Nephroprotective effect of cystathionine is due to its diverse action on the kidney and Ehrlich ascites tumor cells

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Abstract:

Tumor cells, unlike normal cells, are characterized by trace cystathionase (CST) activity and sulfane sulfur levels. The present studies aimed to established whether cystathionine (CT), a substrate of cystathionase, can selectively influence the thiol-dependent antioxidant power of the kidney and Ehrlich ascites tumor (EAT). CT treatment reversed the changes in renal concentrations of non-protein thiols (NPSH), reactive oxygen species (ROS), sulfane sulfur and activities of rhodanese, cystathionase and glutathione S-transferase (GST) in tumor-bearing mice, which returned to the level observed in healthy animals. The results demonstrated that CT corrected all harmful changes in the mouse kidney induced by EAT. In contrast, CT did not elicit such effect in EAT cells, in which it only increased ROS level. It indicates that CT can selectively protect the kidney of tumor-bearing mice against nephrotoxicity of drugs as well as restore biological function of sulfane sulfur. On the other hand, cisplatin (CP) did not affect any of the parameters under study in the kidney of tumor-bearing mice. Interestingly, cisplatin markedly lowered glutathione S-transferase activity and increased sulfane sulfur level and rhodanese activity in tumor cells. It is also worth noting that CP doses devoid of nephrotoxic effect in tumor-bearing mice could enhance cystathionine action on the kidney, causing an additional increase in NPSH and CST and rhodanese activity.
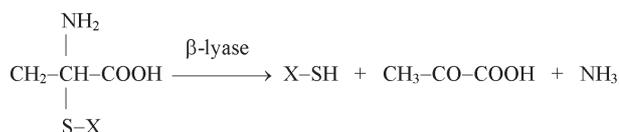
Key words:

cisplatinum, cystathionine, Ehrlich ascites tumor, glutathione, kidney, nephrotoxicity

Abbreviations: CP – cisplatinum, CST – γ-cystathionase, CT – cystathionine, EAT – Ehrlich ascites tumor, γGT – γ glutamyl transpeptidase, GSH – reduced glutathione, GST – glutathione S-transferase, HepG2 – human hepatoma cells, NPSH – non-protein thiols, TNF-α – tumor necrosis factor alpha

Introduction

Nephrotoxicity of drugs may be caused by formation and biodegradation of glutathione S-conjugates in the glutamyl cycle (Scheme 1). Biodegradation of cysteine S-conjugates by β-lyase in the kidney leads to the release of toxic thiols aggravating peroxidative processes (Scheme 1) [6], what was recently found with cisplatin [51].

$$\underset{\substack{| \\ S-X}}{\overset{\substack{NH_2 \\ |}}{CH_2-CH-COOH}} \xrightarrow{\text{β-lyase}} X-SH \; + \; CH_3-CO-COOH \; + \; NH_3$$
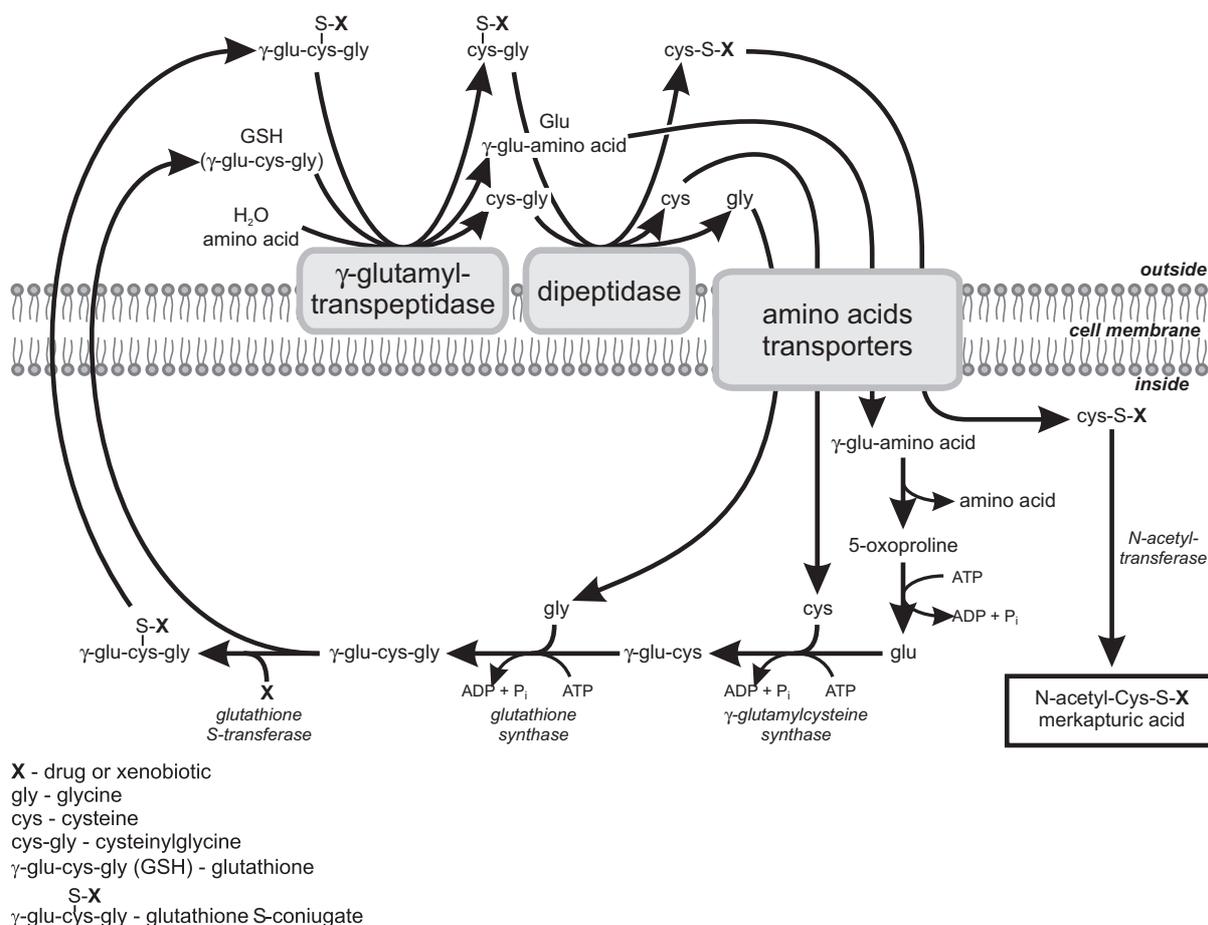
Cisplatin [cis-diamminedichloroplatinum (II)] (CP) belongs to drugs with particularly serious nephrotoxic

liability caused by peroxidative damage. This drug is a potent anticancer agent used especially in treatment of solid tumors. CP molecule easily crosses plasma membrane and migrates to the nucleus, where it elicits changes in DNA structure by forming intra- and interchain adducts [16]. However, clinical use of CP is often limited by its adverse effects, of which renal toxicity is the most serious dose-limiting factor. Signs of injury, such as changes in urine volume, osmolality, and reduction of the glomerular filtration rate characterize the alterations induced by CP in kidney function [27]. For this reason, compounds selectively shielding the kidney but not impairing CP toxicity in tumor cells are actively searched for. Up until now, several compounds have been shown to exhibit nephroprotective effect against CP toxicity, e.g. L-methioninamide, methionyl-L-alanine, (methylthio) acetic acid 4-(methylthio) benzoic acid [21], and metallothioneins [31].

However, in the latter case, chronic CP administration leads to an increase in metallothionein levels also in urinary bladder cancer MBT2 cells, which impairs therapeutic efficacy of the drug [38].

The earliest effect of CP nephrotoxicity is the drop in cellular glutathione (GSH) level and the rise in malondialdehyde level, which indicates acceleration of peroxidative processes [4, 9]. A tripeptide GSH (γ-glutamyl-cysteinyl-glycine) is one of the most efficient cellular antioxidants [37]. Therefore, a majority of studies aiming to enhance antioxidant power of cells, more and more often focus on elevation of GSH level.

