BioMetals **16:** 403–409, 2003.

Effect of metal-based anticancer drugs on wild type and metallothionein null cell lines

H. Mahboobi[1], A. Viarengo[1], D. Colangelo[2] & D. Osella[1,*]
[1]*Dipartimento di Scienze e Tecnologie Avanzate, Universitá del Piemonte Orientale 'A. Avogadro', Corso Borsalino 54, 15100 Alessandria, Italy;* [2]*Dipartimento di Scienze Mediche, Universitá del Piemonte Orientale 'A. Avogadro', Via Solaroli 17, 28100 Novara, Italy;* *Author for correspondence (Fax: +39 0131 287416; E-mail: domenico.osella@mfn.unipmn.it)*

Received 31 May 2002; Accepted 26 June 2002; Published online February 2003

Key words: chemoresistance, metal-based anticancer drugs, metallothionein, mouse embryo fibroblasts

Abstract

Metallothioneins (MT) are ubiquitous low-molecular-weight metal-binding intracellular proteins. We used wild type mouse embryo fibroblasts, GKA1, and its MT-null variant, named GKA2, in order to correlate the presence of MT to the response to a number of different antitumor drugs with different mechanisms of action. We studied sensitivity of GKA1 and GKA2 cells to metal-based compounds having alkylating property, or able to generate reactive oxygen species (ROS); as well as to drugs acting with different mechanisms. The absence of MT in GKA2 cells was correlated to higher sensitivity to the metal-based drugs compared to that of GKA1. No marked differences in sensitivity of two cell lines against gemcitabine, taxol, and vinblastine were observed. No significant change in sensitivity of either GKA1 or GKA2 cells to these non-alkylating drugs was seen after heavy metal pretreatments. In GKA1 cells, MT biosynthesis was induced by copper and cadmium but not by zinc treatment under the conditions of these experiments. Induction of MT was directly proportional to decrease in sensitivity of GKA1 cells to the compounds used in this experiment. In contrast to GKA1 cells, the MT-null cells (GKA2) expressed no detectable metallothionein either constitutively or after treatment with zinc, copper, or cadmium. Nonetheless, heavy metal pretreatment of GKA2 cells did not cause any change in their sensitivity.

Introduction

Metallothioneins (MTs) are defined as low molecular weight (6–7 kDa), cysteine-rich (30%), intracellular proteins with high affinity for certain trace metals including cadmium, mercury, platinum, and silver, as well as biologically essential metals like zinc and copper (Shaw *et al.* 1991). Although metallothionein does not show any enzymatic activities, multifunctional roles have been proposed such as a chelator to harmful heavy metals and excessive essential metals, and a scavenger to various radicals and reactive oxygen species (ROS) (Basu & Lazo 1990; Klaassen *et al.* 1999). These biological roles are manifested through the thiolic functionalities that characterizes MT. The intracellular levels of MT can be readily increased by heavy metals, cytokines, drugs, steroids, and hypoxia via transcriptional activation (Lazo & Pitt 1995). Early research on MT has generally concerned with its role in essential trace element homeostasis and in metal toxicity, or with the molecular control mechanisms of its genetic expression. In the last decade, however, another area has emerged in MT research, which concerns its role in disease states and their therapy. In this regard, the aspect that has received the most attention is the role of MT in tumor chemotherapy. This includes a potential role for MT in drug resistance, as well as in possible rescue therapy to reduce drug toxicity. The nucleophilic properties of MT resulting from its high cysteine content have stimulated several investigators (Kelley *et al.* 1988; Cherian *et al.* 1994; He *et al.* 2000) to examine the protective role of MT against electrophilic anticancer drugs and mutagens. Elevated MT levels have been observed in some

malignant cells with acquired resistance to antineoplastic agents (Kelley *et al.* 1988). Selection for heavy metal resistance produces cells with cross-resistance to anticancer drugs, such as cisplatin and melphalan. Increase in intracellular MT by gene transfer also can produce resistance to electrophilic mutagens and anticancer drugs, such as cisplatin and mitomycin C (Kelley *et al.* 1988). Nevertheless, increases in MT do not always result in a phenotype that is less sensitive to the toxic effects of electrophilic antineoplastic agents and mutagens (Schilder *et al.* 1990).

