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CRITICAL REVIEW

Mammalian metallothioneins: properties and functions

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Metallothioneins (MT) are a family of ubiquitous proteins, whose role is still discussed in numerous papers, but their affinity to some metal ions is undisputable. These cysteine-rich proteins are connected with antioxidant activity and protective effects on biomolecules against free radicals, especially reactive oxygen species. In this review, the connection between zinc(II) ions, reactive oxygen species, heavy metal ions and metallothioneins is demonstrated with respect to effect of these proteins on cell proliferation and a possible negative role in resistance to heavy metal-based and non-heavy metal-based drugs.

1. What are metallothioneins?

Mammalian metallothioneins (MTs) belong to a group of intracellular and low molecular mass proteins (app. 6 kDa), which were discovered in 1957 when Margoshes and Valee isolated them from horse renal cortex tissue.^{1–3} MTs have been implicated in a number of functions, including toxic metal detoxification, as a metal chaperone and in metal ion homeostasis.⁴ These proteins have been isolated and studied in a wide variety of organisms, including prokaryotes, plants, invertebrates and vertebrates.^{5–8} In their primary structure, they are rich in cysteine and have no aromatic amino acids. Cysteine residues are the most highly conserved followed by the basic amino acids lysine and arginine. The metal binding domain of MT consists of 20 cysteine residues juxtaposed with

Lys and Arg arranged in two thiol-rich sites called α and β . Twenty cysteine residues occur in primary sequence in following repetitions: Cys-X-Cys, Cys-Cys-X-Cys-Cys, Cys-X-Cys-Cys, where X is amino acid different from cysteine. Domains are separated by a cysteine-free central part usually called a spacer (Fig. 1). The α -domain often consists of amino-acids residues No. 32–61, β domain No. 1–31. N-terminal part is marked as β -domain with three binding sites for divalent ions, usually for Zn(II) or Cd(II), with nine cysteinyl sulphurs. C-terminal part (α -domain) is capable of binding four divalent metal ions.^{9,10} In summary, the cysteine sulphhydryl groups can bind 7 moles of divalent metal ions per mol of MT, while the molar ratio for monovalent metal ions (Cu and Ag) is twelve.¹¹ Although the naturally occurring protein has Zn(II) in both binding sites, this ion may be substituted for another metal ion that has a higher affinity for thiolate such as Pb(II), Cu(I), Cd(II), Hg(II), Ag(I), Pt(II and IV), and/or Pd(II).^{12–16} On the other hand, both iron

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developing new types of nanomaterials and testing of these materials for nanomedical purposes.

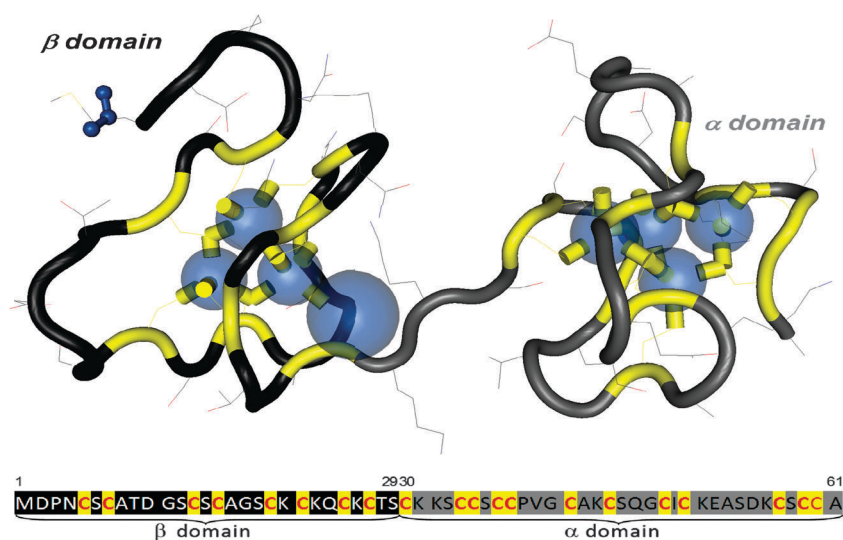


Fig. 1 Structural characterisation of α and β domain of rat MT-2 based on data from ExPasy database. (A) 3D representation of tertiary structure of rat MT-2 (pdb ID 4MT2). (B) The amino acid sequence of rat MT-2 (P04355). Data was obtained from www.expasy.ch.

ions (Fe(II) and Fe(III)) have a lower affinity for thiolate as compared to Zn(II).¹⁷ Structure, folding, assembly, stability, electrochemistry, and catalytic function of MTs are still being investigated, especially in the direction of MTs affinity to metal ions.^{7,18} Well reviewed structural and chemical features are available in the literature.^{19–22}

Four major isoforms (MT-1 through MT-4) have been identified in mammals. In addition, at least thirteen known closely related MT proteins in humans have been described.^{23,24} MTs genes are tightly linked, and at a minimum they consist of eleven MT-1 genes (MT-1A, -B, -E, -F, -G, -H, -I, -J, -K, -L, and -X), and one gene for each of the other MTs isoforms (the MT-2 A gene, MT-3 gene, and MT-4 gene). The nomenclature for MTs isoforms has not been standardized until now.²⁵ A gene called MT-like 5 (MTL-5) that encodes a testis-specific MT-like protein called tesmin was described in the q13 region of chromosome 11.²⁶ Tesmin plays a specific role in both male and female meiotic prophase.²⁶ The specific functional roles of MTs isoforms and their molecular interactions are still unclear.²⁷ MT-1 and MT-2 are the most widely distributed MT isoforms. They are expressed in many cell types in different tissues and organs. Contrariwise, MT-3 and MT-4 demonstrate very limited cell-specific pattern of expression. MT-3 represents a unique metalloprotein called also neuronal-growth inhibitory factor, which inhibits outgrowth of neuronal cells.²⁸ In comparison with MT-1 and MT-2, MT-3 shows distinct chemical, structural and biological properties.^{29–33} MT-4 belongs to noninducible proteins, with its expression primarily confined to certain squamous epithelia.²²