Physiological cellular GSH level is high (2–10 mM), and drops in various pathological states, like neurodegenerative diseases, diabetes, AIDS, malnutrition, etc. [45]. This tripeptide is stable in the cell because it has an atypical peptide bond (in γ position in relation to the amine group), which protects it from hydrolysis by intracellular peptidases. In addition, it is character-

X - drug or xenobiotic
gly - glycine
cys - cysteine
cys-gly - cysteinylglycine
γ-glu-cys-gly (GSH) - glutathione

S-**X**
γ-glu-cys-gly - glutathione S-coniugate

Scheme 1. γ-Glutamyl cycle (according to Meister [27])

ized by high pKa value of -SH group (9.2), which makes it resistant to spontaneous oxidation to disulfide. Consequently, it is present in the cell mostly in the reduced form (GSH), while its disulfide form (GSSG) constitutes only 1% of its total content. Antioxidant GSH action depends on its sulfhydryl group -SH, which directly participates in reactions with reactive oxygen species, e.g. superoxide radical anion ($-O_2^{\cdot-}$), hydroxyl radical ($OH^\bullet$), hydrogen peroxide (H_2O_2) and peroxynitrite ($ONOO^-$). The -SH group in GSH molecule is also involved in regeneration of damaged protein and DNA molecules with free-radical structure. Antioxidant reactions with GSH yield radicals ($-GS^\bullet$) then transformed into the disulfide (GSSG), whose increased cellular level is dangerous because it disrupts pro- and antioxidant balance. An increase in glutathione disulfide level (GSSG) can also lead to facilitation of thiol-disulfide exchange (S-thiolation), i.e. formation of mixed disulfides with proteins [23].

GSH and cysteine levels in cytosol and extracellular space under physiological conditions are regulated by γ-glutamyl cycle (described by Meister) [28], in which GSH is translocated outside the cell, where it is hydrolyzed to its component amino acids by transmembrane peptidases: γ-glutamyl transpeptidase (γGT) and cysteinylglycine dipeptidase. Amino acids released in this process return to the cell, where they again participate in GSH biosynthesis. Therefore, γ-glutamyl cycle functions to maintain cysteine concentrations in the cell, extracellular space and plasma at physiological level. The reaction catalyzed by γGT is known as transpeptidation mechanism. Possibility of hydrolysis was also described [41] (Scheme 1).
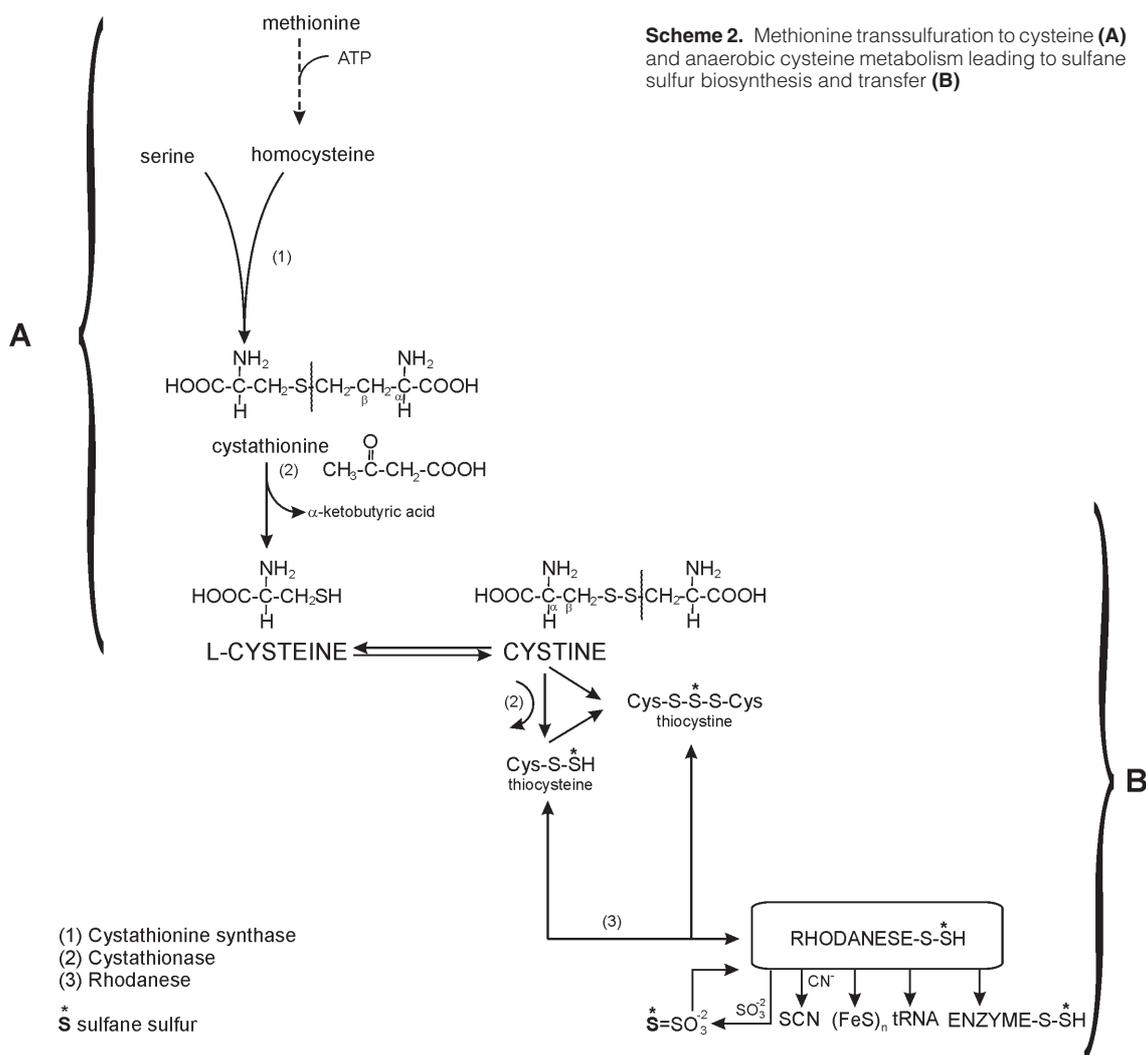
Cysteine is supplied to the organism with dietary proteins and can be synthesized in the hepatic, renal and pancreatic cells in the process of methionine transsulfuration (Scheme 2). Plasma is the only source of cysteine for the remaining tissues and organs. The kidneys are characterized by the highest concentration of cysteine [1] and sulfane sulfur [33], and the highest γGT activity (from 800 to 100 times higher than in the liver in different species) [18]. This indicates their particular role in GSH and cysteine metabolism and in preserving physiological concentrations of plasma thiols. In terminal renal failure, enhanced peroxidative processes are accompanied by changes in concentrations of different redox forms of the most important plasma thiols. i.e. cysteine, GSH, cysteinylglycine and homocysteine [48].

Despite exceptional antioxidant properties and lack of toxicity, GSH cannot be administered as medication because it is not transported across plasma membrane in most cells, except epithelial cells [25]. There are papers describing that oral administration of GSH can increased GSH concentration in several tissues [3]. Cysteine, the rate-limiting substrate in GSH biosynthesis, also cannot be given directly due to its strong neurotoxicity [47]. In addition, methionine, which is a natural cysteine precursor, requires ATP for its metabolism, so it depletes cellular ATP pool [17]. In contrast, cystathionine, a thioether formed in the process of methionine transsulfuration, that does not require ATP, can be used as a cysteine precursor, (Scheme 2). Cystathionine (CT) is transformed by γ-cystathionase (CST) into cysteine and α-ketobutyric acid (Scheme 2). Unique properties of CT have been confirmed by some authors who reported that the CST inhibitor, propargylglycine, enhanced anticancer effect of cisplatin in MBT2 cells [38], which indicates that CST inhibition, i.e. blockade of cysteine synthesis from CT, can increase cytotoxicity of the drug.