Cells grown in culture provide an invaluable tool for examining the importance of MT in the cellular responsiveness to antineoplastic agents. Among the studies aimed at examining the role of MTs, MT-null cells are the most direct and simple model. Therefore, in the current studies we have examined embryonic fibroblasts from wild type and MT-null mice and characterized their sensitivity to metal-based anticancer drugs.

MT can be induced by chemical stimuli such as metals and organic chemicals as well as by ultraviolet radiation and various kinds of stress (Suzuki 1996). Therefore, in this study, prior to exposure of the cells to various anticancer drugs, we pretreated the cells with different concentrations of $ZnCl_2$, $CuSO_4$, and $CdCl_2$ for different time intervals.

Materials and methods

Chemicals

The metal-based drugs, cisplatin (Dhara 1970), carboplatin (Harrison *et al.* 1980), and decamethylferrocenium tetrafluoroborate (DFc^+) (Osella *et al.* 2000) were synthesized according to the published procedures. Gemcitabine was obtained from Eli Lilly, taxol from Bristol–Myers Squibb, and vinblastine as well all other chemicals and solvents, unless noted otherwise, were purchased from Sigma Chemical Co.

Cell culture

The simian virus 40 (SV-40) transformed mouse embryo fibroblasts from MT-I and MT-II double nullizygous (knockout) mice, named GKA2, and normal mice of the same strain, named GKA1, were kindly provided by Prof Dr G. K. Andrews, University of Kansas Medical Center. Cells were maintained in Dulbecco's modified Eagle's medium (MEM) supplemented with 10% (v/v) fetal bovine serum, 2 mM L-glutamine, and antibiotics (penicillin 100 IU/ml and streptomycin 100 IU/ml). Cells were grown at 37 °C in 5% CO_2 and passaged every 3 days.

Pretreatment with heavy metals

For heavy metal pretreatment, cells were exposed to complete medium containing $ZnCl_2$ (10 μM, 2 days), $CuSO_4$ (10 μM, 2 days), or $CdCl_2$ (0.4 μM, 6 days). Higher concentrations of these metals cause evident cytotoxic effects. Control cells were allowed to grow in complete medium under the same experimental conditions.

Cytotoxicity

Cell sensitivity to various drugs were determined using MTT (3-(4-,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) assay (Mosmann 1983). Briefly, exponentially growing (control and heavy metal-pretreated) cells were harvested by means of treatment with 0.1% trypsin/0.02% EDTA for 3 min and the resulting single cell suspension was plated in 6-well plates at 60,000–70,000 cells/well. After 24 h at 37 °C, cells were exposed to 0–100 μM carboplatin, 0–7 μM cisplatin, 0–100 nM gemcitabine, 0–150 nM taxol, or 0–150 nM vinblastine. Moreover, a molecule able to produce radical stress, DFc^+ (Osella *et al.* 2000), was also employed in 0-150 μM range. After 3 days at 37 °C, 100 μl of MTT stock solution (5 mg/ml) was added into each well and the cells were incubated at 37 °C for 1 h. The converted dye was solubilized with acidic propanol (0.1 N HCl in absolute propanol). The viability of cells was quantified by measuring the absorbance of converted dye at 570 nm with background subtraction at 630 nm. The inhibition values were plotted against concentration of drug in medium, and IC_{50} value (defined as the concentration of drug required to inhibit 50% of cell growth) was then calculated.

