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QUARTERLY

Protective action of vitamin C against DNA damage induced by selenium-cisplatin conjugate★✪

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Genotoxicity of anticancer drugs is of a special interest due to the risk of inducing secondary malignancies. Vitamin C (ascorbic acid) is a recognized antioxidant and, since human diet can be easily supplemented with vitamin C, it seems reasonable to check whether it can protect against DNA-damaging effects of antitumor drugs. In the present work the ability of vitamin C to modulate cytotoxic and genotoxic effects of a cisplatin analog, conjugate $(NH_3)_2Pt(SeO_3)$, in terms of cell viability, DNA damage and repair in human lymphocytes was examined using the trypan blue exclusion test and the alkaline comet assay, respectively. The conjugate evoked a concentration-dependent decrease in the cell viability, reaching nearly 50% at 250 μM. $(NH_3)_2Pt(SeO_3)$ at 1, 10 and 30 μM caused DNA strand breaks, measured as the increase in the comet tail moment of the lymphocytes. The treated cells were able to recover within a 30-min incubation in a drug-free medium at 37°C. Vitamin C at 10 and 50 μM diminished the extent of DNA damage evoked by $(NH_3)_2Pt(SeO_3)$ but had no effect on the kinetics of DNA repair. The vitamin did not directly inactivate the conjugate. Lymphocytes treated with endonuclease III, which recognises oxidised pyrimidines, displayed a greater tail moment than those untreated with the enzyme, suggesting that the damages induced by the drug have, at least in part, an oxidative origin. Vitamin C can be considered a potential protective agent against side effects of antitumor drugs, but further research with both normal and cancer cells are needed to clarify this point.

Genotoxicity of anticancer drugs belongs to their most serious side effects due to the possibility of inducing secondary malignancies [1]. Although "genotoxicity" is a useful term whose precise definition is elusive, there is no doubt that DNA damage plays a pivotal role in most mechanisms underlying genotoxicity. DNA damage induced by chemicals, including anticancer drugs, appears primarily in the form of alterations of the phosphate backbone, sugar or base modifications

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Abbreviations: cisplatin, cis-diamminedichloroplatinum; DAPI, 4′,6-diamidino-2-phenylindole; NaCl/P_i phsophate-buffered saline.

such as alkylations, cross-links, or formation of bulky DNA adducts, which are substrates for DNA repair mechanisms. Transient DNA breaks arise in the second step as a consequence of repair and can be considered important markers of genotoxicity [2].

Cis-diamminedichloroplatinum(II) (cisplatin) (Fig. 1A) is a potent anticancer drug widely used in clinical practice. Its therapeutic effect is attributed to formation of adducts with DNA [3]. Adducts that are not removed may block DNA

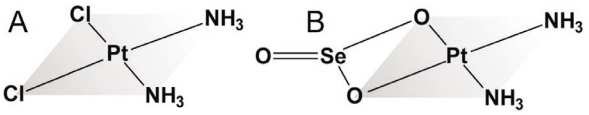

Figure 1. Structures of *cis*-diamminedichloroplatinum(II) (A) and $(NH_3)_2Pt(SeO_3)$ conjugate (B).

replication and transcription. It has also been demonstrated that cisplatin may inhibit the activity of telomerase, an enzyme playing a key role in acquisition by cancer cells of the ability to undergo unlimited cell division [4]. Although cisplatin has curative potential in patients with testicular, head and neck or ovarian cancer it displays also a number of physiological and toxic side effects, among which nephrotoxicity and genotoxicity are the most serious [1].

In order to diminish the unwanted side effects of cisplatin, its conjugates with substances of a known protective action have been synthesized. Selenium has anticarcinogenic activities, which were demonstrated in epidemiological studies, experimental research and clinical trials [5]. It was shown that administration of sodium selenite before cisplatin injection had a protective effect against the nephrotoxicity of the drug [6, 7]. A conjugate of selenite with diammineplatinum [$(NH_3)_2Pt(SeO_3)$] (Fig. 1B) is a novel potentially anticancer drug, which, to our knowledge, has not been extensively tested. It was reported to perturb blood platelets function [8] and we recently showed that it damaged DNA in normal human lymphocytes and could not be applied intravenously without special precautions [9, 10].

