well studied, especially in the case of breast cancer.

Peripheral blood cells such as blood mononuclear cells (PBMC) have been shown to be responsive to zinc-induced MT synthesis (Cao & Cousins, 2000). I hypothesized that zinc-induced upregulation in MT synthesis protects THP-1 cells, a blood mononuclear cell line, against chemotherapy drug-induced cytotoxicity and that MDA-MB-231 cells, a breast cancer cell line, will not be responsive to zinc supplementation. The objectives of this study were: 1) to establish the responsiveness of THP-1 cells and MDA-MB-231 cells to zinc supplementation; 2) to determine whether zinc increased tolerance in THP-1 cells to etoposide, a chemotherapy drug, and to explore the possible cellular defense mechanisms involved; and 3) to explore the potential clinical benefit of zinc supplementation in chemotherapy drug treatment in PBMC cells.
2.2 Materials and methods

Cell culture system and zinc supplementation

MDA-MB-231, an estrogen-receptor negative, metastatic breast cancer cell line, (ATCC, Manassas, VA) was cultured in Dulbecco’s Modified Eagle Medium (DMEM; Gibco, Grand Island, NY) containing 10% fetal bovine serum (FBS), d-glucose (4.5 g/L), L-glutamine (2 mM), sodium pyruvate (110 mg/L), sodium bicarbonate (1.5 g/L) and penicillin/streptomycin (5,000 U/L). THP-1, an acute monocytic leukemia cell line, (ATCC, Manassas, VA) was culture in RPMI-1640 (Gibco, Grand Island, NY) containing 10% FBS, d-glucose (4.5 g/L), L-glutamine (2 mM), sodium pyruvate (110 mg/L), sodium bicarbonate (1.5 g/L), 2-β-mercaptoethanol (50 μM) and penicillin/streptomycin (5,000 U/L). Cells were grown in their corresponding media at 37°C in an atmosphere containing 5% CO₂ for 3 days. To manipulate the cellular zinc status, the medium was supplemented with 0, 25, 50, or 100 μM Zn as zinc sulfate (ZnSO₄) for 24 hours. Experiments have 3 replicates (n = 3) or 6 replicates (n = 6), depending on the assay used. The experiments were duplicated in order to confirm the observations.

Etoposide treatment

Cells were cultured in the system described for 24 hrs followed by the etoposide (Sigma, St. Louis, MO) treatment for a predetermined time according to the individual assay based on the preliminary results (Appendix 2-5).
Growth assay

For THP-1, cells were grown in T25 culture flask at an initial density of 1.0 x 10^6 cells/flask. For MDA-MB-231, cells were grown in 10 cm^2 Petri dishes at an initial density of 5.0 x 10^5 cells/dish. The viability of the cells was assessed by Trypan Blue exclusion assay and a minimum of 95% cell viability was required to perform experiments. At the end of the culture period, cells were washed once with warm PBS (37°C), trypsinized with 2 ml of 0.25 % Trypsin-EDTA. After the cells were detached, trypsin was neutralized with equal volume of the corresponding culture media. The cell suspension was quantitatively transferred to a 15 ml Falcon tube and centrifuged at 300 x g for 5 min, followed by aspiration of the supernatant. The cell pellet was then resuspended in 2 ml of cold PBS. To count the cell numbers, suspended cells (50 µl) were diluted with 10 ml of PBS and counted using a particle counter (Z1 Particle Counter, Beckman Coulter, Fullerton, CA) with a cutoff point set at 8 µm.

Total cellular zinc concentration and the size of the labile intracellular pool of zinc

To determine the total cellular zinc concentration, cells were cultured, harvested, and counted as described above, except that, after re-suspending in 2 ml cold PBS, 1 ml of the re-suspended cells was transferred to a 1.5 ml microcentrifuge tube followed by centrifugation at 300 x g for 5 mins. After aspirating the supernatant, the cells were resuspended in 100 µl of concentrated nitric acid. The cells were allowed to lyse overnight at room temperature with the cap on. Upon complete lysis, double deionized water was added to obtain a final volume of 1 ml. Zinc concentration was determined using a flame atomic absorption spectrophotometer
(Perkin Elmer, Model 2380, Norwalk, CT). The absorbance was extrapolated to concentration using a standard curve constructed with standard zinc solutions and normalized on a per 10^6 cells basis.

To determine the abundance of the labile intracellular pool of zinc, cells were cultured, counted and harvested as described above. Following centrifugation at 300 x g for 5 min, cells were resuspended in HBSS to a cell number of 5.56 x 10^6 cells/ml and 180 µl of cell suspension was pipet into each well of a 96-well plate followed, followed by the addition of 20 µl/well of Zinquin solution (Sigma, St. Louis, MO). The final cell number was 1 x 10^6 cells/well with a final Zinquin concentration of 25 µM. The cells were then incubated at 37°C for 30 min with gentle shake using a thermomixer (Eppendorf, Thermomixer R). The labile intracellular size of zinc was assessed by determining the intensity of the Zinquin-dependent fluorescence at an excitation and emission wavelength of 365 and 485 nm, respectively, using a microplate reader (Spectra, Molecular Devices, Sunnyvale, CA).

RNA isolation and reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA was isolated using Trizol (Invitrogen, Burlington, ON) according to the manufacturer’s instructions. The RNA isolated was stored at -80°C until analysis. The integrity of RNA was determined by electrophoretic separation of total RNA on a 1% agarose gel followed by visualization under UV light. RNA purity was assessed by determining the OD_{260}/OD_{280} ratio using a spectrophotometer. Samples with an OD_{260}/OD_{280} ratio > 1.9 were used for the synthesis of cDNA. RNA sample (0.5 µg RNA/reaction with 25 µl/reaction) was used to synthesize cDNA using SuperScript III (Invitrogen, Burlington, ON) according to the
manufacturer’s instructions. RNase Out Recombinant RNase Inhibitor (40 U/reaction; Invitrogen, Burlington, ON) was used to prevent RNase activity during cDNA synthesis. The cDNAs synthesized were amplified using the primers and conditions reported previously with modifications (Sens et al., 2000; Chung et al., 2006; Appendix 6). Briefly, the resulting cDNA (1 µl) was used for amplification with Platinum Taq Polymerase (0.25 U/reaction; Invitrogen, Burlington, ON) using a thermocycler (Eppendorf Gradient Mastercycler, Brinkman Instruments). The PCR conditions included a hot start (94°C, 2 min), followed by denaturation (95°C for 1 min), annealing (55°C for MT2a, MT3a and β-actin, and 60°C for MT1x for 30 sec), and elongation (72°C for 45 sec). At the end of the last cycle, there was a final extension at 72°C for 7 min. The number of the cycles was optimized with a correlation coefficient of 0.99, 0.98, 0.98, and 0.96 for MT1x, MT2a, MT3a, and β-actin, respectively. Negative control for each PCR run included a no-template control, where cDNA was substituted with equal volume of double deionized water. The PCR product was separated via electrophoresis in an agarose gel (1% for β-actin and 2% for all other targets), stained with ethidium bromide, and visualized with the Kodak Electrophoresis Documentation and Analysis System 290 (EDAS 290, Scientific Imaging Systems Eastman Kodak, New Haven, CT). The optical density of the bands was quantified with Kodak 1D Image Software (release 3.6). The abundance of the mRNA level of the target gene was normalized on the optical density of the corresponding β-actin bands.