Metallothionein sample preparation

Exponentially growing cells (25×10^6) were harvested as described above. The cell suspension was washed 3 times in ice-cold phosphate-buffered saline, and resuspended in 250 μl of buffer containing 50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 0.2% SDS, 2% Triton X-100, 0.5% sodium deoxycholate, 0.01% β-mercaptoethanol, 2 μg/leupeptin, and 2 mM phenylmethylsulfonyl fluoride (PMSF) at 4 °C. Lysates were sheared through a 26-gauge needle and clarified at 4 °C by microcentrifugation. The supernatant was

then treated with ethanol/chloroform as described before (Viarengo *et al.* 1997). Briefly, 1.05 vol of cold (−20 °C) absolute ethanol and 0.08 vol of chloroform was added to the supernatants. The samples were centrifuged at 12.000 × *g* for 5 min at 4 °C. The supernatants were collected and 3 vol of cold ethanol were added. The samples were incubated at −20 °C for 1 h and were centrifuged at 12.000 × *g* for 5 min. Finally, the pellets were resuspended in 50 ml of 50 mM Tris-HCl buffer, pH 7.4. Protein content of the samples was determined by means of the BCA protein assay (Pierce, Rockford, IL) using bovine serum albumin as the standard.

Western immunoblot analysis

The cell extract proteins (30 μg) were loaded on a 12% polyacrylamide gel, electrophoresed, and transblotted to a nitrocellulose membrane. The blot was incubated with a blocking buffer (5% bovine serum albumin in 1× TBS) 2 h at room temperature. The blot was incubated with metallothionein (N-19) antibody (Santa Cruz Biotechnology) overnight at 4 °C on a shaker. The blot was then washed three times with TBS/Tween, and incubated with a 1:1000 dilution of secondary antibody coupled with horseradish peroxidase for 1 h at room temperature on a shaker. The blot was washed three times with TBS/Tween and then incubated with ECL^{TM} Western Blotting detection reagents (Amersham, UK) for 1 min at room temperature. The blot was exposed to x-ray film to visualize the results.

Electrophoretic-fluorimetric assay

To asses the metallothionein content of the samples, electrophoretic-fluorimetric procedure was performed according to method of Viarengo *et al.* (1997) with slight modifications. Briefly, appropriate amounts of 10% SDS (sodium dodecyl solfate) and 92 mM Thiolyte (monobromobimane-mBBr) solutions were added to 50 μl aliquots of samples to obtain final concentrations of 2% and 6 mM for SDS and Thiolyte, respectively. Samples were then incubated for 30 min at 37 °C in the dark. Samples were diluted 1:1 with glycerol, and the thiolyte labeled proteins (30 μg) were separated by SDS polyacrylamide gel electrophoresis (Laemmli 1970) on a 16% polyacrylamide gel. After electrophoresis, the proteins on the gel were fixed in methanol: acetic acid: water (45:10:45 v/v). Fluorescence of the protein bands in the gel was visualized using Fluor-S Max MultiImager System (Biorad).

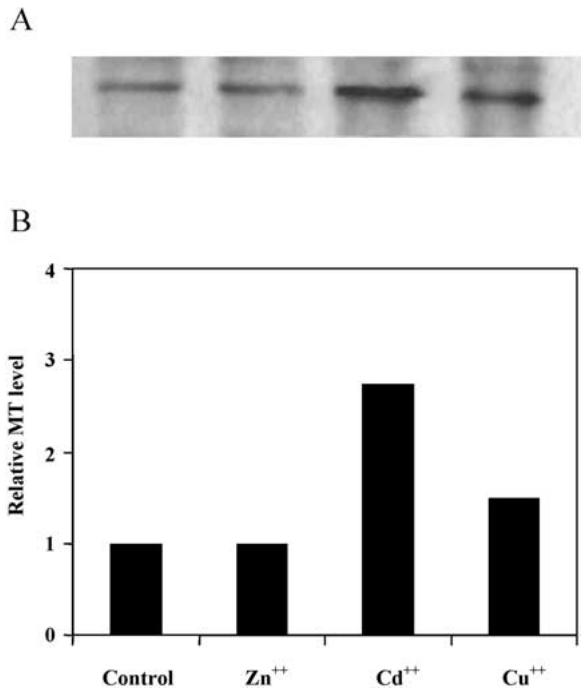

Fig. 1. Western immunoblot analysis of the effect of $ZnCl_2$, $CdCl_2$, and $CuSO_4$ on the level of MT in GKA1 (wild type) cells (A). The protein band densities were quantified by densitometry: the relative MT level is shown as the ratio between heavy metal- treated and control cells (B). Heavy metal treatment conditions were performed as described in *Materials and methods*.