Vitamin C (ascorbic acid) is a water-soluble dietary antioxidant that plays an important role in controlling the oxidative stress [11]. It can also protect DNA against damages induced by various chemicals [12–14] but some reports indicate that vitamin C can be genotoxic [15–17]. Since human diet can be easily supplemented with vitamin C, it is reasonable to check whether it can offer protection against genotoxic effects induced by antitumor drugs.

In the present work the ability of vitamin C to modulate cytotoxic and genotoxic effects of the conjugate $(NH_3)_2$ $Pt(SeO_3)$ in terms of cell viability and DNA damage and repair in human lymphocytes was tested using trypan blue exclusion analysis and the alkaline comet assay, respectively.

MATERIALS AND METHODS

Chemicals. Sodium ascorbate, Tris, RPMI 1640 medium without glutamine, agarose, low melting point agarose, phosphate-buffered saline ($NaCl/P_i$) and DAPI (4′,6-diamidino-2-phenylindole) were obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.). The conjugate $(NH_3)_2Pt(SeO_3)$ was synthesized at the Institute of Pure Chemicals Lachema (Brno, Czech Republic) and was a gift from Dr. Vladimir Kleinwächter of Institute of Biophysics, Czech Academy of Sciences, Brno. Endonuclease III was a gift from Dr. Barbara Tudek of Institute of Biochemistry and Biophysics, Polish Academy of Sciences, Warsaw, Poland. Gradisol L was from Polfa (Kutno, Poland). All other chemicals were of analytical grade and were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.).

Lymphocyte isolation. Blood was obtained from young, healthy, non-smoking donors. Peripheral blood lymphocytes were isolated by centrifugation in a density gradient of Gradisol L (15 min, 280 × ***g***). The viability of the cells was measured by the trypan blue exclusion test and it was consistently found to be about 99%. Lymphocytes accounted for about 91% of leukocytes in the obtained cell suspension. The final concentration

of the lymphocytes was adjusted to 1–3 × 10^5 cells/ml by adding RPMI 1640 medium to the single cell suspension.

Lymphocyte treatment. $(NH_3)_2Pt(SeO_3)$ was dissolved in phosphate-buffered saline to a final concentration of 1 mM and incubated for 24 h at 37°C prior to use. Human peripheral blood lymphocytes were incubated in RPMI 1640 medium for 1 h at 37°C with an aliquot of stock drug solutions to give a desired final platinum concentration.

Assay of cell viability. This was determined by the trypan blue exclusion analysis. Cells were incubated for 2 h at 37°C with the platinum compound at concentrations of 1, 10, 100 or 250 μM with or without vitamin C at concentrations of 10 or 50 μM, washed and resuspended in RPMI 1640 medium. An equal volume of 0.4% trypan blue reagent was added to a cell suspension and the percentage of viable cells was evaluated under a field microscope. Assays were performed in triplicate.

Comet assay. This was performed under alkaline conditions essentially according to the procedure of Singh *et al.* [18] with some modifications [19]. A freshly prepared suspension of lymphocytes in 0.75% low melting point agarose dissolved in phosphate buffered saline was casted onto fully frosted microscope slides (Superior, Germany) precoated with 0.5% normal melting agarose. The cells were then lysed for 1 h at 4°C in a buffer consisting of 2.5 M NaCl, 100 mM EDTA, 1% Triton X-100, 10 mM Tris, pH 10. After the lysis the slides were placed in an electrophoresis unit, allowing DNA to unwind for 40 min in the electrophoretic buffer consisting of 300 mM NaOH, 1 mM EDTA, pH > 13. Electrophoresis was conducted at ambient temperature of 4°C (the temperature of the running buffer not exceeding 12°C) for 30 min at an electric field strength of 0.73 V/cm (30 mA). The slides were then neutralized with 0.4 M Tris, pH 7.5, stained with 2 μg/ml DAPI and covered with cover slips. To prevent additional DNA damage all the steps described above were conducted under a dimmed light or in the dark.

