Anti-Cancer Drugs 1998, 9, pp. 473–478

Preclinical paper

Modulation of cisplatin toxicity in blood platelets by glutathione depletion

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The effect of a specific inhibitor of γ-glutamylcysteine synthetase, L-buthionine sulfoximine (BSO), on the action of cisplatin in pig blood platelets was investigated. We show that the effect of BSO, blocking glutathione (GSH) synthesis, on depletion of GSH level in platelets was time and dose dependent ($p < 0.05$). Exposure of blood platelets to BSO reduced not only the cellular GSH content, but diminished also cytotoxicity effects of cisplatin on platelets. Cytotoxic effects of cisplatin were determined by the extent of oxidative stress in these cells, i.e. lipid peroxidation expressed as thiobarbituric acid reactive substances and oxygen free radicals (superoxide anion). The obtained results confirm that the intracellular level of GSH plays an important role in the action of cisplatin on blood platelets. [© 1998 Lippincott-Raven Publishers.]

Key words: Blood platelets, L-buthionine sulfoximine, cisplatin, glutathione.

Introduction

Platinum compounds are among the most important chemotherapeutic agents for treating cancer. The lead compound, cisplatin (*cis*-diamminedichloroplatinum II, cisplatin), was approved for clinical treatment of testicular and ovarian tumors, and cancers of the head and neck in 1979.[1] A major target for cisplatin in the cell is DNA, but the side chains and terminal amino and carboxylate groups of proteins, small peptides such as glutathione (L-γ-glutamyl-L-cysteinylglycine, GSH), and amino acids are also potentially good ligands for cisplatin.[1-5]

The clinical use of cisplatin is restricted by severe hematological toxicity, ototoxicity and nephrotoxicity.[4-6] The detailed mechanisms by which cisplatin gives rise to renal damage are incompletely understood, although it has been established that cisplatin causes a decrease in renal SH groups.[8-10] Miyama *et al.*[11] suggest that free radical generation may play a crucial role in cisplatin-induced cytotoxicity and that GSH depletors augment this cytotoxicity by enhancing free radical formation in bladder cancer cells.

GSH, the major cellular non-protein thiol, is well known to be important in the protection of cells from free radicals and may play an important role in cellular detoxification of several chemotherapeutic agents including cisplatin.[12] The administration of GSH provides protection against cisplatin-induced nephrotoxicity[13,14] and neurotoxicity.[15]

Our preliminary results indicate that in blood platelets incubated with cisplatin, this compound reacts with free sulfhydryl groups and forms a GS-Pt complex in these cells.[16] This GS-Pt complex is responsible for lipid peroxidation and free oxygen radical production in blood platelets.[17] The GS-Pt complex has also stronger inhibitory effects on platelet function *in vitro* (aggregation, secretory process and thromboxane A_2 formation) than cisplatin alone.[18-20] We suggest that the GS-Pt complex formed in platelets treated with cisplatin may be a metabolite of cisplatin responsible for the toxic effects of cisplatin in these cells.

There is a lack of information about the GSH concentration in platelets and its importance for the activation of these cells. Blood platelets are anucleated cells in which a target molecule of cisplatin action is not DNA but GSH, therefore blood platelets seem to be a suitable model for toxicological studies.

The aims of the present studies were to analyze the role of GSH in the action of cisplatin and its *trans* isomer (*trans*-diamminedichloroplatinum II, TDDP), on pig blood platelets. We treated blood platelets with L-buthionine sulfoximine (BSO), a specific inhibitor of γ-glutamylcysteine synthetase, to block GSH synthesis and studied the response of these cells to decreased levels of GSH caused by BSO preincubation by measuring the oxidative stress, i.e. lipid peroxidation and reactive oxygen radicals generation.

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Materials and methods

Chemicals

Cisplatin, transplatin, 5,5'-dithio-bis(2-nitrobenzoic acid) (DTNB), GSH and BSO were purchased from Sigma (St Louis, MO). Thiobarbituric acid was obtained from Loba Feinchemie (Germany). Other chemicals were of the highest purity available in Poland.

