Effects of flavonoids on glutathione and glutathione-related enzymes in cisplatin-treated L1210 leukemia cells*

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Connections between the ability of quercetin (Qu) and galangin (Ga) to differentially modulate cis-Pt-induced apoptosis and their effects on glutathione system of murine L1210 leukemia cells were studied. The results showed that total glutathione (GSHt) level is increased significantly (~123% of control level), both in cells treated with 10 μM Qu and in cells treated with 4 μM cis-Pt and 10 μM Qu in combination. 10 μM Ga had no effect on GSHt content. Activities of glutathione S-transferase (GST) and glutathione reductase (GR) were not changed significantly when 10 μM flavonoids were used. Significant inhibition of GR activity was observed when flavonoids were used in concentrations higher than 25 μM. The presented data indicate that Qu change the redox state of the cells that is implicated in regulation of apoptosis, due to its ability to increase the GSHt level, and thus may potentiate cis-Pt-induced apoptosis of L1210 cells.

Key words: Glutathione, quercetin, galangin, cisplatin, apoptosis.

The flavonoids are members of a class of natural compounds that recently has been the subject of considerable scientific and therapeutic interest. They have a variety of biological activities, such as anti-allergic, anti-inflammatory, anti-oxidative, free radical scavenging, anti-mutagenic activities and may act as inductors of detoxication enzymes [8, 27]. They inhibit several kinases, promote cell cycle arrest [1, 19, 22] and induce apoptosis [13, 29].

Previous studies showed that alterations in intracellular thiol content regulation and redox state may have significant and widespread effects on many cellular activities, including cell signalizations leading to apoptosis [26]. Because in our previous studies we found that some flavonoids are potent inducers and modulators of apoptosis (Qu enhanced and Ga reduced cis-Pt-induced apoptosis) [5, 6] and some flavonoids may interfere with glutathione [11] and glutathione-related enzymes [17, 20], in this work we intended to look if total glutathione (GSHt) level and changes in glutathione-related enzyme (glutathione S-transferase (GST) and glutathione reductase (GR)) activities are connected with observed effects of flavonoids on cis-Pt-induced apoptosis in murine L1210 leukemia cells.

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Material and methods

Chemicals. Cisplatin (cis-Pt) was obtained from Lachema (Brno, CZ) as solution for injection. Flavonoids (quercetin (Qu) and galangin (Ga)) were purchased from Sigma Chemical Co. (St. Louis, MO, USA) and dissolved in dimethyl sulphoxide (DMSO, Sigma). The stock solutions of flavonoids (0.1 M) were stored at –20 °C. The final concentration of DMSO in the medium was <0.02% and did not affect cell growth [7].

Cell culture and treatment. Murine leukemia cell line L1210 was obtained from the American Type Culture Collection (Rockville, MD, USA). Cells were maintained in RPMI 1640 medium supplemented with 10% heat-inactivated fetal calf serum (Grand Island Biological Co., Grand Island, NY, USA), 100 U/ml penicillin G, 100 μg/ml streptomycin and 2 mM L-glutamine (Sebac, Germany) in an atmosphere of 5% CO_2 in humidified air at 37 °C. The cells were treated with 10 μM flavonoids and 4 μM cis-Pt, or with

their combinations for 24 h. In all experiments the exponentially growing cells were used.

Determination of total glutathione (GSHt) content. Total cellular glutathione (GSHt) was quantified using the glutathione reductase-DTNB recycling assay, modified for 96-well microtiter plates [28]. Cells were washed with PBS (4 °C) and lysed by freezing and thawing in 150 μl of 10 mM HCl. Proteins were precipitated by adding 30 μl of 6.5% SSA. After 10 min, tubes were centrifuged (15 min, 2000 g, 4 °C) and supernatants were stored at –20 °C. 40 μl of the supernatant was mixed with 80 μl of PBS (200 mM, pH 7.5, supplemented with 2 mM EDTA) and 20 μl of 5,5-dithio-bis-(2-nitrobenzoic acid) (0.4 mM) was added. The plate was kept 5 min at room temperature. Following incubation, 10 μl of NADPH (0.2 mM) and 20 μl of GR (8.5 IU/ml) were added. The increase of absorbance at 410 nm was monitored for 10 min and used to determine the amount of GSHt content in the sample by comparison to a reference curve generated with known amounts of GSH. GSHt levels were normalized for protein content (nmol/mg).