Moreover, CST participates in anaerobic sulfur metabolism, that leads to sulfane sulfur formation (Scheme 2) [7]. Sulfane sulfur occurs in zero or –1 oxidation state and is always bound to another sulfur atom. A disulfide, cystine is a γ-cystathionase substrate, while unstable cysteine hydropersulfide CysS-S*-H (thiocysteine) (S*-sulfane sulfur) and stable trisulfide – thiocystine Cys S-S*-S-Cys are sulfane sulfur-containing products of this reaction. Both these compounds are substrates of rhodanese, which is the enzyme transferring sulfane sulfur to different acceptors, which enables it to fulfill its biological role (Scheme 2). Thiocysteine, i.e. cysteine hydropersulfide (R-C-S*SH) is characterized by higher ability to donate protons and electrons, i.e. has stronger antioxidant properties than cysteine -SH groups. Furthermore, perthyil radicals ($RSS^\bullet$) formed concomitantly are more stable and less dangerous than thiyl radicals ($RS^\bullet$) [10].

Lack or trace amount of sulfane sulfur and its metabolic enzymes are a hallmark of all tumor cells [19, 49].

The studies presented in this paper aimed to establish whether cystathionine, which is a potential source of cysteine, can increase antioxidant power of the kidney (by increasing non-protein thiol (NPSH) level), and whether it can influence anaerobic cysteine metabolism. These studies were further justified by the

A

methionine

ATP

serine   homocysteine

(1)

NH_2 NH_2
$HOOC-C-CH_2-S-CH_2-CH_2-C-COOH$
H β α H

cystathionine   O
(2) CH_3-C-CH_2-COOH
α-ketobutyric acid

NH_2 NH_2 NH_2
$HOOC-C-CH_2SH$ $HOOC-C-CH_2-S-S-CH_2-C-COOH$
H   H α β H

L-CYSTEINE ⇌ CYSTINE

(2)   Cys-S-S*-S-Cys
thiocystine

Cys-S-S*H
thiocysteine

B

(3)   RHODANESE-S-S*H

CN^-

$S*=SO_3^{-2}$ ← SO_3^{-2} SCN $(FeS)_n$ tRNA   ENZYME-S-S*H

(1) Cystathionine synthase
(2) Cystathionase
(3) Rhodanese

S* sulfane sulfur

fact that tumor cells, unlike normal cells, are characterized by only trace CST activity [19]. This led us to expect that CT can be a more efficient cysteine precursor in the kidney than in tumor cells, and, thus that it can serve as a nephroprotectant.

The present studies also aimed to determine the changes in thiol antioxidant status of the kidney in the course of Ehrlich ascites tumor (EAT) development. For this purpose, we determined the levels of reactive oxygen species, non-protein thiols and sulfane sulfur, activities of enzymes involved in glutathione S-transferase (GSH) metabolism: γGT and GST, and enzymes participating in sulfane sulfur transfer and metabolism: CST and rhodanese, in the kidney of healthy and EAT-bearing mice. Subsequently, we examined the effect of CT and CP administered alone or in combination on the above-listed parameters in the kidney of EAT-bearing mice and in EAT cells. We expected that results of these studies would demonstrate diverse CT action on thiol antioxidant defense and anaerobic cysteine metabolism in the kidney of EAT-bearing mice and in EAT cells.

Materials and Methods

Animals

Female albino Swiss mice, weighing approximately 20 g, were randomly divided into five groups. Intraperitoneal (*ip*) inoculation of EAT cells was performed in animals of group 2–5, and the mice were maintained for 5 days. Control healthy mice – group 1 and

EAT-bearing mice – group 2 were injected with the same volume of 0.9% sodium chloride solution. Chemicals dissolved in isotonic saline, were *ip* injected at the following doses: group 3 – cystathionine at 10 mg/kg, group 4 – cisplatinum at 5 mg/kg, and group 5 – cystathionine plus cisplatin. Cystathionine dose was taken from the paper by Wada [44] and earlier experiments [24]. Nephrotoxic doses of CP commonly used in rodents are 5–10 mg/kg [2, 29]. On the fifth day, the mice were sacrificed. The EAT cells were collected and washed three times by suspending in cold 0.9% sodium chloride solution, followed by centrifugation at 650 × g for 5 min. The kidneys were isolated, placed in liquid nitrogen and stored at –70°C until biochemical tests were performed.

All procedures were approved by the Ethics Committee for the Animal Research in Kraków (no. 74/OP/2002).

Reagents

1-Chloro-2,4-dinitrobenzene (CDNB), cis-diammine-platinum(II) dichloride, cystathionine, 2',7'-dichlorohydrofluorescein (DCHF-DA), 5,5'dithiobis-2-nitrobenzoic acid, dithiothreitol, homoserine, L-γ-glutamyl-p-nitroanilide, glycylglycine, 3-methyl-2-benzothiazolinone hydrazone, NADH, p-nitroaniline, potassium cyanide, pyridoxal 5'-phosphate, p-phenylenediamine, trichloroacetic acid (TCA), were obtained from Sigma Chemical Company, St. Louis, (USA). The remaining chemicals were purchased from POCh, Gliwice, (Poland).

Determination of non-protein sulfhydryl groups [30]

In this assay 5,5'-dithiobis-2-nitrobenzoic acid is reduced by nonprotein sulfhydryl groups present in trichloroacetic acid (TCA) extract to 2-nitro-5 mercaptobenzoic acid. For the estimation of NPSH, 0.05 ml of TCA extract and 0.1 ml of 6 mM 5,5'dithiobis-2-nitrobenzoic acid were added in succession to 0.850 ml of 0.2 M phosphate buffer pH 8.2, and absorbance was measured at 412 nm. The total content of nonprotein sulfhydryl groups was determined from a standard curve for the glutathione.

Determination of γ-glutamyl transpeptidase activity [34]

In this method, the colorless substrate L-γ-glutamyl--p-nitroanilide was enzymatically converted to p-nitroaniline. The reaction mixture (0.9 ml) contained 5 mM L-γ-glutamyl-p-nitroanilide and 11 mM of $MgCl_2$ dissolved in 111 mM Tris-HCl buffer, pH 9 and was incubated for 5 min at 37°C with 0.1 ml of liver homogenates. Adding 1 ml of 1.5 M acetic acid stopped the reaction. The probes were centrifuged at 12,000 × g for 5 min, and the absorbance of p-nitroaniline that developed during 5 min was measured at 410 nm. The enzyme activity was expressed in μmoles of the product (evaluated from a standard curve for p-nitroaniline) formed during 1 min of incubation per 1 mg of protein.

Determination of glutathione S-transferase activity [14]

Glutathione S-transferase catalyzes reaction between glutathione and 1-chloro-2,4-dinitrobenzene (CDNB) yielding colored conjugate, 2,4-dinitrophenyl-S-glutathione, which shows absorbance maximum at a wavelength of 340 nm. An increase in absorbance after 1 min of incubation was used as a measure of reaction progress. The activity of the enzyme was calculated on the basis of millimolar absorbance index of the formed conjugate, which is 9.6 mmol/cm. The 50 μl of 20 mM GSH solution, the same volume of 20 mM CDNB solution and 50 μl of the homogenate diluted 50 times were added to 850 ml of 0.1 M phosphate buffer, pH 6.5. Thirty and ninety seconds afterwards, an absorbance at 340 nm was measured against the control sample, containing phosphate buffer instead of the homogenate. A difference between an increase in absorbance in the test sample and control sample divided by molar absorbance index was the measure of the enzyme's activity. Activity of the enzyme was expressed in millimoles of the conjugate formed within one minute under the experimental conditions applied in this study.