Blood platelet isolation

Blood collected from a pig into ACD (citric acid: citrate:dextrose, 5:1 v/v) solution was centrifuged for 20 min at 120 *g*. Platelet-rich plasma was then centrifuged for 20 min at 1100 *g*. The platelet pellet was washed gently three times in buffer (10 mM Tris-HCl, 154 mM NaCl, 5 mM glucose) at pH 7.4 and then suspended in the same buffer. Blood platelet suspensions were preincubated with BSO (10, 25 and 50 μM, 1 or 2 h) and then treated with cisplatin or its *trans* isomer at the concentration of 20 μM (0-30 min) at 37°C. The final platelet concentration was 5 mg of platelet protein/ml. Total platelet protein was determined by a modified Lowry method.[21] In control platelets (−BSO) and platelets treated with BSO (+BSO) at the concentrations of 10, 25 and 50 μM during 1 or 2 h the level of glutathione was determined according to Ando and Steiner.[22]

Isolation of acid-soluble fraction GSH and determination of free -SH groups

To frozen control and BSO-treated platelets (1 ml of platelet suspension) 1 ml of precipitating solution (85% H_3PO_4 0.5 ml; 10% EDTA 1 ml; NaCl 15 g) to precipitate proteins were added. The acid-soluble platelet fraction (GSH) was separated according to Ando and Steiner,[22] and the amount of -SH groups was estimated with DTNB. To 0.5 ml of acid soluble fraction 3.2 ml of 0.32 M. H_3PO_4 and 0.25 ml of 4 mM DTNB in 1% sodium citrate were added. After 15 min incubation at room temperature, the absorbance at 412 nm was measured. Standard -SH curve was prepared for reduced glutathione (GSH) at the concentration of 20-100 nmol.