**Apoptosis assay**

THP-1 cells were cultured in 12-well dishes with an initial seeding density of 1.0 x 10^5 cells/well under the same conditions described above, followed by treating the cells with
etoposide (2 μM) for 24 hrs. The cells were then assessed for apoptosis with PE Annexin-V Detection Kit (BD PharMingen, Mississauga, ON) using flow cytometry according to the manufacturer’s instruction. Briefly, the cells are harvested, pelleted, washed with cold PBS, and resuspended in 1x binding buffer at approximately 1 x 10^6 cells/ml. The cell suspension (100 μl) was transferred to a 5 ml FASC tube containing PE Annexin-V (5 μL) and 7-Amino-actinomycin D (7-AAD) (5 μl). After gentle mixing, the reaction mixture was incubated for 15 min at room temperature in the dark. At the end of the incubation period, the binding buffer (400 μl) was added to each tube. The cells were analyzed by flow cytometry (CellQuest, Becton-Dickinson, Franklin Lakes, NJ) within 1 hr. The flow cytometry settings were as the following: Primary laser: 488nm (visible), 250 mW; Annexin V detection: 530/20 BP filter, Fl-1 detector; 7-AAD detection: 660/20 BP filter, Fl-3 detector. Data analysis was evaluated using FlowJo 8.7 software (Ashland, OR)

**Caspase-3 and -9 activities**

Cells were cultured, harvested, and pelleted as described above. The cell pellet was washed once with cold PBS and lysed with a cytoplasmic lysis solution (50 mM HEPES, 100 mM NaCl, 10 mM EDTA, 1.6 mM CHAPS, and 0.1 % trition-X, 5.0 mM DTT; pH 7.4) for 30 min on ice. Cells were then centrifuged at 14,000 x g for 10 min and the supernatant was collected and stored at -80°C till analysis. Protein concentration was determined using DC protein kit (Bio-Rad, Hercules, CA) according to the manufacturer’s instructions. A fraction of the supernatant containing 50 μg of protein was mixed with the reaction buffer containing caspase-3 or -9 substrate (20 μM; Calibiochem, Darmstadt, Germany), HEPES (50 mM; pH 7.4),
NaCl (100 mM), EDTA (10 µM), CHAPS (1.6 mM), glycerol (10%), and DTT (10 mM) in a 96-well plate at 100 µl/well. The blanks were prepared by substituting the supernatant with equal volume of the lysis solution. The caspase substrates contain 7-amino-4-trifluoro methylcoumarin (AFC) sequences, which is recognized and cleaved by specific caspase proteins to cause AFC to fluoresce. The plates were read at an excitation and emission wavelength of 400 and 505 nm, respectively, using the kinetics settings (60 min duration at 10 min interval) of the microplate reader (Spectra, Molecular Devices, Sunnyvale, CA). The spectrophotometer was preheated to 30°C. A linear regression curve was established and Δ fluorescence (RFU) was quantified by extrapolating the readings to a standard curve of known 7-amino-4-trifluoro methylcoumarin (AFC) concentrations.

Comet assay

THP-1 cells were grown in 12-well dishes (2.0 x 10^4 cells/well), harvested, pelleted, and counted as described above. The cells were analyzed for DNA damage using the comet assay as described previously (Collins, 1992) with modifications. Briefly, the cell pellets were resuspended in cold PBS at approximately 1.0 x 10^5 cells/ml. The cell suspension (25 µL) was mixed in 75 µL of 1.0 % low gelling agarose in PBS. The sample-agarose mixture (20 µL) was then transferred to individual well on a 3-well comet slide (Trevigen, Gaithersburg, MD). The gel was allowed to harden at 4°C on a pre-cooled metal plate. The cells were then lysed with a lysis solution (2.5 M NaCl, 0.1 M EDTA, 10 mM Tris, and 1% Triton-X; pH 10.0) for 1 hr at 4°C.
The slides placed in a pre-cooled electrophoresis chamber (4°C) containing an alkaline solution (300 mM NaCl and 1 mM EDTA) for 40 mins allowing the DNA to unwind. After DNA unwinding, the DNA were separated via electrophoresis (14V, 300 mA for 25 min). After separation, the slides were placed in a neutralization solution (0.4M Tris; pH 7.5) for 15 min with the solution being changed every 5 min. The slides were then dipped in 70% ethanol and allowed to dry completely at 37°C. All the processing was performed under dim light.

A modified comet assay was performed via an enzymatic digest using formamidopyrimidine DNA glycosylase (fpg) to cleave oxidative nucleotides from the DNA. This modified assay provides a quantitative estimation of the presence of oxidative stress on the DNA (Collins et al., 1997). THP-1 cells pre-treated with 0 or 100 µM Zn were incubated with 2 µM etoposide for approximately 5 min and was then processed similar to the comet assay as described previously above. After the cells were lysed in the gel on the comet slides, the slides were submerged in the enzymatic buffer (40 mM HEPES, 0.1 M KCl, 0.5 mM EDTA; pH 8.0 using KOH to adjust pH) for 15 min at 4°C with the buffer being changed every 5 mins. After the last washing, the slides were loaded with fpg solution (1:1000 fpg dilution in the enzymatic buffer; 50 µl) or the buffer alone as a control, and then covered with a cover slip. The slides were then incubated at 37°C for 45 mins in a moist box followed by the removal of the cover slips. The slides are then transferred to the pre-cooled electrophoresis chamber and the steps were followed the same as the normal comet assay.

To visualize the comets, the samples were stained with propidium iodide (PI; 50 µL/well; 10 µg/ml; 15 min at room temperature) and visualized under a fluorescence microscope (Zeiss Axiovert 200M, Gottingen, Germany) fitted with Rhodamine filter. The comets were quantified
using CometScore (Sumerduck, VA) and assessed by using tail movement as the measure for DNA damage.

*Colony-formation assay (CFU-GM)*

Human bone marrow mononuclear cells (PBMC; Cat. #2S-101D, Poietic, Walksville, MD) were cultured according to the manufacturer’s instruction. To prepare the cell-medium mixture, etoposide (22 µL) was mixed with the methylcellulose culture medium (4 mL; MethoCult, Cat. #H4534, Stemcell, Vancouver, BC) and IMDM (78 µL; Gibco, Grand Island, NY) at a final concentration of 0, 0.02, 0.2, 0.4, 0.6, 0.8, 1.2 or 2.0 µM in a 10 ml tube. After vortexing for $2 \times 5$ sec, PBMC (300 µL; $1.1 \times 10^6$ cells/ml) were added to each tube with a final cell density of 75,000 cells/tube followed by vortexing for $2 \times 5$ sec. The tubes were then allowed to stand for 5 min on ice to release air bubbles. Finally, the cell-medium mixture was distributed into each of three 35-mm culture dishes using a 1 mL syringe with a 19 Gauge needle. To culture the cells, the culture dishes were placed in a 150 mm Petri dish with a 60 mm Petri dish (without the lid) containing water placed at the center of the 150 mm Petri dish covered with a lid. Cells were cultured at 37 °C, 5% CO$_2$ for 10–14 days. The colonies formed were scored based on the established criteria (Pressina et al., 2001) using an invert microscope.

