Full Paper

Employment of Electrochemical Techniques for Metallothionein Determination in Tumor Cell Lines and Patients with a Tumor Disease

Ivo Fabrik,[a,b] *Sona Krizkova*,[a] *Dalibor Huska*,[a] *Vojtech Adam*,[a] *Jaromir Hubalek*,[c] *Libuse Trnkova*,[d] *Tomas Eckschlager*,[e] *Jiri Kukacka*,[f] *Richard Prusa*,[f] *Rene Kizek*[a]*

[a] Department of Chemistry and Biochemistry, Faculty of Agronomy, Mendel University of Agriculture and Forestry, Zemedelska 1, CZ-61300 Brno, Czech Republic
*e-mail: kizek@sci.muni.cz
[b] Department of Biochemistry, Faculty of Science, Masaryk University, Kotlarska 2, CZ-61137 Brno, Czech Republic
[c] Department of Microelectronics, Faculty of Electrical Engineering and Communication, Brno University of Technology, Udolni 53, CZ-60200 Brno, Czech Republic
[d] Department of Chemistry, Faculty of Science, Masaryk University, Kotlarska 2, CZ-61137 Brno, Czech Republic
[e] Department of Paediatric Haematology and Oncology, 2nd Faculty of Medicine, Charles University, V Uvalu 84, CZ-15006 Prague 5, Czech Republic
[f] Department of Clinical Biochemistry and Pathobiochemistry, 2nd Faculty of Medicine, Charles University, V Uvalu 84, CZ-15006 Prague 5, Czech Republic

Received: December 17, 2007
Accepted: April 4, 2008

Abstract
In the present paper we employed adsorptive transfer stripping technique coupled with chronopotentiometric stripping analysis for determination of metallothionein (MT) in tumor cell lines and differential pulse voltammetry Brdicka reaction for determination of MT in blood serum of patients with head and neck cancer or retinoblastoma, and of rats treated with cisplatin with respect to discuss the role of MT in formation of resistance on treatment with heavy metal based cytostatics. The cisplatin or carboplatin sensitive and resistant neuroblastoma cell lines were derived from the maternal cell line isolated from the bone metastasis of patients with neuroblastoma. Based on the results obtained it can be concluded that level of MT increases with higher dose of platinum based cytostatics at cells. Further we focused on determination of MT in blood serum of rats treated with cisplatin (two doses 1.05 mg and/or 2.1 mg of cisplatin per kg). The highest level of MT at rats treated with 1.05 mg cisplatin was determined after four hours as 4.9 μmol/L. In the case of the second experimental group the maximum was reached even after two hours of the treatment as 4.8 μmol/L. In addition we were interested in the effect of cisplatin or carboplatin treatment of patients with a tumor disease. At patients with tumor in head and neck area treated with cisplatin we observed that the level of MT was going higher due to administration of the drug. This phenomenon was observed at all patients. However at patients with retinoblastoma treated with carboplatin we observed various phenomena including decreasing, increasing or no changes in MT level. Progression of MT levels was therefore individual and probably depended on tumor resistance to carboplatin.

Keywords: Metallothionein, Carboplatin, Cisplatin, Neuroblastoma cell, Retinoblastoma, Head and neck cancer, Resistance, Voltammetry, Brdicka reaction

DOI: 10.1002/elan.200704215

1. Introduction

Cancer is a leading cause of death in the world, particularly in developing countries according to World Health Association and a third of cancers could be cured if detected early and treated adequately. This fact leads to the faster development of new diagnostic, prognostic and therapeutic methods [1, 2]. Based on many clinical studies it is well known that a resistance on a treatment by cytostatic agents is a crucial complication of anticancer therapy [3–5]. The resistance of tumor cell on the cytostatics originates by various mechanisms and the process itself is thought to be multifactorial. The anticancer therapy is complicated especially in the case of forming of multidrug resistance (MDR). There are several mechanisms of the resistance. The decreasing of concentration of the cytostatic in the site of its action is one of the causes of the tumor cells resistance on the therapy [6]. Some membrane proteins like membrane phosphoglycoprotein with ATPase activity called Pgp, MRP (membrane transport protein) and cytosol protein called LRP are involved in such decreasing and thus in formation of MDR [7–11].

A broad range of genes involved in the process of apoptosis or expression of tumor suppressors is changed in the case of MDR. The changed expression of tumor suppressor genes of protein family Bcl-2 is frequently found

 WILEY InterScience DISCOVER SOMETHING GREAT © 2008 WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim

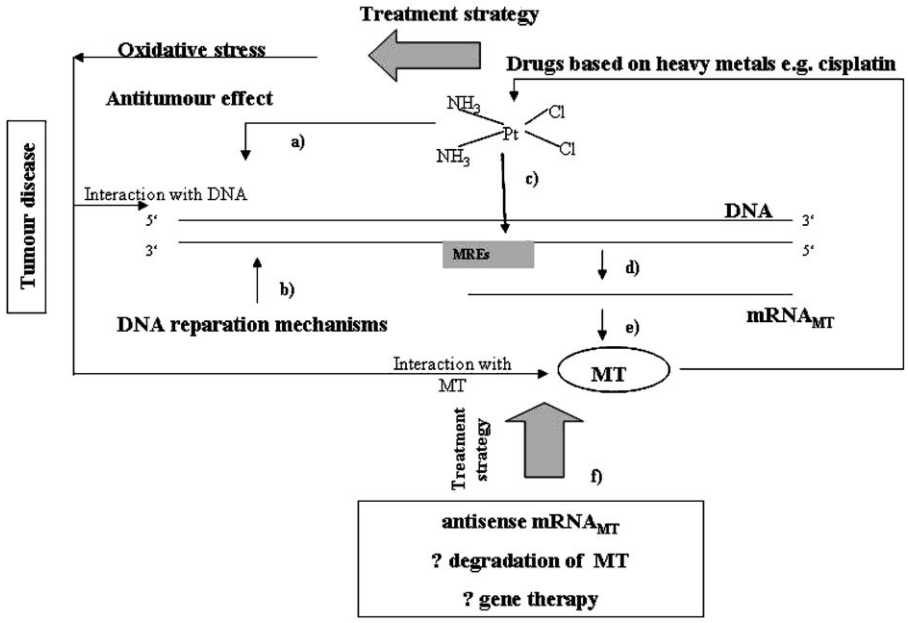

Fig. 1. Influence of anticancer drug based on heavy metal, a) anticancer drug effect (stopping DNA replication); b) mutated genes (reparation of damaged DNA); c) cisplatin binds on metal responsive element (MRE); d) mRNA-MT; e) MT interacts with a drug; f) treatment strategy to suppress MT level.

in chemo resistant tumor cells. It is a common knowledge that the function of tumor suppressors, e.g., p53, is blocked in certain resistant tumor cells [12]. Moreover the multidrug resistance can be associated with increased activity of DNA reparation mechanisms [13].

The direct detoxification of cytostatics belongs to the factors of MDR too. The activity of oxidation and detoxification enzymes which oxidize or conjugate the xenobiotics with endogenous conjugative agents has been increased in most cases of MDR. The microsomal monooxygenases belongs to the most abundant group of enzymes oxidizing xenobiotics. Besides those glutathione-*S*-transferases are conjugative enzymes inactivating cytostatics. The glutathione-transferase system has been studied intensively in context of cytostatics resistance in leukemia cells [14–16]. Except above-mentioned mechanisms the MDR resistance can be connected with increased expression of low-molecular proteins called metallothioneins (MT) [5, 17–20].

Metallothioneins are a group of proteins rich in cysteine with molecular weights ranging from 6 to 10 kDa [21, 22]. Due to high affinity of metallothioneins to heavy metals, e.g., zinc, copper and/or cadmium, homeostatic control and detoxification of the metals are their main physiological function at evolutionary different animal organisms. It is less known about molecular mechanisms of the MT expression. In the case of MT expression caused by heavy metals, metal ions probably have a key role in this process because they are able to bind on specific transcriptional factor called as metal transcription factor 1 (MTF-1). Then, the formed metal-MTF-1 complex interacts with metal-responsive element (MRE) of MT promoter to activate its transcription [17]. Among others, the concentration of MT increases in the moment of administration of heavy metal (e.g., the anticancer drug) [23, 24]. The expressed MT binds the administered anticancer drug rapidly, which results in the decrease of drug concentration below the effective level [24]. The simplified scheme of the forming of a resistance to cytostatic treatment is shown in Figure 1.

