

Comparative Cytotoxicity of Cisplatin, Sodium Selenite and Selenium-Cisplatin Conjugate $[(\text{NH}_3)_2 \text{Pt}(\text{SeO}_3)]$; Changes of Blood Platelet Activation

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Abstract. The cytotoxic effects of a novel compound, conjugate Se-Pt $[(\text{NH}_3)_2 \text{Pt}(\text{SeO}_3)]$ on blood platelet function (aggregation, release of adenine nucleotides) were studied. Contrary to the action of cisplatin or selenite alone, $[(\text{NH}_3)_2 \text{Pt}(\text{SeO}_3)]$ did not inhibit ADP-induced platelet aggregation, thrombin-induced release of adenine nucleotides from platelets, and had no effect on the metabolism of platelet arachidonate. The tested compound seems to be less toxic than cisplatin alone and has no effect on blood platelet activation.

Key words: Blood platelets – Cisplatin – Selenite – Conjugate $[(\text{NH}_3)_2 \text{Pt}(\text{SeO}_3)]$

Introduction

Cisplatin (cis-diamminedichloroplatinum II, CDDP) belongs to the most effective antineoplastic compounds among all platinum-based drugs. The antitumor activity of cisplatin has mainly been attributed to its ability to form adducts with DNA (Keppler 1993, Lindauer and Holler 1996). On the other hand, a variety of adverse effects may accompany the use of this drug. The side effects such as nephrotoxicity, bone marrow toxicity, ototoxicity or haematological toxicity are significant, and prevent the use of high doses of cisplatin (Lowenthal and Eaton 1996). Cisplatin causes haematological toxicity inducing oxidative stress and changing the function of blood cells, it has an inhibitory effect on platelet activation (Wachowicz and Olas 1995, Olas and Wachowicz 1996). The detailed molecular mechanism by which cisplatin can damage the blood cells is incompletely understood, though it has been established that cisplatin induces cell membrane lipid peroxidation (Wachowicz

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1991 Spitz et al 1993 Zhang and Lindup 1994) causes a decrease in -SH groups and forms complex with glutathione (Odenheimer and Wolf 1991 Ishikawa and Ali-Osman 1993 Keppler 1993 Pendyala and Creaven 1993 Wachowicz et al 1995 Olas and Wachowicz 1996) Satoh et al (1992) have previously demonstrated, that selenite coadministration enables the use of increasing doses of CDDP because of reduced toxicity without affecting the antitumor activity of the drug. A decrease in selenium intake via the diet has been reported to enhance the toxicity of cisplatin and it is possible that the differences in the sensitivity of patients to CDDP observed may partly be due to a discrepancy in dietary selenium intake (Baldew et al 1992). The enhancement of Se levels in the cells may play a role in reducing the toxicity of cisplatin. The exact mechanism underlying the protective action of Se on cisplatin induced cellular toxicity remains unclear. It is possible that selenium (given as selenite) and cisplatin or its metabolites may interact with each other and that the toxicity of these compounds may be mutually reduced.

Beaty et al (1992) suggested that cisplatin not only reacts with sulphur containing groups but also with the selenium-containing amino-acid derivative selenol L-methionine. The tested compound - a conjugate selenite with diammineplatinum $[(\text{NH}_3)_2 \text{Pt}(\text{SeO}_3)]$ differs from the commercially available platinum complexes. We lack pharmacological information concerning its cytotoxicity.

Our earlier results revealed that cisplatin reacts with platelet thiols (Wachowicz et al 1995 Olas and Wachowicz 1996) induces platelet lipid peroxidation and inhibits antioxidative enzymes (Wachowicz 1991). Inorganic form of selenium selenite (Se) in nontoxic doses ($1 \mu\text{mol/l}$) has protective effects against cisplatin action on platelets (Wachowicz et al 1995). Therefore the aim of the present investigations was to evaluate in platelets the cytotoxic properties of a novel Se-Pt conjugate $[(\text{NH}_3)_2 \text{Pt}(\text{SeO}_3)]$ and to compare the obtained results with the known action of cisplatin and selenite at nontoxic concentrations on platelet activation. We studied the effects of Se-Pt conjugate on platelet aggregation, secretion and arachidonate metabolism *in vitro*.

Materials and Methods

Chemicals

Cisplatin, sodium selenite, benzamide, phenylmethylsulfonyl fluoride (PMSF), DNase I, DNA type I, guanidine HCL, leupeptin and adenosine triphosphate were obtained from Sigma (St. Louis, MO, USA). Conjugate of selenium with cisplatin $[(\text{NH}_3)_2 \text{Pt}(\text{SeO}_3)]$ synthesised at the Institute of Pure Chemicals, Lachema, Brno (batch no. 290592) was a gift obtained from Prof. V. Klenwachter (Institute of Biophysics, Czech Academy of Sciences, Brno). Bovine thrombin was purchased from Polfa (Poland).

Isolation of blood platelets

Human blood was collected into one-tenth volume of sodium citrate from a forearm vein through an 18 gauge needle. Pig blood was collected into ACD solution (citric acid/citrate/dextrose) 5:1 v/v. Platelets were isolated by differential centrifugation of blood (20 min, at $200 \times g$). The platelet-rich plasma was then centrifuged for 20 min at $1000 \times g$ to sediment platelets. The resulting pellet was resuspended in the modified Ca^{2+} -free Tyrode's buffer (140 mmol/l NaCl, 10 mmol/l glucose and 15 mmol/l Tris/HCl, pH 7.4), and the platelets were subsequently washed three times with the same buffer. The entire washing procedure was performed in plastic tubes and carried out at room temperature. Platelet protein was estimated by a modified Lowry method (Vatassay and Smith 1987).

Human platelet aggregation

The aggregation was measured by platelet turbidity, with 0% aggregation calibrated as the absorbance of platelet-rich plasma (PRP) and 100% aggregation calibrated as the absorbance of platelet-poor plasma (PPP). The aggregation of human platelets in response to ADP was recorded at 37°C at a stirring rate of 1000 rpm using a Labor APPACT dual-channel aggregometer. For this purpose, 275 μl samples of PRP (2.7×10^8 platelets/ml) were transferred into cuvettes after preincubation for 30 min at 37°C with cisplatin at the concentration of 20 $\mu\text{mol/l}$ or conjugate of selenite with cisplatin (Se-Pt) (20 $\mu\text{mol/l}$), selenite (1 $\mu\text{mol/l}$, 10 min) or without these compounds (control), and 10 μl ADP was then added to the final concentration of 10 $\mu\text{mol/l}$, and the extent of platelet aggregation was measured.

Analysis of G-actin content in human platelets

Control human platelets and platelets after 30 min preexposure to conjugate of selenite with cisplatin (20 $\mu\text{mol/l}$), cisplatin alone (20 $\mu\text{mol/l}$, 30 min) or selenite alone (1 $\mu\text{mol/l}$, 10 min) at 37°C were activated by thrombin (0.01 U/mg of platelet protein, 2 min), then lysed with an equal volume of pH 7.4 buffer containing 2% Triton X-100, 10 mmol/l EDTA, 100 mmol/l Tris-HCl, 2 mg/ml leupeptin, 100 mmol/l benzamide and 2 mmol/l PMSF. For inactivated samples, 100 μl of platelet suspension in a small microcentrifuge tube was lysed as described above. Samples were assayed in triplicate immediately after lysis by mixing a small aliquot with a DNase buffer, pH 7.4, at a 1:1 ratio, containing 10 $\mu\text{g/ml}$ deoxyribonuclease I, 0.1 mmol/l CaCl_2 , 10 $\mu\text{mol/l}$ PMSF and 50 mmol/l Tris-HCl. Twenty μl were immediately mixed with 3 ml of a deoxyribonucleic acid buffer, pH 7.4, containing 40 $\mu\text{mg/ml}$ DNA type I, 1.8 mmol/l CaCl_2 , 4 mmol/l MgSO_4 and 100 mmol/l Tris-HCl. The reaction rate was approximately 10^{-3} absorbance units at 260 nm, and the change in absorbance was recorded in a Model UV Beckman DU62 Spectrophotometer. The total actin content of the sample was determined by incubating

a lysed sample with an equal volume of denaturing buffer pH 8.4 containing 1.5 mol/l guanidine HCl, 1 mmol/l sodium adenosine triphosphate, 1 mol/l sodium acetate, 6 mmol/l CaCl_2 , and 20 mmol/l Tris-HCl for 5 to 15 min on ice, and assaying as above. The denaturing buffer causes disassembly of F-actin into G-actin. Percentages of G-actin were calculated by dividing the relative inhibition of DNase activity of the lysed samples by the relative inhibition of the denatured sample (Fox et al. 1981).

Release of adenine nucleotides from pig blood platelets

After preincubation with three different tested compounds, the suspensions of pig platelets were activated by thrombin at the high concentration which induces the platelet secretory process (10 U/mg of platelet protein) for 2 min at 37°C. The platelet suspension without the tested compounds served as a control. Adenine nucleotides were estimated spectrophotometrically in the supernatants obtained after centrifugation of activated platelets. To the supernatant equal volume of HClO_4 (1.2 mol/l) was added, centrifuged, and the absorbance was measured in the clear supernatant at 260 nm. The amount of released adenine nucleotides was expressed as percent of total platelet adenine nucleotides (Wachowicz 1987).

Determination of malonyldialdehyde (MDA) in thrombin stimulated pig platelets

The suspensions of pig platelets in buffered saline (control and preincubated for different times with cisplatin, selenite, and conjugate of cisplatin with selenite) were incubated for 2 min at 37°C with bovine thrombin (1 U/mg of platelet protein) at a concentration able to stimulate the arachidonate cascade. The incubation was stopped by cooling the samples in an ice bath.

Samples of thrombin-activated platelets (in the absence of and after pre-treatment with the tested compounds) were transferred to an equal volume of 20% (v/v) cold trichloroacetic acid in 0.6 mol/l HCl, and centrifuged at $1200 \times g$ for 15 min. One volume of clear supernatant was mixed with 0.2 volume of 0.12 mol/l thiobarbituric acid in 0.26 mol/l Tris at pH 7.0, and immersed in a boiling water bath for 15 min. Absorbance at 532 nm was measured, and the results were expressed as nmoles of malonyldialdehyde (MDA) (Smith et al. 1976, Wachowicz 1984).

The data are presented as means of averaged replicates \pm S.D. Statistical analysis was performed using Student's *t*-test for paired data.

Results

Typical ADP-induced aggregation of human platelets (control platelets and platelets preincubated with CDDP (20 $\mu\text{mol/l}$) and selenite (1 $\mu\text{mol/l}$) or Se-Pt (20 $\mu\text{mol/l}$)) is presented in Fig. 1.4. Cisplatin (20 $\mu\text{mol/l}$) alone caused a significant

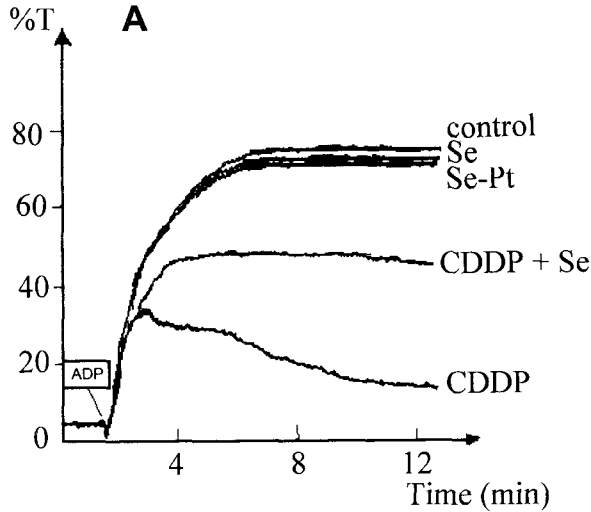


Figure 1A. Typical ADP-induced aggregation of human platelets (control platelets, platelets preincubated with CDDP (20 $\mu\text{mol/l}$), selenite (1 $\mu\text{mol/l}$) (30 min, 37°C) cisplatin after preincubation of platelets with selenite (10 min) or Se-Pt (20 $\mu\text{mol/l}$) ($n = 5$)).

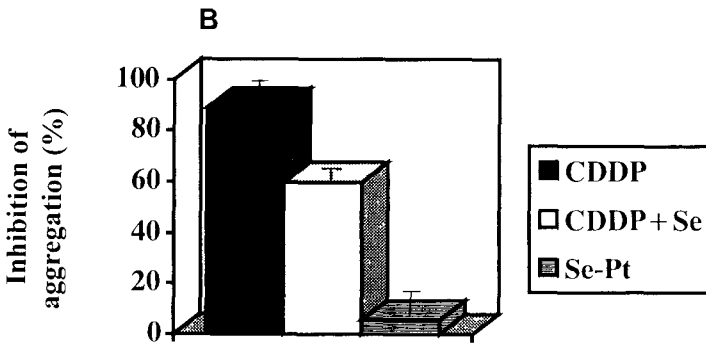


Figure 1B. The effects of cisplatin alone (20 $\mu\text{mol/l}$) (30 min, 37°C), cisplatin after preincubation of platelets with selenite (10 min) and adduct of selenite with cisplatin (Se-Pt, 20 $\mu\text{mol/l}$) on ADP-induced human platelet aggregation ($n = 5$)).

inhibition of the ADP-induced platelet aggregation ($p < 0.01$) (Figs 1A and 1B). After preexposure of platelets to selenite (1 $\mu\text{mol/l}$, 10 min), the inhibitory effect of cisplatin on platelet aggregation was decreased ($p < 0.05$) (Figs 1A and 1B). Selenite alone, and conjugate of selenite with cisplatin (Se-Pt) had no effect on this

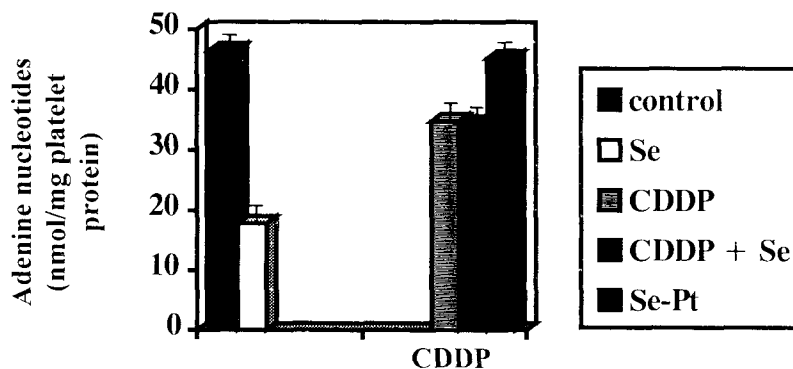


Figure 2. The effects of selenite ($1 \mu\text{mol/l}$) and cisplatin alone ($20 \mu\text{mol/l}$) ($30 \text{ min } 37^\circ\text{C}$), cisplatin after preincubation of platelets with selenite (10 min), and adduct of selenite with cisplatin (Se-Pt $20 \mu\text{mol/l}$) on thrombin-induced release of adenine nucleotides from pig blood platelets ($n = 8$)

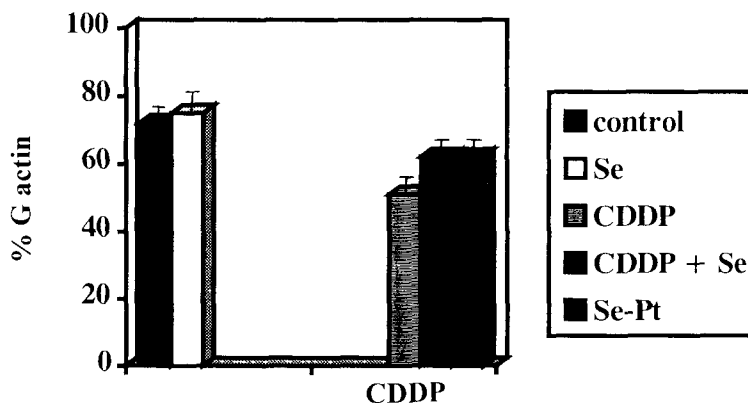


Figure 3. The effects of selenite ($1 \mu\text{mol/l}$) and cisplatin alone ($20 \mu\text{mol/l}$) ($30 \text{ min } 37^\circ\text{C}$), cisplatin after preincubation of platelets with selenite (10 min) and adduct of selenite with cisplatin (Se-Pt, $20 \mu\text{mol/l}$) on the platelet G-actin percent, measured by DNase I inhibition assay ($n = 5$)

process (Fig. 1A). The tested compounds showed different actions on the platelet secretory process. Incubation of platelets with cisplatin ($20 \mu\text{mol/l}$) or selenite ($1 \mu\text{mol/l}$) had an inhibitory effect on thrombin-induced release of adenine nucleotides from the platelets, but the conjugate of selenite with cisplatin ($20 \mu\text{mol/l}$) did not affect the platelet secretory process (Fig. 2). Pre-treatment of platelets with Se-Pt did not change the amount of released adenine nucleotides after stimulation by thrombin. Also after the treatment of platelets with cisplatin alone ($20 \mu\text{mol/l}$,

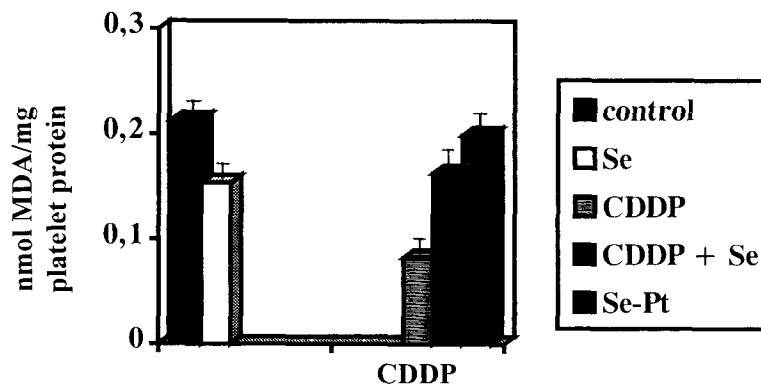


Figure 4. The effects of selenite ($1 \mu\text{mol/l}$) and cisplatin alone ($20 \mu\text{mol/l}$) ($30 \text{ min } 37^\circ\text{C}$) cisplatin after preincubation of platelets with selenite (10 min) and adduct of selenite with cisplatin (Se-Pt $20 \mu\text{mol/l}$) on MDA levels in platelets stimulated by thrombin (1 U/mg platelet protein, $2 \text{ min } 37^\circ\text{C}$) ($n = 8$)

$30 \text{ min}, 37^\circ\text{C}$) the amount of G-actin in the platelets was observed to be reduced ($p < 0.05$) (Fig 3) whereas the tested compound (Se-Pt) had a slightly protective effect on the changes in the platelet G-actin levels (Fig 3)

Thrombin (1 U/mg of platelet protein, $2 \text{ min } 37^\circ\text{C}$) induced the production in control platelets of $0.2 \pm 0.025 \text{ nmol MDA/mg platelet protein}$. The tested Se-Pt compound had no effect on the production of MDA in platelets stimulated by thrombin (Fig 4). Selenite inhibited the thrombin stimulated generation of MDA, and had a protective effect against the inhibitory action of cisplatin on this process (Fig 4).

Discussion

Blood platelets are anuclear cells. Their activation plays an important role in haemostasis. Treatment of platelets with thrombin or other strong platelet agonists activates multiple intracellular signal transduction pathways that are responsible for the physiological response of platelets including changes in platelet shape from discoidal to spherical, extension of filopodia, and changes in the conformation of integrin $\alpha_{\text{IIb}}\beta_3$. This allows the binding of fibrinogen, and leads to aggregation of the platelets. The aggregation response can be monitored in an aggregometer. Activated platelets secrete the contents of their intracellular granules, i.e. proteins and adenine nucleotides. Adenine nucleotides stored in the dense granules do not participate in cellular platelet metabolism, and are released into the extracellular medium. Activated platelets release from membrane phospholipids free arachidonic acid which is then metabolised via cyclooxygenase pathway to thromboxane A_2 .

(TXA₂) and malonyldialdehyde (MDA). In platelets both, TXA₂ and malonyldialdehyde are formed in approximately equimolar amounts, therefore MDA serves as a marker of arachidonate metabolism and TXA₂ synthesis in platelets. Platelet activation is accompanied by reorganisation of the cytoskeleton and a rapid increase of actin filament (F-actin) in platelets.

Many pharmacological agents counteract platelet activation (aggregation, secretion, actin polymerisation, arachidonate cascade) through interference with specific steps in the signal processing cascade. Cisplatin, a widely used chemotherapeutic agent effective against many human cancers, can affect platelet function. Cisplatin was found to inhibit platelet aggregation, secretion, arachidonate pathway, and to change the platelet cytoskeleton protein composition as analysed by polyacrylamide gel electrophoresis (Wachowicz and Olas 1995). This drug induced lipid peroxidation and free radicals generation in platelets (Wachowicz 1992).

Selenium compounds selectively reduce the side-effects of cisplatin without affecting its antitumor activity (Shenberg et al 1989, Baldew et al 1992, Vermeulen et al 1993). Our earlier results showed the protective effects of sodium selenite at nontoxic doses (0.1–1 $\mu\text{mol/l}$) against cisplatin-induced platelet lipid peroxidation and free oxygen radicals generation in these cells (Wachowicz and Szwarocka 1994). Sodium selenite represents an inorganic form of selenium that is widely used in the prevention and therapy of Se deficiency. However, there is a small difference between Se concentrations that are physiologically essential and those that are toxic to cells. In doses higher than 1 $\mu\text{mol/l}$ selenite causes various toxic effects in platelets (Zbikowska et al 1994) and the toxicity may be due to the formation of selenols, since selenite in the cells undergoes reductive metabolism. In the cell, selenite is metabolised to selenols via selenodiglutathione (GS₂SeSG). Both reactions proceed nonenzymatically if sufficient GSH is available. Selenol is sequentially methylated via methylselenol to dimethylselenide and trimethylselenonium (Baldew et al 1992). Selenite does not react with cisplatin (Baldew et al 1992, Beaty et al 1992) but methylselenol (CH₃SeH), a nucleophilic metabolite of selenite is able to form complex with cisplatin *in vitro*. The chemoprotective activities of Se compounds seem to be mainly attributed to the formation of selenols (Spallholz 1994). In our experiments, we used a nontoxic dose of selenite (1 $\mu\text{mol/l}$). The exposure of pig blood platelets to sodium selenite significantly decreased the level of both reduced glutathione and free -SH groups of platelet proteins in a time- and dose-dependent manner (Zbikowska et al 1994). Cisplatin, like selenite, quickly reacts with thiol groups in platelets; competition may exist between selenium compounds and cisplatin for sulfhydryl groups.

Our presented results indicate that selenite at nontoxic concentrations has protective effect against cisplatin-induced inhibition of platelet aggregation (Figs 1A and 1B). When an improved method was used in our experiments to determine the actin content, the levels of G-actin in platelets preincubated with selenite were

not changed, whereas preincubation of platelets with cisplatin caused a decrease of G-actin. The novel Se-Pt conjugate did not change the level of G-actin. Cisplatin interfering with cytoskeletal proteins, probably via -SH groups, seems to affect the state of platelet actin polymerisation (Fig. 3). We suggest an important role of -SH groups in the mechanism of cisplatin and selenite action. Contrary to Se or cisplatin action, the tested Se-Pt conjugate, did neither change endogenous arachidonate metabolism in platelets (Fig. 4). The predominating effect of cisplatin at the studied concentration (therapeutic dose) is the inhibition of platelet responses. Our data show (Figs. 1A, 1B, 2 and 4) that Se-Pt conjugate administered at the same concentration as cisplatin (20 $\mu\text{mol/l}$) had less toxic effects on ADP-induced platelet aggregation, adenine nucleotides release and endogenous arachidonate metabolism in platelets than cisplatin alone (Figs. 1A, 1B, 2 and 4). The differences in the actions of the studied compounds may be due to the different ways of their metabolism in the cell and to reaction with thiol groups. The novel Se-Pt seems to be potentially safe and nontoxic and thus a promising candidate for future evaluations of its chemopreventive and pharmacological activities.

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