*Statistical Analysis*

Data on total cellular zinc concentration, abundance of the labile intracellular pool of zinc, MT mRNA levels, caspase-3 and -9 activities, and DNA damage were analyzed by one-
way ANOVA for zinc supplementation effect followed by Tukey’s HSD test for the significant difference among the means (JMP, Release 5.0; SAS Institute, Inc., Cary, NC; \( p < 0.05 \)). Data on caspase-3 and -9 activities, DNA damage, and abundance of the labile intracellular pool of zinc and MT mRNA levels at each zinc supplementation level were analyzed by one-way ANOVA for etoposide treatment effect followed by Student \( t \)-test for the significant difference between the etoposide treatment and its carrier control \( (p < 0.05) \). For the CFU-GM analysis, each IC\(_{50}\) value was calculated based on the dose–response curve using 4-parameter fitting function in the GraphPad Prism 5.0 software (La Jolla, CA). The \( p \)-value was calculated by performing Student's \( t \)-test. Statistical significance was defined as \( p < 0.05 \).

2.3 Results

*Dose-dependent increase of total cellular zinc concentration and the size of the labile intracellular pool of zinc in THP-1 cells*

In MDA-MB-231 cells, the basal level of total cellular zinc concentration (0 \( \mu \)M zinc supplementation) was 78 nmol/10\(^6\) cells (Figure 2.1A). Zinc supplementation had no effect on the total cellular zinc concentration, except at the highest level of the zinc supplementation (100 \( \mu \)M), where total cellular zinc concentration was increased by 1.6 fold-increase compared to the control \( (p < 0.05; \text{Figure 2.1A}) \). In THP-1 cells, the basal level of total cellular zinc concentration (0 \( \mu \)M zinc supplementation; 74 nmol/10\(^6\) cells; Figure 2.1B) was similar to the level in MDA-MB-231 cells. However, zinc supplementation at 25, 50, and 100 \( \mu \)M resulted in 1.3, 1.8 and 4.2 fold-increases in the total cellular zinc concentration, respectively, compared to the control \( (p < 0.05; \text{Figure 2.1B}) \).
The cellular zinc status was also assessed by the abundance of the labile intracellular pool of zinc. The abundance of the labile intracellular pool of zinc in MDA-MB-231 cells was also not affected by zinc supplementation, except at the highest level of zinc supplementation (100 µM), where the abundance of the labile intracellular pool of zinc was increased by 1.5 fold-increase compared to the control (p < 0.05; Figure 2.2A). In contrast, the abundance of the labile intracellular pool of zinc in THP-1 cells was minimal in the control (Figure 2.2B). However, zinc supplementation at 25, 50, and 100 µM resulted in 10, 39, and 166 fold-increases in the abundance of the labile intracellular pool of zinc, respectively, compared to the control (p < 0.05; Figure 2.2B). The dose-dependent increase in the total cellular zinc concentration and the abundance of the labile intracellular pool of zinc indicated that THP-1 cells were sensitive to zinc supplementation.

*Elevated abundance of MT mRNA in THP-1 cells*

The abundance of MT1x, MT2a and MT3a mRNAs was not affected by zinc supplementations in MDA-MB-231 (Figure 2.3). In contrast, zinc supplementation at 50 and 100 µM resulted in a 46 and 55% increase in the abundance of MT1x mRNA in THP1 cells compared to the control, respectively (p < 0.05; Figure 2.4). Similarly, zinc supplementation at 50 and 100 µM resulted in 47 and 51% increase in the abundance of MT2a mRNA in THP-1 cells, respectively (p < 0.05). In contrast, only zinc supplementation at the highest level tested (100 µM) resulted in an increase (29%) in the abundance of MT3a mRNA (p < 0.05; Figure 2.4). Since zinc supplementation had no effect on the cellular zinc status and MT expression in MDA-
MB-231 cells, the effects of zinc supplementation against etoposide-induced cytotoxicity was focused on THP-1 cells.

_Zinc supplementation reduced apoptotic death in etoposide-treated THP-1 cells_

Etoposide-induced by apoptosis was assessed for the cell morphology using the Annexin-V assay and the biochemical changes by determining caspase-3 and -9 activities. Approximately 30% of the THP-1 cells were stained positive with Annexin-V in the presence of 0 or 25 μM of zinc supplementation (p < 0.05; Figure 2.5a). In contrast, the proportion of Annexin-V positive cells was reduced to 26 and 20% in the presence of zinc supplementation at 50 and 100 μM, respectively (p < 0.05). The proportion of Annexin-V positive cells was inversely correlated to zinc supplementation ($r^2 = 0.99$, p < 0.01; Figure 2.5b), showing an inverse relationship between zinc supplementation and etoposide-induced apoptosis.

In the absence of etoposide treatment, caspase-3 activity was minimal (Figure 2.6A). Etoposide treatment alone (0 μM zinc supplementation group) caused a 110-fold increase in caspase-3 activity compared to the control (p < 0.05; Figure 2.6A). Caspase-3 activity remained unchanged at 25 μM of zinc supplementation compared to etoposide treatment alone; however, caspase-3 activity was reduced by 19 and 38% when the level of zinc supplementation increased to 50 and 100 μM, respectively, compared to the 0 μM zinc supplementation group (p < 0.05).

Similarly, etoposide treatment alone caused a 11-fold increase in caspase-9 activity compared to the control (p < 0.05; Figure 2.6B). Caspase-9 activity remained unchanged at 25 and 50 μM of zinc supplementation compared to the 0 μM zinc supplementation group;
however, caspase-9 activity was reduced by 30% when the level of zinc supplementation was increased to 100 µM compared to the 0 µM zinc supplementation group (p < 0.05).

**Zinc supplementation reduced etoposide-induced DNA and oxidative damage**

Etoposide-induced DNA damage was assessed by its tail movement using the comet assay. In the absence of etoposide treatment, there was minimal tail movement in THP-1 cells, indicating a basal level of DNA damage (Figure 2.7). Etoposide treatment to THP-1 cells for 6 hrs caused a 54-fold increase in tail movement compared to the etoposide treatment control, indicating a dramatic increase in DNA damage (p < 0.05). Zinc supplementation at 25 µM had no effect on the tail movement compared to the 0 µM zinc supplementation group (p < 0.05; Figure 2.7); however, zinc supplementation at 50 or 100 µM decreased etoposide treatment-induced DNA damage by 36% compared to the 0 µM zinc supplementation group (p < 0.05; Figure 2.7).