To detect level of MTs at various biological samples as human body liquids, cells and tissues effective, sensitive and easy to use analytical instruments are needed. The techniques and methods using for these purposes can be divided to several groups on spectrometric [25], immunoassays [26], hyphenated [27, 28], mRNA detection based [29] and electrochemical [30–40]. In the present paper we employ two most sensitive electroanalytical methods, adsorptive transfer stripping technique (AdTS) coupled with chronopotentiometric stripping analysis (CPSA) for determination of MT in tumor cell lines and differential pulse voltammetry Brdicka reaction (DPV Brdicka reaction) for determination of MT in blood serum of patients with head and neck cancer or retinoblastoma, and of rats treated with cisplatin with respect to discuss the role of MT in formation of resistance on treatment by heavy metal based cytostatics.

2. Experimental

2.1. Chemicals and pH Measurements

Rabbit liver MT (MW 7143), containing 5.9% Cd and 0.5% Zn, was purchased from Sigma Aldrich (St. Louis, USA). Tris(2-carboxyethyl)phosphine (TCEP) was produced by

 © 2008 WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim

Molecular Probes (Evgen, Oregon, USA). Other chemicals used were purchased from Sigma Aldrich. The stock standard solutions of MT at 10 μg/mL with 1 mM TCEP were prepared with ACS water (Sigma-Aldrich, USA) and stored in the dark at −20 °C. Working standard solutions were prepared daily by dilution of the stock solutions. The pH was measured using pH meter WTW inoLab (Weilheim, Germany). The pH-electrode (SenTix-H, pH 0–14/3 M KCl) was regularly calibrated by set of WTW buffers (Weilheim, Germany).

2.2. Tumor Cell Lines

The following cell lines obtained from Department of Paediatric Haematology and Oncology, Charles University, Prague, Czech Republic were used: UKF-NB4 – cisplatin or carboplatin sensitive (prepared from recurrence of neuroblastoma into bone marrow, with MYCN amplification, del 1p34.2ter, del 13, ISO 17q) and UKF-NB4 – cisplatin or carboplatin resistant (the line derived from the previous line - UKF-NB4 with in vitro induced resistance to cisplatin). The cell lines were prepared by cultivation with increasing concentration of cisplatin. The cells were cultivated in IMDM medium with 10% of fetal calf serum at 37 °C, the chemo resistant cell lines were cultivated in medium with cisplatin addition [41, 42].

2.3. Human Blood Serum

The samples of intravenous blood was obtained using vein tapping to the closed tapping units without another reagents during therapy of patients suffering from head and neck cancer at Department of Otolaryngology and Maxillofacial Surgery, University Hospital in Brno, Czech Republic, Generally forty five samples of blood obtained from the patients were analyzed. Blood serum samples of patients with retinoblastoma were obtained from the Department of Clinical Biochemistry and Pathobiochemistry, 2nd Faculty of Medicine Charles University, Czech Republic. All patients subscribed informed consent with utilization of their blood samples for the research.

2.4. Rat Blood Serum

Male Wistar rats (Faculty of Medicine, Masaryk University, Brno, Czech Republic), 8 weeks old (270–280 g), were divided into two experimental groups per six specimens. The first experimental group was exposed to one dose of 1.05 mg of cisplatin per kg, the second group to one dose of 2.1 mg of cisplatin per kg. Cisplatin was administered intraperitoneally. Each hour after administration one of the experimental animals were put to death. Blood from the heart of the animal and liver were sampled.

2.5. Preparation of the Samples for Electroanalytical Determination of Metallothionein

2.5.1. Blood Serum Samples

The sample of human and/or rat blood serum was prepared by heat treatment and solvent precipitation. Briefly, the sample was kept at 99 °C in a thermomixer (Eppendorf 5430, USA) for 15 min. with occasional stirring, and then cooled to 4 °C. The denatured homogenates were centrifuged at 4 °C, 15000 g for 30 min. (Eppendorf 5402, USA). The supernatant (5 μL) was analyzed by differential pulse voltammetry Brdicka reaction [23, 38, 43, 44].

2.5.2. Tumor Cell Lines Samples

The harvested cells were transferred to a test tube and then deep frozen by liquid nitrogen to disrupt cells. The frozen cells were mixed with extraction buffer (100 mM potassium phosphate, pH 8.7) to a final volume of 1 mL and homogenized using hand-operated homogenizer ULTRA-TURRAX T8 (IKA, Germany) placed in an ice bath for 3 min at 25000 rpm [45]. The homogenate was centrifuged at 10000 g for 15 min and at 4 °C (Eppendorf 5402, USA). The supernatant were processed in the same way as blood serum samples mentioned in Section 2.5.1. The processed samples were measured by adsorptive transfer stripping technique coupled with chronopotentiomeric stripping analysis [33].

2.6. Electrochemical Measurements

2.6.1. Electroanalytical Determination of Metallothionein by Adsorptive Transfer Stripping Technique Coupled with DPV Brdicka Reaction

An adsorptive transfer stripping technique (AdTS) coupled with DPV Brdicka reaction was employed for the determination of metallothionein in cell lines extract. The electrochemical measurements were performed using an AUTOLAB analyzer (EcoChemie, The Netherlands) connected to VA-Stand 663 (Metrohm, Switzerland), using a standard cell with three electrodes. The three-electrode system consisted of hanging mercury drop electrode as working electrode, an Ag/AgCl/3 M KCl reference electrode and a glassy carbon auxiliary electrode. For smoothing and baseline correction the software GPES 4.9 supplied by EcoChemie was employed [46]. The Brdicka supporting electrolyte containing 1 mM $Co(NH_3)_6Cl_3$ and 1 M ammonia buffer ($NH_3(aq) + NH_4Cl$, pH 9.6) was used; surface-active agent was not added. AdTS DPV Brdicka reaction parameters were as follows: initial potential of −0.6 V, end potential −1.6 V, modulation time 0.057 s, time interval 0.2 s, step potential of 1.05 mV, modulation amplitude of 250 mV, $E_{ads} = 0$ V. Temperature of the supporting electrolyte was 4 °C. For other experimental conditions see [36].

 © 2008 WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim

2.6.2. Automated Electroanalytical Determination of Metallothionein by DPV Brdicka Reaction

DPV Brdicka reaction was employed for the determination of metallothionein in human blood serum samples. The electrochemical measurements were performed with 747 VA Stand instrument connected to 746 VA Trace Analyzer and 695 Autosampler (Metrohm, Switzerland), using a standard cell with three electrodes and cooled sample holder (4 °C). A hanging mercury drop electrode (HMDE) with a drop area of 0.4 mm^2 was the working electrode. An Ag/AgCl/3 M KCl electrode was the reference and glassy carbon electrode was auxiliary electrode. The supporting electrolyte (1 mM $[Co(NH_3)_6]Cl_3$ and 1 M ammonium buffer; NH_3(aq) and NH_4Cl, pH 9.6) was changed after five measurements [23, 47]. The DPV parameters were as follows: initial potential of − 0.7 V, end potential of − 1.75 V, modulation time 0.030 s, time interval 0.8 s, step potential 2 mV, modulation amplitude − 25 mV, $E_{ads} = 0$ V. All experiments were carried out at temperature 4 °C (Julabo F25, Germany). The data obtained were collected by using of VA-Database 2.2 (Metrohm G.B, Switzerland). Further the data were transferred and processed by GPES 4.9 supplied by EcoChemie. For smoothing and baseline correction the software GPES 4.9 supplied by EcoChemie was employed [46]. Other details on automated analysis of MT will be published elsewhere.

