Amino Acids (2011) 41:131–139
DOI 10.1007/s00726-010-0593-4

ORIGINAL ARTICLE

Potential therapeutic advantage of ribose-cysteine in the inhibition of astrocytoma cell proliferation

H. Jurkowska · T. Uchacz · J. Roberts · M. Wróbel

Received: 31 December 2009 / Accepted: 2 April 2010 / Published online: 23 April 2010

Abstract It has been observed that astrocyte and astrocytoma cells differ in their response to D-ribose-L-cysteine (RibCys) in the culture medium. RibCys, a prodrug of L-cysteine, elevates the level of cysteine and glutathione in both astrocytoma and astrocyte cultures. It also affects the activity of two sulfurtransferases, 3-mercaptopyruvate sulfurtransferase and rhodanese, involved in the metabolism of sulfane sulfur-containing compounds and in consequence exerts an effect on the level of sulfane sulfur. Under conditions, in which the raised level of sulfane sulfur was accompanied by an elevated activity of 3-mercaptopyruvate sulfurtransferase, the proliferation of the human astrocytome U373 line was decreased. The experiments were simultaneously performed with murine astrocytes to compare the behavior of normal cells under similar conditions. In murine astrocytes, RibCys was capable of increasing cellular proliferation, and was accompanied by a diminished level of sulfane sulfur and unchanged activity of the two sulfurtransferases. Thus, RibCys might offer a therapeutic advantage in the inhibition of astrocytoma cell proliferation. Besides, in the absence of oxidative stress, measured as the ratio of GSH/GSSG, the obtained results confirm that the fall in the level of sulfane sulfur is associated with increasing proliferation of cells, whereas a rise in the level causes a decrease in the proliferation of U373 cells.

Keywords Astrocytes · Cysteine · Glutathione · 3-Mercaptopyruvate sulfurtransferase · Ribose-cysteine · Rhodanese · Sulfane sulfur · U373 cells

Introduction

Considerable evidence has accumulated from clinical studies in support of a role of glutathione (GSH) depletion in a variety of chronic diseases (Lang et al. 2000), cancers (Estrela et al. 1992, 2006) or diabetes (Livingstone and Davis 2007). GSH, one of the body's most important natural antioxidants and detoxifiers, represents an easily mobilized system for the removal of peroxides by a reaction that results in the generation of oxidized glutathione (GSSG). The product of GSH oxidation, GSSG, is known to be toxic and has been found to be an inhibitor of protein synthesis and a variety of enzymes (Kosower 1970); it is rapidly converted back to GSH by the enzyme glutathione reductase. As a consequence, the cytoplasmic redox ratio of GSH/GSSG is held at about 30:1–100:1. Multidrug and radiation resistance of many tumors, as compared with normal tissues, appears to be associated with higher GSH levels in cancer cells (Stavrovskaya 2000). It has been reported that elevation of intracellular GSH is associated with mitogenic stimulation (Shaw and Chou 1986), and GSH may regulate DNA synthesis (Suthanthiran et al. 1990). GSH depletion decreases the rate of cell proliferation and inhibits cancer growth (Terradez et al. 1993). Conversely, cell proliferation is enhanced by the supply of precursors for L-cysteine and GSH (Zmuda and Friedenson 1983; Atzori et al. 1990; Iwata et al. 1994).

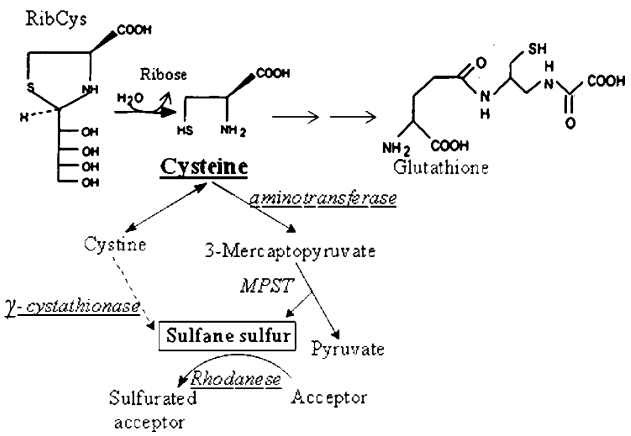
This article is part of the Special Issue on Sulfur- and Selenocontaining Amino Acids.

H. Jurkowska · T. Uchacz · M. Wróbel (✉)
Chair of Medical Biochemistry, Jagiellonian University Medical College, Kopernika 7 St., 31-034 Kraków, Poland
e-mail: mbwrobel@cyf-kr.edu.pl

J. Roberts
School of Pharmacy, University of Wisconsin, Madison, USA

Tissue GSH concentration varies as a function of L-cysteine availability, the limiting substrate for GSH synthesis (Droge and Breitkreutz 2000). To date, a significant challenge in the art has been to provide L-cysteine to cells at sufficiently high levels to drive glutathione biosynthesis and reduce the unwanted side effects of chemo- or radiotherapy of cancer, slow the aging process, prevent mutagenesis, alter gene expression and enhance cellular repair processes. Prodrugs of L-cysteine, i.e., chemical compounds converted to L-cysteine in the cell, such as *N*-acetyl-L-cysteine (NAC) or 2(R,S)-D-ribo-(1′,2′,3′,4′-tetrahydroxybutyl)thiazolidine-4(R)-carboxylic acid (RibCys) can be used by the cell to drive glutathione biosynthesis (Roberts et al. 1987; Oz et al. 2007) (Scheme 1). *N*-acetyl-L-cysteine, deacetylated after transport into cells, has long been used as an antidote for paracetamol overdose to prevent liver damage resulting from glutathione depletion. NAC can inhibit the action of the transcription factor NF-κB, which is believed to be involved in the mechanism whereby oxidative stress leads to cellular pathology (Mihm et al. 1995).