GR activity assay. Glutathione reductase (GR) activity was determined by monitoring the oxidation of NADPH at 340 nm [4]. The assay mixture contained 0.1 M PBS (pH 7.15), 1 mM EDTA, 50 μM NADPH, 1 mM GSSG and 50–100 μl of cell lysate. GR activity was calculated as nmol of NADPH oxidised/min/mg protein using a molar extinction coefficient $\varepsilon_{340} = 6.22\ mM^{-1}.cm^{-1}$.

GST activity assays. Glutathione S-transferase (GST) activity was determined according to the method of Habig and Jakoby [12]. Cells were washed with PBS (4 °C) and lysed by freezing and thawing in 150 μl of H_2O. Tubes were centrifuged for 15 min at 10 000 g and 4 °C. GST activity of cell lysate was determined by measuring activity towards the GST substrate 1-chloro-2,4-dinitrobenzene (CDNB) by following the rate of increase of absorbance at 340 nm. The assay mixture contained 0.1 M PBS (pH 6.5), 0.4 mM GSH, and 0.2 mM CDNB and 50–100 μl of cell lysate. The ε_{340} of DNPSG is 9.6 $mM^{-1}.cm^{-1}$. GST activity was expressed as nmol of CDNB conjugated with GSH/min/mg protein.

Results

Effects of flavonoids on GSHt level of cis-Pt-treated cells. To determine GSHt concentration changes in L1210 cells treated with combination of 10 μM flavonoid and 4 μM cis-Pt, cells were plated at $0.5x10^6$ cells/ml and analyzed for GSHt. After 24 h, cis-Pt alone decreased level of GSHt to 83.1% of GSHt content of control cells. Among tested flavonoids only Qu increased the level of GSHt to 122.4% of GSHt of untreated cells. Cis-Pt alone decreased the level of GSHt, however combinational treatment of cells with Qu and cis-Pt led to increase of GSHt to the level of GSHt of Qu alone (125.1% of control level) (Fig. 1). Ga alone or in combination with cis-Pt did not affect the GSHt level. Content of oxidized glutathione (GSSG) increased in cells treated with Qu alone or in cells treated with Qu and cis-Pt in combination, but this effect was not significant (data not shown).

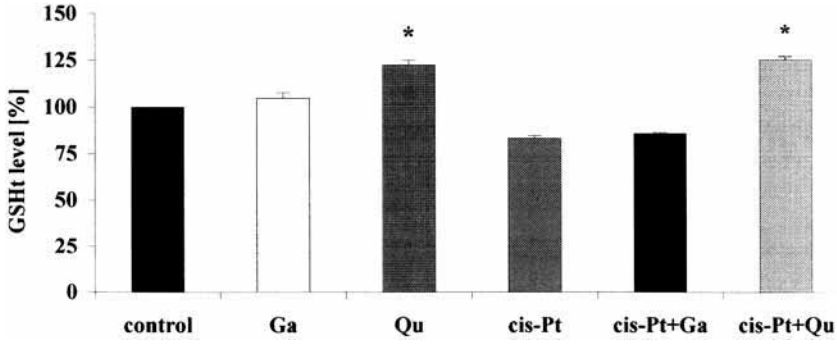

Figure 1. Effects of flavonoids (10 μM) and cis-Pt (4 μM) on total glutathione (GSHt) level in L1210 cells treated for 24 h. The bars represent the mean value ± S.D. from three separate experiments. GSHt content in control cells was 50.89 ± 1.34 nmol/mg protein.
*** p<0.05 as compared with control.**

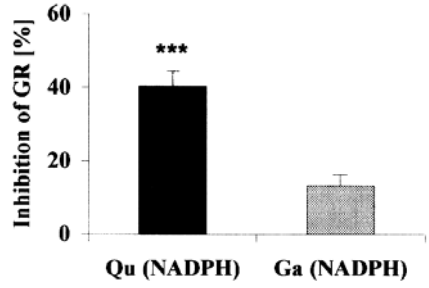

Figure 2. Effects of flavonoids (25 μM) on activity of GR in cell lysate of untreated L1210 cells. The bars represent the mean value ± S.D. from three separate experiments. Activity of GR without inhibitors was 6.04 ± 0.09 nmol/min/mg protein.
***** p<0.005 as compared with control.**