Statistical analysis

The data were analyzed according to Student *t*-test. Significance was established at $P < 0.05$ level. The results of at least three measurements were presented as mean ± S.D.

Results

Western immunoblot analysis of the GKA1 and GKA2 lines confirmed the different patterns of MT expression, i.e. the wild type cell line showed detectable amounts of MT while the knockout cells were MT-negative.

The continuous treatment with $ZnCl_2$ for two days under our experimental conditions did not induce any change in MT content of GKA1 (Figure 1A). On the other hand, an increase of MT level was experimentally demonstrated after $CuSO_4$ or $CdCl_2$ exposure and was calculated as 1.5 and 2.8-fold, respectively as compared with the untreated cells used as control (Figure 1B). As expected, these heavy metal treatments caused no MT expression in GKA2 cells.

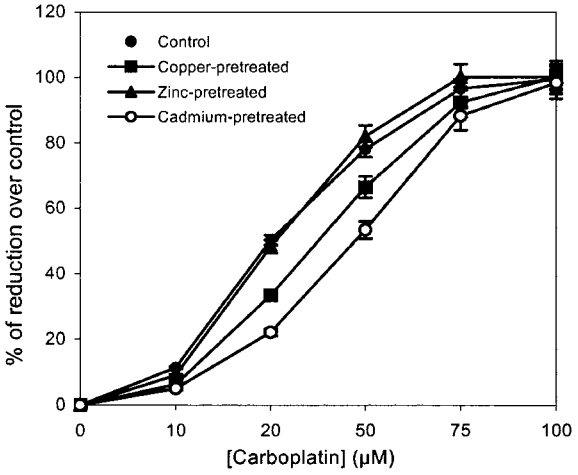

Fig. 2. Dose - cytotoxic response of carboplatin on GKA1 (wild type) mouse fibroblasts from which IC_{50} are determined. Concentration and duration of heavy metal pretreatments are described in *Materials and methods*.

The survival ratio of metal-pretreated GKA1 and GKA2 cells (under the mild conditions employed) were $\geq 99\%$ with respect to control cells, as determined by the MTT assay.

Our group has already set a method for MT determination in molluscs (Viarengo *et al.* 1997). This procedure had a good sensitivity, but it was not enough to provide accurate measurements of MT content in the mice fibroblasts used in this work. Using this electrophoretic-fluorimetric assay, it was possible to qualitatively reveal a MT increase in GKA1 cells only for exposure to $CdCl_2$ (0.4 μM for 6 days), while no apparent variation in MT level was seen for $ZnCl_2$ or $CuSO_4$ treatments. GKA2 line showed no detectable MT content before and after metal induction treatment.

The effects of several antineoplastic drugs on the two cell lines were also examined by MTT vitality assay. The non-alkylating drugs, gemcitabine, taxol, and vinblastine induced similar cytotoxic effects for both cell lines before (control) and after metal treatment, as evidenced by the IC_{50} values. These data are resumed in Table 1.

A different response was observed when the wild type (GKA1) and MT-null (GKA2) cell lines were treated with metal-based alkylating drugs, carboplatin and cisplatin, and with the ROS producing agent, DFc^+.

Both cell lines showed a concentration dependent response to carboplatin (Figure 2), cisplatin and DFc^+. The sensitivity of GKA2 cell line to carboplatin, cisplatin, and DFc^+ was always higher (from 2.6- to 3.3-fold) compared to GKA1 (Table 2 and 3, refer to the control), and this sensitivity was not affected at all by the pretreatment with metals.

The data presented in Table 2 point out that $ZnCl_2$ pretreatment is not able to significantly modify the IC_{50} values for all the metal-based drugs, thus confirming the data obtained from the content of MT. Pretreatment of GKA1 cells with $CuSO_4$, however, gave rise to a statistically significant decrease (≥ 1.5-fold) in sensitivity of the cells to these agents. Pretreatment with $CdCl_2$ led to a higher decrease (≥ 2-fold) in sensitivity of GKA1 cells to these agents (Table 2).