2. Biochemical, molecular-biological and biological importance of metallothionein in mammalia

These cysteine-rich proteins are localised in cytoplasm and some organelles, predominantly in mitochondria, where their presence is sensitively and strictly regulated by the oxidative

state induced by mitochondrial respiration.³⁴ Reciprocal regulation of mitochondrial reactive oxygen species (ROS) production is evident.^{35,36} In addition, MTs are involved in the regulation of permeability of inner mitochondrial membrane.³⁷ Cell and tissue specific regulation of cellular respiratory and energy metabolism in liver mitochondria is still discussed.^{38–40} From mitochondria, whose outer membrane pores admit molecules up to 10 kDa, MTs can be transported to cytoplasm and other target organelles. Lysosomes represent another place of MTs localisation. The presence of MT, namely MT-3, is related to lysosomal changes and cell death in neurons under oxidative stress.⁴¹ Relation between iron-catalysed intralysosomal peroxidative reactions, MT protective effect and oxidative stress is suggested in a study by Baird *et al.*⁴² Depending on the cell state, but especially presence of oxidative stress, MTs are rapidly translocated to the nucleus through nuclear pore complexes.⁴³ MT localized in the nuclei is oxidized there and it is transported to cytosol; this system is balanced.⁴⁴ Translocation of MTs to the nucleus is probably connected with protection of the cell against DNA damage and apoptosis and gene transcription during different stages of cell cycle,^{45–51} but also with high extracellular concentration of glucose in certain cell types, as was demonstrated using umbilical vein endothelial cells (HUVEC).^{52,53} In these cells, MT expression is regulated by endothelin ET-1, where elevated levels were evidenced in diabetes mellitus patients.

Regulation of zinc(II) ions by MTs was demonstrated in numerous papers, especially in association with cell cycle regulation and proliferation.^{54,55} MT can provide a pool of zinc(II) ions that can be released and donated to other metalloproteins and transcription factors.^{47,48} Translocation of MT into the nucleus plays an important role in genoprotection mediated by zinc(II) in the HaCaT cell line.⁵⁶ The role of nitric oxide in enhancement of MT nuclear localization is connected with scavenging ability of MT with subsequent formation of nitrosothiol, which reduces nuclear as well as cytoplasmic damage by nitric oxide.⁵⁷ Clathrin-mediated endocytosis of MT was determined in human hepatocellular carcinoma HepG2 cells. This fact supports not only the presence of intracellular, but also extracellular MT and its role

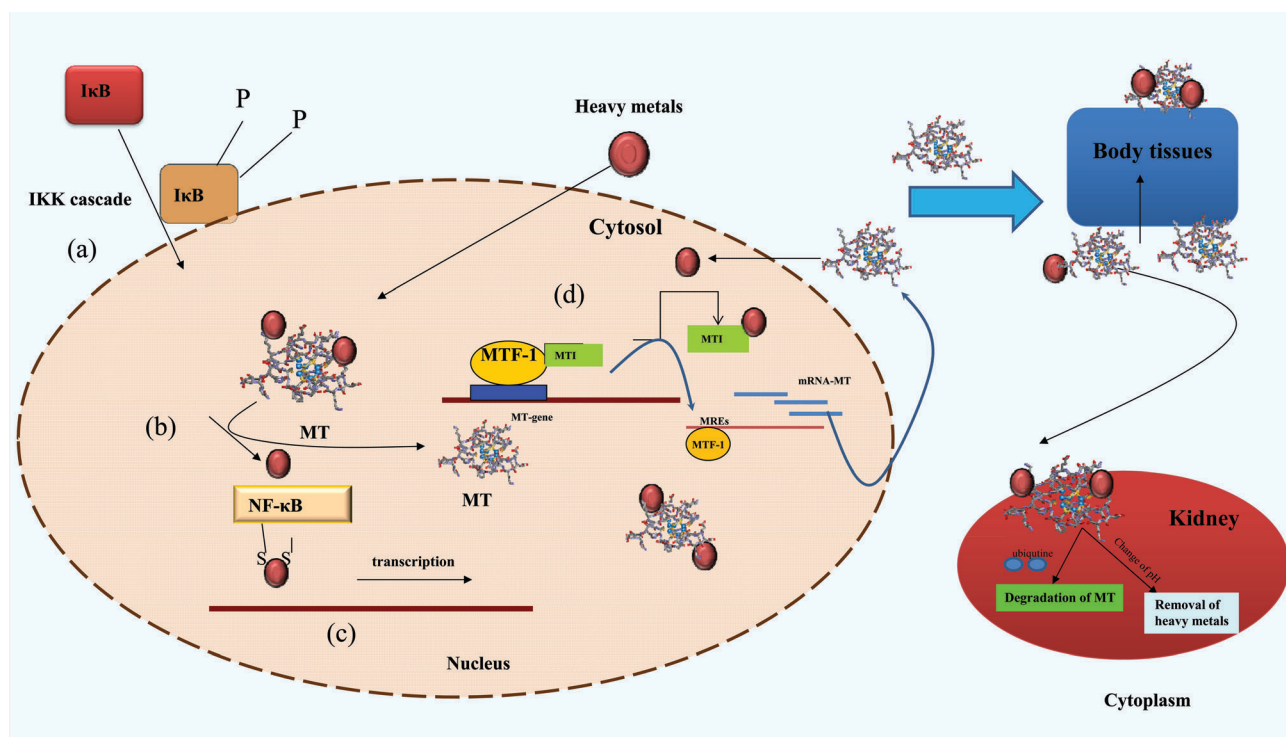


Fig. 2 Simplified scheme of MT interaction with platinum-based cytostatics. (a) Platinum based cytostatic agent application into organisms, (b) transport into cell nucleus, (c) its bounding to MTF-1 and release from complex with MTF-1 followed by MTF-1 bounding to metal responsive element (MRE). This step initiates mRNA MT synthesis. mRNA is consequently translated and active MT bound molecules of platinum-based cytostatics.

in cell communication.^{58,59} Extracellular MTs originate from necrotic cells.