Effects of flavonoids on GR activity of L1210 cells. The above mentioned results show that there is increase in GSHt content in cells treated with Qu alone or in cells treated with Qu and cis-Pt in combination. Although there were no significant changes in GSSG contents, we looked if flavonoids may affect the activity of GR of L1210 cells. We found that GR activity was not significantly changed even during treatment of cells with 10 μM flavonoids and cis-Pt (data not shown). Inhibition of GR activity by the flavonoids was observed (experiments done with lysate of untreated L1210 cells) when flavonoids were used in concentrations higher than 25 μM. 25 μM Qu had significant effect on activity of GR (>40% of inhibition of GR activity), when

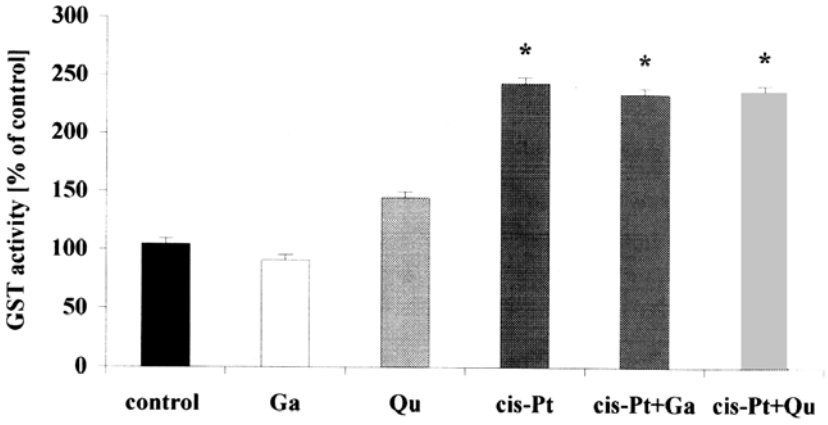

Figure 3. Effects of flavonoids (10 μM) and cis-Pt (4 μM) on activity of GST in L1210 cells treated for 24 h. The bars represent the mean value $\pm$ S.D. from three separate experiments. Activity of GST in control cells was 1.47 $\pm$ 0.04 nmol/min/mg protein.
*** $p<0.05$ as compared with control.**

comparing with Ga that only slightly inhibited GR activity (~13% of inhibition of GR activity) (Fig. 2).

Effects of flavonoids on GST activity of cis-Pt-treated cells. Previous studies have suggested that cells response to stress (e.g. chemotherapy) by induction of detoxification enzymes [16, 21]. Thus we looked for the activity of GST of the treated cells. We found that activity of this enzyme is significantly increased (>230% of GST activity of untreated cells) in cells treated with cis-Pt alone. Flavonoids alone or in combinations with cis-Pt did not significantly increased/decreased the activity of GST as compared with cis-Pt-treated cells (Fig. 3).

Discussion

Several studies were focused on how glutathione changes (its concentration and/or an oxidation to its disulfide (GSSG)) are connected to expression of detoxification enzymes, to impairs of cell proliferation, and to apoptosis [2, 14, 15, 24].

In the light of our previous findings that flavonoids may differentially affect therapeutic efficacy of cis-Pt due to changes in apoptosis, in present work we looked if GSHt and GSH-related enzymes are connected to flavonoid effects on cis-Pt-induced apoptosis [5, 6]. It has been shown that GSH concentration undergoes an initial decrease followed by an increase in response to agents that induce GST [23]. Our studies have shown that the activity of GST in cis-Pt-treated cells that undergo apoptosis is increased. Tested flavonoids alone or in combination with cis-Pt did not significantly influence activity of GST (see Fig. 3). To inhibit the GST activity by flavonoids, they must be used in concentration 10-fold higher than that used in our experiments [30].

On the other hand, we found that the GSHt concentration is increased both in cells treated with Qu and in cells treated with cis-Pt and Qu in combination (see Fig. 1). In the latter treated cells (Qu + cis-Pt) the apoptosis was significantly increased [6]. Ga treatment had no effect on GSHt concentration and additionally it decreased the apoptosis of cis-Pt-treated cells [5, 6]. These findings suggest that oxidative stress may play an important role in flavonoid (Qu) effects on cis-Pt-induced apoptosis. During interaction of cis-Pt with DNA, the radicals ($O^{2-\cdot}$) are generated and activity of oxidative stress response factors is increased. Thus oxidative stress may be involved in regulation of apoptosis [18]. Additionally, Qu was found to act as dual agent, it can act as anti-oxidant or pro-oxidant and it can induce or down regulate the expression of p53, caspase-3, c-myc and K-ras, as well [3, 29]. Our results, supported even by studies of Shacter et al [25], indicate that Qu acts as pro-oxidant (increase the GSHt level). Fernandes and Cotter showed that redox environment (GSH/GSSG) may be the final determinant for the execution of apoptosis. Apoptosis may occur with moderate but lethal oxidative stimuli, whereas necrosis would result from severe oxidative challenges [10]. Redox state of the GSH is regulated mainly through availability of precursors for GSH synthesis and by activity of GCS and GR. GR is NADPH-dependent reductase and some flavonoids may inhibit GR activity (IC_{50} for Qu is 73.2 μM) [31]. We found that to inhibit significantly the activity of GR (GR pre-incubated with NADPH), it is needed to apply the Qu in concentrations higher than 25 μM (see Fig. 2). Activity of GR in cells treated with 10 μM flavonoids and 4 μM cis-Pt was not inhibited significantly. GSSG level was not increased in thus treated cells (data not shown). Elliott et al suggested the role of ROS in inhibition of GR (type IV from baker's yeast) but SOD did not block the inhibitory effect of Qu [9]. Analysis of enzyme kinetics showed, that pre-incubation of yeast GR with NADPH (>50 μM) enhanced the inhibitory activity of Qu but kinetic parameters did not show for an uncompetitive inhibition (unpublished data).

In summary, the present data indicate the possible involvement of antioxidant/oxidant activity of Qu (effect on GSHt level) in its ability to potentiate cis-Pt-induced apoptosis. At present, the exact mechanisms through which Qu and Ga potentiate or reduce cis-Pt-induced apoptosis are not fully understood due to multi-target effects of flavonoids but possible implication of flavonoids in therapy of cancer is perspective, as these drugs are able to modulate the redox state of the cell and thus may regulate the important cellular processes, e.g. apoptosis.

References

[1] Agullo G, Gamet-Payrastre L, Manenti S, Viala C, Remesy C, Chap H, Payrastre B. Relationship between flavonoid structure and inhibition of phosphatidylinositol 3-kinase: a comparison with tyrosine kinase and protein kinase C inhibition. Biochem Pharmacol 1997; 53: 1649–1657.

[2] Allen RG, Tresini M. Oxidative stress and gene regulation. Free Radic Biol Med 2000; 28: 463–499.

[3] Avila MA, Cansado J, Harter KW, Velasco JA, Notario V. Quercetin as a modulator of the cellular neoplastic phenotype. Effects on the expression of mutated H-ras and p53 in rodent and human cells. Adv Exp Med Biol 1996; 401: 101–110.

[4] Carlberg I, Mannervik B. Glutathione reductase. Methods Enzymol 1985; 113: 484–490.

[5] Cipak L, Novotny L, Cipakova I, Rauko P. Differential modulation of cisplatin and doxorubicin efficacies in leukemia cells by flavonoids. Nutr Res 2003; 23: 1045–1057.

[6] Cipak L, Rauko P, Miadokova E, Cipakova I, Novotny L. Effects of flavonoids on cisplatin-induced apoptosis of HL-60 and L1210 leukemia cells. Leuk Res 2003; 27: 65–72.

[7] Courage C, Budworth J, Gescher A. Comparison of ability of protein kinase C inhibitors to arrest cell growth and to alter cellular protein kinase C localisation. Br J Cancer 1995; 71: 697–704.

[8] Di Carlo G, Mascolo N, Izzo AA, Capasso F. Flavonoids: old and new aspects of a class of natural therapeutic drugs. Life Sci 1999; 65: 337–353.

[9] Elliott AJ, Scheiber SA, Thomas C, Pardini RS. Inhibition of glutathione reductase by flavonoids. A structure-activity study. Biochem Pharmacol 1992; 44: 1603–1608.

[10] Fernandes RS, Cotter TG. Apoptosis or necrosis: intracellular levels of glutathione influence mode of cell death. Biochem Pharmacol 1994; 48: 675–681.

[11] Galati G, Moridani MY, Chan TS, O'Brien PJ. Peroxidative metabolism of apigenin and naringenin versus luteolin and quercetin: glutathione oxidation and conjugation. Free Radic Biol Med 2001; 30: 370–382.