L-Cysteine, whether formed from methionine in tissues or supplied from a culture medium, serves as a precursor for synthesis of proteins and several other essential molecules, e.g. GSH, coenzyme A, taurine, and inorganic sulfur. Astrocytoma, neuroblastoma, and glioblastoma cells are characterized by trace expression and trace activity of cysteine dioxygenase (CDO) (Qusti et al. 2000); thus, the main pathway of cysteine transformation in these cells except glutathione synthesis and protein synthesis is non-oxidative, desulfurative cysteine transformation, which leads to the formation of sulfane sulfur-containing compounds (Ogasawara et al. 1994).



Scheme 1 D-ribose-L-cysteine conversion to glutathione and sulfane sulfur-containing compounds. *MPST* 3-mercaptopyruvate sulfurtransferase, *RibCys* D-ribose-L-cysteine. In brain cells, MPST plays the main role in generating sulfane sulfur-containing compounds from L-cysteine and for this reason, cystine conversion by CST, to sulfane-sulfur compounds is presented by a *dotted line*

One of the strategies for preventing injuries associated with GSH depletion is to boost the levels of GSH within the cells. On the other hand, L-cysteine delivered to cells is catabolized by several nonoxidative desulfuration pathways including desulfuration of cyst(e)ine by reactions catalyzed by γ-cystathionase (CST) (Szczepkowski and Wood 1967), and by aminotransferase in conjunction with 3-mercaptopyruvate sulfurtransferase (MPST) (Ubuka et al. 1977). The reactions catalyzed by these enzymes are important in producing sulfane sulfur-containing compounds (Toohey 1989). Although the biological role of reduced sulfur, such as sulfane sulfur, is not completely understood, according to Toohey (1989), malignant cell proliferation may be related to a deficiency of sulfane sulfur and the uncontrolled operation of a set of enzymes normally inactivated by sulfane sulfur. In both astrocytoma U373 cells and astrocytes, no CST activity was detected (Jurkowska and Wróbel 2008) and it can be suggested that the pathway of sulfane sulfur formation from cyst(e)ine through the CST reaction, do not play a significant role in the investigated cells (Scheme 1, dotted line). Based on previous investigations performed in U373 cells, it is reasonable to expect that metabolic processes leading to an increased sulfane sulfur level are inhibited in neoplastic cells (Jurkowska and Wróbel 2008).

Investigations described in this paper focused on the relationship between sulfane sulfur levels and the proliferation of brain cancer cells, as well as on potential applications of sulfane sulfur donors in cancer therapy. The purpose of the present study was to determine whether L-cysteine supplementation, in the form of RibCys, is sufficient to result in an inhibition of proliferation of human astrocytoma (U373) cells under the condition of an increased level of sulfane sulfur and in the absence of oxidative stress, measured as the ratio of GSH/GSSG. To compare the behavior of normal cells under similar experimental conditions, the experiments were simultaneously performed with murine astrocytes. The cells were cultured up to 48 h (U373) and 72 h (astrocytes) with a medium containing RibCys. Cellular GSH and GSSG were measured by HPLC.

Materials and methods

Sources of chemicals

RibCys synthesis has been described previously (Roberts et al. 1995). The yield for RibCys was 85%, and the purity was established by nuclear magnetic resonance (NMR) spectroscopy and elemental analysis (Roberts et al. 1995). Folin–Ciocialteau reagent, NADPH (Na_4), NADH, lactate dehydrogenase (LDH), pyridoxal phosphate (PLP),

1,4-dithio-bis-(2-nitrobenzoic acid) (DTT), *N*-ethylmaleimide (NEM), bathophenanthrolinedisulfonic acid (BPDS), 2,4-dinitrofluorobenzene (DNFB), acetonitrile, *N*-acetyl-L-cysteine (NAC), poly-L-lysine, trypsin, sodium pyruvate, and DMEM (Dulbecco's Modified Eagle Medium) were obtained from Sigma Chemical Company (St. Louis, MO, USA). Fetal bovine serum was obtained from GIBCO Laboratories (Grand Island, NY), potassium cyanide (KCN) from Merck (Darmstadt, Germany), sodium 3-mercaptopyruvate, trifluoroacetic acid (TFA), and 2-mercaptoethanol from Flucka Chemie GmbH. Nε-methyl-L-lysine was purchased from Bachem, Bubendorf, Switzerland. The Cytotoxicity Detection Kit (LDH) and Cell Proliferation ELISA, BrdU (colorimetric) test were obtained from Roche Applied Science. All other chemicals were of reagent grade and purchased from common commercial suppliers.

Cell cultures

All experiments were performed using the human astrocytoma U373 cell line and the murine cortical astrocyte primary culture. Human astrocytoma U373 cells were grown in monolayer in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum and antibiotics (100 U/ml penicillin and 100 μg/ml streptomycin), in plastic culture dishes (100 mm in diameter), at 37°C, in a humidified atmosphere containing 5% CO_2. After the trypsinization (0.25% trypsin/EDTA), the cells were diluted with complete medium (DMEM with 10% FBS) (Ponten and Macintyre 1968). Murine cortical astrocyte primary cultures established from 1-day-old pups (Swiss Albino strain) were grown to confluence in DMEM supplemented with 10% fetal bovine serum, pyruvate (110 mg/l), glucose (4,500 mg/l), and antibiotics (100 U/ml penicillin and 100 μg/ml streptomycin), at 37°C, in a humidified atmosphere containing 5% CO_2. The cells were plated in poly-L-lysine-coated flasks (75 cm^2) (McCarthy and de Vellis 1980). The cells were collected after one to two passages. All the cultures used in the experiments were about 2 weeks old.