Atomic absorption spectrometry (AAS) was performed for determination of Pt uptake (AAS; model 3300 Perkin Elmer) according to the method described elsewhere (Perego *et al.* 1999). The results evidenced no significant difference in the intracellular Pt content of GKA1 and GKA2 cells as the consequence of the treatment, in the same experimental conditions, with cisplatin (data not shown).

Discussion

Since all the cells have various physiological levels of MT, the possibility for having a MT-null cell system, as previously reported in literature (Kondo *et al.* 1995), gives the chance to improve the knowledge of the role of MTs. The two cell lines used in this study showed no significant difference in growth pattern and viability under normal cell culturing.

In the current study, several antineoplastic drugs with different mechanism of action and different resistance machinery were used.

Gemcitabine, taxol, vinblastine were used as non-alkylating agents. Gemcitabine acts as an inhibitor of ribonucleoside diphosphate reductase and inhibits DNA synthesis (Matsui & Fukuoka 1992). Taxol and vinblastine (a vinca alkaloid) act on the mitotic fusion. In particular, taxol promotes microtubule assembly *in vitro* and induces the reorganization of the cytoskeleton into unusual microtubule arrays in cultured cells (Green & Goldman 1983) and vinblastine inhibits tubulin polymerization into microtubules (Zhou & Rahmani 1992). The cytotoxicity results of non-alkylating agents used in this work revealed no significant difference between sensitivity of GKA1 and GKA2 cells to these drugs. Moreover, heavy metal pretreatment did not alter the sensitivities of these cells to the non-alkylating agents.

Table 1. Effect of non-alkylating agents (gemcitabine, taxol, and vinblastine) on survival of control and heavy metal-pretreated GKA1 and GKA2 cells. Concentration and duration of pretreatments are described in *Materials and methods*. Data are expressed as the average concentration of drug required to kill 50% of cells (IC_{50}), obtained from three experiments ±SD.

Cell Line	Pretreatment	Gemcitabine (nM)	Taxol (nM)	Vinblastine (nM)
GKA1	Control	22.0 ± 2.0	39.8 ± 2.4	42.6 ± 3.4
	$ZnCl_2$	21.5 ± 1.9	38.6 ± 2.4	44.3 ± 2.7
	$CuSO_4$	21.9 ± 2.1	42.1 ± 2.0	41.3 ± 4.6
	$CdCl_2$	22.3 ± 2.4	40.9 ± 3.1	43.7 ± 4.5
GKA2	Control	20.0 ± 1.0	42.5 ± 1.8	40.5 ± 3.5
	$ZnCl_2$	19.5 ± 1.5	42.9 ± 2.5	43.5 ± 3.3
	$CuSO_4$	20.5 ± 1.8	44.5 ± 2.1	42.3 ± 4.2
	$CdCl_2$	20.8 ± 2.0	43.4 ± 2.7	43.1 ± 4.5

Table 2. Effect of alkylating agents (carboplatin and cisplatin) and ROS- producer (DFc^+) on survival of control and heavy metal-pretreated GKA1 (wild type) cells. Concentration and duration of pretreatments are described in *Materials and methods*. Data are expressed as the average concentration of drug required to kill 50% of cells (IC_{50}), obtained from three experiments ± SD.

Pretreatment	Carboplatin (μM)	Resistance ratio[a]	Cisplatin (μM)	Resistance ratio[a]	DFc^+ (μM)	Resistance ratio[a]
Control	23.0 ± 2.0		1.8 ± 0.1		23.1 ± 2.7	
$ZnCl_2$	22.0 ± 2.7	0.96	2.0 ± 0.2	1.11	25.6 ± 2.5	1.11
$CuSO_4$	35.8 ± 3.0	1.56	2.9 ± 0.2	1.61	35.3 ± 3.0	1.53
$CdCl_2$	46.6 ± 4.1	2.03	3.7 ± 0.3	2.06	46.7 ± 2.3	2.02

[a]Resistance ratio is IC_{50} of heavy metal-pretreated cells divided by IC_{50} of control cells.

Carboplatin and cisplatin were chosen as a model for alkylating agents. The cellular toxicity of these electrophilic agents occurs primarily through their ability to generate covalent DNA inter- and intra-strand cross-links, hence, preventing DNA replication and transcription (Lippert 1999).