MTs have many important and crucial functions. The most important of them includes detoxification of essential as well as non-essential heavy metal ions, such as Cd(II) or Hg(I,II), homeostasis and control of Zn(II) and Cu(II) ions and metal-transfer reactions.⁵⁴ The scheme of detoxification is shown in Fig. 2. Study of reduction and oxidation of MT revealed formation of some higher molecular fraction, MT oligomers called MT aggregates. Formation of MT aggregates with increasing zinc concentration is also observed. We found that reduced MT forms aggregates more readily than oxidized MT.^{60,61}

Storage MT function for essential metals is also demonstrated. A special role of MT in Zn(II) transport and its sequestration at binding places localised on biomembranes was evidenced.⁶² Due to a wide range of MTs functions, its connections with immune defence responses, protein-protein and protein-nucleotide interactions, zinc fingers and zinc-based transcriptional factor regulation, mitochondrial function and energetic metabolism regulation, angiogenesis, cell cycle, cell differentiation and apoptosis are suggested. It seems that expression of MT is sensitively and strictly regulated by the oxidative state induced in mitochondrial respiration.⁶³ Deregulation of this state may be connected with many diseases. Some of MT-1 and MT-2 actions have therapeutic possibilities in treatment of acute as well as chronic diseases, where oxidative stress plays a crucial role.⁴⁷ MTs are believed to participate in some pathological processes, such as carcinogenesis as well as emergence of radioresistance or chemoresistance

of tumour cells.^{64–66} MT expression is connected with response to brain injury or during neurodegenerative disease progression as down-expression during Parkinson's, Alzheimer's or Creutzfeldt-Jacob diseases.^{67–70} MT-3 deficient mice exhibit abnormalities of psychological behaviour.⁷¹ MT-3 was also detected in some cells of the glomerulus and the collective tubules in the kidney, some cells in the glandular epithelium of the dorsolateral lobe of the prostate, some Sertoli cells and Leydig cells in the testis, and taste bud cells in the tongue of rats.⁷² MT-3 in adipocytes demonstrates important protective role against hypoxic damage.^{73,74} MT-4 was found to be specifically expressed in stratified squamous epithelia, where it plays essential, but an until now poorly defined role.⁷⁵ Role of MTs in Wilson's and Menke's diseases is intensely studied because of marked changes in content of free copper(II) ions in the blood of the patients, which may relate to MT expression.³¹

3. Induction of metallothioneins, role of cadmium and other metal ions

Expression of MTs is induced by many factors including physical stress, chemical stress and endogenous factors.⁷⁶ In the case of physical factors, role of cold treatment, X-irradiation, and UV-radiation is discussed and increased MT expression due to the above-mentioned factors especially in liver, kidney and thymus tissues is demonstrated.⁷⁷ Chemical stress includes plenty of toxicologically important compounds, such as solvents like carbon tetrachloride, chloroform, and ethanol, drugs like acetaminophen, menadione, but also important anti-tumour drugs as bleomycin, adriamycin and cisplatin, and alkylating

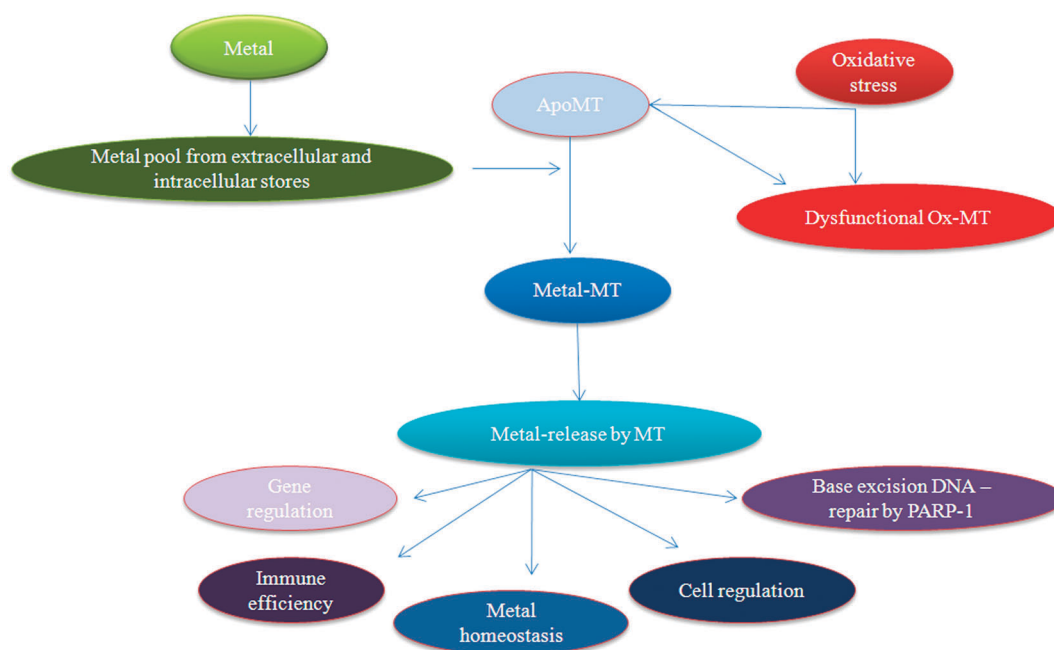


Fig. 3 Scheme of the impacts of metallothioneins, stress and metal ions interplay. Infections and stress trigger an inflammatory response by the increasing the production of pro-inflammatory cytokines which, in turn, stimulate the gene expression of metallothioneins (which are produced as apo-MT). These proteins can be activated, however, by increasing metal ions concentration. These proteins need zinc to properly absolve their function of zinc “releasers” during stressing condition. Releasing of zinc and other essential metal ions has numerous impacts including gene regulation and increase in immune efficiency. Adopted and modified according to Mocchegiani *et al.*²¹⁴

agents as bromobenzene, diethyl maleate, or phorone. Radical-generating agents, which are able to induce free radicals, especially reactive oxygen species, belong to the group of the most important MT gene expression inducers.⁷⁸ Induction of MTs in relation to heavy metals, important environment pollutants, has been demonstrated in many tissues including liver, kidney, intestine or pancreas.^{79–81} The most important MTs induction was observed in gills and liver, because of their crucial role in metal uptake and bioaccumulation, respectively detoxification.⁸² These results suggest an important role of MT in marine as well as freshwater organisms as a biomarker.⁸³ Heavy metals including metals used in treatment of cancer or metals with a promising future in cancer therapy such as platinum, palladium and rhodium accumulate in mammalian kidneys, mostly in proximal tubule cells with subsequent damage of reabsorptive and secretory functions, which is closely connected with MTs (Fig. 3). Recent studies have indicated oxidative stress with associated lipid peroxidation, apoptosis, and necrosis as common phenomena in nephrotoxicity due to these metals.⁸⁴ Cd(II) ions are determined as the most potent MTs inducer.⁸⁵