Etoposide can induce DNA damage through causing DNA base oxidization, which is considered as an early event in DNA damage (Collins et al., 1997). The DNA base oxidization can be detected using a modified comet assay involving digesting the DNA with fpg, a repair endonuclease (Collins et al., 1997). To assess the extent of DNA oxidation in etoposide treatment induced DNA damage, THP-1 cells were treated with etoposide briefly (≈ 5 min). In the absence of etoposide treatment and zinc supplementation, the tail movement in the THP-1 cells was very low (p < 0.05; Figure 2.8), indicating a low level of DNA base oxidation in these cells. When the cells were treated with etoposide alone, there was a 39-fold increase in tail
movement compared to the control (p < 0.05; Figure 2.8), indicating a dramatic increase in DNA base oxidation. However, when the cells pretreated with 100 µM of zinc followed by the treatment of etoposide, there was a 5-fold increase in tail movement compared to the control (p < 0.05). There was an 88% reduction in the tail movement of the 100 µM zinc-pretreated etoposide treatment compared to the etoposide treatment. This indicates that zinc pretreatment dramatically reduced etoposide treatment-induced DNA base oxidation in THP-1 cells (p < 0.05).

_Etoposide altered the size of the labile intracellular pool of zinc_

In the absence of etoposide treatment and zinc supplementation, the abundance of the labile intracellular pool of zinc was 12 RFU in THP-1 cells (Figure 2.9). The abundance of the labile intracellular pool of zinc remained unchanged at 25 µM of zinc supplementation compared to the 0 µM zinc supplementation group. When zinc supplementation was increased to 50 and 100 µM, there was a 4- and 14-fold increase in the abundance of the labile intracellular pool, respectively, compared to the 0 µM zinc supplementation group (p < 0.05).

When the cells were treated with etoposide treatment alone, the abundance of the labile intracellular pool of zinc remained the same as in the 0 µM zinc supplementation group (Figure 2.8). When the cells were treated with etoposide in combination with zinc supplementation at 25 µM, the abundance of the labile intracellular pool of zinc remained unchanged compared to that in the cells treated with etoposide alone. When zinc supplementation was increased to 50 and 100 µM, there was a 4- and 19-fold increase in the abundance of the labile intracellular pool of
zinc, respectively, compared to that in the cells treated with etoposide alone (p < 0.05).

Etoposide treatment had no effect on the abundance of the labile intracellular pool of zinc in THP-1 cells when zinc supplementation level was at 0, 25, or 50 µM (Figure 2.9). However, when zinc supplementation was increased to 100 µM, etoposide treatment significantly increased the abundance of the labile intracellular pool of zinc by 25% (p < 0.05).

*Etoposide had no effect on the abundance of MT mRNA*

In the absence of etoposide treatment, zinc supplementation at 25 µM resulted in 42% increase in the abundance of MT1x mRNA level in THP-1 cells compared to the zinc supplementation control (p < 0.05; Figure 2.10). Zinc supplementation at 50 µM had no effect on the abundance of MT1x mRNA level; however, further increase in the zinc supplementation level to 100 µM resulted in 85% increase in MT1x mRNA level compared to the control (p < 0.05). Similarly, in THP-1 cells treated with etoposide, zinc supplementation at 25, 50, and 100 µM resulted in 2, 2.7, and 3 folds increase in the abundance of MT1x mRNA level, respectively, compared to the 0 µM zinc supplementation group (p < 0.05; Figure 2.10). Interestingly, etoposide treatment reduced the abundance of MT1x mRNA level by 63, 47, and 38% at 0, 25, and 100 µM of zinc supplementation, respectively, compared to their corresponding etoposide treatment control (p < 0.05; Figure 2.10). The abundance of MT1x mRNA level remained between the etoposide treatment and the control at 50 µM of zinc supplementation.

The abundance of MT2a mRNA level was affected by zinc supplementation, but not etoposide treatment. Zinc supplementation at 50 and 100 µM, but not at 25 µM, resulted in
approximately 2 and 4 folds increase in the abundance of MT2a mRNA level compared to the zinc supplementation control in both THP-1 cells treated with etoposide and their corresponding etoposide treatment controls (p < 0.05; Figure 2.11). In contrast, neither etoposide treatment nor zinc supplementation had effect on the abundance of MT3a mRNA level (Figure 2.12).

**Zinc supplementation reduced etoposide-induced cytotoxicity in PMBC**

Etoposide treatment-induced cytotoxicity in PBMC cells was assessed by determining the colony formation assay (CFU-GM). In the absence of etoposide treatment, zinc supplementation had no effect on the colony formation in PBMC (Figure 2.13a). When the cells were treated with etoposide, the number of colony formed was reduced by 60 at zinc supplementation control (0 µM) compared to its corresponding etoposide treatment control (p < 0.05; Figure 2.13a). When zinc supplementation level was increased to 50 and 100 µM, etoposide treatment resulted in a 38 and 41% reduction in the number of colony formed compared to their corresponding etoposide treatment controls (p < 0.05). Among etoposide-treated cells, zinc supplementation at 50 and 100 µM resulted in 50 and 57% increase in the number of colony formed compared to the zinc supplementation control (p < 0.05). The number of colony formed was the same between the cells received zinc supplementation at 50 and 100 µM. In addition, zinc supplementation at 50 µM and 100 µM also increased IC₅₀ by 36 and 27%, respectively, compared to the zinc supplementation control (p < 0.05; Figure 2.13b).
2.4 Discussion

*Zinc supplementation elevated zinc status and MT mRNA levels in THP-1 cells, but not in MDA-MB-231*

In the present study, zinc supplementation resulted in a dose-dependant increase in the total cellular zinc concentration in THP-1 cells, indicating elevated zinc status in these cells. In addition, zinc supplementation also resulted in a dose-dependent increase in the size of the labile intracellular pool of zinc in THP-1 cells. In contrast, zinc supplementation had no or little effect on both total cellular zinc concentration and the size of labile intracellular pool of zinc in MDA-MB-231 cells. The mechanisms that cause this difference in responsiveness between these two types of cells towards zinc supplementation are not clear. However, it is well established that cellular zinc concentration is homeostatically regulated. Zinc homeostasis is achieved through the interplay between zinc importers and exporters (Cousins et al., 2006). Furthermore, cellular uptake of zinc is known to be tissue specific (Huber & Cousins, 1992; Satoh et al., 2000). It is possible that the difference in responsiveness reported here reflect differences in zinc homeostasis between these two types of cells.

Zinc supplementation at 50 and 100 µM also resulted in an increase in the levels of MT1x and MT2a mRNA in THP-1 cells while MT3a mRNA level remained unaffected. In contrast, zinc supplementation had no effect on the mRNA levels of all three MT isoforms assessed in MDA-MB-231 cells. MT synthesis can be induced by a number of factors, including metals, glucocorticoids and, different types of stress signals (Satoh & Bremner, 1993). Zinc has been recognized as a potent inducer of MT synthesis. The labile intracellular pool of zinc consists of free zinc ions and the zinc that is loosely bound to macromolecules such as proteins.
A larger size of the labile intracellular zinc pool represents a higher availability of intracellular zinc to induce MT synthesis and to exert its physiological functions such as regulating zinc-dependent components in the signaling pathways (Cousins et al., 2006). Thus, the effects of zinc supplementation on MT synthesis were consistent with its effects on zinc status and the size of the labile intracellular pool of zinc in THP-1 and MDA-MB-231 cells.