2.6.3. Electroanalytical Determination of Metallothionein by CPSA

An AdTS coupled with chronopotentiometric stripping analysis (CPSA) was employed for the determination of metallothionein in cell lines extract through recording the inverted time derivation of potential $(dE/dt)^{-1}$ as a function of potential E [33]. These electrochemical measurements were performed with AUTOLAB Analyzer (EcoChemie, Netherlands) connected to VA-Stand 663 (Metrohm, Switzerland), using a standard electrochemical cell with three electrodes. The working electrode was a HMDE with a drop area of 0.4 mm^2. The reference electrode was an Ag/AgCl/3 M KCl electrode and the auxiliary electrode was a graphite stick electrode. For smoothing and baseline correction the software GPES 4.9 supplied by EcoChemie was employed [46]. CPSA parameters were as follows: I_{str} of − 1 μA, temperature of supporting electrolyte 20 °C, supporting electrolyte 0.1 M H_3BO_3 + 0.05 M $Na_2B_4O_7$ (pH 9.2). Other experimental details on CPSA analysis of MT were published in [33, 40, 48, 49].

2.7. Determination of Esterase Activity

The culture was treated by trypsin for 2 min and then shaken (60 rpm) to release the sessile cells. The cells were separated by centrifugation (50 × g, 5 min) and washed one times with PBS (pH 7.4). The cell lysis was carried out on ice with Triton X100 in result concentration 0.1% (v/v) for 20 min. The mechanical impurities were removed from the lysate by the centrifugation (10 000 g, 15 min, 4 °C). The supernatant was then processed immediately. The intracellular esterase activity determination was carried out by using fluorescein-diacetate test with modifications: for incubation of the reaction mixture was used 37 °C [50 – 53]. The whole proteins were evaluated according Bradford [54]. The cell density (cell per 1 mL of suspension) was determined by using the Fuchs – Rosenthal counting cell.

2.8. Polyacrylamide Electrophoresis

The cell samples were also analyzed using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The electrophoresis was performed according to Laemmli [55] using a Biometra maxigel apparatus (Biometra, Germany). First 15% (w/v) running, then 5% (w/v) stacking gel was poured, the gels were prepared from 30% (w/v) acrylamide stock solution with 1% (w/v) bisacrylamide concentration; the polymerization of the running gel was carried out at room temperature for 1 h and 30 min for the stacking gel. Prior to analysis the samples were mixed with sample buffer containing 5% (v/v) 2-mercaptoethanol in a 1:1 ratio. The samples were boiled for 2 min, and then loaded onto a gel in 60 μL aliquots. For determination of MW, the protein ladder 'Precision plus protein standards' from Biorad was used. The electrophoresis was run at 150 V for 3 hours with cooling until the front dye reached the bottom of the gel. The silver staining of the gels was performed according Blum [56]. After silver staining the gels were scanned and analyzed by Biolight software (Vilber-Lourmat, USA).

2.9. Descriptive Statistics

MICROSOFT EXCEL (USA) was used for statistical analyses. Results are expressed as mean ± SD unless noted otherwise. Accuracy, precision and recovery of metallothioneins were evaluated with homogenates (human blood serum) spiked with standard. Before extraction, 100 μL metallothioneins standards and 100 μL water were added to human blood serum samples. Homogenates were assayed blindly and metallothioneins concentrations were derived from the calibration curves. The spiking of metallothioneins was determined as a standard measured without presence of real sample. Calculation of accuracy (% Bias), precision (% CV, coefficient of variation) and recovery was carried out as indicated by Causon [57] and Bugianesi et al. [58].

3. Results and Discussion

Biologically active molecules with free −SH groups called thiols play numerous roles in organisms and can affect a treatment [5, 6, 19, 20, 59 – 64]. Via free sulfhydryl groups the thiols can be conjugated to various molecules, however

 © 2008 WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim

their affinity to the metal ions can be considered as one the highest. One may suggest that these compounds could play role in resistance on cancer treatment by heavy metal based cytostatics, because cisplatin and others are still frequently in use. Due to the reactivity of free –SH the electrochemical methods belong to the most sensitive ones employing for their determination [33, 36, 65 – 69].

3.1. Electroanalytical Methods in Metallothionein Analysis

Almost all methods used for detection of metallothionein in tissues are based on immunohistochemical principles [27, 59, 61, 64]. These procedures require biopsy tissues and labor and time consuming sample handling and can not be used for exact quantification of the protein of interest. Recently we have proposed sensitive and selective procedure for sample preparation and consequent analysis to determine metallothionein in blood [23, 49, 70]. The sample preparation method is based on heat treatment and solvent precipitation, which can effectively denature and remove high molecular weight proteins out from samples [33, 40, 48, 49, 70 – 72]. The principle of the detection technique called AdTS DPV Brdicka reaction bases in adsorption of thermostable metallothionein directly with the surface of the mercury electrode. The procedure is shown in Figure 2A. The transfer technique can be considered as so-called 'sample purifying step' separating low-molecular compounds from larger ones. Therefore metallothionein is adsorbed and low-molecular thiols, such as cysteine or glutathione, do not affect the measurements, other details will be published elsewhere. The measuring electrode system is in the connection with potentiostat/galvanostat and controlling computer (Fig. 2B). Typical DP voltammograms of the same MT (100 ng/mL) sample of are shown in Figure 2C. Two well developed catalytic signals called Cat1 and Cat2 are observable. It is shown in inset in Figure 2C that nine times measured Cat2 signal of MT (100 ng/mL) gave well repeatable response with relative standard deviation below 5%. However due to necessity of the manual control of all mentioned steps we have looked for the possibility to automate the measurements. To automate measurements 747 VA Stand instrument connected to 746 VA Trace Analyzer and 695 Autosampler with cooled sample holder was employed. A measurement is carried out automatically under the control of microprocessor within five minutes. For the quantification of MT the catalytic signal Cat2 is used. Typical voltammograms of MT (1.5, 3 and 6 μM) measured by automated electrochemical analyzer are shown in Figure 3A. The MT signals depended on MT concentration. The continuous line shows on reduction of cobalt(III) ions. The electrochemical analysis of MT resulted in appearing of three signals in voltammograms obtained. The signal of MT complex with cobalt ions called RS2Co appeared at -1.0 V. This signal shifted to more positive potentials with increasing MT concentration. Two other signals called Cat1 (-1.2 V) and Cat2 (-1.4 V) were catalytic. The calibration curve measured is shown in Figure 3B. The dependence of Cat2 height signal of MT on its concentration was linear within the studied range ($y = 2.0009x + 1.6673$, $R^2 = 0.9965$). *RSD* was below 5%. Recovery was tested by three additions of MT standard into human blood serum sample (Table 1). The results were within 95 – 100%.

3.2. Neuroblastoma Cell Lines

Primarily we aimed our attention to determination of MT in samples of neuroblastoma cell lines resistant and sensitive to platinum based drugs. The pictures of the cell lines resistant and sensitive to cisplatin are shown in Figure 4A. The single cell lines have been characterized previously according chromosomal aberrations [41]. However the changes in metabolic activity of the cell lines are still unclear. To reveal the activity of metabolism we employed fluorimetric determination of activity of intracellular esterase [50 – 53]. The same volumes of the cell samples with the exact and same count of cells were analyzed (Fig. 4B). We found that the esterase activity of cisplatin sensitive cell lines was higher in comparison with the activity of resistant ones. This phenomenon can be associated with the toxic effects of cisplatin on the cells. In addition we attempted to utilize fluorimetric determination for evaluation of the number of living cells in the samples of neuroblastoma cell lines. Based on known cell number homogenized in defined volume the equivalents of cell number from 0 to 7000 were transferred into reaction mixture for esterase activity determination. The detected intracellular esterase activity was directly proportional to cell number and the detection limit was about 600 cells for the sensitive neuroblastoma cultures and about 1000 cells for the resistant cultures (Fig. 4B).

In the following experiments the neuroblastoma cell extracts were measured by using two different techniques AdTS DPV Brdicka reaction and AdTS CPSA. The dependence of the height of Cat2 signal measured by Brdicka reaction on the count of the cells is shown in

Table 1. Recovery of MT for analysis of the human blood serum sample ($n = 5$).