DNA repair. To examine DNA repair the cells after the treatment as well as control samples were washed and incubated in fresh, platinum-free RPMI 1640 medium for 1 h at 37°C. Aliquots of the suspension were taken immediately and at 10, 15, 30 and 60 min later. The repair incubation was stopped by placing the samples in an ice bath.

Inactivating potential of vitamin C against $(NH_3)_2Pt(SeO_3)$ conjugate. In order to check the possibility of chemical inactivation of the conjugate, two sets of experiments were performed. In the first set, the lymphocytes after incubation with vitamin C were washed before incubation with the conjugate. This procedure ensured the absence of free vitamin C during the incubation with the conjugate. In the second set, the conjugate was incubated either with buffer alone or with buffer containing vitamin C for 15, 30 and 60 min before being added to the cells. This procedure allowed checking for direct chemical inactivation of the conjugate.

Endonuclease III treatment. After lysis of the cells in 2.5 M NaCl, 0.1 M Na_2EDTA, 10 mM Tris/HCl, pH 10.0, 1% Triton X-100, for 1 h at 4°C, the slides were washed three times in endonuclease III buffer comprising 40 mM Hepes/KOH, 0.1 M KCl, 0.5 mM EDTA, 0.2 mg/ml bovine serum albumin, pH 8.0, and drained, and the agarose was covered with 25 μl of either buffer or endonuclease III at 1 μg/ml in buffer, sealed with a cover glass, and incubated for 30 min at 37°C [20]. Further steps were performed as described above.

Comet analysis. The objects were observed at 200× magnification in a Labophot-2 fluorescence microscope (Nikon, Japan) attached to a Pulnix video camera (Kinetic Imaging, Liverpool, U.K.) equipped with a UV-1A filter block (an excitation filter of 365/10 nm and a barrier filter of 435 nm) and connected to a personal computer-based image analysis system Comet v. 3.0 (Kinetic Imaging, Liverpool, U.K.). Fifty images were randomly selected from each sample and the comet tail moment (a product of fraction of DNA in tail and tail length) was measured. Two parallel tests with aliquots of the same sample of cells were performed for a total of 50 cells and the mean comet tail moment was calculated. The comet tail moment is positively correlated with the level of DNA breakage in a cell [18]. The mean value of

the tail moment in a particular sample was taken as an index of DNA damage in this sample.

Statistics. All the values in this study were expressed as means ± S.E.M. from two separate experiments. The data were analyzed using STATISTICA (StatSoft, Tulsa, OK, U.S.A.) statistical package. If no significant differences between variations were found, as assessed by Snedecor-Fisher test, the differences between means were evaluated by applying Student's *t*-test. Otherwise, Cochran-Cox test was used.

RESULTS

Cell viability. The results of cell viability assays after incubation of human lymphocytes with the conjugate $(NH_3)_2Pt(SeO_3)$ at different concentrations are displayed in Table 1. The conjugate at a concentration of 100 μM and higher evoked a dramatic decrease in lymphocyte viability – at 100 μM only about 68% of the cell were viable ($P < 0.05$), at 250 μM – less than 53% ($P < 0.001$). The viability of the platinum-treated cells was not changed by treatment with vitamin C at 10 or 50 μM concentration.

DNA damage. Figure 2 shows the mean comet tail moment of the lymphocytes exposed for 1 h to the $(NH_3)_2Pt(SeO_3)$ conjugate at 1, 10 and 30 μM in the absence or in the presence of vitamin C at 10 or 50 μM. The conjugate significantly increased the comet tail moment of the lymphocytes in a dose-dependent manner. At the highest concentration of the conjugate, 30 μM, the increase was almost 30 times as high as the control value (62.27 μm ± 7.51 μm *vs.* 2.11 μm ± 0.29 μm, $P < 0.001$). The increase in the tail moment induced by the conjugate can be considered evidence of its strand-breaking activity. Vitamin C at concentrations of 10 or 50 μM decreased, in a dose-dependent manner, the comet tail moment of the lymphocytes exposed to $(NH_3)_2Pt(SeO_3)$.