Lipid peroxidation and O_2^- generation in blood platelets

The process of lipid peroxidation induced by isomers

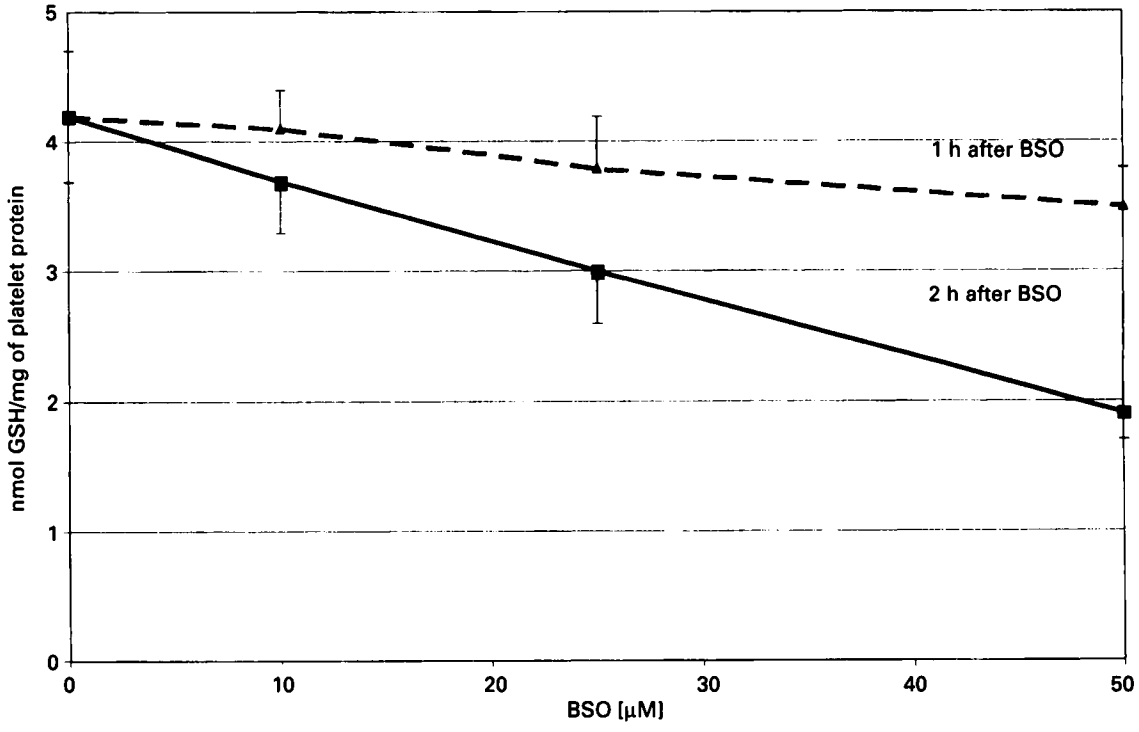

Figure 1. The effect of BSO exposure (10, 25 and 50 μM) on the level of GSH in pig blood platelets after 1 and 2 h ($p<0.05$, $n = 5$).

of diamminedichloroplatinum (*cis* or *trans*) in control platelets and platelets after pre-treatment with BSO was determined by the thiobarbituric acid technique,[23] and expressed as TBARS.

Generation of superoxide anion (O_2^-·) radicals in control platelets and in platelets preincubated with BSO and treated with cisplatin or transplatin was measured by cytochrome *c* reduction, as described by Jahn and Hansch.[24] Briefly, an equal volume of Tris-buffered saline containing cytochrome *c* (160 μM) was added to a 1 ml suspension of platelets. After incubation, the platelets were sedimented by centrifugation at 2000 *g* for 5 min and the supernatants were transferred to cuvettes. Reduction of cytochrome *c* was measured spectrophotometrically at 550 nm. To calculate the molar concentration of O_2^-· an extinction coefficient for cytochrome *c* of 18700 M^{-1} cm^{-1} was used.

GS–Pt complex formation

GSH (20, 40 and 80 nmol) was incubated with cisplatin at 37°C. The generation of O_2^-· radicals during reaction of cisplatin with GSH at different glutathione:cisplatin ratios (1:2, 2:1 and 4:1) was estimated by cytochrome *c* reduction[24] at different time points (0, 2, 4, 6, 8 and 10 min).

Statistical analysis

The data are presented as the means of the averaged replicates ± SD. Statistical analysis was performed using Student's *t*-test for paired data.

Results and discussion

Untreated control platelets had a cellular GSH concentration of 4.1 ± 0.3 nmol GSH/mg of platelet protein. BSO depleted the levels of GSH in the pig blood platelets in a dose- and time-dependent manner (Figure 1). This inhibitor at the concentration of 25 μM depleted GSH by 5% after 1 h incubation and 30% after 2 h. A significant decrease (by 50%) was observed after 2 h incubation of platelets with BSO at