There is an increasing body of evidence in the literature concerning antioxidant property of MT (Basu & Lazo 1990; Viarengo *et al.* 1999). To evaluate the involvement of MT in prevention of free radical injury, we examined the cytotoxicity of DFc^+, an iron-based complex able to produce reactive oxygen species (ROS), especially OH radicals (Osella *et al.* 2000).

The cytotoxicity results of alkylating and radical producing compounds used in this work revealed significant differences between sensitivity of GKA1 and GKA2 cells to these drugs, and the heavy metal pretreatment further enhanced these differences.

The response to the treatment with a number of anticancer drugs allowed us to confirm the importance of MT in protection against the metal-based agents (Lazo & Pitt 1995). This is evident from the comparison of the level of sensitivity of the two cell lines to Pt-based drugs used in this study (Tables 2 and 3).

Knockout of the MT gene renders animals/cells more vulnerable to oxidative stress and DNA alkylating agent-induced toxicity (Klaassen & Liu 1998). In the current study, since both cell lines were treated in the same way with heavy metals, any possible resistance mechanism(s) other than induction of MT should have occurred to the same extent for both cell lines. Thus, the decrease in sensitivity observed for metal-pretreated GKA1 cells with respect to GKA2 is, most probably due to the increase in MT level. Moreover, similar Pt uptake in the two cell lines after cisplatin treatment, as evidenced by AAS, supports the conclusion that the difference in the drug sensitivity

Table 3. Effect of alkylating agents (carboplatin and cisplatin) and ROS-producer (DFc^+) on survival of control and heavy metal-pretreated GKA2 cells. Concentration and duration of pretreatments are described in *Materials and methods*. Data are expressed as the average concentration of drug required to kill 50% of cells (IC_{50}), obtained from three experiments ± SD. Resistance ratio is IC_{50} of heavy metal-pretreated cells divided by IC_{50} of control cells.

Pretreatment	Carboplatin (μM)	Cisplatin (μM)	DFc^+ (μM)
Control	6.9 ± 0.8	0.7 ± 0.1	8.5 ± 1.0
$ZnCl_2$	7.2 ± 0.9	0.8 ± 0.09	8.7 ± 0.9
$CuSO_4$	7.1 ± 0.8	0.7 ± 0.1	8.3 ± 1.1
$CdCl_2$	6.8 ± 0.7	0.8 ± 0.1	8.9 ± 1.0

(IC_{50}) is not due to different drug permeability of the cells.

In conclusion this work confirms that MT is a factor in determining the responsiveness of normal cells to electrophilic platinum-containing agents, such as carboplatin and cisplatin, as well as to oxidative stress induced by the iron-based compound, DFc^+.

Acknowledgements

This work was financially supported by MIUR (Roma) and CNR (Roma). We are indebted to Johnson Matthey Ltd. for a generous loan of K_2PtCl_4. We thank Drs Nadia Ceratto and Annalisa Ghiglia for useful advice concerning the cell growth, and Dr Francesco Dondero for electrophoretic-fluorimetric assay. This research was carried out in the frame of the EU Cost D20 action (metal compounds in the treatment of cancer and viral diseases).

References

Basu A, Lazo JS. 1990 A hypothesis regarding the protective role of metallothioneins against the toxicity of DNA interactive anticancer drugs. *Toxicol Lett* **50**, 123–135.

Cherian MG, Howell SB, Imura N *et al.* 1994 Contemporary issues in toxicology. Role of metallothionein in carcinogenesis. *Toxicol Appl Pharmacol* **126**, 1–5.

Dhara SC. 1970 A rapid method for the synthesis of *cis*-$[Pt(NH_3)_2Cl_2]$. *Indian J Chem* **8**, 193–194.

Green KJ, Goldman RD. 1983 The effects of taxol on cytoskeletal components in cultured fibroblasts and epithelial cells. *Cell Motil* **3(4)**, 283–305.

Harrison RC, McAuliffe CA, Zaki AM. 1980 An efficient route for the preparation of highly soluble platinum (II) antitumor agents. *Inorg Chim Acta* **46**, L15–L16.