	Homogenate (μM) [a]	Spiking (μM) [a]	Homogenate + spiking (μM) [a]	Recovery (%)
MT addition 1	6.5 ± 0.2 (3.1)	1.10 ± 0.03 (2.7)	7.5 ± 0.2 (2.7)	99
MT addition 2	6.5 ± 0.2 (3.1)	2.20 ± 0.05 (2.2)	8.4 ± 0.3 (3.6)	97
MT addition 3	6.5 ± 0.2 (3.1)	4.40 ± 0.15 (3.4)	10.6 ± 0.3 (2.8)	97

[a] MT concentration; expressed as mean $\pm$ *SD* (CV%)

 © 2008 WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim

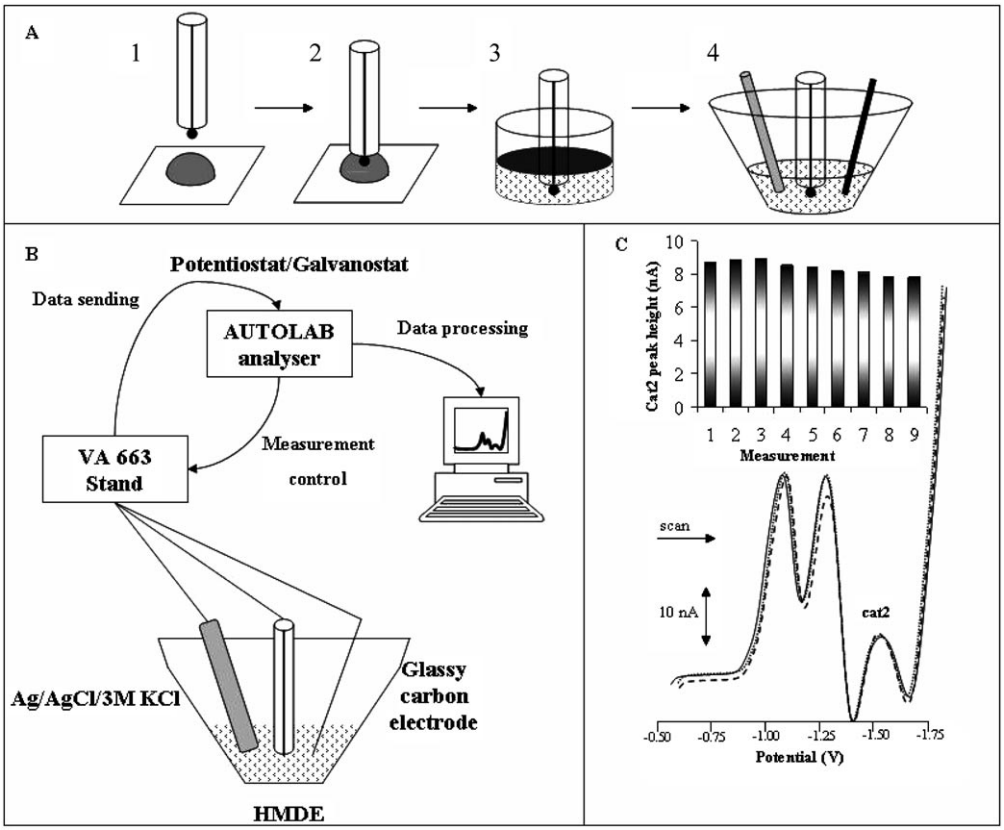

Fig. 2. A) Scheme of adsorptive transfer stripping technique used for MT detection; 1) renewing of the hanging mercury drop electrode (HMDE) surface; 2) adsorbing of MT in a drop solution onto the HMDE surface at open circuit; 3) washing electrode in ACS water; 4) measuring of MT by DPV in the presence of the Brdicka supporting electrolyte. B) Electrochemical cell with three electrodes in the connection with potentiostat/galvanostat and controlling device. C) Typical AdTS DP voltammograms of metallothionein (100 ng/mL) measured in triplicate.

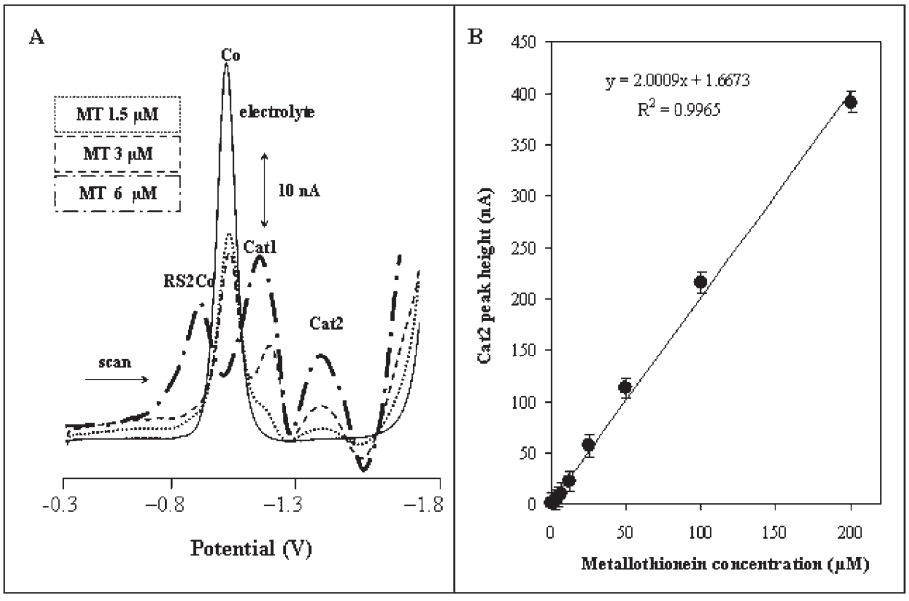

Fig. 3. A) DP voltammograms of MT (1.5, 3 and 6 μM) measured by using 747 VA Stand instrument connected to 746 VA Trace Analyzer and 695 Autosampler ($n = 3$). B) The dependence of the height of Cat2 signal on the MT concentration.

 © 2008 WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim

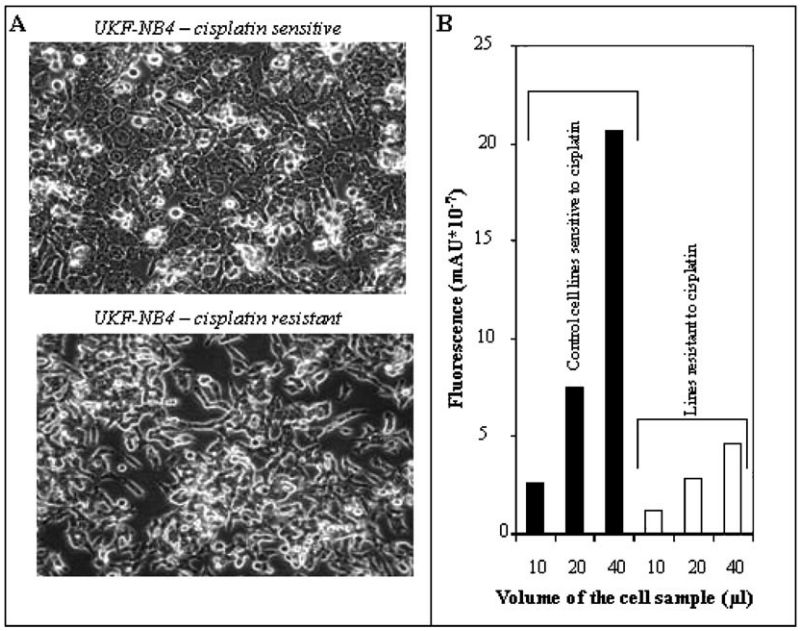

Fig. 4. A) Images of tumor cell lines UKF-NB4 cisplatin sensitive and resistant. B) Changes of esterase activity in UKF-NB4 cell lines.