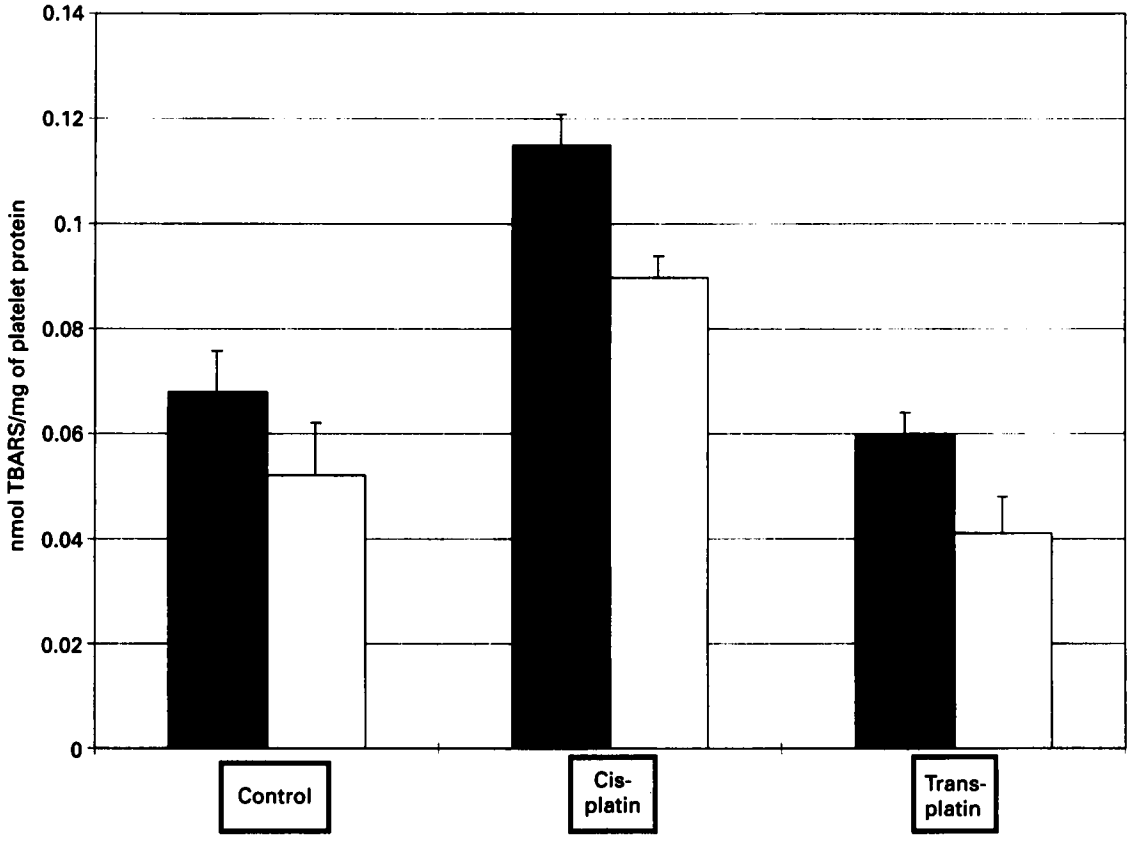

Figure 2. The effect of BSO preincubation (50 μM, 2 h, 37°C) on the level of TBARS in pig blood platelets induced by cisplatin or transplatin (20 μM, 30 min, 37°C) ($p<0.05$, $n = 10$). ■, BSO (−); □, BSO (+).

the concentration of 50 μM (Figure 1). The decreased level of GSH was accompanied by the reduced production of TBARS and $O_2^{-\cdot}$ (Figures 2 and 3, and Table 1).

Cisplatin induced oxidative stress in the cells (Figures 2 and 3, and Table 1). We showed that at the concentration of 20 μM it induced lipid peroxidation (Figure 2 and Table 1) and oxygen radical generation (Figures 3 and 4, and Table 1) in pig blood platelets. After preincubation of these cells with BSO (50 μM, 2 h, 37°C) when the total cellular GSH level was markedly depleted, the production of TBARS was reduced by about 25% in both, in control platelets and in platelets treated with cisplatin (20 μM, 30 min, 37°C). In the case of platelets incubated with transplatin (20 μM, 30 min, 37°C) the level of TBARS decreased by about 33% (Figure 2 and Table 1).

Using the method of cytochrome *c* reduction we have shown that in control platelets and in platelets treated with isomers of diamminedichloroplatinum (20 μM, 37°C) the reduced production of $O_2^{-\cdot}$ was accompanied by the decrease of GSH level caused by BSO at the concentration 50 μM (Figure 3 and Table 1). It indicates that $O_2^{-\cdot}$ generation is due to reaction of cisplatin or its *trans* isomer with GSH leading to the formation of decreased amounts of GS-Pt complex.

We noticed that the reaction of cisplatin with GSH *in vitro* resulted in the formation of $O_2^{-\cdot}$ (Figure 4)