[12] Habig WH, Jakoby WB. Glutathione S-transferases (rat and human). Methods Enzymol 1981; 77: 218–231.

[13] Choi JA, Kim JY, Lee JY, Kang CM, Kwon HJ, Yoo YD, Kim TW, Lee YS, Lee SJ. Induction of cell cycle arrest and apoptosis in human breast cancer cells by quercetin. Int J Oncol 2001; 19: 837–844.

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

[15] Kamata H, Hirata H. Redox regulation of cellular signalling. Cell Signal 1999; 11: 1–14.

[16] Krishna R, Mayer LD. Multidrug resistance (MDR) in cancer. Mechanisms, reversal using modulators of MDR and the role of MDR modulators in influencing the pharmacokinetics of anticancer drugs. Eur J Pharm Sci 2000; 11: 265–283.

[17] Kurata M, Suzuki M, Takeda K. Effects of phenol compounds, glutathione analogues and a diuretic drug on glutathione S-transferase, glutathione reductase and glutathione peroxidase from canine erythrocytes. Comp Biochem Physiol B 1992; 103: 863–867.

[18] Maldonado V, Melendez-Zajgla J, Ortega A. Modulation of NF-kappa B, and Bcl-2 in apoptosis induced by cisplatin in HeLa cells. Mutat Res 1997; 381: 67–75.

[19] McVean M, Weinberg WC, Pelling JC. A p21(waf1)-independent pathway for inhibitory phosphorylation of cyclin-dependent kinase p34(cdc2) and concomitant G(2)/M arrest by the chemopreventive flavonoid apigenin. Mol Carcinog 2002; 33: 36–43.

[20] Nagata H, Takekoshi S, Takagi T, Honma T, Watanabe K. Antioxidative action of flavonoids, quercetin and catechin, mediated by the activation of glutathione peroxidase. Tokai J Exp Clin Med 1999; 24: 1–11.

[21] Oguri T, Fujiwara Y, Katoh O, Daga H, Ishikawa N, Fujitaka K, Yamasaki M, Yokozaki M, Isobe T, Ishioka S, Yamakido M. Glutathione S-transferase-π gene expression and platinum drug exposure in human lung cancer. Cancer Lett 2000; 156: 93–99.

[22] Panaro NJ, Popescu NC, Harris SR, Thorgeirsson UP. Flavone acetic acid induces a G2/M cell cycle arrest in mammary carcinoma cells. Br J Cancer 1999; 80: 1905–1911.

[23] Pinkus R, Weiner LM, Daniel V. Role of quinone-mediated generation of hydroxyl radicals in the induction of glutathione S-transferase gene expression. Biochemistry 1995; 34: 81–88.

[24] Rigacci S, Iantomasi T, Marraccini P, Berti A, Vincenzini MT, Ramponi G. Evidence for glutathione involvement in platelet-derived growth-factor-mediated signal transduction. Biochem J 1997; 324: 791–796.

[25] Shacter E, Williams JA, Hinson RM, Sentürker S, Lee YJ. Oxidative stress interferes with cancer chemotherapy: inhibition of lymphoma cell apoptosis and phagocytosis. Blood 2000; 96: 307–313.

[26] Schafer FQ, Buettner GR. Redox environment of the cell as viewed through the redox state of the glutathione disulfide/glutathione couple. Free Radic Biol Med 2001; 30: 1191–1212.

[27] Stavric B. Antimutagens and anticarcinogens in foods. Food Chem Toxicol 1994; 32: 79–90.

[28] Vandeputte C, Guizon I, Genestie-Denis I, Vannier B, Lorenzon G. A microtiter plate assay for total glutathione and glutathione disulfide contents in cultured/isolated cells: performance study of a new miniaturized protocol. Cell Biol Toxicol 1994; 10: 415–421.

[29] Wang IK, Lin-Shiau SY, Lin JK. Induction of apoptosis by apigenin and related flavonoids through cytochrome c release and activation of caspase-9 and caspase-3 in leukemia HL-60 cells. Eur J Cancer 1999; 35: 1517–1525.

[30] Zhang K, Wong KP. Glutathione conjugation of chlorambucil: measurement and modulation by plant polyphenols. Biochem J 1997; 325: 417–422.

[31] Zhang K, Yang EB, Tang WY, Wong KP, Mack P. Inhibition of glutathione reductase by plant polyphenols. Biochem Pharmacol 1997; 54: 1047–1053.