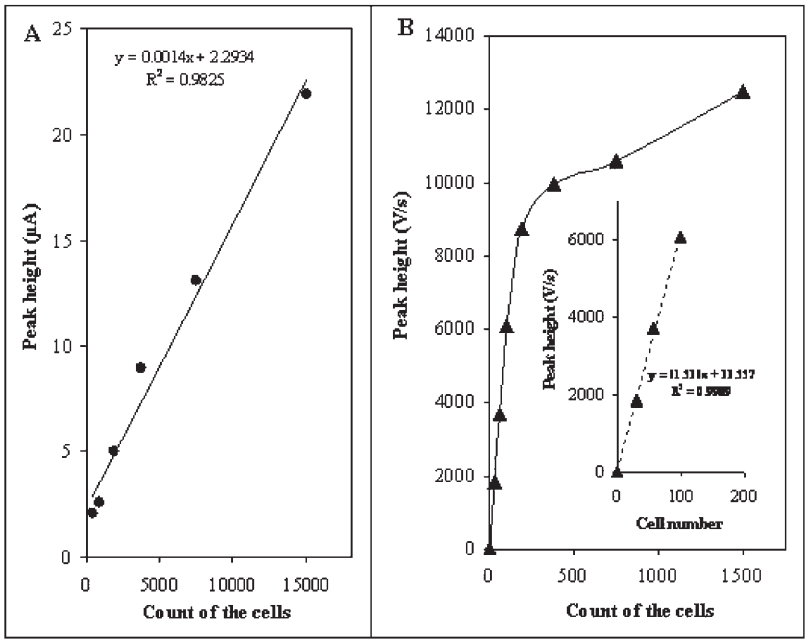

Fig. 5. Dependence of peak heights on count of tumor cells measured by A) AdTS DPV Brdicka reaction or B) peak H; in inset: the dependence for lower counts of the cells.

Figure 5A. The obtained dependence is linear with the equation $y = 0.0014x + 1.733$, $R^2 = 0.9747$. The lowest count of the cells, which we were able to detect, was 100. The same samples were also analyzed using chronopotentiometry. The typical concentration dependence is shown in Figure 5B. The height of the peak increased up to the number of the cells about 500 and then rose more gradually. Within the range from 0 to 100 cells the dependence was strictly linear with the equation $y = 61.536x + 33.557$, $R^2 = 0.9989$. Using this procedure the detection of less than 30 neuroblastoma cells was possible. In spite of the fact that CPSA is more sensitive than the Brdicka reaction we employed the Brdicka reaction due to faster and more reproducible analysis. Based on the results obtained above we always analyzed the equivalent of 1000 cells in the following experiments.

 © 2008 WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim

3.3. The Changes of Metallothionein Level in the Tumor Cells

The cisplatin or carboplatin sensitive and resistant neuroblastoma cell lines are derived from the maternal cell line isolated from the bone metastasis of patients with neuroblastoma. The resistant tumor cell line is constantly cultivated in the cultivation media containing anticancer drug (cisplatin or carboplatin). The typical AdTS DP voltammograms of cellular extracts obtained from maternal, sensitive and resistant cell lines are shown in Figure 6A. In the voltammograms the well distinguishable catalytic signals Cat2 are observed. These signals correspond to the presence of free –SH groups in MT molecule. To confirm that we measured MT in cell extracts we carried out PAGE electrophoresis of cellular homogenates followed by silver staining. The very slight band indicating the presence of MT was detectable only in cell line resistant to cisplatin (Fig. 6A). By using PAGE we successfully confirmed presence of MT in cell extracts and also shown that the Brdicka reaction is much more sensitive to presence of this protein. The concentration of MT in the cell extracts determined by the electrochemical method is shown in Figure 6B. The concentration of MT in the cell line resistant to cisplatin (the cells were cultivated in the presence of 1 μM cisplatin for 72 h) increased for more than 60% compared to the maternal cell line. The MT level in the cisplatin sensitive cell line (the cells were cultivated without cisplatin for 72) was also slightly higher compared to maternal line (Fig. 6B). The changes of MT level in cell lines in the exponential growth phase treated with cisplatin and carboplatin for 24 h are shown in Figure 6C,D. The cisplatin concentration 0.01 μM caused negligible rise in MT concentration compared to the sensitive cells. The applied dose of 0.1 μM of cisplatin lead to the MT concentration increase for 10% compared to the sensitive cells, however the higher cisplatin concentration induced greater increase of MT concentration. The very similar phenomenon was observed in the case of carboplatin, concentrations from 0.1 to 1 μM resulted in slightly enhanced MT biosynthesis compared to the sensitive cells. The concentration of carboplatin higher than 1 μM led to very rapid MT biosynthesis. Based on the results obtained it can be concluded that level of MT increases with higher dose of platinum based cytostatics at cells. Thus we were interested in the effect of various treatment strategies on MT level at patient with malignant tumors.

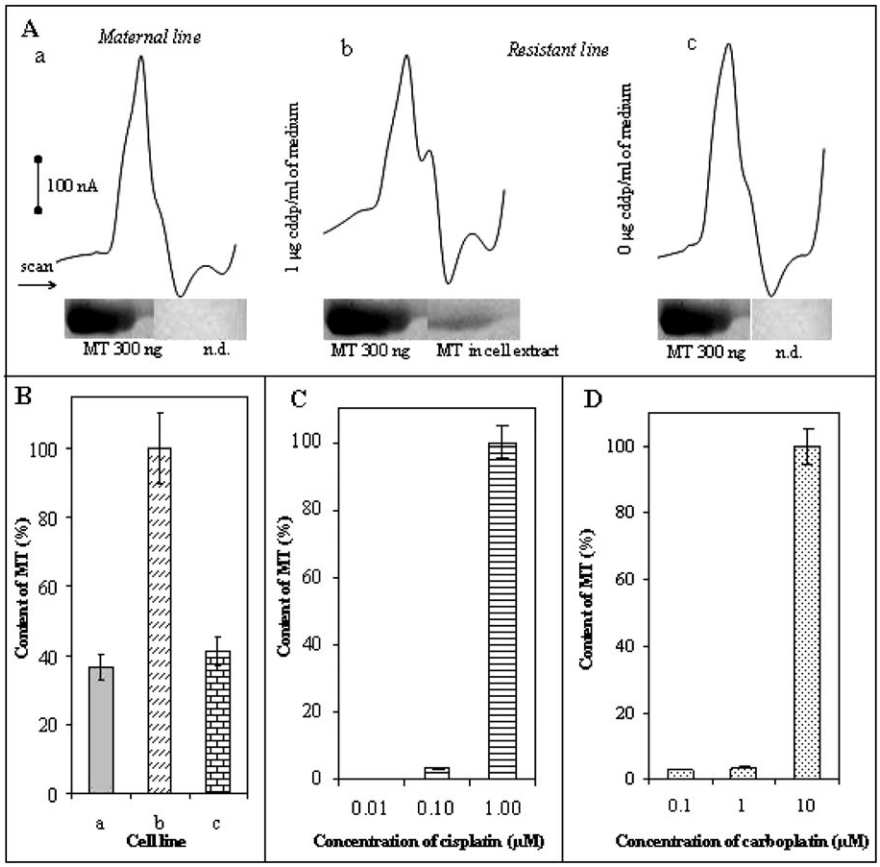

Fig. 6. A) AdTS DP voltammograms of the cell lines UKF-NB4 a) maternal line, b) resistant line (cultivated in the presence of 1 μM cisplatin), c) sensitive line (cultivated without cisplatin). PAGE analysis of the samples, on the left part of the panel it is a signal of 300 ng of MT standard, on the right part of the panel the signal of the sample is shown. B) The changes of MT signal measured in UKF-NB4 cell lines, a) maternal line, b) resistant line, c) sensitive line. The MT peak height measured in the cells cultivated in the presence of various concentrations of C) cisplatin and/or D) carboplatin.