Treatment of cells with RibCys

The cells were seeded at 1×10^6/100 mm dish the day before treatment. RibCys (2 and 15 mM) was dissolved in cell culture media and filtered through a 0.22-μm filter for sterilization. The cells were incubated for 6, 12, 24, 48 or 72 h and then washed three times with 3 ml of cold PBS (10 mM potassium phosphate buffer, pH 7.4, containing 150 mM NaCl) to completely remove sulfhydryl-group-containing compounds that might interfere with the GSH assay. Then the cells were harvested in cold PBS, centrifuged at 5,000 rpm at 4°C during 10 min and solubilized in phosphate buffer, pH 7.5 for homogenization.

Cell homogenization

U373 cells and astrocytes ($1–7 \times 10^6$) were suspended in 0.1 M phosphate buffer pH 7.5, in proportion 1 million cells/0.04 ml of the buffer, and sonicated 3×5 s at 4°C (Bandelin Sonoplus GM 70). After centrifugation at 5,000 rpm at 4°C for 10 min, the supernatant was used for the determination of protein concentration, sulfane sulfur levels and the activity of rhodanese and MPST. For GSH, cysteine and their disulfides determination, cells were suspended in 0.1 ml 10% perchloric acid/1 mM BPDS. The sediment was separated by centrifugation at 3,000 rpm for 10 min, and supernatant was saved at −76°C until used for HPLC analyses.

Enzyme assay

MPST activity was assayed according to the method of Valentine and Frankelfeld (Valentine and Frankenfeld 1974), with some modifications as described by Wróbel et al. (2004). The enzyme activity was expressed as nmoles of pyruvate produced during 1 min incubation at 37°C per 1 mg of protein. Rhodanese activity was assayed by Sőrbo's method (1955), following a procedure described in Wróbel et al. (2004). The enzyme activity was expressed as nmoles SCN—formed during 1 min incubation at 20°C per 1 mg of protein.

Sulfane sulfur was determined by the method of Wood (1987), based on cold cyanolysis and colorimetric detection of ferric thiocyanate complex ion, and protein was determined by the method of Lowry et al. (1951) using crystalline bovine serum albumin as a standard.

HPLC (High Performance/Pressure Liquid Chromatography) method was used to determine the levels of such metabolites as cysteine, cystine, reduced (GSH) and oxidized glutathione (GSSG) in the investigated cells and in culture media based on the method described by Dominick et al. (2001). Samples were separated on a 4.6 mm × 250 mm Luna C18 (5u) column with a Phenomenex Security Guard column filled with the same packing material. A mobile phase consisting of solvent A (water/0.1% TFA) and solvent B (acetonitrile/0.1% TFA) was used to elute samples. The samples were eluted with 20% B after injection followed by 35 min linear gradient to 55% B and a 10-min isocratic period at 55% B, then a 15-min linear gradient to 100% B and a 10-min isocratic period. The column was then re-equilibrated to the initial conditions for 15 min. Analyses of 20 μl of sample were performed at a flow rate of 1.0 ml/min at 20°C temperature with diode array detection at 365 nm. Standard curves were generated in the supernatant obtained from cells ranging from 13 to

75 nmoles of each compound per milliliter. Cells were homogenized in the presence of the metal ion chelator, bathophenanthrolinedisulfonic acid (BPDS), to prevent auto-oxidation of thiols and thiol–disulfide exchange.

Cell proliferation was examined using the Cell Proliferation ELISA, BrdU (colorimetric) test (Roche Applied Science). The assays were performed according to the manufacturer's protocol (BrdU test). Each test (or its paired control) was performed in triplicate for each experiment. For the determination of cellular proliferation, the cells were seeded into 96-well plates at a concentration of 1.5–2 $\times$ 10^3 cells/well (U373 cells), or 1.5–5 $\times$ 10^3 cells/well (astrocytes) in DMEM supplemented as reported above. Twenty-four hours after the initial seeding, the culture medium was replaced with 100 µl of complete medium (control cultures) or 100 µl of medium containing various RibCys concentrations (2, 4, 5, 8, 10 and 15 mM) and the plates were cultured for various time intervals (U373 cells 6, 12, 24, and 48 h; astrocytes 48 and 72 h).

Determination of cell viability

Cell viability was investigated by measuring the leakage of LDH from dead or dying cells into the culture medium using the Cytotoxicity Detection Kit (LDH). Briefly, the cells were cultured in 96-well plates at a density of 1–2 $\times$ 10^3 U373 cells per well or 1.5–5 $\times$ 10^3 astrocytes per well. After treatment, LDH was determined spectrophotometrically by measuring its activity in an aliquot of cell-free medium. One hundred microliters of the culture medium collected from the dishes after the incubation of the cells was added to 100 µl of the incubation mixture and incubated for 30 min at room temperature, in the dark. After the incubation, 50 µl of 1 M HCl was added and the absorbance was measured with a microculture plate reader at 490 nm. The leakage was calculated as a percentage of total activity after lysis of the cells by 1% Triton X-100.

Presentation of data and statistical analysis

The statistical significance of the antiproliferative effect of RibCys, changes in sulfane sulfur levels and activities of enzymes was determined using the Student's t test. $p < 0.05$ was considered significant.