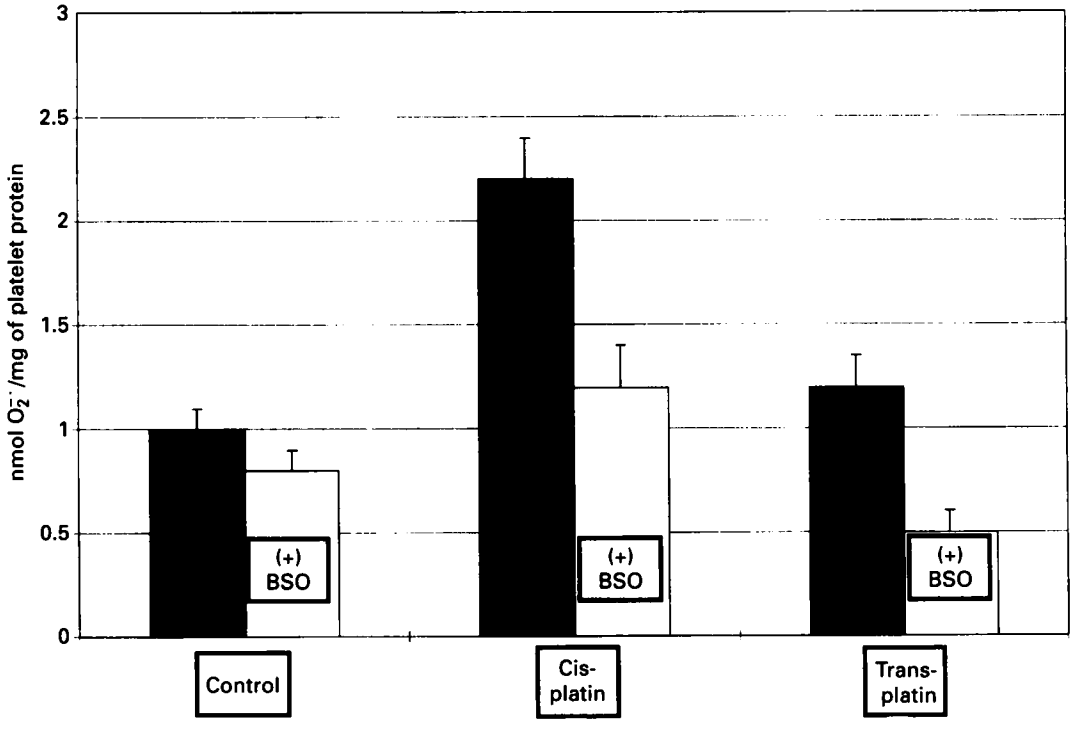

Figure 3. The effect of BSO pretreatment (50 μM, 2 h, 37°C) on the level of $O_2^{-\cdot}$ in pig bloood platelets stimulated by cisplatin or transplatin (20 μM, 2 min, 37°C) ($p<0.05$, $n = 8$).

Table 1. The effect of BSO preincubation (50 μM, 2 h, 37°C) on the generation of both TBARS ($p<0.05$, $n = 10$) and $O_2^{-\cdot}$ ($p<0.05$, $n = 8$) in pig blood platelets treated with cisplatin and transplatin

	nmol TBARS/mg of platelet protein		nmol $O_2^{-\cdot}$/mg of platelet protein	
	−BSO	+BSO	−BSO	+BSO
Control	0.068±0.009	0.052±0.010	1.0±0.12	0.78±0.11
Cisplatin	0.115±0.006	0.090±0.004	2.2±0.23	1.2±0.21
Transplatin	0.060±0.004	0.041±0.007	1.2±0.19	0.5±0.12