 © 2008 WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim

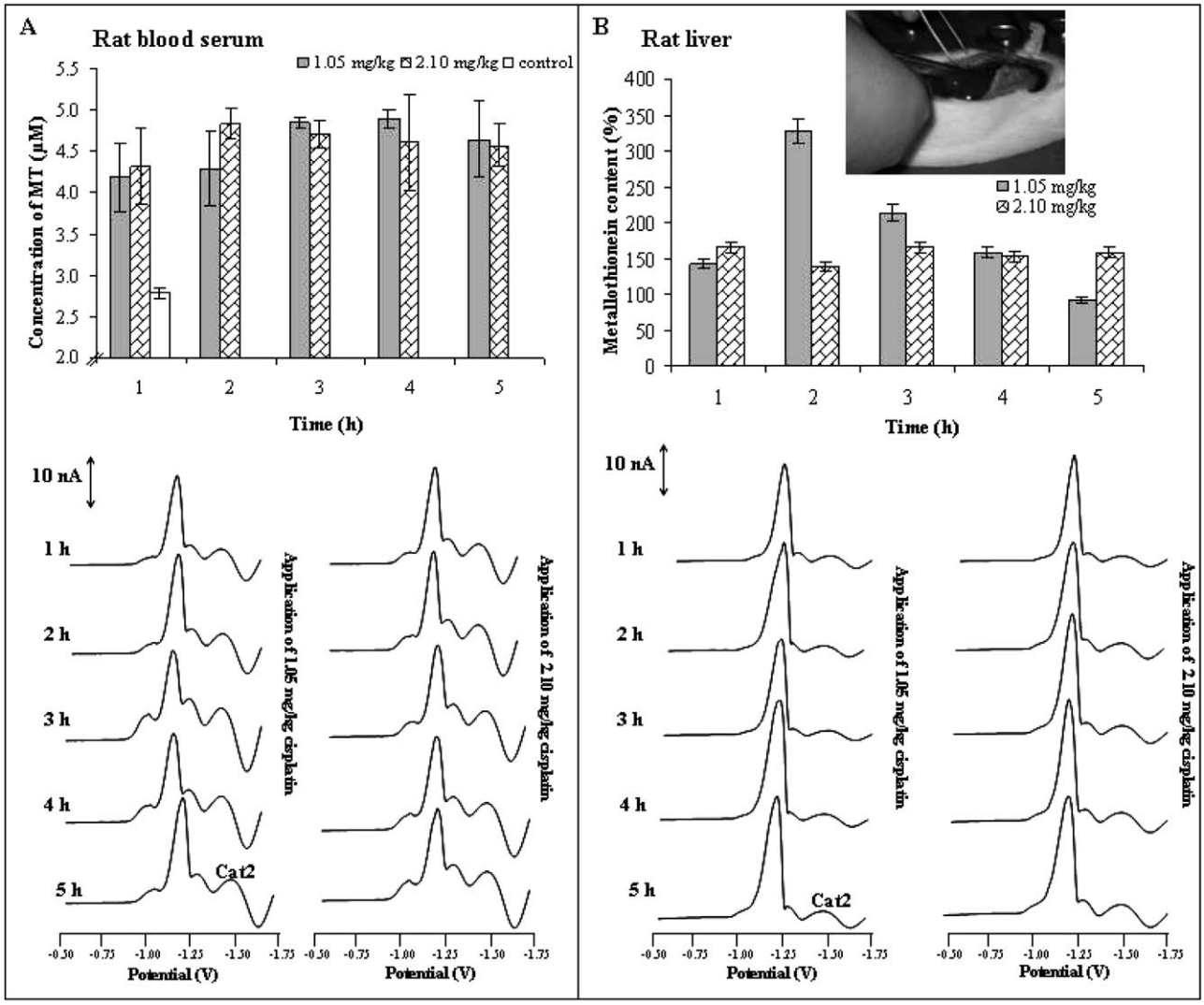

Fig. 7. Changes in MT level in A) blood serum or B) liver of rats treated with 1.05 mg of cisplatin per kg or 2.1 mg of cisplatin per kg. In bottom insets: typical DP voltammograms. In upper inset: photography of sampling of blood serum and liver.

3.4. Changes of Metallothionein Levels at Rats after Intraperitoneal Application of Cisplatin

In addition we tested the automated electrochemical instrument on analysis of MT in blood serum of rats treated with cisplatin. We found out that synthesis of MT enhanced quickly. Even one hour after administration of cisplatin the level of MT increased at both experimental groups. Particularly, the level of MT in blood serum of untreated rats was app. 2.9 μmol/L. At rats treated with 1.05 mg and/or 2.1 mg of cisplatin for one hour the MT level was 4.2 and/or 4.3 μmol/L, respectively. The highest level of MT at rats treated with 1.05 mg cisplatin was determined after four hours as 4.9 μmol/L. In the case of the second experimental group the maximum was reached even after two hours of the treatment as 4.8 μmol/L. Moreover we quantified MT level in rats' liver (the levels MT determined in control tissues were taken as 100%). It clearly follows from the results obtained that lower dose of cisplatin (1.05 mg/kg) induced highest MT expression in second hour after injection then MT level decreased. Higher cisplatin dose (2.1 mg/kg) resulted in enhancing of MT synthesis, which did not change it during the experiment (Fig. 7).

3.5. Changes of Metallothionein Level at Treated Patients with Malignant Tumors in Head and Neck Area

A monitoring of MT level at patients with a tumor disease could be useful from various points of view. One of the points of view is the role of MT as marker of resistance to tumor disease treatment by heavy metal based cytostatics. Due to evaluation of this assumption the samples of blood of patients with a tumor in head and neck area were collecting during drug the treatment. The changes in MT level at the patients in the time manner is shown in Figure 8A. The levels of MT is several times higher compared to MT levels in blood serum of healthy volunteers [73]. Typical DP

 © 2008 WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim

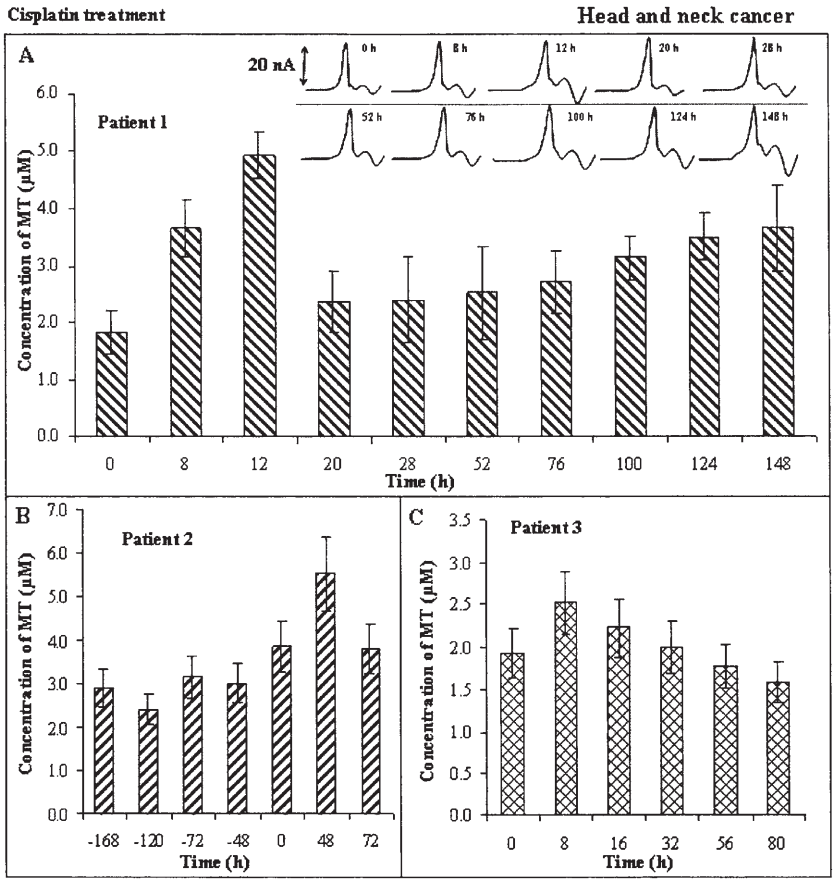

Fig. 8. Monitoring of MT level at three patients with a tumor in head and neck area treated with cisplatin. A) Patient 1 (0–148 h), in inset: typical DP voltammograms; B) patient 2, (−168, −120, −72, −48 are hours before administration of cisplatin); C) patient 3 (0–80 h).

voltammograms of blood serum of patient with head and neck cancer treated with cisplatin are shown in inset in Figure 8A. The effect of cisplatin on MT level before and after administration of this drug is clearly shown in Figure 8B. The level of MT was going higher due to administration of the drug. This phenomenon was observed at all patients (Fig. 8A,B,C). Moreover the MT level rapidly decreased even after tens of hours after administration. Then the changes in MT level were negligible.