Results

D-ribose-L-cysteine cytotoxicity to U373 cells and mouse astrocytes

Basal cytotoxicity test for predicting starting concentrations for the investigation of RibCys effect on U373 cells proliferation showed that the viability of the cells studied after 24 and 48 h was between 91 and 100% for 2, 4, 5 and 8 mM RibCys concentrations in culture medium (Fig. 1a). The addition of RibCys to U373 cells produced a concentration-dependent loss of cell viability. 10 mM RibCys was regarded cytotoxic, with the viability of the cells after 24 and 48 h dropping to 82 and 84%, respectively. In the case of mice astrocytes (Fig. 1b), the cytotoxic effect of RibCys was observed at lower concentrations as compared to U373 cells. RibCys concentrations above 8 mM were regarded cytotoxic to mice astrocytes.

D-ribose-L-cysteine effect on the level of cellular cysteine and glutathione

The RP-HPLC method was used to investigate changes in the level of cysteine, cystine, GSH and GSSG in U373 cells and mice astrocytes cultured in the presence of RibCys.

In U373 cells cultured for 24 and 48 h in the presence of 2 and 5 mM RibCys, an increased level of cysteine and/or cystine was detected (Fig. 2a). The highest elevation in the level of cysteine was determined after 48 h of incubation of U373 cells in the presence of 5 mM RibCys. The results obtained for U373 cells presented in Fig. 2a show a stimulating, concentration-dependent effect of RibCys on the level of GSH at both duration times, 24 and 48 h. The 5 mM RibCys in the culture media led to an increased level of GSH in U373 cells at both time points (Fig. 2a). The presently employed method of RP-HPLC allows for a simultaneous determination of GSH and GSSG levels in a sample, what in turn allows for determining the concentration ratio of GSH/GSSG (Table 1). For U373 cells incubated for 24 and 48 h without RibCys, the GSH/GSSG ratio was almost identical, amounting to 10. The ratio did not change for 2 mM RibCys after 24 h, but it was higher after 48 h, amounting to 14, and for 5 mM, it was 15.

In astrocytes cultured for 48 and 72 h in the presence of 4 mM RibCys (Fig. 2b), an increased level of cystine was detected (Fig. 2b). The level of cystine was several times higher after 72 h of incubation as compared to 48 h. In both control astrocytes and astrocytes cultured in the presence of RibCys, the level of cysteine was undetectable. The RibCys presence in the culture media, at 4 mM concentration, led to an increased level of GSH in astrocytes after 72 h of incubation (Fig. 2b). In the control astrocytes, the GSH/GSSG ratio was 10 after 6 h of incubation and decreased with increasing incubation—after 48 h, it was 5 and after 72 h, it was somewhat above 1. In astrocyte cells cultured in the presence of 4 mM RibCys, the ratio of GSH/GSSG was very stable and increased threefold only after 72 h.

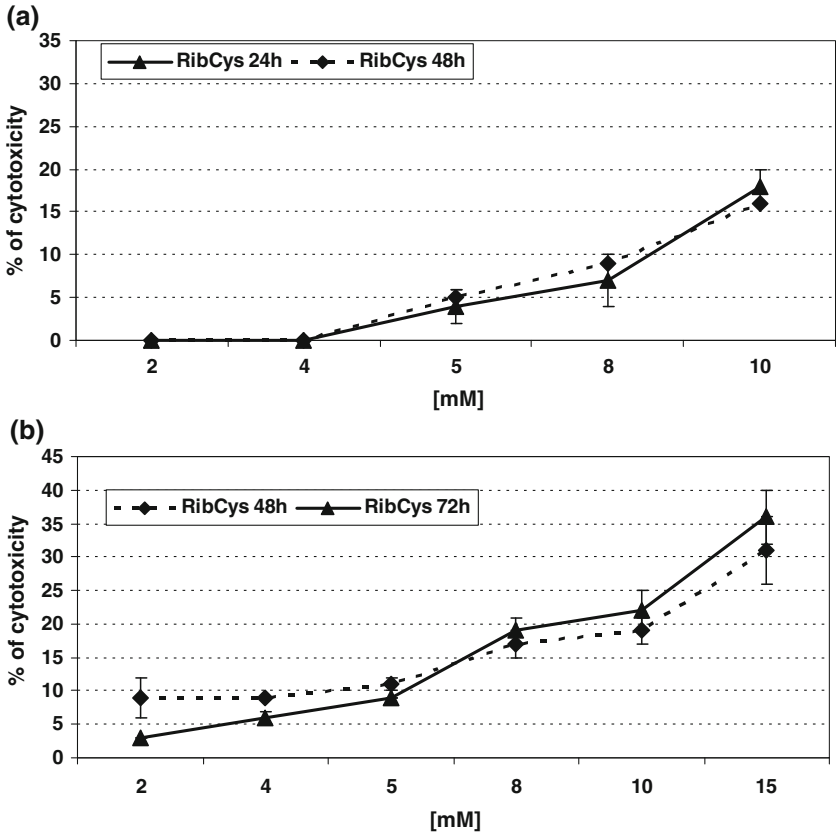


Fig. 1 D-ribose-L-cysteine cytotoxicity to U373 cells (**a**) and mouse astrocytes (**b**). D-ribose-L-cysteine cytotoxicity was investigated by measuring the leakage of lactate dehydrogenase from cells into the culture medium. The absorbance was measured at 490 nm. The leakage was calculated as a percentage of total activity after lysis of the cells by 1% Triton X-100. For each RybCys concentration, four to five determinations were performed in the three independent experiments. Percent of cytotoxicity was calculated based on mean values. Each *point* represents mean ± SEM

D-ribose-L-cysteine effect on MPST and rhodanese activity and the level of sulfane sulfur

The results presented in Table 2 show changes in rhodanese and MPST activities, as well as sulfane sulfur levels in U373 cells and mice astrocytes in the presence of RibCys. A significantly increased, about 72%, activity of rhodanese was observed in U373 cells cultured for 24 h with 2 mM RibCys as compared to control cells. However, longer duration, even in the presence of higher concentration of RibCys (5 mM) did not cause any changes. The activity of MPST after 24 h of incubation with 2 mM RibCys was significantly lower in comparison to the controls (by about 14%) and the effect was accompanied by an approximately 22% decrease in the level of sulfane sulfur. However, it was found that a longer duration (48 h) and higher RibCys concentration caused an elevation of the activity of MPST (by about 10%) and an increase in the level of sulfane sulfur by about 34% in comparison to the control cultures. Astrocytes seem to be more stable with respect to the activity of rhodanese and MPST–RibCys in 4 mM concentration did not influence these values after both 48 and 72 h of incubation. However, a significant decrease in the level of sulfane sulfur was detected after 48 h (11%) and after 72 h (20%) in astrocytes cultured in the presence of 4 mM RibCys, as compared to the control cultures.