Determination of reactive oxygen species [5]

2'7'-Dichlorohydrofluorescein diacetate (DCFH-DA) was deesterified in homogenates to dichlorohydrofluorescein, and was then oxidized to fluorescent dichlorofluorescein by ROS. To 1.2 ml of 0.1 M phosphate

buffer, pH 7.4, 0.01 ml of homogenate and 0.01 ml of DCFH-DA were added. Mixtures were incubated in a water bath for 30 min at 37°C and then centrifuged at 12,000 × g for 8 min. Fluorescence was measured with a Hitachi F-2000 fluorescence spectrometer with an excitation of 488 nm and an emission of 525 nm. ROS were evaluated from a standard curve for dichlorofluorescein.

Determination of sulfane sulfur level [49]

The level of the sulfur in kidney homogenate was determined by cold cyanolysis according to Wood [49]. To 0.1 ml of liver homogenate 0.08 ml of 1 M NH_3, 0.72 ml distilled water and 0.1 ml of 0.5 M KCN were added. The samples were incubated at room temperature for 45 min. Then 0.02 ml of 38% formaldehyde and 0.2 ml of Goldstein's reagent [Fe(NO)₃ + HNO₃ + H₂O] were added. After centrifugation at 12,000 × g for 10 min, the absorbance at 460 nm was determined. A standard curve was prepared with 1 mM KSCN.

The pool of sulfane sulfur in EAT cells was assayed by the modification of Ogasawara's method [32]. In this method, bound sulfur is easily liberated as sulfide after reduction by dithiotreitol. The released sulfide is converted into a fluorescent derivate, thionine, in the reaction with p-phenylenediamine and ferric ion. Thionine is determined by fluorimetric method.

Determination of rhodanese activity [40]

Enzymatic activity of rhodanese was determined according to Sörbo. Assay mixture containing 100 μl of homogenate diluted 100 times, 60 mM thiosulfate, 47.5 mM sodium phosphate buffer pH 7.4 and 71.5 mM cyanide was incubated in final volume of 525 μl at room temperature for 5 min. The reaction was stopped by addition of formaldehyde. Reaction product, i.e. thiocyanide and ferric ions formed colored complex, whose absorbance was measured spectrophotometrically at 460 nm. The enzyme activity was expressed in μmoles of the product (evaluated from a standard curve for thiocyanide) formed during 1 min of incubation per 1 mg of protein.

Determination of γ-cystathionase activity [39]

Enzymatic activity of cystathionase was determined according to Soda [39] with modifications. The assay mixture contained 0.02 μmol of pyridoxal 5'-phos-

phate, 4 μmol of homoserine, 0.065 mmol of potassium phosphate buffer and liver homogenate in final volume of 1 ml. The mixture was incubated at 37°C for 30 min and the reaction was stopped by adding 50% TCA. After centrifugation (14,000 × g/10 min) to 0.5 ml supernatant 1 ml of acetate buffer pH 8.0 and 0.4 ml of 0.1% 3-methyl-2-benzo-thiazolinone hydrazone were added. Incubation was carried out at 50°C for 30 min. Absorbance at 320 nm was measured after cooling the reaction mixture. The enzyme activity was expressed in μmoles of the product formed during 1 min of incubation per 1 mg of protein.

Determination of protein content using Lowry's method [26]

Statistical analysis

The results are presented as the means ± SD, and statistical significance of differences was evaluated using ANOVA followed by the Least Significance Difference test for *post-hoc* comparisons. The differences were considered statistically significant when $p < 0.05$.

Results

Renal NPSH level in EAT mice was significantly decreased in comparison with healthy animals (Fig. 1A). Cisplatin administration increased NPSH level *vs.* control tumor-bearing mice but it still remained lower than in healthy animals. Cystathionine elevated NPSH level but the largest increase to the level comparable to healthy mice was seen in animals treated with a combination of CP and CT (Fig. 1A). In contrast, in EAT cells, cisplatin did not affect NPSH level, cystathionine decreased it, while NPSH level in CP + CT-treated animals was equal to the control, what means that CP abolished CT action (Fig. 1B).

No significant differences were noted between EAT-bearing mice and healthy animals in renal γGT activity (Fig. 2A). CP did not influence renal γGT activity in tumor-bearing mice, whereas CT significantly increased activity of this enzyme. The combined treatment with CP and CT also in this case caused the highest and significant rise in γGT activity *vs.* control group. In EAT cells, neither CP nor CP sig-
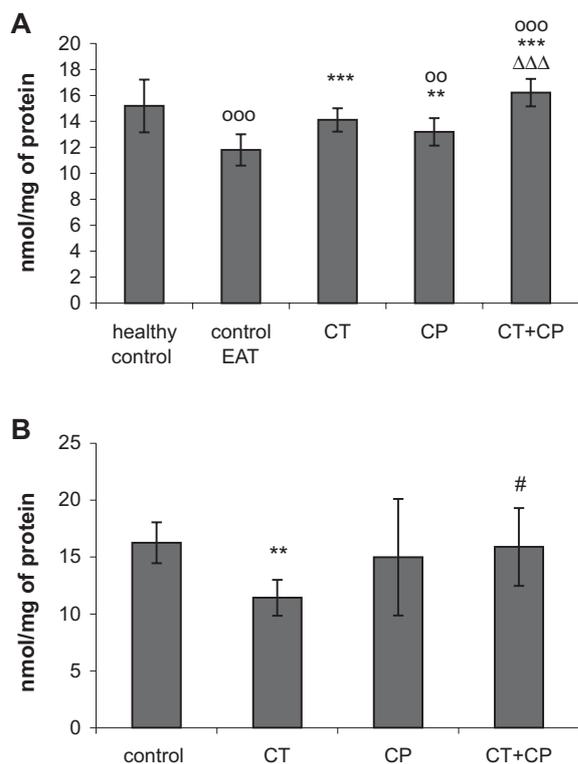
Fig. 1. Level of non-protein sulfhydryl groups (NPSH) in the kidney of healthy and EAT-bearing mice **(A)** and in EAT cells **(B)**. NaCl (control groups), cystathionine (CT) at 10 mg/kg, cisplatinum (CP) at 5 mg/kg and cystathionine plus cisplatin were *ip* injected for 5 days. °° p < 0.01 *vs.* healthy control, °°° p < 0.001 *vs.* healthy control, ** p< 0.01 *vs.* control EAT, *** p < 0.001 *vs.* control EAT, # p < 0.05 *vs.* CT, ΔΔΔ p < 0.001 *vs.* CP
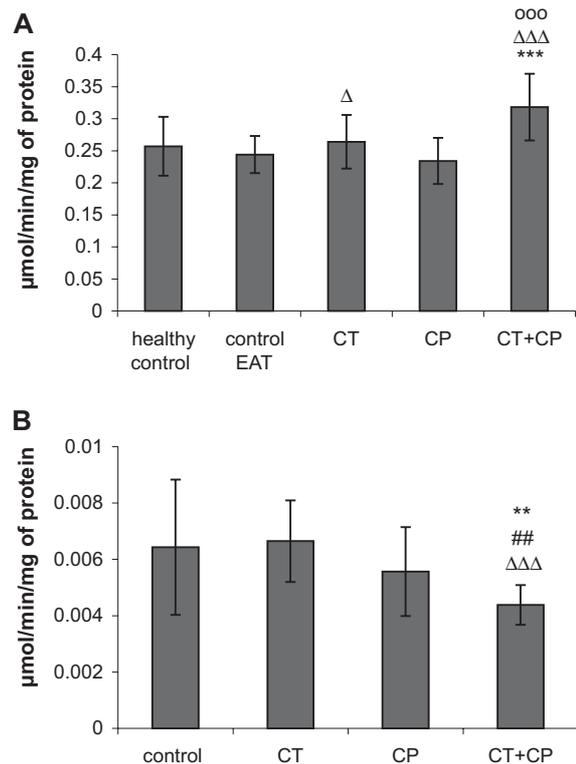
Fig. 2. γ-Glutamyltranspeptidase (γGT) activity in the kidney of healthy and EAT-bearing mice **(A)** and in EAT cells **(B)**. NaCl (control groups), cystathionine (CT) at 10 mg/kg, cisplatinum (CP) at 5 mg/kg and cystathionine plus cisplatin were *ip* injected for 5 days. °°° p < 0.001 *vs.* healthy control, ** p < 0.01 *vs.* control EAT, *** p < 0.001 *vs.* control EAT, ## p < 0.01 *vs.* CT, Δ p < 0.05 *vs.* CP, ΔΔΔ p < 0.001 *vs.* CP

nificantly altered γGT activity but their combination drastically decreased activity of this enzyme (Fig. 2B).

Renal GST activity was significantly lowered in EAT-bearing mice *vs.* healthy control group (Fig. 3A). It was significantly increased only after treatment with cisplatin and cystathionine in combination (Fig. 3A). In EAT cells, GST activity was diminished by CP, unchanged by CT and significantly increased by their combination (Fig. 3B).

Renal reactive oxygen species (ROS) level in tumor-bearing mice was significantly increased in comparison with healthy animals (Fig. 4A), which indicates acceleration of peroxidative processes. ROS level was still elevated after CP treatment but CT given alone or jointly with CP lowered it. In EAT cells, ROS level was significantly lower than in normal kidney cells and significantly rose after CT and CT + CP treatment (Fig. 4B).

Sulfane sulfur level in EAT-bearing mice was equal to the healthy group. Cystathionine given either alone or jointly with CP to EAT-bearing mice statistically significantly increased sulfane sulfur above the control level (Fig. 5A). In EAT cells, CT had no effect on sulfane sulfur level, whereas CP administered alone or with CT markedly and significantly increased bound sulfane sulfur level (Fig. 5B).

Renal activity of rhodanese, the enzyme participating in transfer of sulfane sulfur-containing compounds was significantly diminished in EAT-bearing animals *vs.* healthy mice. Activity of this enzyme rose to the level observed in healthy animals both after CT alone and in combination with CP (Fig. 6A). On the other hand, in EAT cells, CT had no effect on rhodanese activity, while CP augmented activity of this enzyme, what was not observed after combined treatment with CT + CP (Fig. 6B).
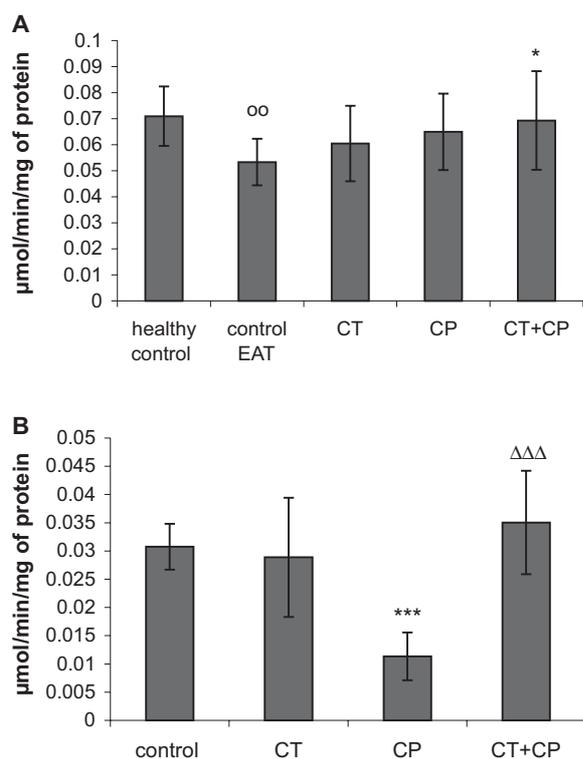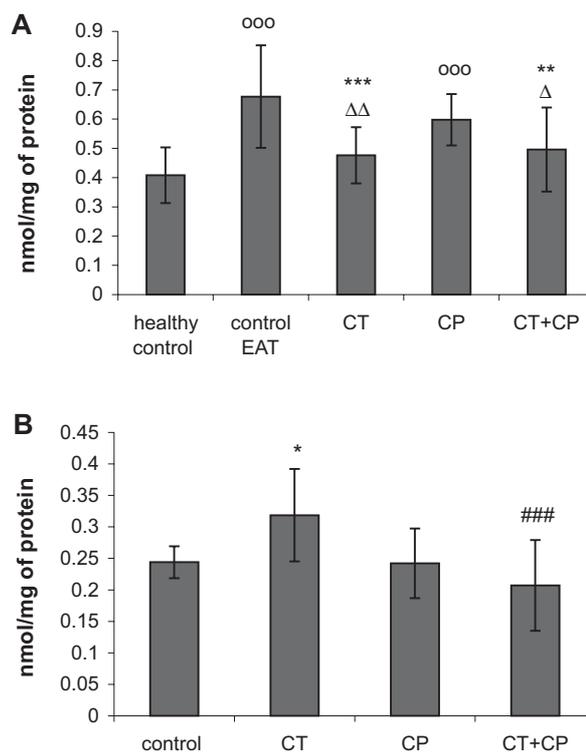
Fig. 3. Glutathione S-transferase (GST) activity in the kidney of healthy and EAT-bearing mice **(A)** and in EAT cells **(B)**. NaCl (control groups), cystathionine (CT) at 10 mg/kg, cisplatinum (CP) at 5 mg/kg and cystathionine plus cisplatin were *ip* injected for 5 days. $^{\circ\circ}$ p < 0.01 *vs.* healthy control, * p < 0.05 *vs.* control EAT, *** p < 0.001 *vs.* control EAT, $^{\Delta\Delta\Delta}$ p < 0.001 *vs.* CP

Fig. 4. Level of reactive oxygen species (ROS) in the kidney of healthy and EAT-bearing mice **(A)** and in EAT cells **(B)**. NaCl (control groups), cystathionine (CT) at 10 mg/kg, cisplatinum (CP) at 5 mg/kg and cystathionine plus cisplatin were *ip* injected for 5 days. $^{\circ\circ\circ}$ p < 0.001 *vs.* healthy control, * p < 0.05 *vs.* control EAT, ** p < 0.01 *vs.* control EAT, *** p < 0.001 *vs.* control EAT, $^{\#\#\#}$ p < 0.001 *vs.* CT, $^{\Delta}$ p < 0.05 *vs.* CP, $^{\Delta\Delta}$ p < 0.01 *vs.* CP

Renal γ-cystathionase activity was reduced in tumor-bearing mice *vs.* healthy control group. Cystathionine alone and in combination with CP increased CST activity to the control level (Fig. 7A). Trace CST activity in EAT cells was even more lowered by CT or CP given alone, but, interestingly, it was increased almost to the control level after the CT + CP combination (Fig. 7B).

Discussion

It is assumed that nephrotoxic action of many drugs and other xenobiotics, including CP, is most often caused by acceleration of peroxidative processes. There are different hypotheses explaining the mechanism of oxidative stress generation by cisplatin. Since renal proximal tubules express NADPH oxidase, it was

suggested to be responsible for excessive ROS production elicited by CP [12]. It was substantiated by the observation that the NADPH oxidase inhibitor (diphenyleneiodonium chloride), lowered ROS level and prevented renal cell dysfunction [22]. Superfluous ROS formation was also attributed to a potential CP-induced Ca^{2+} influx into mitochondria due to a decrease in inner mitochondrial membrane potential [43]. This hypothesis was corroborated by studies on renal epithelial cell culture, which indicated that CP enhanced Ca^{2+} release and peroxidative cell damage (measured by lactate dehydrogenase release) [22]. The increased concentration of calcium ions can also provoke necrosis by activation of proteases and phospholipases.

Low CP doses constantly present in renal tubule cell culture induced apoptosis, while its high doses caused necrosis. In the latter case, it was accompanied by excessive generation of NO, $O_2^{\bullet-}$, TNF-α, and peroxynitrite ($ONOO^-$), which is considered to be a marker of nephropathy induced by high CP doses [20].

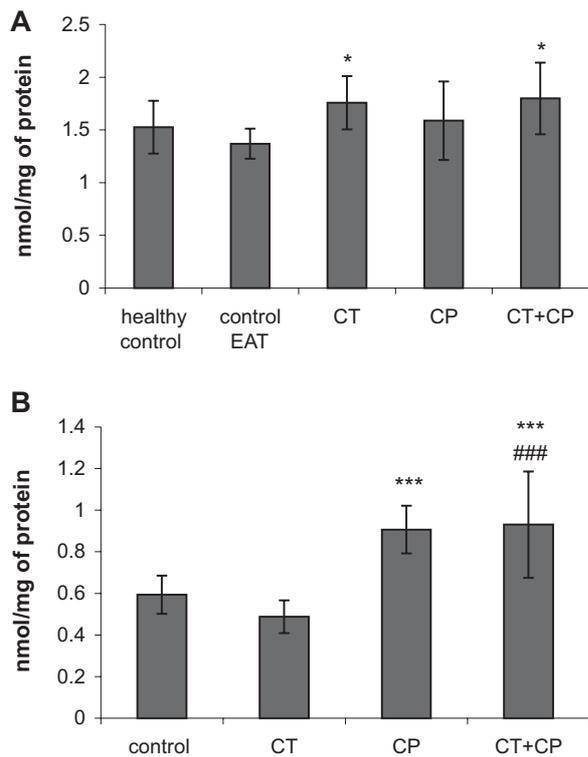**Fig. 5.** Sulfane sulfur in the kidney of healthy and EAT-bearing mice **(A)** and in EAT cells **(B)**. NaCl (control groups), cystathionine (CT) at 10 mg/kg, cisplatinum (CP) at 5 mg/kg and cystathionine plus cisplatin were *ip* injected for 5 days. * $p < 0.05$ *vs.* control EAT, *** $p < 0.001$ *vs.* control EAT, ### $p < 0.001$ *vs.* CT
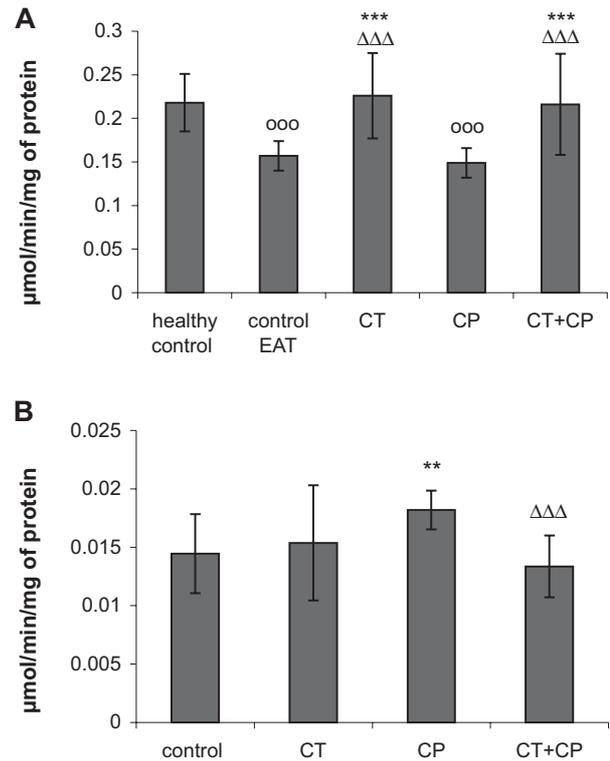

Fig. 6. Rhodanese activity in the kidney of healthy and EAT-bearing mice **(A)** and in EAT cells **(B)**. NaCl (control groups), cystathionine (CT) at 10 mg/kg, cisplatinum (CP) at 5 mg/kg and cystathionine plus cisplatin were *ip* injected for 5 days. °°° $p < 0.001$ *vs.* healthy control, ** $p < 0.01$ *vs.* control EAT, *** $p < 0.001$ *vs.* control EAT, ΔΔΔ $p < 0.001$ *vs.* CP

The present studies showed the marked weakening of GSH-related antioxidant and detoxifying mechanisms in the kidneys of EAT-bearing mice in comparison with healthy mice, as demonstrated by the decrease in NPSH level (of which GSH constitutes over 95% under physiological conditions) (Fig. 1A), diminution of GST activity (Fig. 3A) and rise in ROS (Fig. 4A). Physiological GSH level is essential for normal kidney function, particularly for proximal tubules, characterized by accelerated oxygen metabolism [25]. However, progression of carcinogenesis in the body makes the kidney even more "defenseless" against nephrotoxic action of drugs. We observed similar situation earlier in livers of tumor-bearing mice, which was the cause of enhanced hepatotoxicity of drugs [24].

EAT-bearing mice had also significantly lower renal activity of CST (Fig. 7A), the enzyme involved in sulfane sulfur formation, and rhodanese, participating in sulfane sulfur transfer to different acceptors (Fig. 6A). Sulfane sulfur occurs in cells mostly in the form of protein hydropersulfides and trisulfides, which enables it to fulfill the regulatory role. This means that EAT growth disrupts anaerobic cysteine metabolism in the kidney, impairing regulatory mechanisms dependent on sulfane sulfur.

CP doses used in his study did not enhance peroxidative processes in EAT-bearing mice (ROS level did not change) (Fig. 4A), which was accompanied by a slight but significant rise in NPSH level (Fig. 1A). It indicates that CP at these doses did not induce peroxidative damage in the kidney. These observations are in line with other reports which demonstrated that chronic administration of low CP doses, in contrast to acute treatment, did not disturb antioxidant status of the kidney [13]. Administration of CP alone also did not cause any changes in γGT, GST, rhodanese and CST activities (Fig. 2, 3, 6, 7) or in the concentrations of sulfane sulfur-containing compounds (Fig. 5A) in the kidney of EAT-bearing mice. It means that CP at
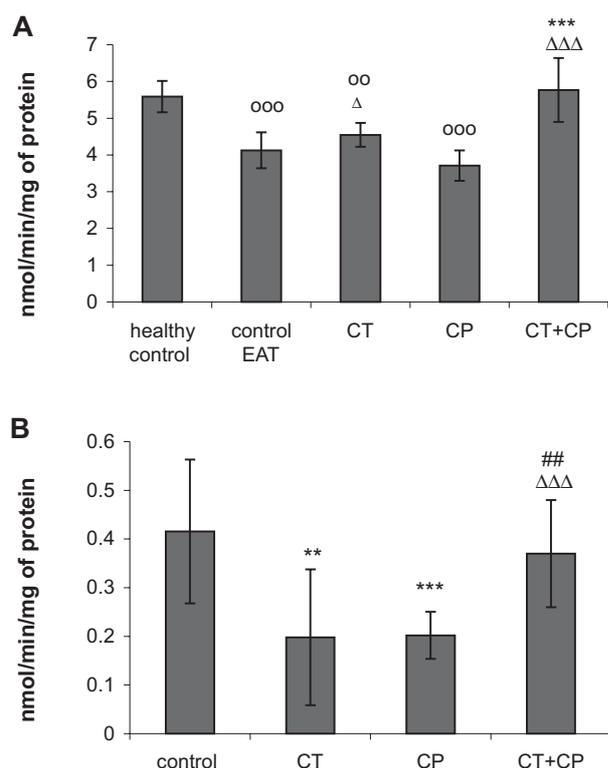
Fig. 7. γ-Cystationase activity in the kidney of healthy and EAT-bearing mice **(A)** and in EAT cells **(B)**. NaCl (control groups), cystathionine (CT) at 10 mg/kg, cisplatinum (CP) at 5 mg/kg and cystathionine plus cisplatin were *ip* injected for 5 days. $^{\circ\circ}$ p < 0.01 *vs.* healthy control, $^{\circ\circ\circ}$ p < 0.001 *vs.* healthy control, ** p < 0.01 *vs.* control EAT, *** p < 0.001 *vs.* control EAT, $^{\#\#}$ p < 0.01 *vs.* CT, $^{\Delta}$ p < 0.05 *vs.* CP, $^{\Delta\Delta\Delta}$ p < 0.001 *vs.* CP

the doses used in these experiments elicited no changes in any of the parameters under study in the kidney of EAT-bearing mice.

Cystathionine administration to tumor-bearing mice significantly increased renal NPSH level (Fig. 1A) and lowered ROS concentration *vs.* healthy animals (Fig, 4). It demonstrates beneficial effect of CT, owning to unique properties of this compound, namely its ability to be a source of cysteine, indispensable for GSH biosynthesis, and free radical scavenger properties (mostly superoxide radical anion $O_2^{\cdot-}$ scavenging ability) [45]. Moreover, CT is capable of reducing GSH efflux from the cells [11]. It has been shown that this mechanism underlies its protective antiapoptotic action on HepG2 cells and monocytes, since cells extrude GSH before the development of apoptosis [11]. The obtained results indicate that this compound can fulfill a protective role in the kidney, therefore, nephroprotective potential of CT appears promising.

Furthermore, CT administered alone elevated renal level of sulfane sulfur (Fig. 5A) and activity of rhodanese and CST (Fig. 6A and 7A). It means that CT could restore proper anaerobic cysteine metabolism and biological function of sulfane sulfur.

Unique properties of CT were also noted in earlier reports, which demonstrated the induction of cystathionine synthase activity under oxidative stress conditions in tumor (HLE, HepG2) and normal cells (crystalline lenses) [35]. Cisplatin-induced oxidative stress, implicating TNF-α, also increased cystathionine synthase activity in HepG2 cells [52]. It indicates that prooxidant conditions stimulate CT biosynthesis what can be considered to be a defensive mechanism of the cell.

Noteworthy, the administration of CP and CT in combination more strongly augmented renal NPSH level in tumor-bearing mice than CT alone (Fig. 1A). The non-toxic CP doses administered jointly with CT exerted such synergistic effect also on GST, γGT and CST activities (Fig. 2A, 3A, 7A).

In contrast, CT significantly reduced NPSH level (Fig. 1B) and enhanced ROS generation in the EAT cells themselves (Fig. 4B). It indicates that CT effect is selectively beneficial in the kidney and harmful in EAT cells. It is most probably due to the marked difference in CST activity between renal and EAT cells, because of that CT can efficiently protect the kidney from peroxidative action of drugs.

High γGT activity in tumor cells is more and more often considered to be a source of a moderate oxidative stress, which can play a regulatory role [8, 36]. Recent reports have revealed the role of oxidative stress in the regulation of cell proliferation and apoptosis in tumor cells. For his reason, the drop in ROS level, observed in our study, may have diverse effect in tumor cells.

Glutathione S-transferase is an enzyme responsible for biodegradation of drugs by formation of their glutatione S-conjugates. Its activity in tumor cells is increased [15]. For his reason, inhibitory effect of CP on GST activity in EAT cells seems very interesting (Fig. 3B).

Like other tumors, EAT cells are characterized by a very low sulfane sulfur level, which occurs only in the "bound" form. Sulfane sulfur deficit and lack of its regulatory effect is considered by Toohey to be one of causes of uncontrolled growth of tumor cells [42]. In our studies, CT alone did not affect the bound sulfane sulfur level in EAT cells (Fig. 5B). However, its

combined administration with CP increased sulfane sulfur level in EAT cells (Fig. 5B), which was accompanied by the increase in rhodanese activity (Fig. 6B). It indicates that the anticancer drug, CP, can increase sulfane sulfur level in tumor cells and facilitates its transfer to different acceptors (Scheme 2). This observation is interesting because such effect has never been achieved by supplementation of different sulfur compounds and cysteine precursors [46, 50]. It should be remembered that sulfane sulfur level and rhodanese activity in the cells under study was very low, therefore, the quantitative changes were small. On the other hand, sulfane sulfur is believed to act as a precise and efficient regulator with very high potential activity.

In summary, the present studies demonstrated that CT was able to correct some harmful changes in the mouse kidney induced by progressing EAT, i.e. weakened thiol antioxidant defense and disrupted sulfur metabolism. It indicates that CT, which is a natural cysteine precursor, can protect the kidney against potential nephrotoxicity of drugs and restore biological function of sulfane sulfur. Although we did not detect nephrotoxic effect of CP, we demonstrated that this drug could influence rhodanese and GST activities and sulfane sulfur level in EAT cells. Interestingly, CP doses devoid of nephrotoxic effect, in tumor-bearing mice could enhance CT actions on the kidney, causing the increase in NPSH level and CST and GST activity.

References:

1. Aebi E, Lauterberg BH: Divergent effects of intravenous GSH and cysteine on renal and hepatic GSH. Am J Physiol, 1992, 263, R348–352.
2. Ajith TA, Nivitha V, Usha S: *Zingiber officinale Roscoe* alone and in combination with alpha-tocopherol protect the kidney against cisplatin-induced acute renal failure. Food Chem Toxicol, 2007, 45, 921–927.
3. Aw TY, Wierzbicka G, Jones DP: Oral glutatione increases tisuse glutathione in vivo. Chem Biol Interact, 1991, 80, 89–97.
4. Behling EB, Sendao MC, Francescato HD, Antunes LM, Costa RS, Bianchi MP: Comparative study of multiple dosage of quercetin against cisplatin-induced nephrotoxi-
5. Bondy SC, Guo SX: Effect of ethanol treatment on induction of emulative oxidative stress. Eur J Pharmacol, 1994, 270, 349–355.
6. Cooper AJ, Pinto JT: Cysteine S-conjugate beta-lyases. Amino Acids, 2006, 30, 1–15.
7. Cooper AJ: Biochemistry of sulfur-containing amino acids. Annu Rev Biochem, 1983, 52, 187–222.
8. Del Bello B, Paolicchi A, Comporti M, Pompella A, Maellaro E: Hydrogen peroxide produced during gamma-glutamyl transpeptidase activity is involved in prevention of apoptosis and maintenance of proliferation in U937 cells. FASEB J, 1999, 13, 69–79.
9. Dillioglugil MO, Maral Kir H, Gulkac MD, Ozon Kanli A, Ozdogan HK, Acar O, Dillioglugil O: Protective effects of increasing vitamin E and A doses on cisplatin-induced oxidative damage to kidney tissue in rats. Urol Int, 2005, 75, 340–344.
10. Everet SA: Antioxidant drug design: a comparison of thiol and prooxidant reaction mechanisms. In: Biothiols in Health and Disease. Ed. Parker L, Cadenes E, Ed. New York, 1995, 49–64.
11. Ghibelli L, Fanelli C, Rotilio G, Lafavia E, Coppola S, Colussi C, Civitareale P, Ciriob MR: Rescue of cells from apoptosis by inhibition of active GSH extrusion. FASEB J, 1998, 12, 479–486.
12. Gill PS, Wilcox CS: NADPH oxidases in the kidney. Antioxid Redox Signal, 2006, 8, 1597–1607.
13. Gonzales R, Ramay C, Borrego A, Hernandez F, Merino N, Zamora Z, Rojas E: Lipid peroxides and antioxidant enzymes in cisplatin-induced chronic nephrotoxicity in rats. Mediat Infkamm, 2005, 14, 139–143.
14. Habig WH, Pabst MJ, Jakoby WB: Glutathione S-transferase: the first enzymatic step in mercapturic acid formation. J Biol Chem, 1974, 248, 7130–7139.
15. Hades PC, May L, Hayes JD, Harrison DJ: Glutathione S-transferases in human liver cancer. Gut, 1991, 32, 1546–1549.
16. Hah SS, Stivers KM, de Vere White RW, Henderson PT: Kinetics of carboplatin – DNA binding in genomic DNA and bladder cancer cells as determined by accelerator mass spectrometry. Chem Res Toxicol, 2006, 19, 622–626.
17. Hardwick DF, Applegarth DA, Cockraft DM, Ross PH, Calder JR: Pathogenesis of methionine-induced toxicity. Metabolism, 1970, 19, 381–391.
18. Hinchman CA, Ballatori N: Glutathione-degrating capacities of liver and kidney in different species. Biochem Pharmacol, 1990, 40, 1131–1135.
19. Iciek M, Włodek L: Biosynthesis and biological properties of compounds containing highly reactive, reduced sulfane sulfur. Pol J Pharmacol, 2001, 53, 215–225.
20. Ikari A, Nagatani Y, Tsukimoto M, Harada H, Miwa M, Takagi K: Sodium dependent glucose transporter reduces peroxynitrite and cell injury caused by cis-platin in renal tubular epithelial cells. Biochim Biophys Acta, 2005, 1717, 109–117.
21. Jones MM, Basinger MA, Holscher MA: Thioeter suppression of cisplatin nephrotoxicity in the rat. Anticancer Res, 1991, 11, 449–453.