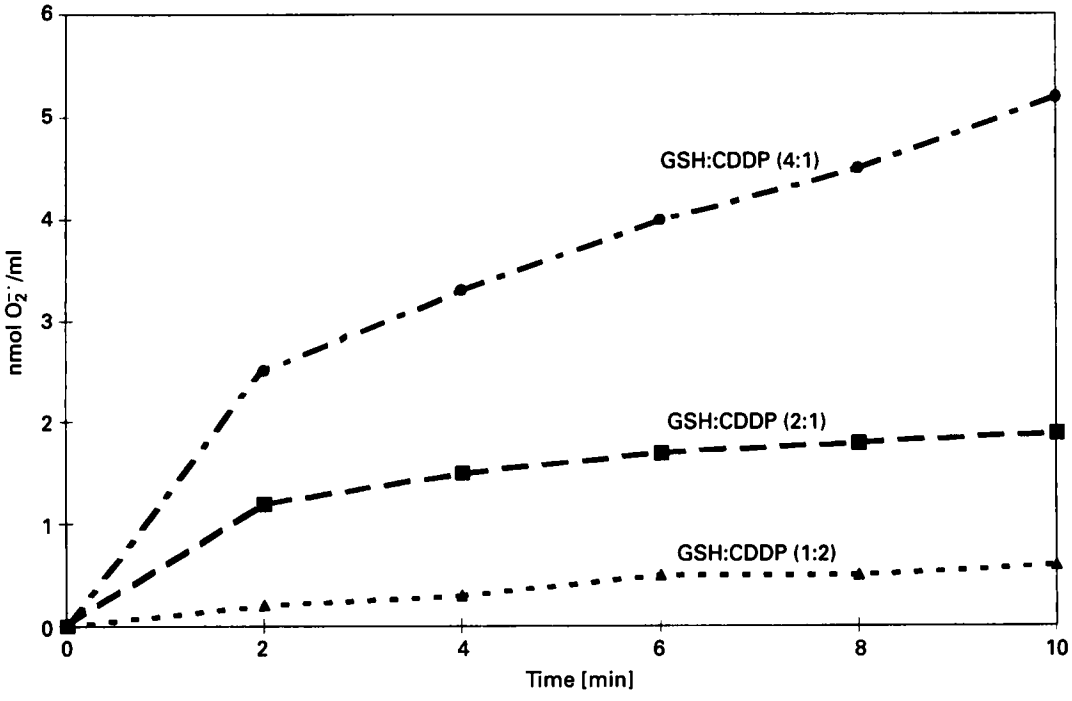

Figure 4. Generation of $O_2^{\cdot-}$ during reaction of cisplatin with GSH at different molar ratios of GSH and cisplatin (10:20, 20:10 and 40:10 μM) (n=5).

and therefore GSH was chosen as a model thiol compound to study the reactivity of cisplatin towards thiols. The formation of superoxide radicals *in vitro* during the reaction of cisplatin with GSH at different GSH:cisplatin ratios is presented in Figure 4. In the reaction of cisplatin with GSH where thiols were in excess (4:1), $O_2^{\cdot-}$ was formed more rapidly than when the reagents were present in ratio 2:1 (Figure 4). Formation of $O_2^{\cdot-}$ in the cells incubated with cisplatin was demonstrated by Masuda *et al.*[25] and by Gautier *et al.*[26]

Blood platelets are highly reactive cells derived from fragmentation of bone marrow megakaryocytes. They play an essential role in the first phases of the hemostatic process. Platelets from patients with cancer exhibit a variety of functional abnormalities. Moreover, chemotherapeutic drugs can affect blood platelet function. Our studies have shown that cisplatin is an antiplatelet drug; however, the mechanism by which this anticancer drug interferes with platelets is not entirely understood.[27]

Our preliminary results demonstrated that a conjugate of cisplatin with cellular GSH (GS-Pt complex) formed in blood platelets may be responsible for the cytotoxic effects of cisplatin in these blood cells, i.e. inhibition of platelet aggregation and release reaction.[19] The results presented here confirm our previous observations. Depletion of GSH by BSO leads to decreased amounts of GS-Pt conjugate and the reduction of the oxidative stress induced by cisplatin in blood platelets.

The modulation of the GSH concentration in different type of cells including tumor cells has also been reported to influence the cytotoxicity of cisplatin.[13,28,29]

GSH plays an important role in the defence mechanism of the cell by acting as an antioxidant or by reacting with electrophiles. It can react with toxic agents including cisplatin to form conjugates. In blood platelets, contrary to tumor cells (human melanoma cells, bladder cancer cells),[11,28] depletion of GSH does not increase but diminishes cytotoxic effects of cisplatin. The *trans* isomer has the same effect. It seems that the geometry of platinum compounds has no effect on their reactivity with GSH. Because we suggest a link between the GSH cycle and lipid peroxidation, and generation of free radicals in blood platelets incubated with cisplatin, further studies are required to elucidate the mechanism of formation the GS-Pt complex and its action on these cells.

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(Received 22 January 1998; revised form accepted 17 March 1998)