He T, Wei D, Fabris D, Fenselau C. 2000 Intracellular sequestration of anti-tumor drugs by metallothionein. *Cell Mol Biol* **46(2)**, 383–392.

Klaassen CD, Liu J. 1998 Metallothionein transgenic and knockout mouse models in the study of cadmium toxicity. *J Toxicol Sci* **23 (Suppl 2)**, 97–102.

Klaassen CD, Liu J, Choudhuri S. 1999 Metallothionein: an intracellular protein to protect against cadmium toxicity. *Annu Rev Pharmacol Toxicol* **39**, 267–294.

Kelley SL, Basu A, Teicher BA, Hacher MP, Hamer DH, Lazo JS. 1988 Overexpression of metallothionein confers resistance to anticancer drugs. *Science* (Washington DC). **241**, 1813–1815.

Kondo Y, Woo ES, Michalska AE, Choo KHA, Lazo JS. 1995 Metallothionein null cells have increased sensitivity to anticancer drugs. *Cancer Res* **55**, 2021–2023.

Laemmli UF. 1970 Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227**, 680–685.

Lazo JS, Pitt BR. 1995 Metallothioneins and cell death by anticancer drugs. *Annu Rev Pharmacol Toxicol* **35**, 635–653.

Lazo JS, Kondo Y, Dellapiazza D, Michalska AE, Choo KHA, Pitt BR. 1995 Enhanced sensitivity to oxidative stress in cultured embryonic cells from transgenic mice deficient in MT I and II genes. *J Biol Chem* **270**, 5506–5510.

Lippert B, ed. 1999 *Cisplatin. Chemistry and biochemistry of a leading anticancer drug*. Weinheim: Wiley – VCH.

Matsui K, Fukuoka M. 1992 New antitumor antimetabolites-Gemcitabine and DMDC. *Gan To Kagaku Ryoho* **19(13)**, 2127–2132.

Mosmann T. 1983 Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. *J Immunol Meth* **65**, 55–63.

Osella D, Ferrali M, Zanello P, Laschi F, Fontani M, Nervi C, Cavigiolio, G. 2000 On the mechanism of the antitumor activity of ferrocenium derivatives. *Inorg Chim Acta* **306**, 42–48.

Cassino C, Cavigioglio G, Colangero D, Galeotti F, Ghiglia A, Osella D, Tabbí G. 2001 Water stability and cytotoxic activity relationship of a series of ferrocenium salts. Characterization of the radical production during the degradation process. *J Inorg Biochem* **86**, 171.

Perego P, Gatti L, Caserini C, Supino R, Colangelo D, Leone R, Spinelli S, Farrell N, Zunino F. 1999 The cellular basis of the efficacy of the trinuclear platinum complex BBR 3464 against cisplatin-resistant cells. *J Inorg Biochem* **77(1–2)**, 59–64.

Shaw CF, Stillman MJ, Suzuki KT. 1991 An overview of metal-thiolate complex formation in metallothioneins. In: Stillman

MJ, Shaw III CF, Suzuki KT, eds. *Metallothioneins: synthesis, structure and properties of metallothioneins, phytochelatins and metal-thiolate complexes.* Weinheim: VCH Verlagsgellschaft; 1–30.

Suzuki KT. 1996 Structure and function of metallothionein. *Nippon Rinsho* **54(1)**, 33–39.

Viarengo A, Ponzano E, Dondero F, Fabbri R. 1997 A simple spectrophotometric method for metallothionein evaluation in marine organisms: an application to Mediterranean and Antarctic molluscs. *Mar Environ Res* **44(1)**, 69–84.

Viarengo A, Burlando B, Cavaletto M, Marchi B, Ponzano E, Blasco J. 1999 Role of metallothionein against oxidative stress in the mussel *Mytilus galloprovincialis. Am J Physiol* **277**, R1612–R1619.

Zhou XJ, Rahmani R.1992 Preclinical and clinical pharmacology of vinca alkaloids. *Drugs* **44(Suppl 4, 1–16)**, 66–69.