D-ribose-L-cysteine effect on the proliferation of U373 cells and mouse astrocytes

The proliferation of U373 cells was investigated for 2, 4, 5, 8 and 10 mM RibCys concentrations in the culture medium after 24 and 48 h (Fig. 3a). Low, 2 mM RibCys in culture stimulated cell proliferation by 12% after 24 h and 19% after 48 h as compared to the control cultures. The reverse effect was observed for higher RibCys concentrations. Cells cultured for 48 h in the presence of 5 mM RibCys and for 24 or 48 h in the presence of 8 mM RibCys showed a decreased rate of proliferation, respectively, by 9, 14 and 24% in comparison to the control values. A decreased proliferation observed for 10 mM RibCys can be regarded as the cytotoxic effect of high RibCys concentration, as it is presented in Fig. 1a. The proliferation of mouse astrocytes investigated for non-cytotoxic RibCys concentrations: 2, 4 and 5 mM after 48 and 72 h was increased (Fig. 3b); especially, the increase for 2 mM RibCys after 48 h (more than 85%), or for 4 mM (60%), is dramatic. Higher RibCys concentrations, 8, 10 and 15 mM, regarded as cytotoxic (Fig. 1b), decreased the number of cells.

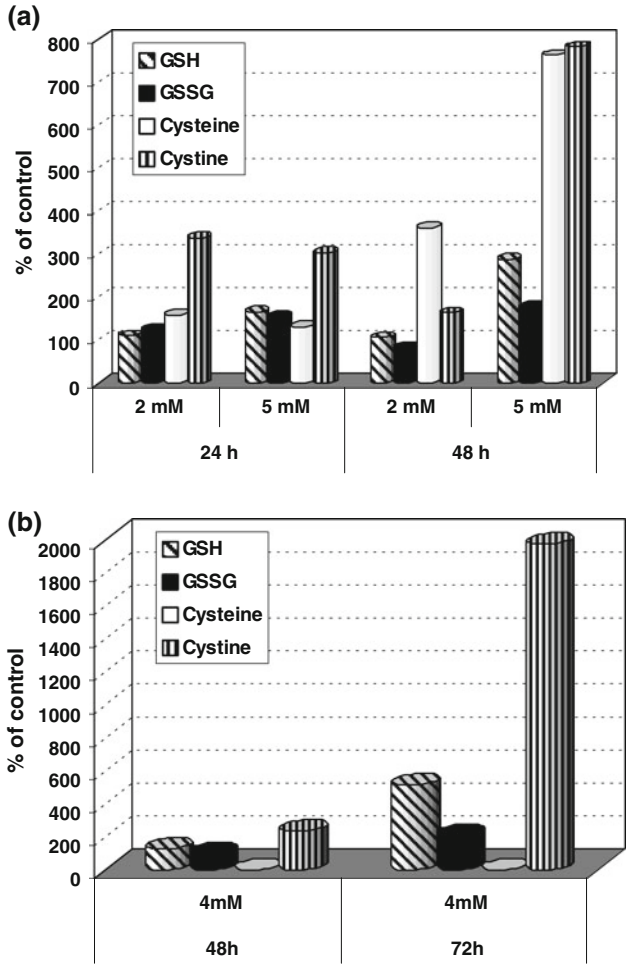


Fig. 2 D-ribose-L-cysteine effect on the intracellular level of GSH, GSSG, cysteine and cystine in U373 cells (**a**) and mouse astrocytes (**b**). Every value is a mean of three independent experiments. RibCys was dissolved in cell culture media. U373 cells were incubated for 24 and 48 h, astrocytes for 48 and 72 h. Cysteine, cystine, GSH and GSSG levels determined in control U373 cells equaled, respectively, to 0.3, 2.5, 14.9 and 1.7 nmole/10^6 cells, and in control astrocytes 0, 0.5, 5.8 and 1 nmole/10^6 cells

Table 1 GSH/GSSG ratios in cells homogenates cultured without and with RibCys

RibCys (mM)	Hours	U373	Astrocytes
Without	6	11	11
	12	ND	ND
	24	10	ND
	48	9	5
	72	ND	1.2
2	24	9.5	ND
	48	14	ND
4	6	10	9
	12	ND	ND
	24	17	ND
	48	8	5
	72	ND	3.3
5	24	11	ND
	48	15	ND
8	24	ND	6
	48	ND	3.8
	72	ND	3.2

Results are the mean from three independent experiments

ND not determined

Discussion

GSH has a central function in redox regulation (Jones 2002), as the most abundant low-molecular-weight thiol within the cell. GSH modulates the redox status of thiol groups in signaling proteins, through which it can influence a variety of cell functions, e.g. gene expression (Arrigo 1999), cell proliferation (Hwang and Sinskey 1991; Gardiner and Reed 1995), and programmed cell death (Yoshida et al. 1995; Hall 1999a, b), though the precise mechanisms are unknown.