22. Kawai Y, Nakao T, Kunimura N, Kohoda Y, Gemba M: Relationship of intracellular calcium and oxygen radicals to cis-platin-related cell injury. J Pharmacol Sci, 2006, 100, 65–72.

23. Klatt P, Lamas S: Regulation of protein function by S-glutathiolation in response to oxidative and nitrosative stress. Eur J Biochem, 2000, 267, 4928–4944.

24. Kwiecień I, Michalska M, Włodek L: The selective effect of cystathionine on doxorubicin hepatotoxicity in tumor bearing mice. Eur J Pharmacol, 2006, 550, 39–46.

25. Lash LH: Role of glutathione transport processes in kidney function. Toxicol Appl Pharmacol, 2005, 2004, 329–342.

26. Lowry O, Rosebrough NJ, Farr AL, Randall RJ: Protein measurement with the folin phenol reagent. J Biol Chem, 1951, 193, 265–275.

27. Matsushima H, Yonemura K, Ohishi K, Hishida A: The role of oxygen free radicals in cisplatin-induced acute renal failure in rats. J Lab Clin Med, 1998, 131, 518–526.

28. Meister A: Metabolism and transport of glutathione and other γ-glutamyl compounds. In: Function of Glutathione: Biochemical, Physiological, Toxicological and Clinical Aspects. Ed. Larsson A, Orrenius S, Holmgren A, Mannervik B, Raven Press, New York, 1983, 1–22.

29. Mora Lde O, Antunes LM, Francescato HD, Bianchi Mde L: The effects of oral glutamine on cisplatin-induced nephrotoxicity in rats. Pharmacol Res, 2003, 47, 517–522.

30. Moron MS, Depierre JW, Mannervik B: Levels of glutathione, glutathione reductase and glutathione S-transferase activities in rat lung and liver. Biochim Biophys Acta, 1979, 67, 582–589.

31. Naganuma A, Imura N: Role of metallothionein in cancer chemotheraphy. Gan To Kagaku Ryoho (abstract), 1994, 21, 301–306.

32. Ogasawara Y, Ishii K, Togawa T, Tanabe S: Determination of bound sulfur in serum by dialysis/high-performance liquid chromatography. Anal Biochem, 1993, 215, 73–81.

33. Ogasawara Y, Isoda S, Tanabe S: Tissue and subcellular distribution of bound and acid labile sulfur and the enzymatic capacity of sulfide production in the rat. Biol Pharm Bull, 1994, 17, 1535–1542.

34. Orłowski M, Meister A: γ-glutamyl-p-nitroanilidine. A new convenient substrate for determination and study of L-and D-γ-glutamyltranspeptidase activities. Biochim Biophys Acta, 1963, 73, 679–681.

35. Persa C, Pierce A, Ma Z, Kabil O, Lou MF: The presence of a transsulfuration pathway in the lens: a new oxidative stress defense system. Exp Eye Res, 2004, 79, 875–886.

36. Pieri L, Dominici B, Del Bello E, Maellaro M, Comporti A, Paolicchi A, Pompella A: Redox modulation od protein kinase/phosphatase balance in melanoma cells: the role of endogenous and γ-glutamyltransferase-dependent H$_2$O$_2$ production. Biochim Biophys Acta, 2003, 1621, 76–83.

37. Pompella A, Visvikis A, Paolichi A, De Tat V, Casini AF: The changing faces of glutathione, a cellular protagonist. Biochem Pharmacol, 2003, 66, 1499–1503.

38. Saga Y, Hashimoto H, Yachiku S, Iwata T, Tokumitsu M: Reversal of acquired cisplatin resistance by modulation of metallothionein in transplanted murine-tumors. Int J Urol, 2004, 11, 407–415.

39. Soda K: A spectrophotometric microdetermination of ketoacids with 3-methyl-2-benzothiazolone hydrazone. Agric Biol Chem, 1967, 31, 1054–1060.

40. Sörbo BH: Rhodanese. In: Methods in Enzymology, vol. II. Ed. Collowick SP, Kaplan NO, Academic Press, New York, 1955, 334–337.

41. Stark AA, Porat N, Volohonsky G, Komlosh A, Bluvshtein E, Tubi C, Steinberg P: The role of gamma-glutamyl transpeptidase in the biosynthesis of glutathione. Biofactors, 2003, 174, 139–149.

42. Toohey F: Sulfane sulfur in biological systems: a possible regulatory role. Biochem J, 1989, 264, 625–632.

43. Van der Water B, Zoeteweij JP, de Bont HJGM, Mulder GJ, Nagelkerke JF: Role of mitochondrial Ca^{2+} in the oxidative stress induced dissipation of the mitochondrial membrane potential. J Biol Chem, 1994, 269, 14546–14552.

44. Wada K, Kamisaki Y, Nakamoto K, Itoh T: Effect of cystathionine as a scavenger of superoxide generated from human leukocytes or derived from xanthine oxidase in vitro. Eur J Pharmacol, 1996, 296, 335–340.

45. White AC, Tannical VJ, Fanburg BL: Glutathione deficiency in human disease. J Nutr Biochem, 1994, 5, 218–226.

46. Włodek L, Wróbel M, Czubak J: Transamination and transsulfuration of L-cysteine in Ehrlich ascites tumor cells and mouse liver. Int J Biochem, 1993, 25, 107–112.

47. Włodek L: Causes of L-cysteine neurotoxicity. Acta Biol Crac, 2002, 44, 15–24.

48. Włodek PJ, Smoleński OB, Chwatko G, Iciek MB, Miłkowski A, Bald E, Włodek L: Disruption of thiol homeostasis in plasma of terminal renal failure patients. Clin Chim Acta, 2006, 366, 137–145.

49. Wood JL: Sulfane sulfur. Methods Enzymol, 1987, 143, 25–29.

50. Wróbel M, Włodek L: Effects of thiazolidine-4-(R)carboxylates and other low molecular-wieght sulfur compounds on the activity of mercaptopyruvate sulfurtransferase, rhodanese and cystationase in Ehrlich ascites tumor cells and tumor-bearing mouse liver. Amino Acids, 1997, 12, 309–314.

51. Zhang L, Cooper AJ, Krasnikov BF, Xu H, Bubber P, Pinto JT, Gibson GE, Hanigan MH: Cisplatin-induced toxicity is associated with platinum deposition in mouse kidney mitochondria in vivo and with selective inactivation of the α-ketoglutar dehydrogenase complexes in LLC-PK1 cells. Biochemistry, 2006, 45, 8959–8971.

52. Zou CG, Banerjee R: Tumor necrosis factor-alpha-induced targeted proteolysis of cystathionine beta-synthase modulates redox homeostasis. J Biol Chem, 2003, 278, 16802–16808.