*Zinc supplementation reduced etoposide-induced apoptosis and DNA damage in THP-1 cells.*

The maximum tolerated dose (MTD) of chemotherapy drugs is defined as the highest dose that causes no long-term side effects leading to chronic illness (Becker 2007). Peripheral blood cells are often used as indicators of MTD since complications in peripheral blood cell function can lead to immunodeficiency disorders. In this study, zinc supplementation reduced etoposide-induced apoptosis in a dose-dependent manner (Figure 2.5). Consistent with the presence of apoptotic cells, the activity of caspase-9, a initiator of apoptosis, and caspase-3, the executioner of apoptosis, were reduced in response to zinc supplementation in THP-1 cells (Figure 2.6). These observations are consistent with previous observations showing that zinc supplementation suppress H2O2-induced apoptosis in human premonocytic U937 cells (Futamachi et al., 1998) and reduces drug-induced apoptosis in skin fibroblast, and endothelial and neuronal cells (Lessia et al., 1999, Meeraani et al., 2000; Milton et al., 2001). Importantly, the doses of zinc supplementation used in those studies involved drug-induced apoptosis are low to moderate (≤100 µM), which are very similar with the doses used in present study.

In addition to the reduction of apoptosis, zinc supplementation also induced necrosis in THP-1 cells in the present study using the Annexin-V assay. In general, low-to-moderate levels of zinc (0-100 µM) do not influence cell viability or necrosis while high levels of zinc
supplementation (≥ 200 µM) can induce necrosis in vitro (Iguchi et al., 1998). In this study, necrosis was evident even at low levels of zinc supplementation (25 and 50 µM; Figure 2.5a), suggesting that the necrosis may be a false-positive due to the Annexin-V assay. Further studies are required to affirm these observations and to investigate the role of zinc in chemotherapy drug-induced necrosis.

Etoposide is a phenol derivative that can act as a pro-oxidant due to the production of phenoxy radical metabolites, which leads to oxidative stress (Kagan & Tyurina, 1998; Kagan et al., 2001). Enzymes such as cytochrome p450, myeloperoxidase, prostaglandin synthetase, and tyrosinase are involved in the production of the phenoxy radical metabolites and a high level of cellular activity of these enzymes can enhance etoposide-induced cytotoxicity (Haim et al., 1987; Kalyanaram et al., 1989; Usui et al., 1990). Certain antioxidants such as ascorbate and α-tocopherol (Miyoshi et al., 2005, Gokhale at al., 2006) are not able to influence etoposide-induced apoptosis, whereas other antioxidants such as glutathione (GSH), N-acetyl cysteine (NAC), butylated hydroxyanisole and quercetin have been shown to prevent etoposide-induced apoptosis in peripheral blood cell lines (Verhaegen et al., 1995; Lizard et al., 1998; Kapiszewksa et al., 2007). This indicates that only certain antioxidants have the appropriate antioxidant properties to quench phenoxy radicals and thus prevent etoposide-induced apoptosis. In particular, phenoxy radicals can be reduced by endogenous thiols to prevent etoposide-induced apoptosis (Fan et al., 2006), which further supports the potential involvement of MT in etoposide resistance.

MT has a high antioxidant capacity due to its proportionally higher abundance of sulfhydryl-rich (thiol) residues to scavenge free radicals. Its zinc-thiol residues (-SZn) can be oxidized to form disulfide bonds (-SS-) between the sulfur groups. This oxidized MT is known
as thionin (T₀). The oxidation of MT can cause the release of zinc from the zinc-thiol site, which can influence other zinc-dependent pathways (Krezel et al., 2007). It should be mentioned that zinc ion itself is redox inert and is unable to directly interact with free radicals (Krezel et al., 2007). These zinc-thiol residues can be oxidized by various free radicals including hydrogen peroxide, superoxide, and nitric oxide (Fliss & Menard, 1992; Kroncke et al., 1994). Furthermore, the zinc-thiol residues in MT provide better protection against radiation-induced DNA damage compared to copper- or cadmium-thiol residues in MT (Cao & Cherian, 2003). In present study, acute etoposide treatment induced oxidative DNA damage in THP-1 cells, indicating that THP-1 cells were susceptible to phenoxy radical-induce oxidative stress. Interestingly, zinc-supplementation almost completely prevented this etoposide-induced oxidative DNA damage after a short incubation period. In contrast, zinc-supplementation reduced etoposide-induce DNA damage only by 35% when the cells were treated with etoposide for a longer period. The lack of a better protection against DNA damage at longer time periods indicates that etoposide treatment could be caused by: (i) inefficiencies of the MT redox cycle to regenerate MT (Tᵣ) from metal-free MT (T₀) could diminish MT redox capacity and/or (ii) etoposide could induce DNA damage through its primary mode of action by inhibiting topoisomerase, that may contribute to the DNA damage after longer incubation times.

MT is known to directly sequester cytotoxic drugs due to the high binding affinity of the zinc-thiol residues towards drugs that are electrophilic, which are primarily alkylating (i.e. melphalan) and platinum-based drugs (i.e. cisplatin) used in chemotherapy (Yu et al., 1995; Zaia et al., 1996). Etoposide itself is not considered an electrophilic compound and, therefore, is unlikely that MT interferes with etoposide to prevent etoposide-induced apoptosis.
Oxidation of MT causes the release of zinc from the thiol sites into the cytosol where it can regulate other zinc-sensitive processes. For example, elevated zinc levels can inhibit caspase-3 (Perry et al., 1997) and calcium-dependent endonuclease (Lohmann & Beyersmann, 1993), which are critical proteins involved in apoptosis. In the present study, etoposide caused the elevation in the size of the labile intracellular pool of zinc in the zinc-pretreated THP-1 cells, suggesting that phenoxy radicals displace zinc ions from MT. Oxidative stress has been shown to upregulate MT transcription (Chun et al., 2004, Chang et al., 2006) and protein levels (Shimoda et al., 2003) presumably by releasing zinc from MT resulting in an elevated abundance of labile intracellular pool of zinc, which, in turn, promotes MT synthesis. MT transcription is particularly sensitive to changes in the size of the labile intracellular pool of zinc due to the presence of metal-transcriptional response regions on its promoter region on MT genes. The lack of up-regulation in the MT isoforms observed in this study indicated that the elevation in the size of the labile intracellular pool of zinc due to the etoposide treatment had little influence on MT levels and other zinc-dependent processes. It is unlikely that zinc-dependent processes are influenced by the alteration in the size of the intracellular pool of zinc since MT genes are very responsive to changes in the size of the labile pool of zinc.

*Potential clinical benefits of zinc supplementation to protect blood mononuclear cells from etoposide-induced cytotoxicity.*

Antioxidant administration has been shown to have little effect on blood progenitor cell survival against cytotoxic drugs. Elevated GSH levels did not influence CFU counts using the CFU-GM assay on bone marrow cells in animals and humans treated with alkylating chemotherapy drug, busulphan (Hassan et al., 2002). Other studies have reported that NAC...
administration can protect peripheral blood cells against free radical-induced cytotoxicity; however, some of these studies failed to take into account that antioxidants can induce differentiation of PMBC and, therefore, the apparent protective effects associated with NAC administration is not necessarily due to antioxidant effect of NAC (Cortelezzi et al., 2000). Other antioxidants such as retinoic acid, a metabolite of vitamin A, induces the differentiation of granulocyte/macrophage; however, only managed to have a subtle increase CFU when the cells are challenged with free radical generators (van Bockstaele et al., 1992; Cortelezzi et al., 2000). Therefore, it appears the effect of antioxidants on chemotherapy drug-induced cytotoxicity is complex and varies from the type of antioxidant and the drug used and more investigations are needed before antioxidant is used as part of the chemotherapy regime.