In malignant tumors, as compared with normal tissues, that resistance is associated with higher GSH levels within cancer cells (Stavrovskaya 2000; Estrela et al. 2006). The results presented in this paper confirm higher glutathione content in cancerous, U373 cells than in normal astrocytes (see Legend to Fig. 2). The synthesis of glutathione is dependent on the extracellular availability of cysteine, the key rate-limiting substrate (Meister 1989) and its concentration depends, in turn, on the transport into astrocytes; Na^+-dependent systems as the major route and Na^+-independent uptake representing a minor component of total transport (Shanker et al. 2001). RibCys delivers cysteine into cells (Scheme 1) (Roberts et al. 1987). It should also be mentioned here that we determined a large quantities of cysteine in the culture media containing RibCys after 48 and 72 h of incubation, which may suggest that it undergoes solvolysis there. On the other hand, astrocytes export cysteine, either in tissue or in culture; the direct efflux of cysteine is a highly significant biological mechanism responsible for cysteine delivering to neurons (Guebela and Torresb 2004).

Mouse astrocytes, maintaining a very low, intracellular level of cysteine (Fig. 2b) seemed to be resistant to 4 mM RibCys in the culture medium, whereas the level of cysteine in U373 cells remained unchanged in 24 h incubation, but a longer exposure caused a significant increase in the intracellular level in the culture media (Fig. 2a). The level of cystine increased manyfold in U373 cultured for 48 h in the presence of 5 mM RibCys (Fig. 2a), as well as in astrocytes cultured for 72 h with 4 mM RibCys (Fig. 2b).

In the investigated cells, the level of GSH, similarly as the level of cysteine, was also fairly stable and only

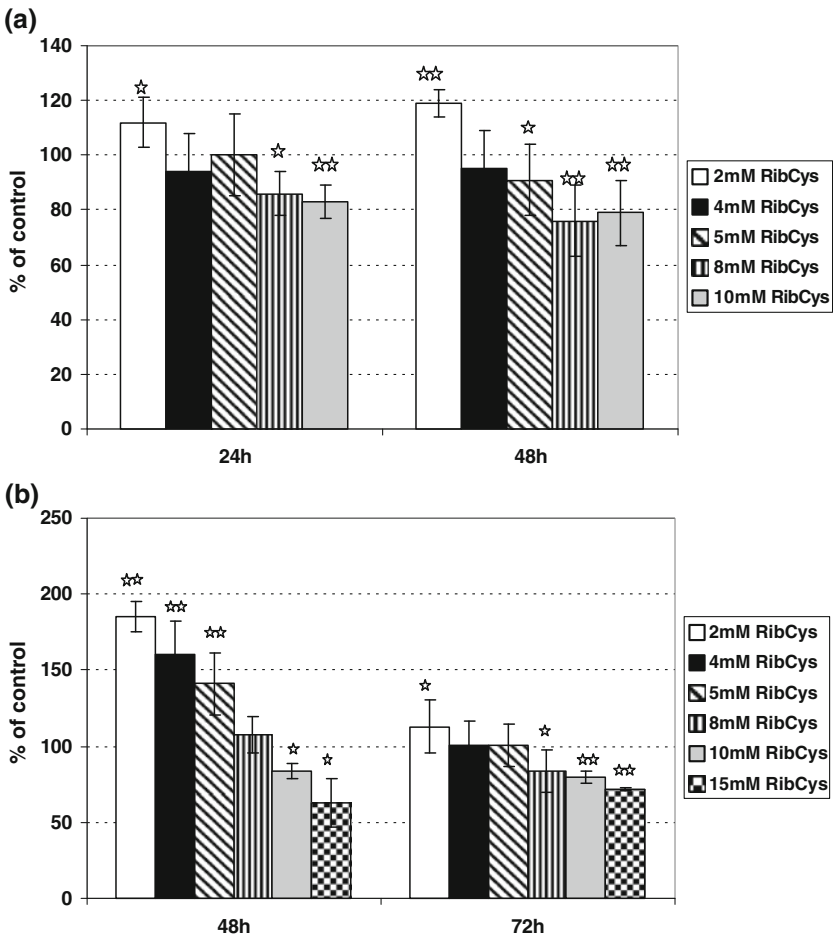


Fig. 3 The effect of D-ribose-L-cysteine on U373 cells (**a**) and mouse astrocytes (**b**) proliferation (BrdU test). Each *point* represents mean ± SD of three independent experiments

incubation of U373 cells with 5 mM RibCys for 48 h disturbed GSH homeostasis, leading to an almost threefold increase in its level (Fig. 2a). In astrocyte cells, the level of GSH was observed to be increased several times only after 72 h of incubation with 4 mM RibCys (Fig. 2b).

The drop in cell proliferation with a drop in the level of cellular GSH (Hamilos et al. 1989) and cell proliferation enhancement by a supply of precursors for cysteine and GSH (Iwata et al. 1994) provide evidence of a link between a limited sulfur amino acid supply, decreased GSH concentration and oxidized GSH/GSSG redox state. With respect to the studies presented in the paper, one may thus assume that under conditions, 5 mM RibCys, 48 h, in which an increased GSH level (Fig. 2a) and GSH/GSSG ratio occur (Table 1), inhibition of proliferation (Fig. 3a) should not be an effect of oxidative stress. Such conditions should also protect cells from apoptosis, as it was observed when cystathionine or methionine was used as glutathione inducer in U937 and HepG2 cells (Ghibellia et al. 1998). In the absence of oxidative stress, measured as the ratio of GSH/GSSG, the obtained results confirm that the fall in the level of sulfane sulfur (2 mM RibCys, 24 h, Table 1) is associated with increasing proliferation of cells (2 mM RibCys, 24 h, Fig. 3a). Thus, the present study showed that L-cysteine supplementation, in the form of RibCys, is sufficient to result in an inhibition of proliferation of astrocytoma (U373) cells under the condition of an increased level of sulfane sulfur and in the absence of oxidative stress.