3.6. Changes of Metallothionein Level at Patients with Retinoblastoma Treated by Carboplatin

We were interested in the issue if the administration of second generation of the platinum based drugs called carboplatin to patients with a tumor disease could influence MT level. Patients with rare tumor disease – retinoblastoma ($n = 9$) were treated by carboplatin. We determined MT level during 24 hours after the administration (Fig. 9). Average concentration of MT in plasma of patients with retinoblastoma before treatment with carboplatin was 3.4 μM. During analysis of MT level we can observe various phenomena. At patients Nos. 2, 4 and 6 MT level did not change. On the contrary level of MT determined at patients Nos. 1, 3, 5 and 9 decreased up to 4 hours after administration. This can be related with binding of MT to cytostatic. Treatment of patients Nos. 7 and 8 resulted in almost twofold MT level enhancing as same as at patients and at rats treated with cisplatin (Figs. 7, 8 and 9). Progression of MT levels was individual and probably depended on tumor resistance to carboplatin.

4. Conclusions

Electrochemical instrument belongs to the one of the most sensitive instrument for thiols determination [31, 37]. The whole electroanalytical process is very simple and fast. In the present paper we employed the electrochemical methods for evaluation of role of metallothionein in resistance on tumor disease treatment. Particularly we aimed our attention on tumor cell lines, patients with tumor in head and neck area and with retinoblastoma, and rats treated with cisplatin. In conclusion MT could participate in resistance to platinum based cytostatics and therefore should be investigated more deeply.

 © 2008 WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim

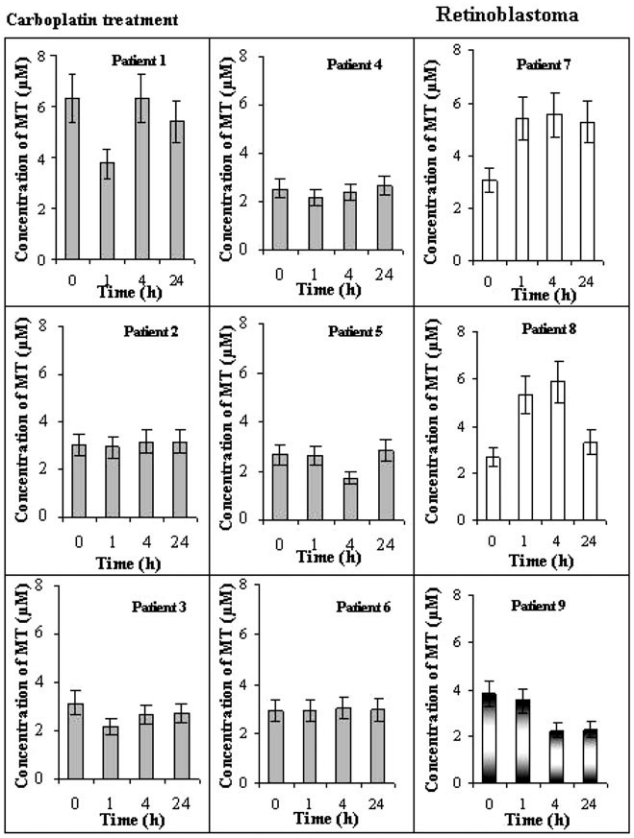

Fig. 9. Changes in MT level at patients with retinoblastoma treated with carboplatin.

5. Acknowledgements

We gratefully acknowledge the Grant Agency of the Academy of Sciences of the Czech Republic (grant No. GA AV IAA401990701) and the Ministry of Education, Youth, and Sports (Grant No. MSMT 0021620813) for the financial support to this work. The authors wish to also express their thanks to Dr. Hana Binkova for providing of clinical samples.

6. References

[1] K. R. Benson, *Hist. Philos. Life Sci.* **2003**, *25*, 440.
[2] A. Ullrich, A. Waxman, V. Silva, D. Bettcher, G. Vestal, C. Sepulveda, R. Beaglehole, *Ann. Oncol.* **2004**, *15*, 249.
[3] G. D. Girnun, E. Naseri, S. B. Vafai, L. Qu, J. D. Szwaya, R. Bronson, J. A. Alberta, B. M. Spiegelman, *Cancer Cell*. **2007**, *11*, 395.
[4] J. Helleman, H. Burger, I. H. L. Hamelers, A. W. M. Boersma, A. de Kroon, G. Stoter, K. Nooter, *Cancer Biol. Ther.* **2006**, *5*, 943.
[5] J. Goodisman, D. Hagrman, K. A. Tacka, A. K. Souid, *Cancer Chemother. Pharmacol.* **2005**, *57*, 257.
[6] P. Surowiak, V. Materna, I. Kaplenko, M. Spaczynski, M. Dietel, H. Lage, M. Zabel, *Virchows Arch.* **2005**, *447*, 626.
[7] Y. C. Awasthi, R. Sharma, S. Yadav, S. Dwivedi, A. Sharma, S. Awasthi, *Curr. Drug Metab.* **2007**, *8*, 315.
[8] C. Atalay, *Expert Opin. Ther. Patents.* **2007**, *17*, 511.
[9] S. Y. Sung, P. A. S. Johnstone, *Curr. Probl. Cancer*. **2007**, *31*, 36.
[10] M. K. Choi, D. D. Kim, *Arch. Pharm. Res.* **2006**, *29*, 1067.
[11] L. O'Driscoll, M. Clynes, *Curr. Cancer Drug Targets* **2006**, *6*, 365.
[12] A. J. Levine, J. Momand, C. A. Finlay, *Nature* **1991**, *351*, 453.
[13] P. A. Jones, S. B. Baylin, *Nat. Rev. Genet.* **2002**, *3*, 415.
[14] R. Kerb, S. Hoffmeyer, U. Brinkmann, *Pharmacogenomics* **2001**, *2*, 51.
[15] A. E. Salinas, M. G. Wong, *Curr. Med. Chem.* **1999**, *6*, 279.
[16] B. K. Sinha, *Drugs* **1995**, *49*, 11.
[17] G. Nordberg, T. Jin, P. Leffler, M. Svensson, T. Zhou, M. Nordberg, *Analysis* **2000**, *28*, 396.
[18] S. Krizkova, V. Adam, J. Petrlova, O. Zitka, K. Stejskal, J. Zehnalek, B. Sures, L. Trnkova, M. Beklova, R. Kizek, *Electroanalysis* **2007**, *19*, 331.
[19] Y. Doki, M. Monden, *J. Gastroenterol.* **2004**, *39*, 1228.
[20] J. Holford, P. J. Beale, F. E. Boxall, S. Y. Sharp, L. R. Kelland, *Eur. J. Cancer* **2000**, *36*, 1984.
[21] J. H. R. Kagi, A. Schaffer, *Biochemistry* **1988**, *27*, 8509.
[22] D. H. Hamer, *Annu. Rev. Biochem.* **1986**, *55*, 913.
[23] J. Petrlova, D. Potesil, J. Zehnalek, B. Sures, V. Adam, L. Trnkova, R. Kizek, *Electrochim. Acta* **2006**, *51*, 5169.
[24] J. Kukacka, J. Petrlova, R. Prusa, V. Adam, B. Sures, M. Beklova, L. Havel, R. Kizek, *Faseb J.* **2006**, *20*, A75.
[25] J. Szpunar, *Analyst* **2005**, *130*, 442.
[26] M. M. Chu, Z. Q. Guo, N. Muto, N. Itoh, K. Tanaka, H. W. Ren, *Front. Biosci.* **2006**, *11*, 2113.
[27] K. Nostelbacher, M. Kirchgessner, G. I. Stangl, *J. Chromatogr. B* **2000**, *744*, 273.
[28] J. Szpunar, R. Lobinski, A. Prange, *Appl. Spectrosc.* **200**3, *57*, 102A.
[29] S. Ganguly, E. Taioli, B. Baranski, B. Cohen, P. Toniolo, S. J. Garte, *Cancer Epidemiol. Biomarkers Prev.* **1996**, *5*, 297.
[30] V. Adam, O. Blastik, S. Krizkova, P. Lubal, J. Kukacka, R. Prusa, R. Kizek, *Chem. Listy* **2008**, *102*, 51.
[31] M. Dabrio, A. R. Rodriguez, G. Bordin, M. J. Bebianno, M. De Ley, I. Sestakova, M. Vasak, M. Nordberg, *J. Inorg. Biochem.* **2002**, *88*, 123.
[32] C. Harlyk, O. Nieto, G. Bordin, A. R. Rodriguez, *J. Electroanal. Chem.* **1998**, *458*, 199.
[33] R. Kizek, L. Trnkova, E. Palecek, *Anal. Chem.* **2001**, *73*, 4801.
[34] R. Kizek, J. Vacek, L. Trnkova, B. Klejdus, L. Havel, *Chem. Listy.* **2004**, *98*, 166.
[35] R. W. Olafson, R. G. Sim, *Anal. Biochem.* **1979**, *100*, 343.
[36] J. Petrlova, D. Potesil, R. Mikelova, O. Blastik, V. Adam, L. Trnkova, F. Jelen, R. Prusa, J. Kukacka, R. Kizek, *Electrochim. Acta* **2006**, *51*, 5112.
[37] I. Sestakova, T. Navratil, *Bioinorg. Chem. Appl.* **2005**, *3*, 43.
[38] B. Raspor, M. Paic, M. Erk, *Talanta* **2001**, *55*, 109.
[39] D. Ivankovic, J. Pavicic, B. Raspor, I. Falnoga, T. Tusek-Znidaric, *Int. J. Environ. Anal. Chem.* **2003**, *83*, 219.
[40] M. Strouhal, R. Kizek, J. Vecek, L. Trnkova, M. Nemec, *Bioelectrochemistry* **2003**, *60*, 29.
[41] J. Bedrnicek, A. Vicha, M. Jarosova, M. Holzerova, J. Cinatl, M. Michaelis, J. Cinatl, T. Eckschlager, *Neoplasma* **2005**, *52*, 415.
[42] R. Prusa, M. Svoboda, O. Blastik, V. Adam, O. Zitka, M. Beklova, T. Eckschlager, R. Kizek, *Clin. Chem.* **2006**, *52*, A174.
[43] M. Erk, D. Ivankovic, B. Raspor, J. Pavicic, *Talanta* **2002**, *57*, 1211.
[44] B. Raspor, *J. Electroanal. Chem.* **2001**, *503*, 159.
[45] V. Supalkova, J. Petrek, J. Baloun, V. Adam, K. Bartusek, L. Trnkova, M. Beklova, V. Diopan, L. Havel, R. Kizek, *Sensors* **2007**, *7*, 743.