3.1 Cadmium

Cadmium is an industrial and environmental pollutant which is released during smelting of ores, burning of fossil fuels, waste incineration, and from urban traffic pollution and as a co-product of phosphate fertilizers. Cadmium(II) is shown to be involved in the development of certain diseases. The primary routes of cadmium(II) exposure are uptake of cadmium(II) contaminated food and inhalation. Due to high amounts of cadmium(II) in cigarettes, both smokers and passive smokers are

exposed to high amounts of cadmium(II).^{86–89} Induction of MTs expression is given by dosage of applied cadmium(II) as well as method of application. In oral acute cadmium intoxication, the primary target organ is the gastrointestinal tract (GIT).⁹⁰ Necrosis of GIT epithelia is demonstrated. Tissue damage in liver, kidneys and testes is given by the redistribution of cadmium(II) from gastrointestinal tract. Due to its similarity with divalent ions, especially calcium(II) and zinc(II), cadmium(II) can replace these ions from their binding sites. On the other hand, protective effect of manganese(II) as an inhibitor of cadmium(II) entry into cells through divalent ions transporters is suggested.^{91,92} The intracellular effect of cadmium(II) includes generation of free radicals, especially reactive oxygen species, which are connected with lipid peroxidation, DNA damage and protein denaturation.⁹³ Copper(II) supplementation may have a protective effect against Cd-induced oxidative stress in liver, kidney and placental tissues of pregnant rats.⁸⁹ After cadmium uptake, this heavy metal is redistributed and preferentially accumulated in liver and kidneys with subsequent formation of hepatic and renal lesions.^{93,94} Histopathological changes of placentas of pregnant smokers resulted in placental necrosis with serious foetal toxic effects. Cadmium(II) distribution and metabolisation is connected with liver levels of iron. Positive correlation between hepatic iron and malondialdehyde as marker of lipid peroxidation was demonstrated in work of Djukic-Cosic *et al.*⁹⁵ As a final consequence, carcinogenicity is a known outcome of chronic inflammation. Of the many harmful constituents in smokeless tobacco, oral tissue metallothionein gradients suggest that metals contribute to the toxicity from smokeless tobacco use and possibly sensitization.⁹⁶ An activation of protective

cellular mechanisms after cadmium(II) exposure follows. Induction of glutathione S-transferase (GST) appears up to 24 hours after cadmium(II) uptake.⁹⁷ This enzyme catalyses conjugation of glutathione with a variety of xenobiotic and endobiotic compounds with electrophilic functional groups; thus, GST expression is considered to be an important factor in protection against toxic effects of toxicants including phenols, *N*-heterocyclic compounds, phenobarbital or antitumor drugs.⁹³ In the case of Wistar rats, parenteral cadmium(II) application led to increasing MT expression in liver and testes as 2.16 fold increased MT expression was detected six hours after cadmium(II) application.⁹⁸ Exposition to cadmium(II) ions may be detected in human peripheral blood lymphocytes. MT expression shows correlation between Cd(II) exposition and renal dysfunction.⁹⁹ After uptake, cadmium(II) is redistributed and in tissues it is bound to metallothioneins.¹⁰⁰ Toxicity of free cadmium(II) ions and Cd-MT is discussed.^{101,102} Free MT level measured using anti-MT antibodies was carried out in numerous studies. Renal dysfunction correlated with urinary cadmium levels and two markers of renal dysfunction as β 2-microglobulin and N-acetyl- β -D-glucosaminidase. These results indicate that MT represents a suitable marker for renal dysfunction related to cadmium exposure and toxicity of Cu-based pesticide applicators. Oxidative stress biomarkers, ceruloplasmin (Cpl), MTs, copper(II), haematological parameters, and biochemical markers for pancreatic, hepatic and renal functions were measured in plasma. Results suggest that the incorporation of oxidative stress biomarkers to biochemical/clinical tests should be considered for validation and included in human health surveillance protocols.¹⁰³

3.2 Other metal ions

Free metal ions, such as Cu(I,II) and Fe(II,III), are well known as free oxygen radicals inducers. However, they also play important physiological roles, so their homeostasis must be strictly controlled.^{33,104,105} At a cellular level, Cu(I, II) and Fe(II,III) uptake involves high-affinity transporters. These metal ions almost never exist in the cytoplasm as free forms, because they readily generate free oxygen radicals connected with oxidation of cellular components due to their redox activity. Copper(II) ions are immediately transferred to glutathione and MT providing effective and safe mechanisms of intercellular storage and transport of this metal. In addition, places with excess of Cu-MT may be susceptible to oxidative stress.¹⁰⁶ Administration of essential as well as non-essential heavy metals (Zn, Cu, Cd, Hg,) increases biosynthesis of MT-1 and MT-2 by inducing the transcription initiated after binding of metal to the MRE-binding transcription factor-1 (MTF-1), or by cis-acting DNA elements. MTF-1 is multiple zinc finger protein and the only known mediator of metal responsiveness of MT-1 and MT-2.^{51,67,107–113} Study of Cheuk *et al.* demonstrated induction of MT without induction of MTF-1 for many metal ions including Zn(II) and Cu(II) in two zebra fish cell lines.¹¹⁴ Correlation between serum strontium(II) concentration and MT level is also determined.¹¹⁵