The results obtained for *N*-acetylocysteine (NAC) (Jurkowska and Wróbel 2008) suggest a relationship between neoplastic cell proliferation and a level of sulfane sulfur. NAC, as a cellular precursor of cysteine, has allowed for determining such conditions (NAC concentration value and time of exposure), under which the U373 cell proliferation was inhibited, while the cells demonstrated an elevated activity of MPST and an increased sulfane sulfur level as compared to the controls.

In this paper, we intend to demonstrate that RibCys, an L-cysteine precursor, affects MPST activity which is the main enzyme responsible for the production of sulfane sulfur from cysteine in astrocytoma U373 line (Scheme 1), and thereby affects the level of sulfane sulfur. As Table 1 shows, in U373 cells, a relationship exists between the level of sulfane sulfur and the MPST activity. In addition, a decreased level of sulfane sulfur after 24 h of incubation

Table 2 Sulfurtransferases activity and sulfane sulfur levels in astrocytoma U373 cells and mice astrocytes

	Rhodanese (nmol mg^{-1} min^{-1})	MPST (nmol mg^{-1} min^{-1})	Sulfane sulfur (nmol/mg protein)
U373 cells			
24 h			
Control	36 ± 4	146 ± 14	139 ± 24
2 mM RibCys	*62 ± 15	**126 ± 10	*108 ± 29
48 h			
Control	52 ± 12	145 ± 18	111 ± 18
5 mM RibCys	44 ± 12	*158 ± 21	**149 ± 24
Mouse astrocytes			
48 h			
Control	189 ± 46	812 ± 121	191 ± 43
4 mM RibCys	171 ± 23	763 ± 184	*170 ± 28
72 h			
Control	241 ± 46	788 ± 123	190 ± 27
4 mM RibCys	227 ± 17	826 ± 146	**152 ± 34

Values are mean from 8 to 24 determinations in three to five independent experiments

* $p < 0.05$, ** $p < 0.001$ (Student's t test)

with 2 mM RibCys is correlated with a decreased MPST activity and also with an increased rhodanese activity, the enzyme responsible for the metabolism of sulfane sulfur (Ubuka et al. 2008; Westley et al. 1983). The observed decrease in U373 cell proliferation was correlated with an increase in MPST activity and an increase of the level of sulfane sulfur. At the same time, in astrocyte cultures, no effect of RibCys on the activity of MPST and rhodanese was observed, and the level of sulfane sulfur decreased (Table 1), which was accompanied by an increase or no change in cell proliferation (Fig. 3b). It appears, therefore, that the observed different effect of RibCys on cancer cells and normal cells may be of therapeutic significance. In neoplastic cells, RibCys through restoration of the sulfane sulfur level is able to play a part in inhibition of their proliferation.

Acknowledgments This work was supported by a grant from the Polish Committee for Scientific Research (KBN) K/ZDS/000450 and K/ZBW/000147.

References

Arrigo AP (1999) Gene expression and thiol redox state. Free Radic Biol Med 27:936–944

Atzori L, Dypbukt JM, Sundqvist K, Cotgreave I, Edman CC, Moldeus P, Grafstrom RC (1990) Growth-associated modifications of low-molecular-weight thiols and protein sulfhydryls in human bronchial fibroblasts. J Cell Physiol 143:165–171

Dominick PK, Cassidy PB, Roberts JC (2001) A new and versatile method for determination of thiolamines of biological importance. J Chromatogr B 761:1–12

Droge W, Breitkreutz R (2000) Glutathione and immune function. Proc Nutr Soc 59:595–600

Estrela JM, Hernandez R, Terradez P, Asensi M, Puertes RI, Vina J (1992) Regulation of glutathione metabolism in Ehrlich ascites tumor cells. Biochem J 286:257–262

Estrela JM, Ortega A, Obrador E (2006) Glutathione in cancer biology and therapy. Crit Rev Clin Lab Sci 43(2):143–181

Gardiner CS, Reed DJ (1995) Synthesis of glutathione in the preimplantation mouse embryo. Arch Biochem Biophys 318:30–36

Ghibellia L, Fanellia C, Rotilioa G, Lafaviaa E, Coppolaa S, Colussia C, Civitarealea P, Ciriolob MR (1998) Rescue of cells from apoptosis by inhibition of active GSH extrusion. FASEB J 12:479–486

Guebela DV, Torresb NV (2004) Dynamics of sulfur amino acids in mammalian brain: assessment of the astrocytic-neuronal cysteine interaction by a mathematical hybrid model. Biochim Biophys Acta 1674:12–28

Hall AG (1999a) Glutathione and the regulation of cell death. Adv Exp Med Biol 457:199–203

Hall AG (1999b) The role of glutathione in the regulation of apoptosis. Eur J Clin Invest 29:238–245

Hamilos DL, Zelarney P, Mascali JJ (1989) Lymphocyte proliferation in glutathione-depleted lymphocytes: direct relationship between glutathione availability and the proliferative response. Immunopharmacology 18:223–235

Hwang C, Sinskey AJ (1991) The role of oxidation-reduction potential in monitoring growth of mammalian cultured cells. In: Spier RE, Griffiths JB, Meignier B (eds) Production of biologicals from animal cells in culture. Halley Court, Oxford, pp 548–657

Iwata S, Hori T, Sato N, Ueda-Taniguchi Y, Yamabe T, Nakamura H, Masutani H, Yodoi J (1994) Thiol-mediated redox regulation of lymphocyte proliferation: possible involvement of adult T cell leukemia-derived factor and glutathione in transferring receptor expression. J Immunol 152:5633–5642