Zinc supplementation has consistently been shown to reduce the cytotoxicity of free radical generators using the CFU-GM assay (Gogu & Agrawal, 1996; Rossen et al., 1994; Doz et al., 1992). Observations obtained from the present study further supports the notion that zinc supplementation reduces hematopoietic toxicity induced by cytotoxic agents such as etoposide. Zinc administration resulted in greater IC₅₀ in peripheral blood cells when treated with etoposide compared to the control. Zinc did not induce the differentiation of CFU-GM counts at baseline indicating that differentiation did not influence IC₅₀ and, rather, the protective effect of zinc administration is observed when cells are treated with etoposide. The protective effect of zinc administration to etoposide in PBMC using the CFU-GM was similar to the results seen in the THP-1 cells. Collectively, these observations suggest that zinc could protect peripheral blood cells against chemotherapy drug-induced cytotoxicity.

In summary, zinc administration resulted in selective uptake of zinc and the size of labile intracellular pool of zinc in blood mononuclear cells, whereas breast cancer cells were not
influenced by zinc supplementation. The consequential increase in intracellular zinc and the
elevation in MT synthesis were associated with a reduction in etoposide-induced apoptosis and
DNA damage. The antioxidant properties of MT likely reduced the oxidative stress of etoposide
resulting in the inhibition of apoptosis. These results indicate that zinc plays a potentially pivotal
role in against chemotherapy (i.e. etoposide)-induced cytotoxicity in normal cells.
Figure 2.1: Differential responsiveness in total cellular zinc levels to zinc supplementation between MDA-MB-231 (A) and THP-1 (B) cells. MDA-MB-231 and THP-1 cells were cultured in medium supplemented with 0, 25, 50, or 100 µM zinc for 24 hr. Values represent mean ± SEM (n=3; the experiment was repeated once). Means with different letters are significantly different (p<0.05).
Figure 2.2: Differential responsiveness in the abundance of the labile intracellular pool of zinc to zinc supplementation between MDA-MB-231 (A) and THP-1 (B) cells. MDA-MB-231 and THP-1 cells were cultured in medium supplemented with 0, 25, 50, or 100 µM zinc for 24 hr. Values represent mean ± SEM (n=3; the experiment was repeated once.). Means with different letters are significantly different ($p<0.05$).
Figure 2.3: Effect of zinc supplementation on the up-regulation of MT isoforms in MDA-MB-231. MDA-MB-231 cells were cultured in medium supplemented with 0 (empty bar), 25 (light gray bar), 50 (dark gray bar) or 100 µM (lined bar) zinc for 24 hr. (A) Representative ethidium bromide-stained agarose gel showing MT1x, MT2a, MT3a and β-actin mRNA bands. (B) Abundance of MT1x, MT2a, and MT3a mRNA was normalized on the optical density of β-actin band. Values represent mean ± SEM (n=3).
Figure 2.4: Effect of zinc supplementation on the up-regulation of MT isoforms in THP-1. THP-1 cells were cultured in medium supplemented with 0 (empty bar), 25 (light gray bar), 50 (dark gray bar) and 100μM (lined bar) Zn for 24hr. (A) Representative ethidium bromide-stained agarose gel showing MT1x, MT2a, MT3a and β-actin mRNA bands. (B) Abundance of MT1x, MT2a, and MT3a mRNA was normalized on the optical density of β-actin band. Values represent mean ± SEM (n=3). Within each MT isoform, means with different letters are significantly different (p<0.05).
Figure 2.5a: Effect of zinc supplementation on apoptotic death in etoposide-treated cells. THP-1 cells were cultured in medium supplemented with 0 (A), 25 (B), 50 (C), or 100 µM (D) zinc for 24 hr followed by etoposide treatment (2 µM) for 24 hr. Cell death was determined by Annexin-V staining for apoptotic cells and 7-AAD staining for necrotic cells using flow cytometry. Live, apoptotic, and necrotic cells are located in the left bottom quadrant, right bottom quadrant, and right top quadrant, respectively.
Figure 2.5b: Correlation between zinc supplementation and etoposide-induced apoptosis in THP-1 cells. Data from the Annexin-V assay shown in Figure 5a was used in this analysis.
Figure 2.6: Effect of zinc supplementation on caspase-3 and -9 activity of THP-1 treated with etoposide. THP-1 cells were cultured in medium supplemented with 0, 25, 50, or 100 µM zinc for 24 hr followed by treating the cells with 2µM etoposide for 24 hr. The control is not treated with either zinc or etoposide. Caspase-3 (A) and -9 (B) activity was determined by fluorescent caspase substrates using fluorescence spectrophotometer. Fold difference was calculated by divided the treatment caspase activity values by the control caspase activity values. Values represent mean ± SEM (n=3; the experiment was repeated once). Means with different letters are significantly different (p<0.05). Asterisk indicates a significant difference (p<0.05) between control and etoposide-treated cells (0 µM zinc).
Figure 2.7: Effect of zinc supplementation on etoposide-induced DNA damage. THP-1 cells were cultured in medium supplemented with 0, 25, 50, or 100 µM zinc for 24 hr followed by treating the cells with 2 µM etoposide for 6 hr. The control is not treated with either zinc or etoposide. DNA damage was quantified using the comet assay with tail movement as the measure of DNA damage. Values represent mean ± SEM (n=6). Means with different letters are significantly different (p<0.05). Asterisk indicates a significant difference (p<0.05) between control and etoposide-treated cells (0 µM zinc).
Figure 2.8: Effect of zinc supplementation on etoposide-induced oxidative DNA damage. THP-1 cells were cultured in medium supplemented with 0 or 100 µM of zinc for 24 hr followed by treating the cells with 0 (Control) or 2 µM (Etoposide) etoposide for 5 min. A modified comet assay was performed with DNA enzyme, Fpg to detect the presence of specific oxidized bases on the DNA. Tail movement was calculated by subtracting the Fpg-treated DNA tail movement by the non Fpg-treated DNA tail movement of each treatment group. Values represent mean ± SEM (n = 3; the experiment was repeated once). Means with different letters are significantly different (p<0.05).
Figure 2.9: Effect of etoposide treatment on the abundance of the labile intracellular pool of zinc (LIPZ) in response to zinc supplementation. THP-1 cells were cultured in medium supplemented with 0, 25, 50, or 100 µM of zinc for 24 hr followed by treating the cells with 0 (solid bar) or 2 µM (lined bar) etoposide for 6 hr. Abundance of the labile intracellular pool of zinc was determined by Zinquin-dependent fluorescence intensity. The RFU was normalized on per 1.0 x 10^6 basis. Values are mean ± SEM (n=3; the experiment was repeated once). Means among different zinc supplementation levels with different letters are significantly different (p<0.05). Lower-case and upper-case letters signifies control and etoposide treatment groups, respectively. Asterisk indicates a significant difference between the etoposide-control and etoposide-treated cells at the same zinc supplementation level (p<0.05).
Figure 2.10: Effect of etoposide to alter MT1x expression. THP-1 cells were cultured in medium supplemented with 0, 25, 50, or 100 µM zinc for 24 hr, followed by treating the cells with 0 (solid bars) or 2 µM (lined bars) etoposide for 6 hr. Abundance of MT1x and β-actin mRNA was assessed by RT-PCR. (A) Representative ethidium bromide-stained agarose gel showing MT1x and β-actin mRNA bands. (B) Abundance of MT1x mRNA was normalized on the optical density of β-actin band. Values represent mean ± SEM (n=3). Means among the zinc supplementation levels within the same etoposide treatment level with different letters are significantly different (p < 0.05). Lower-case and upper-case letters signifies control and etoposide treatment groups, respectively. Asterisk indicates a significant difference between the etoposide-control and etoposide-treated cells at the same zinc supplementation level (p < 0.05).
Figure 2.11: Effect of etoposide to alter MT2a expression. THP-1 cells were cultured in medium supplemented with 0, 25, 50, or 100 µM zinc for 24hr, followed by treating the cells with 0 (solid bars) or 2µM (lined bars) etoposide for 6 hr. Abundance of MT2a and β-actin mRNA was assessed by RT-PCR. (A) Representative ethidium bromide-stained agarose gel showing MT1x and β-actin mRNA bands. (B) Abundance of MT1x mRNA was normalized on the optical density of β-actin band. Values represent mean ± SEM (n=3). Means among the zinc supplementation levels within the same etoposide treatment level with different letters are significantly different (p<0.05). Lower-case and upper-case letters signifies control and etoposide treatment groups, respectively.
Figure 2.12: Effect of etoposide to alter MT3a expression. THP-1 cells were cultured in medium supplemented with 0, 25, 50, or 100 µM zinc for 24hr, followed by treating the cells with 0 (solid bars) or 2µM (lined bars) etoposide for 6 hr. Abundance of MT3a and β-actin mRNA was assessed by RT-PCR. (A) Representative ethidium bromide-stained agarose gel showing MT1x and β-actin mRNA bands. (B) Abundance of MT1x mRNA was normalized on the optical density of β-actin band. Values represent mean ± SEM (n=3).
Figure 2.13a: Effect of zinc supplementation to alter etoposide-induced cytotoxicity. PBMC cells were cultured in medium supplemented with 0 (light gray bars), 50 (dark gray bars), or 100 µM (lined bars) zinc in the presence of etoposide at 0 (Control) or 0.2 µM (Etoposide) for 14 days. Values are mean ± SEM (n=3; the experiment was repeated once). Means among the drug treatments within the same zinc treatment level with different letters are significantly different ($p<0.05$). Asterisks indicate a significant difference between the zinc supplementation control and the zinc supplemented groups within the same etoposide treatment ($p<0.05$).
Figure 2.13b: Effect of zinc supplementation to alter etoposide-induced cytotoxicity. PBMC cells were cultured in medium supplemented with 0, 50, or 100 µM zinc in the presence of etoposide for 14 days. Values are mean ± SEM (n=3; the experiment was repeated once). The IC$_{50}$ was derived from a dose-response curve. Asterisks indicate a significant difference between control and the zinc supplemented groups ($p<0.05$).
2.5 References


CHAPTER 3
General Discussion, Limitations, and Future Directions

3.1 General discussion

Antioxidant administration during chemotherapy or radiation therapy is thought to be an effective co-therapy to protect against treatment-related side effects. However, its use has been limited due to inconsistent effectiveness among different antioxidants used and large variable findings in previous studies. Several randomized controlled clinical studies have shown that some antioxidants can reduce the side effects of chemotherapy drugs; however, other studies have shown that some antioxidants protect both tumor as well as normal cells against chemotherapy drugs (Satoh et al., 2000; Lissioni et al., 2003; Mills et al., 2005), thus lowering the effectiveness of chemotherapy drugs. MT has been suggested to be a potential antioxidant candidate for reducing the side effects of chemotherapy drugs due to its strong antioxidant capacity and, depending on the type of inducer (i.e., zinc, copper, and bismuth), can be selectively elevated in normal cells compared to tumor cells (Satoh et al., 2000).

My thesis research has shown that zinc supplementation selectively induced MT synthesis in THP-1 cells, a blood monocyte cell line, while MT synthesis remained unaffected in MDA-MB-231 cells, a breast cancer cell line. Furthermore, zinc supplementation protected the THP-1 cells from etoposide-induced apoptosis. Presumably, the increase in MT synthesis induced by zinc supplementation protected the blood monocyte cells against etoposide-induced apoptosis. Etoposide has a relatively low toxicity towards normal mammary tissues because the dosage required for inducing cytotoxicity in normal human mammary epithelial cells is higher than the dosage for anti-proliferation effect in breast cancer cell lines, such as MDA-MB-231.
and HL60 (Li et al., 2006). Rather, etoposide mainly exerts strong cytotoxicity on peripheral blood mononuclear cells (PBMC; Li et al., 2006), in which the cytotoxicity can be reduced by elevation of MT synthesis in PMBC. In clinical settings, the dosages of some anticancer drugs such as camptothecin, doxorubicin, colchicine, and paclitaxel are kept at low due to their higher cytotoxicity towards PBMC (Li et al., 2006). Since zinc is a potent inducer of MT synthesis in PMBC, zinc supplementation might also be beneficial in protecting PBMC against the cytotoxicity of these drugs. If this were proven in vivo, zinc preloading could permit the use of higher dosage of these anticancer drugs resulting in enhanced effectiveness of chemotherapy.

MT reduces chemotherapy drug-induced cytotoxicity primarily through directly binding to the drug or quenching free radicals produced by the drug (Cherian et al., 1993; Doz et al., 1993; Kang, 2006). The relatively high abundance of the hydrogen in the sulfur hydro (-SH) residues in MT provides a strong antioxidant capacity against various free radical metabolites (Kang, 2006; Krezel et al., 2007) and is considered as a more potent antioxidant than GSH (Thornalley & Vasak, 1985; Abel & Ruiter, 1989; Cao & Cousins, 2000). Etoposide can induce oxidative stress by the formation of phenoxy radical metabolites (Kagan & Tyurina, 1998; Kagan et al., 2001). In the present study, the exposure of THP-1 cells to etoposide caused the rapid oxidation of nucleotides in peripheral blood cells that is likely responsible for the induction of apoptosis. Zinc administration protected against oxidative DNA damage caused by etoposide, which coincided with the repeated antioxidant effect of MT to scavenge free radical metabolites (Krezel et al., 2007). The oxidation of the zinc-thiol residues to quench free radicals can also cause the release of zinc ions, which in turn stimulate the upregulation of stress-response proteins (Gunes et al., 1998; Lichtlen et al., 2001). The current study reported etoposide increases the size of the labile intracellular pool of zinc at high zinc supplementation, which may
potentially regulate other proteins involved in chemotherapy drug-induced cytotoxicity. However, etoposide did not increase MT synthesis, which is highly responsive to changes in intracellular zinc levels, indicating that it is unlikely that the elevation of labile intracellular zinc levels exerted additional drug detoxification effect.

3.2 Limitations

There are several limitations to this study. This thesis research project used two established cell lines as the model systems. It, therefore, is difficult to establish if peripheral blood cells, not breast cancer cells, would also be responsive to zinc supplementation in vivo or in humans. Thus, direct extrapolation of the observations reported in this thesis to the context of the human body could be misleading. There are some reports suggesting that zinc administration causes the elevation of intracellular zinc and MT levels in vitro (Leccia et al., 1999, Meerarani et al., 2000; Milton et al., 2001); however, these reports vary greatly depending on the type of cell line used in each study. Nonetheless, there is clear evidence that zinc supplementation will upregulate MT levels in PBMC in humans (Cao & Cousins, 2000, Cousins et al., 2003). Also, it should be mentioned that THP-1 cells and PBMC have similar responses to zinc supplementation (Cousins et al., 2003), which further complements the use of THP-1 cells in this study. However, the effects of zinc supplementation on tumor cells in vivo are not known.

Another limitation is the degree of MT involvement in drug resistance with zinc supplementation. MT is not the only zinc-sensitive factor that may influence apoptotic resistance and oxidative stress mechanisms as zinc can also regulate other drug resistance processes (i.e., GSH) and biological functions (i.e., kinase activity). Conversely, MT induction by other metals
(i.e., cadmium, bismuth, and copper) has been shown to exert drug resistance indicating the zinc administration acts merely as an inducer on MT (Satoh et al., 2000; Shimoda et al., 2003).

Finally, the effect of zinc supplementation on the efficacy of etoposide-induced cytotoxicity in breast cancer cells was not investigated. Preliminary results found that MDA-MB-231 cells required a significantly higher etoposide concentration (>100 µM) and longer incubation time (>24 hours) to exert its cytotoxicity. A longer incubation time would indicate that etoposide employed a different mode of action, likely through anti-proliferation pathways rather than through induction of apoptosis, to exert its cytotoxicity in MDA-MB-231 cells. Because of this, it would be technically challenge to make direct comparison between MDA-MB-231 cells and THP-1 cells.

3.3 Future directions

Further investigations are needed to detail the precise role of MT in drug-induced oxidative stress. There are three states of MT (MT, TR and TO) that relate to the zinc-binding capacity of MT (MT/TR ratio) and its redox state (TR/TO ratio), which have important implications to redox potential. Mild oxidative stress in human ovarian cells results in an elevated abundance of the labile intracellular pool of zinc, a greater MT/TR ratio and a lower TR/TO ratio (Krezel & Maret, 2007). The lower TR/TO ratio suggests that in an oxidative environment, there is a build-up of TO due, at least in part, to ineffective reduction of TO to TR. The reduction of TO to TR requires reducing agents such as GSH/GSSG and selenium-containing proteins (i.e., thioredoxin). Selenium has a pivotal role in catalyzing the reduction of TR (Chen & Maret, 2001) and has been shown to have protective function against cytotoxic drugs (Lin et al., 1992; Fakih et al., 2005).
Cancer patients have low selenium and zinc serum levels that are even more deficient in these minerals during chemotherapy treatment (Ma & Jiang, 1993; Shenberg et al, 1995; Federico et al., 2001). Oral selenium and zinc supplementation in patients with cancers of the digestive tract do not show a further worsening of nutritional status and experienced a significant decrease of asthenia (body weakness) with an increase of appetite (Federico et al., 2001). I speculate that selenium supplementation can enhance the redox state of MT and improve the effectiveness of zinc-induced MT metabolism to protect peripheral cells against drug-induced apoptosis.
3.4 References


Figure A.1: Interaction between chemotherapy-induced apoptosis and metallothionein synthesis.
Figure A.2: Dose- and time-dependent reduction in etoposide-induced cell viability in THP-1 cells. THP-1 cells were treated with 0, 2, 5, 10, or 20 µM etoposide for 0, 6, 12, or 24 hr. Cell viability was assessed using the thiazolyl blue tetrazolium bromide (MTT) assay. The ODs were normalized on the OD of the control (0 µM etoposide). Values are mean ± SEM (n=6).
Appendix 3

Figure A.3. Time-dependent increase in etoposide-induced apoptosis in a THP-1 cells. THP-1 cells were treated with 2 μM etoposide for 0 (A), 6 (B), 12 (C), 24 (D) hr in RPMI medium. Cells were stained with Annexin-V and 7-AAD for assessing apoptotic and necrotic cell deaths, respectively using flow cytometry. Live, apoptotic, and necrotic cells are located in the left bottom quadrant, right bottom quadrant, and right top quadrant, respectively.
Figure A.4: Time-dependent increase in etoposide-induced caspase-3 activity in THP-1 cells. THP-1 cells were treated with 2 µM etoposide for 0, 6, 12, or 24 hr. Caspase-3 activity was determined by fluorescent caspase substrates using fluorescence spectrophotometer. Values are mean ± SEM (n=3). Means with different letters are significantly different (p<0.05).
Appendix 5

Figure A.5. Time-dependent effect of etoposide on DNA damage in THP-1 cells. THP-1 cells were treated with 2 µM etoposide for 0, 3, 6, 12, 24 hr. Caspase-3 activity was determined by fluorescent caspase substrates using fluorescence spectrophotometer. Values are mean ± SEM (n=3). Means with different letters are significantly different ($p<0.05$).
### Appendix 6

#### Table A.6. PCR primers and conditions for RT-PCR

<table>
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<tr>
<th>Gene</th>
<th>Primers</th>
<th>Size (bp)</th>
<th>Cycles†</th>
<th>Reference</th>
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<tr>
<td>MT1x</td>
<td>S- TCTCCTTGCCTCGAAATGGAC</td>
<td>151</td>
<td>23/30</td>
<td>Chung et al., 2006</td>
</tr>
<tr>
<td></td>
<td>AS- GGGCACACTTGGACACAGC</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MT2a</td>
<td>S- CCGACTCTAGCCGCCTCTTT</td>
<td>259</td>
<td>20</td>
<td>Chung et al., 2006</td>
</tr>
<tr>
<td></td>
<td>AS- GTGGAAGTCGGTTCTTTACA</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MT3a</td>
<td>S- CCGTTTCACGGCCCTCCAG</td>
<td>325</td>
<td>27/40</td>
<td>Sens et al., 2000</td>
</tr>
<tr>
<td></td>
<td>AS- CACCAGCCACACTTCACCACA</td>
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<tr>
<td>β-actin</td>
<td>S- TATGGAGAAGATTTGGCACC</td>
<td>786</td>
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<td>Mahnke et al., 1997</td>
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<tr>
<td></td>
<td>AS- CCACCAATCCACACAGTA</td>
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*Primers are written 5’ → 3’; S = sense; AS = anti-sense; † = first and second number indicates the number of cycles required for THP-1 and MDA-MB-231, respectively, and the one number indicates the same cycles for both cell lines.*