4. Metallothioneins and relation to free radicals

ROS increases MT-1 and MT-2 transcriptional responses in a dose-dependent manner, and subsequently mRNA expression

and its levels, as it was demonstrated in study of Klassen *et al.*¹¹⁶ MT-1 and MT-2 content is increased by glucocorticoids (signal transcription through glucocorticoid response elements GREs).¹¹⁷ Catecholamines are also able to activate MT-1 and MT-2 gene transcription.^{118,119} MT genes are transcriptionally activated also by redox fluctuations. MT-1 gene expression is activated as a response to hypoxia.¹²⁰

Moreover, it is clear that MT is induced by oxidative stress. Thus, one of the most important MT functions consists in cell protection against free radicals.⁴⁰ Free oxygen radicals are associated with ubiquitous cell functions, especially by the mitochondrial electron transport system and by NADPH oxidase in leucocytes and macrophages. The danger of free radicals consists of the ability of damage to biomolecules, including DNA, proteins and polyunsaturated fatty acids, major components of cell biomembranes.⁶³ There are several protective systems including antioxidants, such as catalase (CAT) for hydrogen peroxide, superoxide dismutase (SOD) for superoxide, and glutathione peroxidases (GPx) for hydrogen peroxide and lipid peroxide. Copper chaperone (CCS) specifically delivers copper(II) to zinc superoxide dismutase (SOD1) in cytoplasm of mammalian cells. This suggests that the amount of CCS protein exceeds that required to supply Cu(II) to SOD1 in the cells. Further, the CCS knockdown induces Cu(II) accumulation in cells; however, Cu(II) accumulation is ameliorated by MT induction, a decrease of Ctr1 expression and an increase in Atp7a expression to maintain Cu(II) homeostasis.¹²¹ Mitochondria, which are permanently exposed to oxidative stress, display very effective mechanisms as antioxidant enzymes: phospholipids hydroperoxide glutathione peroxidase (PHGPx), classical glutathione peroxidase, and manganese-superoxide dismutase (Mn-SOD). Non-specific antioxidants, such as reduced glutathione, ceruloplasmin, ascorbic acid, α -keto acids (pyruvate), purine derivatives (urate), and transferrin participate in maintenance of oxidative cell status. Mechanisms of MTs as antioxidant molecules are still unknown, but the antioxidant role of mammalian MTs is well documented. *In vitro* experiments with DNA incubated with MT demonstrated that MT is capable of scavenging free hydroxyl radicals.¹²² Scavenging MTs ability is demonstrated for organic radicals. Some studies focused on demonstrating the role of MT and its antioxidant abilities, *e.g.* hydroxyl radicals scavenging, in aquatic organisms; aquatic organisms are exposed to environmental pollution which leads to induction of MT biosynthesis in certain tissues, especially in liver. This fact is supported by localisation of MT in inter-membrane space which is the main place of electron transport *via* cytochrome c during mitochondrial respiration. This fact is probably connected to the increased role of MT in the case of aquatic organisms.^{123,124} Role of environmental pollution, MT biosynthesis and its antioxidant abilities must be further investigated.¹²⁵ Connection between hydrogen peroxide and MT level was evidenced.¹²⁶ Zn-MT has been shown to be a potent protective and stabilization agent of biomembranes undergoing lipid peroxidation. Released Zn(II) ions themselves can serve as regulators and inhibitors of this process, mainly in the presence of oxidative stress.¹²⁷ Zn(II) ions can antagonize the catalytic properties of the redox-active transition metals, such as Cu(I, II) and Fe(II, III), which are connected to the formation of superoxide and hydroxyl radicals.^{128–130} MT thiol clusters are capable of chelating

iron(II, III), as has been demonstrated in *in vitro* experiments with subsequent prevention of Fenton-dependent oxidative reaction catalysis.^{42,131–134} Iron(II, III) is closely connected with zinc(II) ions that significantly reduce iron-mediated generation of hydroxyl radicals and competitively inhibit intestinal cytosolic aconitase activity, which plays an important role in the determination of the labile iron pool, the pool of iron participating in the Fenton reaction.¹³⁵ There are only limited results indicating direct MT ability to scavenge free radicals *in vivo*. The published results are inconsistent. MT-null mice treated with saline and zinc(II) exposed to oxidative stress induced by γ -radiation and 2-nitropropane demonstrated no alterations in antioxidant-defensive systems in comparison with wild-type mice.¹³⁶ On the other hand, a study using the same experimental model as MT-null mice demonstrated a protective MT effect against ozone-induced lung inflammation *via* regulation of oxidative stress.¹³⁷ Glutathione itself does not play in this case so important a role, thus, this protective effect is fully attributed to MTs.¹³⁸

4.1 Reduced glutathione

The role of GSH synthesis, MTs and antioxidant properties was investigated.¹³⁹ MT cooperates with GSH in maintaining the cellular redox state. It may function as a secondary antioxidant in the cellular protection system that exerts its antioxidant action only under extreme conditions of oxidative stress.^{140,141} In cell lines with blocked GSH synthesis, the enhanced role of MTs was found.¹⁴² Pre-induction of MT synthesis leads to significant inhibition of oxidative-stress induced lipid peroxidation.^{143,144} Supplementation of experimental mice by Zn(II) ions led to induced cardiac MT synthesis.¹⁴⁵ In connection with previously published results, role of Zn(II) supplementation and its relationship with Zn-MT and antioxidant properties requires further investigation.

4.2 Apoptosis

Apoptosis plays a crucial role in a number of physiological processes, where unwanted cells are physiologically eliminated from multicellular organisms.¹⁴⁶ Abnormal apoptosis or a disruption of apoptosis (when cells lose competence to undergo apoptosis) is connected with autoimmune disorders or malignant tumour diseases. Connection between free radicals, p53 expression – which is related to oxidative stress – and apoptosis has been demonstrated by Apostolova *et al.* and by other authors.^{52,147} In this case, MTs play a negative role due to prevention against p53 activation and associated apoptotic effects.¹⁴⁸ Oxidative stress, namely ROS, are considered to be apoptosis inducers.^{149,150} The connection between MTs and free radicals has been discussed above. Experimental works connect loss of MT expression with enhanced sensitivity to apoptosis. The results indicate that signals to apoptosis, such as ROS, are eliminated and a negative effect of MT in cell proliferation appears due to the antioxidant activity of MT. High MT levels and the presence of mutated p53 are associated with high tumour grade. Increased MT level with the related association of histological tumour grade has been demonstrated in invasive breast ductal carcinoma in work of Yap *et al.*⁶⁶ A recent paper shows a relationship between tumour subclassification and MT expression.¹⁵¹ Despite the fact that more detailed information is missing, direct

interaction between MTs and p53 are highly probable.¹⁵² This fact is demonstrated in a human breast cancer epithelial cell line NM-1, where the formation of complex p53-apo-MT was demonstrated.^{153–155} Galizia *et al.* focused on role of MT expression and p27 down-regulation in gastric cancer. p27 down-regulation, which controls cell cycle progression at G1, correlated with tumour progression and enhanced MT expression.¹⁵⁶

5. Changes in MT levels during cell proliferation

At present, there is no knowledge about the significance of MT distribution in different cellular compartments. The highest cytoplasmic MT concentration was found in the late G1 and G1/S cell cycle phases.¹⁵⁷ Depending on the cell cycle phase, cell differentiation or in the case of toxicity, MT-1 and MT-2 are translocated into the nucleus.^{25,50} High rates of MT synthesis have been detected in rapidly proliferating tissues. This fact suggests an important role of MT in cell growth.¹⁵⁸ When MTs bind to zinc(II) or copper(II), they serve as reservoirs of metals for synthesis of apoenzymes and zinc-finger transcription regulators.^{159–161} Zinc(II) is a cofactor for a lot of enzymes and therefore it is important for cell growth, protein, nucleic acid, carbohydrate, and lipid metabolism. It has catalytic and structural functions in more than three thousands zinc proteins. Zinc(II) can modulate cellular signal recognition, second messenger metabolism, protein kinase and protein phosphatase activities. It may modulate activities of transcription factors. Zinc(II) modifies the metabolism of cGMP, the activities of protein kinase C and MAPK, and the activity of MTF-1 which regulates the transcription of the genes for MT.¹⁶²

Cellular zinc levels are regulated by two families of specific transport proteins as ZnT family and ZIP family. Proteins of the ZnT family transport Zn from cells and into intracellular compartments from the cytoplasm. ZIP proteins transport zinc into the cells.¹⁶³ Breast cancer as well as tumours that spread to lymph nodes express zinc transporter LIV-1 associated with an oestrogen receptor.^{163–165}

A second essential element is copper(II), a component of metalloenzymes important for electron transfer, oxygen transport, and oxygenation reactions. Copper(II) has three main transporters as ceruloplasmin, albumin, and transcuprein.¹⁶⁶ MTs interact with different proteins important for cell cycle regulation. They are the p50 subunits of NF- κ B, kinase domain of PKC δ , and GTPase Rab3A. MT can modulate the biological activity of p53 by zinc(II) exchange. MT-1 and MT-2 regulate the level, activity and cellular location of the transcription factor NF- κ B.^{167–170} NF- κ B is essential for cell protection from apoptosis induced by TNF and other stimuli. NF- κ B activates antiapoptotic genes such as Bcl-2, c-myc, and TRAF-1. Apo-MT-1 (metal-free form of MT-1), but not MT-1 (MT-1 with metal ion) forms a complex with p53 and, thus increases metal-dependent expression of MREs.^{154,169,171} A novel pathway for MT expression was discovered in the papillary thyroid cancer cell line (KAT5) where MT expression is predated by elevated calcium ions and ERK1/2. Inhibition of calcium as well as ERK1/2 led to blocking of MT expression and progression of G0/G1 to G2/M cell phase.¹⁷² Lim *et al.* demonstrated the role of MT-2A in modulation of cell cycle progression from G1 to S phase

via the ATM/Chk2/cdc25A pathway.¹⁷³ Intra-tumour hypoxia is another feature of various types of cancers, including prostate carcinoma; it is associated with tumour progression, acquisition of anti-apoptotic potential and therapeutic resistance.¹⁷⁴ Proliferation of prostate tumour cells LNCaP and PC-3 was related to the protective effect of MT under hypoxia and its up-regulation is demonstrated.¹⁷⁵ Connection between reactive oxygen species and cell cycle progression has been demonstrated repeatedly.¹⁷⁶ They are produced during cell proliferation. Several biological events can be considered, such as the electron transport in energy metabolism.¹⁷⁶ Endogenous ROS accumulation leads to inhibition of cell proliferation. Antioxidant effects of MT, especially in the nuclei of observed (MT)-null (MT^{-/-}) cells, is obvious. Hypoxia itself is accompanied by oxidative stress and ROS generation. In conclusion, MT regulates cell proliferation through its antioxidant activity and zinc(II) level control.

6. Metal based drugs and their effect on MT level – potential risk of resistance

According to the World Health Organisation (WHO), cancer represents the leading cause of death in developed countries. Anti-tumour drugs, based on platinum, are used in the treatment of malignant diseases. These cytostatics include cisplatin, carboplatin, and recently oxaliplatin. Clinical studies demonstrate the origin of platinum-based drug resistance which causes significant complications during anti-tumour therapy. Resistance of cancer cells to cytostatic drugs is usually a complex multifactorial phenomenon which involves combination of more than one mechanism.¹⁷⁷ There is no single predominant mechanism even within histological type tumours.¹⁷⁷ The most important mechanisms are increased drug efflux resulting in reduced drug accumulation, activation of detoxification mechanisms in tumour cells, altered activities of certain enzymes, such as reductases and aldehyde dehydrogenases, increased DNA repair, increased tolerance to DNA damage, changed O6-alkylguanine DNA alkyltransferase activity, methyladenine DNA glycosylase activity, nucleotide repair system pathway, changes in target structure, or increased levels of intracellular thiols such as glutathione and metallothionein, which binds compounds toxic for cells.¹⁷⁸

6.1 Clinical relevance of MT

Study and comprehension of these mechanisms will enable the design of suitable techniques and procedures for malignant tumour treatment. Increased MT levels were detected in many tumour types and cells such as breast, kidney, nasopharynx, lungs, prostate, testes, urinary bladder, cervix, endometrium, salivary glands, pancreas, acute lymphoblastic leukaemia or melanoma. MT level is directly connected with the grade and prognosis of disease.^{179,180} The intensity of metallothionein (MT-1/2) expression in various histological types of non-small cell lung cancer (NSCLC) was evaluated and the expression intensity was correlated with clinical/pathological parameters and Ki-67 and minichromosome maintenance protein 2 (MCM-2) proliferation markers. A positive correlation was noted between expression of MT-1/2 and expressions of Ki-67 and MCM-2 in NSCLC overall. MT-1/2 expression is evident in proliferating NSCLC neoplastic cells, pointing to the prognostic importance

of parallel expression of MT-1/2 and Ki-67.¹⁸¹ In addition, MT expression in mobile tongue squamous cell carcinoma (SCC) was investigated. All of the examined mobile tongue SCC cases showed MT positivity in tumour cells; however, neither MT overexpression nor staining intensity was significantly associated with clinical and pathological parameters. MT cellular distribution was significantly associated with histopathological grade of differentiation and depth of invasion. MT staining intensity was identified as a significant predictor of overall patient survival. MT positivity correlated with depth of invasion, vascular invasion and the existence of lymph node metastases. MT may be implicated in the development and progression of mobile tongue SCC and may be considered as a useful clinical marker for patient management and prognosis.³ An analysis of MT-3 staining in urinary cytology showed that a subset of both active and non-active patients with urothelial cancer shed positive cells in their urine, but that control patients only rarely shed MT-3 positive cells. The MT-3 gene is silenced in non-transformed urothelial cells by a mechanism involving histone modification of the MT-3 promoter. In contrast, transformation of the urothelial cells with either Cd(II) or As(III) modified the chromatin of the MT-3 promoter to a bivalent state of promoter readiness. Urinary cytology for MT-3 positive cells would not improve the diagnosis of urothelial cancer, but might represent a potential biomarker for tumour progression.¹⁸² Further, this method was applied to detect MTs in blood serum obtained from patients with breast cancer and in neuroblastoma cells resistant and sensitive to cisplatin in order to show the possible role of metallothioneins in carcinogenesis. It was found that MT level in blood serum was almost twice higher as compared to the level determined in healthy individuals.^{183,184}

MT levels were also determined in blood of patients suffering from primary malignant tumours in the head and neck areas. The obtained data suggests that reference MT level in the blood of healthy humans is within the interval from 0.2 to 0.8 μM . In the tumour blood samples, the most extended group was represented by patients suffering from oropharyngeal cancer, laryngeal cancer, hypopharyngeal cancer, and oral cavity cancer and occurring nasal cavity and paranasal sinus cancer and parotid carcinoma. MT levels determined in the blood of patients varied from 1.08 to 6.39 μM , whereas average values differed in accordance with tumour localization. MT levels are closely associated with the rate of tumour differentiation, stage of tumour disease and tumour cell characteristics.¹⁸⁵ As far as the MT level in tumour tissues is concerned, the highest MT level was determined in the tissues of oral tumours followed by hypopharynx and larynx. MT level related to the size of primary tumour focus oscillates with increasing tumour size. Notwithstanding this fact, the increasing tendency with tumour disease progression is observable. A similar tendency was determined for MT level related to the metastatic activity of tumours, where the MT level increased with tumour spreading into local lymph nodes.¹⁸⁶

Systematic comparison of PSA and metallothionein (MT) levels in blood serum of prostate cancer-diagnosed patients was performed. The obtained results indicate that the potential of MT as an additional prostate cancer marker reducing the probability of false positive/negative diagnosis in combination

with PSA.¹⁸⁷ Another study was focused on the determination of putative tumour markers of aggressive high-grade forms of prostate cancer. Alpha-methylacyl-CoA racemase (AMACR), metallothionein classes 1A and 2A (MT1A and MT2A) were determined and compared to prostate specific antigen (PSA) levels. In the case of serum level, a significantly enhanced MT level (4.5-fold) in patients' sera was found. No significant changes were observed in the case of AMACR. These findings indicate a possible alternative role of MT to a PSA prostate cancer marker.¹⁸⁸ Also no major relationships between serum caveolin-1 level and serum MT levels were found. No trend was observed when MT was associated with AMACR. When cluster analysis on MT and caveolin-1 was carried out in the same way as with PSA or markers of oxidation, it was found that when both of these serum markers are at high levels, worse prognosis is expected because of the greater proportion high grade tumours.¹⁸⁹

Moreover, serum metallothionein levels of patients with childhood solid tumours were determined. A more than 5-fold increase in the amount of metallothionein was found in sera of patients suffering from cancer disease, compared with those in the sera of healthy donors.¹⁹⁰ Results from this work indicate that the MT level in blood serum may be considered as a promising marker for diagnostics, prognosis and estimation of therapy efficiency in childhood tumours.¹⁹¹

6.2 Chemoresistance of anticancer drugs

6.2.1 Metal-based drugs. Metallothionein as protein with high affinity to metals is capable due to its -SH groups to bind platinum-based cytostatics through platinum and thereby reduce their cytotoxic effect. Chemoresistance to main platinum antitumor compounds like cisplatin and carboplatin is mediated through two broad mechanisms, first, a failure of a sufficient amount of platinum to reach the target DNA and, second, a failure to achieve cell death after binding of platinum to DNA.¹⁹² Transfer of platinum from cisplatin and carboplatin to MTs results in inactivation of those drugs.¹⁹³ A newly developed platinum-derived cytostatic compound, heptaplatin, is more effective against the MT-overexpressing gastric cancer cell line resistant to cisplatin than either cisplatin or carboplatin. In addition, pre-treatment with zinc inducing MT's reduced cytotoxicity of cisplatin and carboplatin but not heptaplatin was observed.¹⁹⁴ The influence of the zinc pre-treatment on the increase in resistance to cancer treatment can be viewed in two ways: (a) as an inducer of MTs, and (b) as a protector itself by obstructions of ionic channels. Ionic channels are used to facilitate heavy metal ion entry into a cell. This mechanism was demonstrated as preventing cadmium(II) ion toxicity. MT plays a crucial role in limiting cadmium entry to the cell by transporting essential zinc to the membrane and the binding of toxic cadmium. Similar mechanisms of toxicity reduction is supposed in platinum-based cytostatics which may be pumped out of the cells by several transport systems.¹⁹⁵ We showed that cultivation of neuroblastoma-derived cell lines resistant to cisplatin in medium with cisplatin or carboplatin significantly increased MT levels measured by adsorptive transfer technique.^{196,197} On the other hand, in a sensitive cell line we detected only insignificant MT increase after cultivation with the same concentrations of