 © 2008 WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim

[46] M. U. A. Bromba, H. Ziegler, *Anal. Chem.* **1981**, *53*, 1583.
[47] V. Adam, S. Krizkova, O. Zitka, L. Trnkova, J. Petrlova, M. Beklova, R. Kizek, *Electroanalysis* **2007**, *19*, 339.
[48] S. Krizkova, O. Zitka, V. Adam, M. Beklova, A. Horna, Z. Svobodova, B. Sures, L. Trnkova, L. Zeman, R. Kizek, *Czech J. Anim. Sci.* **2007**, *52*, 143.
[49] R. Prusa, O. Blastik, D. Potesil, L. Trnkova, J. Zehnalek, V. Adam, J. Petrlova, F. Jelen, R. Kizek, *Clin. Chem.* **2005**, *51*, A56.
[50] J. Vitecek, V. Adam, J. Petrek, P. Babula, P. Novotna, R. Kizek, L. Havel, *Chem. Listy* **2005**, *99*, 496.
[51] J. Vitecek, V. Adam, J. Petrek, J. Vacek, R. Kizek, L. Havel, *Plant Cell Tissue Organ Cult.* **2004**, *79*, 195.
[52] J. Vitecek, J. Petrlova, V. Adam, L. Havel, K. J. Kramer, P. Babula, R. Kizek, *Sensors* **2007**, *7*, 222.
[53] J. Vitecek, J. Petrlova, J. Petrek, V. Adam, L. Havel, K. J. Kramer, R. Kizek, *Biol. Plant.* **2007**, *51*, 551.
[54] M. M. Bradford, *Anal. Biochem.* **1976**, *72*, 248.
[55] U. K. Laemmli, *Nature* **1970**, *227*, 680.
[56] H. Blum, H. Beier, H. J. Gross, *Electrophoresis* **1987**, *8*, 93.
[57] R. Causon, *J. Chromatogr. B.* **1997**, *689*, 175.
[58] R. Bugianesi, M. Serafini, F. Simone, D. Y. Wu, S. Meydani, A. Ferro-Luzzi, E. Azzini, G. Maiani, *Anal. Biochem.* **2000**, *284*, 296.
[59] P. Surowiak, V. Materna, A. Maciejczyk, M. Pudelko, E. Markwitz, M. Spaczynski, M. Dietel, M. Zabel, H. Lage, *Virchows Arch.* **2007**, *450*, 279.
[60] Z. E. Gagnon, A. Patel, *J. Environ. Sci. Health Part A-Toxic/ Hazard. Subst. Environ. Eng.* **2007**, *42*, 381.
[61] T. Endo, M. Yoshikawa, M. Ebara, K. Kato, M. Sunaga, H. Fukuda, A. Hayasaka, F. Kondo, N. Sugiura, H. Saisho, *J. Gastroenterol.* **2004**, *39*, 1196.
[62] D. Hagrman, J. Goodisman, J. C. Dabrowiak, A. K. Souid, *Drug Metab. Dispos.* **2003**, *31*, 916.
[63] Y. Yamabe, Y. Kondo, W. Endo, K. Sasaya, N. Imura, T. Hasegawa, Y. Seko, S. Himeno, *J. Health Sci.* **2001**, *47*, 378.
[64] E. Wrigley, H. W. Verspaget, G. C. Jayson, A. T. McGown, *J. Cancer Res. Clin. Oncol.* **2000**, *126*, 717.
[65] E. Palecek, M. Masarik, R. Kizek, D. Kuhlmeier, J. Hassmann, J. Schulein, *Anal. Chem.* **2004**, *76*, 5930.
[66] J. Petrlova, R. Mikelova, K. Stejskal, A. Kleckerova, O. Zitka, J. Petrek, L. Havel, J. Zehnalek, V. Adam, L. Trnkova, R. Kizek, *J. Sep. Sci.* **2006**, *29*, 1166.
[67] D. Potesil, J. Petrlova, V. Adam, J. Vacek, B. Klejdus, J. Zehnalek, L. Trnkova, L. Havel, R. Kizek, *J. Chromatogr. A* **2005**, *1084*, 134.
[68] K. Stejskal, S. Krizkova, V. Adam, B. Sures, L. Trnkova, J. Zehnalek, J. Hubalek, M. Beklova, P. Hanustiak, Z. Svobodova, A. Horna, R. Kizek, *IEEE Sens. J.* **2008**, in press.
[69] J. Vitecek, J. Petrlova, J. Petrek, V. Adam, D. Potesil, L. Havel, R. Mikelova, L. Trnkova, R. Kizek, *Electrochim. Acta* **2006**, *51*, 5087.
[70] R. Prusa, R. Kizek, L. Trnkova, J. Vacek, J. Zehnalek, *Clin. Chem.* **2004**, *50*, A28.
[71] J. Kukacka, D. Vajtr, D. Huska, R. Prusa, L. Houstava, F. Samal, V. Diopan, K. Kotaska, R. Kizek, *Neuroendocrinol. Lett.* **2006**, *27*, 116.
[72] V. Adam, J. Petrlova, D. Potesil, J. Zehnalek, B. Sures, L. Trnkova, F. Jelen, R. Kizek, *Electroanalysis* **2005**, *17*, 1649.
[73] I. Fabrik, V. Adam, S. Krizkova, J. Kukacka, R. Prusa, L. Trnkova, R. Kizek, *Klinicka Onkologie* **2007**, *20*, 384.

 © 2008 WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim