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Cisplatin-induced changes in biological activity of blood platelets: thiol-related mechanisms

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The effects of cisplatin (*cis*-diamminedichloroplatinum (II), CDDP) and transplatin (*trans*-diamminedichloroplatinum(II), TDDP), and their complexes with glutathione (GS–Pt) *in vitro* on oxygen free radical generation ($O_2^{-\cdot}$), lipid peroxidation and ADP-induced aggregation in pig blood platelets were studied. Incubation of pig blood platelets with cisplatin or transplatin caused a loss of both protein -SH and the thiol groups of GSH. In pig blood platelets exposed to cisplatin or transplatin (20 μM) the GS–Pt complex was formed as a major metabolite. The formation of GS–Pt complexes in platelet cytosol was time dependent and the intracellular content of this complex reached a maximal level after 24 h. GS–Pt complexes were found to induce platelet lipid peroxidation, measured as thiobarbituric acid reactive substance level, and $O_2^{-\cdot}$ generation, and it was more active than cisplatin or transplatin alone. Cisplatin and its GS–Pt complex had an inhibitory effect on ADP-induced platelet aggregation. These results showed that GS–Pt complexes affect platelet metabolism and function. It seems that glutathione-associated metabolism of platinum compounds plays an important role in the cytotoxicity and biological activity of these drugs.

Key words: Blood platelet, cisplatin, glutathione, transplatin.

Introduction

Our research has focused on the action of cisplatin, a chemotherapeutic agent commonly used for a wide spectrum of human malignancies, and its *trans* isomer on the metabolism and function of blood platelets.

There are indications that blood platelets contribute to cancer metastasis.[1] Various human and animal tumor cells were found to be capable of inducing platelet activation, and this ability in most cases was associated with tumor growth and metastasis.[1–6] Platelet participation in the process of metastasis is likely to result from direct platelet binding to tumor cells or/and from the release of reaction products following platelet activation.

Many antiplatelet and anticoagulation agents have been tested for potential antimetastatic effects in animal models, and some antiplatelet agents have demonstrated potent antimetastatic effects.[7,8]

It is generally accepted that the antitumor activity and cytotoxicity of cisplatin is attributed primarily to its ability to form DNA–Pt adducts.[9–13] Cisplatin reacts not only with DNA, but may also react with sulfhydryl groups of proteins and with reduced glutathione.[14] The modulation of the glutathione concentration in mammalian cells has also been reported to influence the cytotoxicity of cisplatin.[15–17]

Blood platelets lack a nucleus and therefore DNA is not the target of cisplatin action.

The aims of the present studies were to provide further evidence supporting thiol-related alteration as a mechanism of cisplatin action on blood platelets and to elucidate the mechanism by which platelet activation *in vitro* is changed.

Preliminary date from our laboratory show that cisplatin *in vitro* has a stimulatory effect on platelet lipid peroxidation and oxygen radical generation.[18–20] It causes an inhibition of platelet activation and affects the enzymatic transformation of platelet endogenous arachidonate.[21]

Methods

Chemicals

Cisplatin, transplatin, DTNB (5,5′-dithio-bis(2-nitrobenzoic acid)), glutathione (GSH) and protein standards were purchased from Sigma. QAE–Sephadex was obtained from Pharmacia and thiobarbituric acid from Loba Feinchemie. Other chemicals were of the highest purity available.

Blood platelet isolation

Platelets obtained from fresh pig blood by differential centrifugation were washed and suspended in Ca^{2+} free Tyrode's buffer (pH 7.4). The platelet sus-

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pensions (10 mg of platelet protein/ml) were incubated at 25 or 37°C with cisplatin or transplatin at final concentrations of 20, 100 and 200 μM (2, 5 and 10 nmol Pt/mg of platelet protein, respectively). The total protein concentration in these cells was determined using a modified technique which employs sodium dodecyl sulfate (10%) for solubilizing proteins.[22]

Isolation of acid-soluble and acid-precipitable fractions

To frozen control and cisplatin (or transplatin)-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 protein was added. Acid-soluble (glutathione) and acid-insoluble (proteins) platelet fractions were separated according to Ando and Steiner,[23,24] and the amount of -SH groups was estimated with DTNB.

Determination of the free -SH groups

In the acid-soluble fraction. To 0.5 ml of the acid-soluble fraction, 3.2 ml of 0.32 M Na_2HPO_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.

In the acid-precipitable fraction. To the pellet, 5 ml H_2O and 3 ml 10% SDS were added. After solubilization, 0.5 ml samples were taken and free -SH groups were determined as described above. A standard -SH curve was prepared for GSH at a concentration of 20–100 nmol.

Isolation of GS–Pt complexes by anion-exchange chromatography

After incubation of platelets with cisplatin or transplatin (20 μM) the GS–Pt complexes from the acid-soluble fraction were isolated by anion-exchange chromatography.[16] Briefly, to the acid-soluble fraction obtained as described above, 7.5% perchloric acid was added (v/v) and after centrifugation (16 000 *g*, 10 min) 1 ml of supernatant was applied to a QAE–Sephadex column equilibrated with 10 mM Tris–HCl (pH 7.5). The column was washed with 5–7 ml deionized water. The GS–Pt complexes bound to the QAE–Sephadex were eluated with 0.2 M HCl. In the eluent the GS–Pt complex was estimated spectrophotometrically (at 280 nm). The molar extinction coefficient at 280 nm was 8.05 mM^{-1} cm^{-1}.[16]

Effects of GS–Pt complexes on platelet lipid peroxidation and O_2 generation

The biological activity of the GS–Pt complexes was examined *in vitro*. Platelets were incubated (30 min, 25°C) with 10 μM GS–Pt. The process of platelet lipid peroxidation induced by GS–Pt was measured by the thiobarbituric acid technique,[25] expressed as TBARS and compared with platinum-induced platelet lipid peroxidation and with control (platelets incubated with the buffer). Generation of $O_2^{-\cdot}$ radicals in control platelets and in platelets after incubation with GS–Pt complexes or platin alone was measured by cytochrome *c* reduction, as described by Jahn and Hansch.[26] 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 $O_2^{-\cdot}$, an extinction coefficient for cytochrome *c* of 18 700 M^{-1} cm^{-1} was used.

Platelet aggregation

The aggregation of platelets in response to ADP was recorded at 37°C at a stirring rate of 1000 r.p.m. using a Labor APPACT dual-channel aggregometer. For this purpose, 275 μl platelet-rich plasma (2.7×10^8 platelets/ml) was preincubation for 5 min at 37°C with cisplatin, transplatin or GS–Pt (20 μM) without these compounds (control). Then 10 μl ADP was added to a final concentration of 10 μM and the extent of platelet aggregation was measured.

Statistics

Statistical analysis was performed by Student's *t*-test for paired data.

Results

After incubation of pig blood platelets with platin compounds (cisplatin and transplatin) the amount of free thiols in both acid-soluble and acid-precipitable fractions significantly decreases (Figures 1–4). Figures 1 and 2 show the time-course of loss of both platelet protein -SH and platelet GSH caused by cisplatin and transplatin (20 μM) ($p < 0.001$). The concentrations of protein -SH and reduced glutathione in control platelets were about 75 ± 10 and 4.1 ± 0.4 nmol/mg of platelet protein, respectively. These concentrations remained constant during the time of incubation (until 24 h). Platelets incubated with cisplatin or transplatin suffered a dramatic depletion of free -SH groups of both protein ($p < 0.01$) (Figure 4) and reduced glutathione ($p < 0.01$) (Figure 3). A significant decrease ($p < 0.001$) was observed after 1 h of incubation. After 24 h of incubation of platelets with platinum compounds, the protein -SH was decreased to about 20 nmol/mg of platelet protein (Figure 2) whereas -SH of glutathione was decreased to about 1 nmol/mg of platelet protein (Figure 1).

The depletion of -SH groups in the platelet glutathione fraction occurred when the SH/Pt ratio was about 2:1 and at the higher concentration of drugs a further loss of GSH was not observed (Figure 3). The decrease of the amount of platelet protein -SH was observed when -SH groups were in excess (Figure 4). Our experiments showed that the Gs–Pt complexes were formed in pig blood platelets after incubation with cisplatin or transplatin at a concentration of 20 μM (molar ratio of GSH/platinum was about 2:1) (Figure. 5).

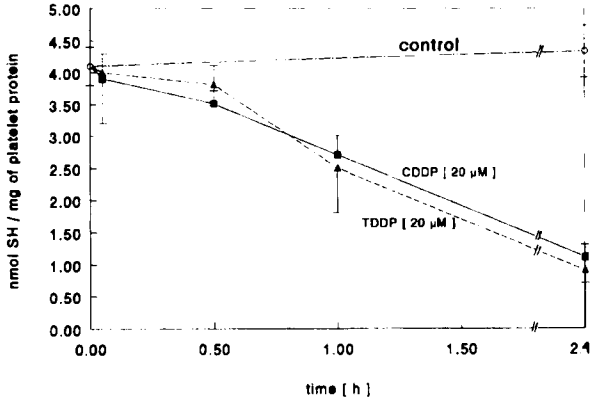

Figure 1. Time-course of the loss of reduced glutathione -SH groups in acid soluble fraction of platelets after incubation with 20 μM cisplatin (■) or transplatin (▲). Platelets were incubated at 25°C.

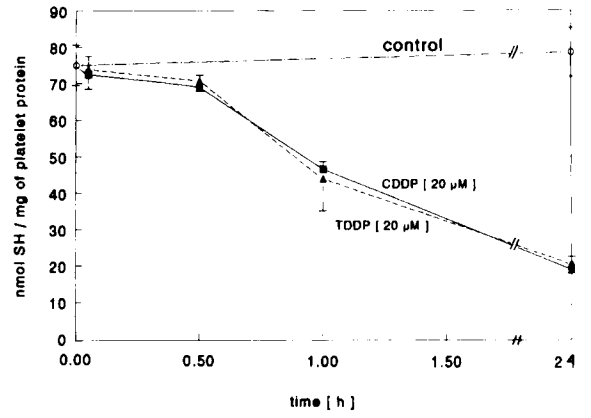

Figure 2. Time-course of the loss of platelet protein -SH groups after incubation of platelets with 20 μM cisplatin (■) or transplatin (▲). Platelets were incubated at 25°C.

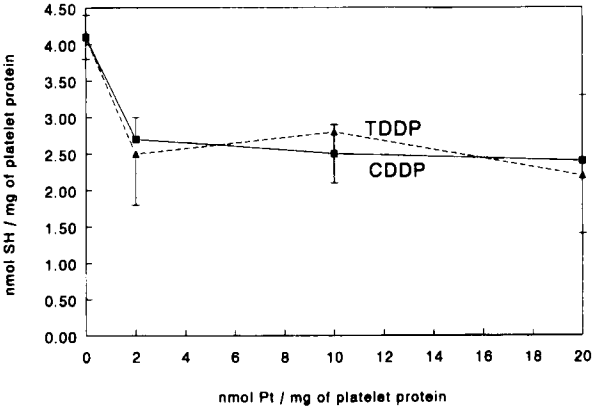

Figure 3. The effects of cisplatin (■) and transplatin (▲) concentration on free thiols level in the platelet acid-soluble fraction (1 h; 25°C).

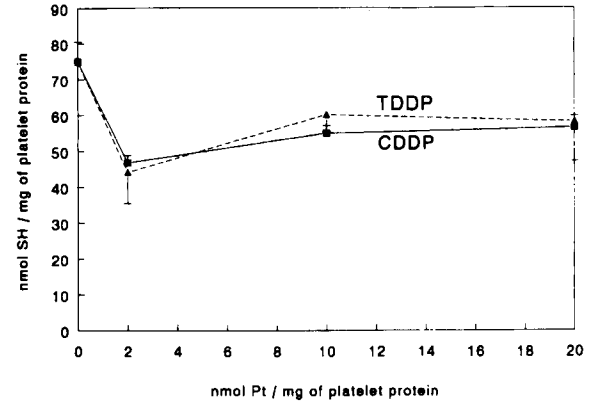

Figure 4. The effect of cisplatin (■) and transplatin (▲) concentration on free thiols level in the platelet acid-precipitable fraction (1 h; 25°C).

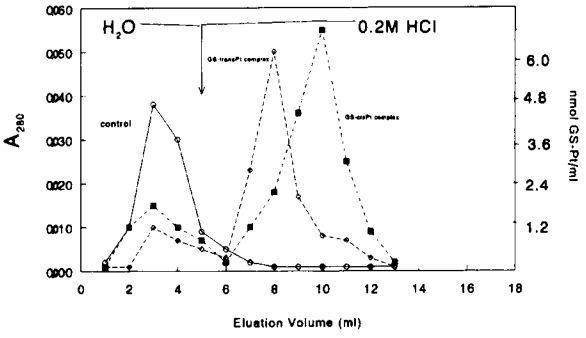

Figure 5. Anion-exchange column chromatography of GS–Pt complexes from control platelets (control) and from platelets after incubation with cisplatin or transplatin. Platelets were incubated without or with isomers of platinum (20 μM) at 37°C for 1 h. Then, 1 ml of the acid-soluble fraction was mixed with 1 ml of 7.5% perchloric acid and centrifuged at 16 000 *g* for 10 min. The supernatant was applied to a QAE–Sephadex column equilibrated with 10 mM Tris–HCl, pH 7.5. The column was washed with 6 ml of distilled water and subsequently with 10 ml of 0.2 M HCl and 0.5 ml fractions were collected. The GS–Pt complex was estimated spectrophotometrically at 280 nm.

GS–Pt complexes from the platelet extracts were isolated by anion-exchange column chromatography (Figure 5). Figure 6 shows the intracellular content of GS–Pt complexes in platelets after incubation of the cells with cisplatin (Figure 6A) and transplatin (Figure 6B) at the concentration of 20 μM. Complexes reached their maximal level after 24 h ($p < 0.01$) (Figure 6A and B). The formation of GS–Pt in plasma was not observed. Treatment of washed pig platelets with isomers of platinum or with their complexes (GS–Pt) at the same concentration (10 μM) produced a significant increase of TBARS expressed as malonyldialdehyde (Figure 7). In relation to the platelets incubated with cisplatin or transplatin alone, the level of TBARS increased significantly in platelets treated with GS–Pt complexes (Figure 7). GS–Pt complexes also produced generation of free oxygen radicals ($O^{2-\cdot}$) in platelets (Figure 8).

The studies demonstrated that platinum compounds and the complex cisplatin with GSH had inhibitory effects on ADP-induced platelet aggregation. Cisplatin caused a significant inhibition of this process (Figure 9).

Discussion

Among the platinum coordination complexes with antineoplastic activity, cisplatin is the most widely used and is effective against a variety of malignan-

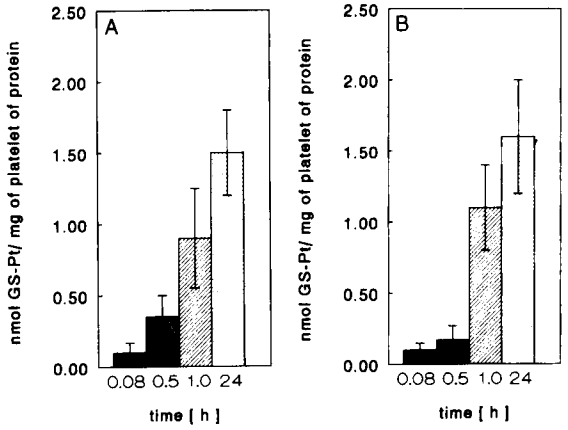

Figure 6. Time-dependent formation of intracellular GS–Pt complexes in blood platelets after incubation with cisplatin (20 μM; 25°C) (A) or with transplatin (20 μM; 25°C) (B).

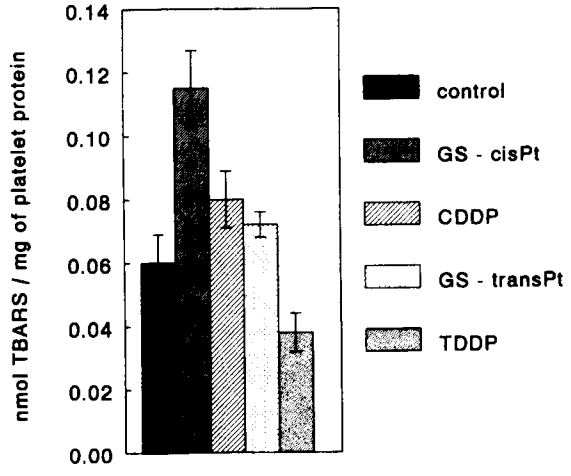

Figure 7. The effects of GS–Pt complexes, cisplatin and transplatin (10 μM, 30 min, 25°C) on TBARS production in washed pig platelets.

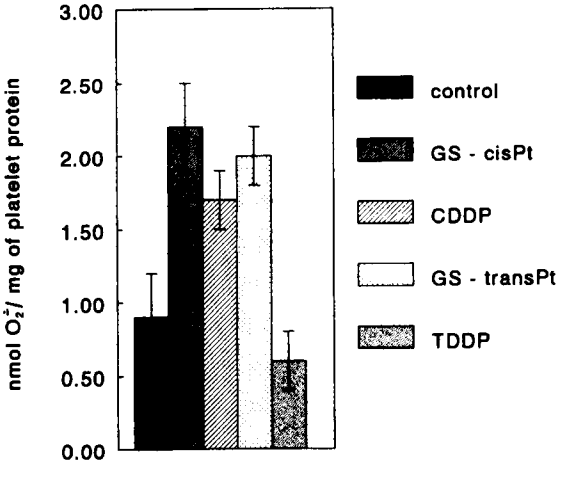

Figure 8. The effects of GS–Pt complexes, cisplatin and transplatin (10 μM, 2 min, 25°C) on $O_2^{-\cdot}$ generation in washed pig platelets.

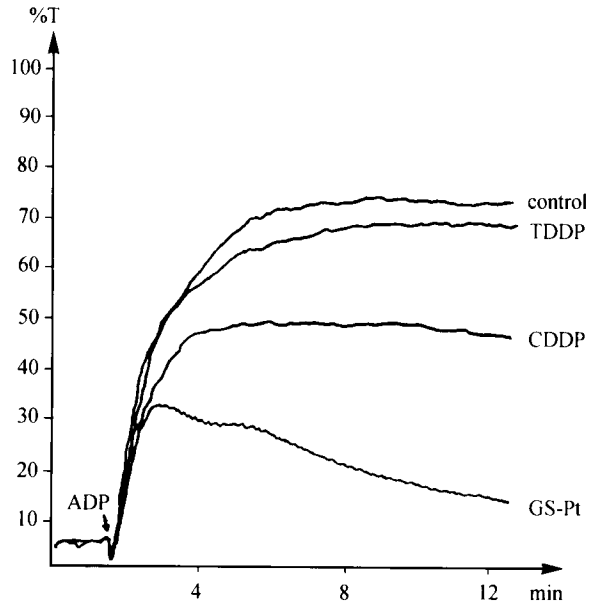

Figure 9. The effects of cisplatin, GS–Pt (cisplatin) complex and transplatin (20 μM, 5 min, 37°C) on ADP-induced platelet aggregation.

cies.[9,27] The clinical use of cisplatin is limited by severe toxicity to several normal tissues, especially the kidney, the gastrointestinal tract and the bone marrow.[10]

Cisplatin-based chemotherapy is also associated with hematological toxicity, such as anemia, leukopenia and thrombocytopenia.[27–29] These effects may become important dose-limiting factors and must be taken into consideration in the clinical application of this anticancer drug. The average platelet life-span in circulation after release from megakaryocytes is estimated to be 8–10 days. Patients who have received cisplatin therapy for treatment of malignancy may develop severe thrombocytopenia that is potentially life threatening because of the risk of hemorrage. In patients receiving cisplatin, not only the number of platelets is decreased but circulating platelets show a changed and limited hematological function, which results in failure in the maintenance of normal hemostasis. Cisplatin affects blood platelets;[18,20,21] however, little information is available about the changes in platelet metabolism and function induced by cisplatin. The exact relationship of these changes to the hemostatic effectiveness of platelets following cisplatin treatment remains unclear. An agent protecting circulating platelets against toxicity of cisplatin would be extremely useful. It seems that among different compounds, selenium and GSH may play an important role. Co-administration of sodium selenite enables the use of increasing doses of cisplatin and enhances the antitumor effect of cisplatin by depressing its side effects. The mechanism underlying the protective action of selinium against cisplatin-induced cellular toxicity remains unclear. Although the exact mechanism of cisplatin action on blood platelets is not well understood, it is assumed that -SH groups in these cells are the critical target of cytotoxicity for this drug. In nucleated cells, a competition of cisplatin for thiols and DNA takes places, i.e. about 1% of cisplatin reacts with genomic DNA and forms adducts with guanine and cytosine, whereas most of the drug interacts with proteins, RNA and small thiol compounds.[12,30,31].

Since platelets are anucleated cells which lack DNA, DNA is not a target of cisplatin action in this cell.

The results of the present work show that platinum compounds significantly decreased the concentration of platelet free -SH groups in both proteins and non-proteins—mainly GSH. It is known that the depletion of protein -SH usually occurs by its oxidation, the formation of protein disulfides and mixed disulfides. The mechanism by which cisplatin depletes protein -SH groups and reduces the level of GSH in platelet cytosol may be its direct binding with -SH rather than oxidation. Cisplatin reacts spontaneously with a variety of nucleophiles, including glutathione.[14]

Extracellular reaction of GSH with cisplatin prior to treating the cells reduced the toxicity of the compound, indicating that this reaction is capable of participating in the detoxification of cisplatin.[32] Glutathione was used to protect against cisplatin-induced nephrotoxicity.[33] In rodents the administration of GSH in excess protected against the cisplatin-induced nephrotoxicity[33] and also neurotoxicity.[34] Litterst *et al.*[35] showed that in the cytosol of tissues from cisplatin-treated rats, 30% of cisplatin was associated with glutathione. The mechanism of this protective effect of high doses of reduced glutathione is unknown. Glutathione is the most abundant intracellular non-protein thiol known to have an important role in cellular protection as well as in the detoxification of numerous xenobiotics. Some tumor cells, particularly those of human origin, contain very high levels of GSH,[13,36–39] suggesting that GSH may be a key factor limiting the therapeutic efficacy of cancer treatment. Glutathione is the major physiological substrate for γ-glutamyl transpeptidase (EC 2.3.2.2) (GGT). GGT is essential in generating a nephrotoxic form of cisplatin. This enzyme catalyzes the initial steps in the metabolism of gluta-

thione-conjugated drugs to mercapturic acids, some of which are severely toxic. Hanigan *et al.*[33] suggest that the high concentration of glutathione may protect against cisplatin toxicity by serving as a competitive inhibitor of GGT activity. Reduced GSH diminishes cisplatin-induced neurotoxicity *in vivo*[34] and protects against the lipid peroxidation induced by cisplatin in blood platelets *in vitro*.[40] From our previous studies it appeared that pretreatment of platelets not only with GSH but also with selenium protected blood platelets against cisplatin-induced lipid peroxidation and $O^{2-\cdot}$ generation.[20,40]

Recent studies suggest a critical role of GSH in the mechanisms of tumor cell resistance to alkylating agents, such as cisplatin.[15,28,39] It seems that the resistance to chemotherapeutic agents may be due to the elevated cellular GSH levels. In leukemia cells incubated with cisplatin the intracellular GSH level was elevated (about 5-fold).[16]

The formation of the GS–Pt complexes is considered as a significant part of the cellular metabolism of cisplatin.[16] Ishikawa and Ali-Osman[16] treated L1210 leukemia cells with cisplatin and found that 60% of platinum in the cells was in the form of a GS–Pt complex. Stochiometric analysis showed a 2:1 molar ratio of GSH to cisplatin for the reaction. In this reaction, each GSH molecule acts as a chelating ligand, coordinating to platinum via cysteinal sulfur and nitrogen atoms. The molecular mass of the GS–Pt complex determined by mass spectroscopy was 809 Da. Transplatin has a different biological activity than cisplatin. The ineffectiveness of transplatin as a therapeutic agent also results from its inability to form intrastrand DNA cross-links both *in vivo* and *in vitro*.[10] The *trans* geometry of the platinum compound does not possess antineoplastic activity, but is responsible for the reaction with free -SH groups present in the blood platelets. We have shown that transplatin, like cisplatin, interacts with platelet -SH groups of proteins and reduced glutathione. The GS–transplatin complex like the GS–cisplatin complex induces platelet lipid peroxidation and $O_2^{-\cdot}$ generation. It seems that the geometry of the platinum compounds has no effect on their reactivity with thiols. The platinum compounds (*cis* and *trans*) possess distinct abilities to bind with DNA, but their interaction with -SH groups in the cell seems to be almost the same.

The present study shows that GS–Pt complexes were formed in the cytosol fraction of pig blood platelets as a major metabolite in these blood cells exposed *in vitro* to cisplatin. The complex was found to be potentially active in oxygen free radical generation, in the induction of platelet lipid peroxidation and the inhibition of platelet aggregation. We suggest that complexes between platinum and GSH, which are formed in platelets after treatment with cisplatin, may be of biological importance and could be responsible for the alteration of platelet function. How cisplatin and its isomer transplatin might alter cellular metabolism and inhibit platelet activation remains to be elucidated.

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