cisplatin or carboplatin. MT levels both in chemosensitive and chemoresistant cancer cells were low if they were cultivated in platinum free medium. Cisplatin-resistant neuroblastoma cell lines were established by exposing parental neuroblastoma lines to increasing concentrations of cisplatin.¹⁷⁷ Other experimental studies showed that a cisplatin-resistant ovarian cancer cell line exposed to cisplatin manifested a nuclear MT expression, detected by immunocytochemistry, while this line was cultivated in medium without platinum and a cisplatin-sensitive line expressed MTs only in cytoplasm.¹⁹⁸ In hepatoblastoma patients treated with carboplatin, it was verified that non-responders had higher percentage immunohistochemistry detected MT positive tumour cells.^{199,200} The significance of MT expression for resistance of gastric cancer to cisplatin was verified by Suganuma *et al.* who examined expression of 6300 genes by an oligonucleotide microarray method in gastric cancer samples.²⁰¹ They reported that MTs may cause resistance to some "non-metal cytostatic drugs". They reduce apoptosis induced by etoposide in lung and liver cancer cell lines and this effect is amplified by induction of MTs by pre-treatment with zinc or cadmium. In this study, cellular MT concentrations were estimated using a cadmium-hemoglobin radioassay method.¹⁴⁷

6.2.2 Non-metal-based drugs. Due to etoposide induced apoptosis by uncorrected DNA damage it may be supposed that MTs play a role in prevention of apoptosis. The exact mechanism by which MTs inhibit cell death from etoposide exposure was unknown until now. Microarray analyses on paired gastric cancer cells collected before and after irinotecan treatment identified five MT isoforms having significantly higher signal in non-responders than in responders. When compared with control cells, a human gastric adenocarcinoma-derived cell line transfected by MT-1X showed significantly increased IC₅₀ for irinotecan.²⁰² The role of MTs and pro-apoptotic gene down-regulation in medulloblastoma and rhabdomyosarcoma cell lines with induced resistance to the alkylating drug BCNU was demonstrated using microarray gene expression analysis.²⁰³ Esophageal squamous cell carcinomas, non-expressing MTs, respond well to chemoradiotherapy (5-fluorouracil and cisplatin) while cancer with high MT expression are resistant.²⁰⁴ Women with invasive breast ductal carcinoma treated with adjuvant chemotherapy (cyclophosphamide/methotrexate/5 fluorouracil- or doxorubicin based regimes) had significantly longer event free survival if their tumour had lower MT expression. MT was detected both by immunohistochemistry and by real time (RT) PCR.^{66,205} Similar results were described by Hishikawa and co-workers in oesophageal cancer. They found more frequent 5-year survival of patients suffering from squamous cell oesophageal cancer treated with cisplatin in MT negative than MT positive tumours. This finding confirmed that MT expression in squamous cell oesophageal cancer detected by immunohistochemistry is a predictive factor for cisplatin efficiency.²⁰⁶ Experimental study showed that increased MT concentration in the heart prior to exposure to doxorubicin prevents anthracycline induced cardiotoxicity. MTs in myocardial tissue may be pharmaceutically induced by bismuth subnitrate, isoproterenol, and tumour necrosis factor.²⁰⁷ Studies using transgenic mice with MT overexpression showed that MT protection against anthracycline cardiotoxicity

is related to its anti-apoptotic effect by inhibiting both mitochondrial cytochrome c-release-mediated and p38-MAPK-mediated apoptotic signal pathways. It seems that MTs interfere with oxidative stress caused by anthracycline metabolism in the heart.²⁰⁸

The affinity of bismuth for binding to sulphur compounds has been reported; one such target biomolecule is the cysteine-rich metalloprotein metallothionein. Renal mammalian metallothionein is shown to be induced by Bi salts, with the Bi³⁺ binding to the renal MT. However, the exact metal-to-metallothionein stoichiometric ratios for the 2-domain beta alpha mammalian protein and the individual beta and alpha domain fragments remains unknown.²⁰⁹ MT cooperates with GSH/GST system; glutathione S-transferases are a family of enzymes catalysing conjugation of anti-tumour drugs⁷ such as melphalan, cyclophosphamide and chlorambucil with tripeptide glutathione, the most abundant intracellular thiol.²¹⁰

6.2.3 X-rays. The incidence of X ray induced lymphoma is significantly lower in wild mice compared to MT1/2 null mice. The levels of 8-hydroxy-2'-deoxyguanosine (an indicator of oxidative damage DNA) after irradiation were increased more in MT deficient mice than in wild strain.⁶⁵ Shibuya and co-workers also detected more severe X-ray induced hematotoxicity in MT deficient mice than in wild strains.²¹¹ Zinc(II) sulphate and bismuth nitrate pre-treatment prevented X-ray induced myelosuppression in wild strain mice but not in MTI/II null ones. These results indicate that MTs play important role in protection against carcinogenesis and cell injury induced by radiation and against oxidative damage of DNA induced by X-rays application. Bismuth compounds are currently used to treat gastric ailments and to prevent toxic side effects from cancer treatments.

7. Conclusions

There are various types of thiols that play crucial roles in a cell.^{212,213} Metallothioneins thanks to their unique structure are able to bind metal ions and to scavenge reactive oxygen species. This feature is used in numerous reactions and, thus, MT can play important role in various biochemical pathways. The above mentioned results, both experimental and clinical, showed that MTs play an important role in chemoresistance to platinum-based cytostatics and probably also in some "non-metal cytostatic drugs". Therefore MT detection in tumours may be useful as a predictive marker. Their inhibition seems to be promising in acquiring improvement to therapeutic treatment of some malignant tumours. Also, important is the protective role of MT in the prevention of radiation induced cell damage.

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