Jones DP (2002) Redox potential of GSH/GSSG couple: assay and biological significance. Methods Enzymol 348:93–112

Jurkowska H, Wróbel M (2008) *N*-acetyl-L-cysteine as a source of sulfane sulfur in astrocytoma and astrocyte cultures: correlations with cell proliferation. Amino Acids 34:231–237. doi:10.1007/s00726-007-0471-2

Kosower EM (1970) A role for glutathione in muscle contraction. Experientia 26:76–77

Lang CA, Mills BJ, Mastropaolo W, Liu MC (2000) Blood glutathione decreases in chronic diseases. J Lab Clin Med 135:402–405

Livingstone C, Davis J (2007) Targeting therapeutics against glutathione depletion in diabetes and its complications. Br J Diabetes Vasc Dis 7:258–265

Lowry OH, Rosenbrough NJ, Farr AL, Randal RI (1951) Protein measurement with the Folin phenol reagent. J Biol Chem 193:265–275

McCarthy KD, de Vellis J (1980) Preparation of separate astroglial and oligodendroglial cell cultures from at cerebral tissue. J Cell Biol 85:890–902

Meister A (1989) Metabolism and function of glutathione. In: Dolphin D, Poulson R, Avramovic O (eds) Glutathione: chemical and biochemical and medical aspects. Wiley, New York, pp 367–474

Mihm S, Galter D, Droge W (1995) Modulation of transcription factor NF-kB activity by intracellular glutathione levels and by

variations of the extracellular cysteine supply. FASEB J 9:246–252

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

Oz HS, Chen TS, Nagasawa H (2007) Comparative efficacies of 2 cysteine prodrugs and a glutathione delivery agent in a colitis model. Transl Res 150(2):122–129

Ponten J, Macintyre EH (1968) Long term culture of normal and neoplastic human glia. Acta Pathol Microbiol Scand 74:465–486

Qusti S, Parsons RB, Abouglila KDH, Waring RH, Williams AC, Ramsden DB (2000) Development of in vitro model for cysteine dioxygenase expression in the brain. Cell Biol Toxicol 16:243–255

Roberts JC, Nagasawa HT, Zera RT, Fricke RF, Goon DJW (1987) Prodrugs of L-cysteine as protective agents against acetaminophen-induced hepatotoxicity. 2-(polyhydroxyalkyl)- and 2-(polyacetoxyalkyl)thiazolidine-4(R)-carb oxylic acids. J Med Chem 30:1891–1896

Roberts JC, Koch KE, Detrick SR, Warters RL, Lubec G (1995) Thiazolidine prodrugs of cysteamine and cysteine as radioprotective agents. Radiat Res 143:203–213

Shanker G, Allen JW, Mutkus LA, Aschner M (2001) The uptake of cysteine in cultured primary astrocytes and neurones. Brain Res 902:156–163

Shaw JP, Chou IN (1986) Elevation of intracellular glutathione content associated with mitogenic stimulation of quiescent fibroblasts. J Cell Physiol 129:193–198

Sőrbo B (1955) Rhodanese. Methods Enzymol 2:334–337

Stavrovskaya AA (2000) Cellular mechanisms of multidrug resistance of tumor cells. Biochemistry (Mosc) 65:95–106

Suthanthiran M, Anderson ME, Sharma VK, Meister A (1990) Glutathione regulates activation-dependent DNA synthesis in highly purified normal human T lymphocytes stimulated via the CD2 and CD3 antigens. Proc Natl Acad Sci USA 87:3343–3347

Szczepkowski TW, Wood JL (1967) The cystathionase-rhodanese system. Biochim Biophys Acta 139:469–478

Terradez P, Asensi M, Lasso delareza MC, Puertes IR, Vina J, Estrela JM (1993) Depletion of tumor glutathione in vivo by buthionine sulfoximine: modulation by the rate of cellular proliferation and inhibition of cancer growth. Biochem J 292:477–483

Toohey JI (1989) Sulphane sulphur in biological systems: a possible regulatory role. Biochem J 264:625–632

Ubuka T, Yuasa S, Ishimoto Y, Shimomura M (1977) Desulfuration of L-cysteine through transamination and transsulfuration in rat liver. Physiol Chem Phys 9:241–246

Ubuka T, Okada A, Nakamura H (2008) Production of hypotaurine from L-cysteinesulfinate by rat liver mitochondria. Amino acids 35(1):53–58

Valentine WN, Frankenfeld JK (1974) 3-Mercaptopyruvate sulfurtransferase (E.C.2.8.1.2): a simple assay adapted to human blood cells. Clin Chim Acta 51:205–210

Westley J, Adler H, Westley L, Nishida C (1983) The sulfurtransferases. Fundam Appl Toxicol 3:377–382

Wood JL (1987) Sulfane sulfur. In: Jakoby WB, Griffith OW (eds) Methods in enzymology, vol 143. Academic Press, San Diego, pp 25–29

Wróbel M, Jurkowska H, Śliwa L, Srebro Z (2004) Sulfurtransferases and cyanide detoxification in mouse liver, kidney and brain. Toxicol Mech Methods 14:331–337

Yoshida K, Hirokawa J, Tagami S, Kawakami Y, Urata Y, Kondo T (1995) Weakened cellular scavenging activity against oxidative stress in diabetes mellitus: regulation of glutathione synthesis and efflux. Diabetologia 38:201–210

Zmuda J, Friedenson B (1983) Changes in intracellular glutathione pool levels in stimulated and unstimulated lymphocytes in the presence of 2-mercaptoethanol or cysteine. J Immunol 130:362–364