DNA repair. Figure 3 shows the comet tail moments of lymphocytes exposed to the conjugate $(NH_3)_2Pt(SeO_3)$ at 30 μM in the absence and presence of vitamin C at 10 and 50 μM immediately after the exposure as well as 10, 15, 30 and 60 min thereafter. The comet tail moment of the control lymphocytes was constant during the entire repair incubation time, indicating that preparation and subsequent processing of the lymphocytes did not introduce a significant damage to their DNA. The lymphocytes exposed to the conjugate either in the presence and in the absence of vitamin C were able to remove the DNA damage within 30 min.

Inactivating potential of vitamin C. Significant ($P < 0.05$) differences were observed between DNA-damaging effects of the $(NH_3)_2Pt(SeO_3)$ conjugate when this compound was applied without pre-treatment of the lymphocytes with vitamin C and after 30 min preincubation of the cells with the vitamin and subsequent washing (Fig. 4). This suggests that the protective action of vitamin C resulted from its interaction with DNA or the conjugate bound to DNA rather than from its direct interaction with the conjugate. To check further this hypothesis, the $(NH_3)_2Pt(SeO_3)$ conjugate at 30 μM was incubated with vitamin C at

Table 1. Influence of vitamin C on the effect of the conjugate $(NH_3)_2Pt(SeO_3)$ on viability of human lymphocytes measured by the trypan blue exclusion method

$(NH_3)_2Pt(SeO_3)$ concentration [μM]	Viability (%)		
	No vitamin C	10 μM vitamin C	50 μM vitamin C
0	97.2 ± 0.8	99.2 ± 0.6	98.4 ± 0.65
1	96.7 ± 0.7	96.6 ± 0.7	98.7 ± 0.3
10	94.2 ± 0.4	95.7 ± 0.3	92.0 ± 0.5
100	67.8 ± 1.0*	72.3 ± 1.2*	62.3 ± 1.2*
250	52.2 ± 3.2*	59.0 ± 0.5**	57.3 ± 1.4**

[a]Mean ± S.E.M. of three independent measurements; *$P < 0.05$; **$P < 0.001$ as compared with control

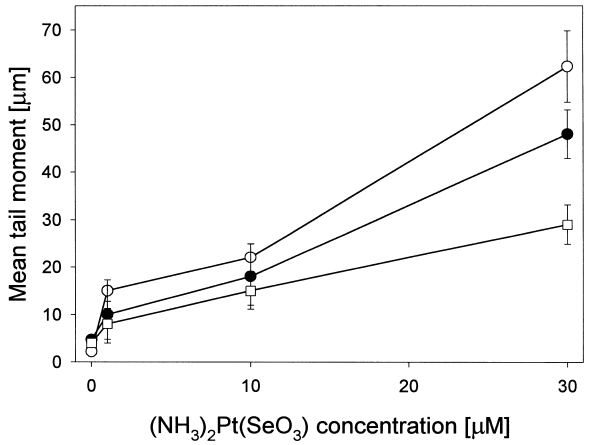

Figure 2. Mean comet tail moment of human lymphocytes exposed for 1 h at 37°C to $(NH_3)_2Pt(SeO_3)$ in the absence (○) or in the presence of vitamin C at 10 (●) and 50 μM (□) as compared with unexposed cells (■).

The number of cells scored for each treatment was 100. Error bars denote S.E.M.

10 μM for 15, 30 and 60 min. The results indicate that the conjugate remained stable during the applied preincubation times (Fig. 5). When preincubation was conducted with vitamin C, the extent of the DNA-damaging effect evoked by the conjugate was constant and independent of the length of incubation time ($P < 0.05$). This confirms the hypothesis that direct chemical inactivation of the $(NH_3)_2Pt(SeO_3)$ conjugate by vitamin

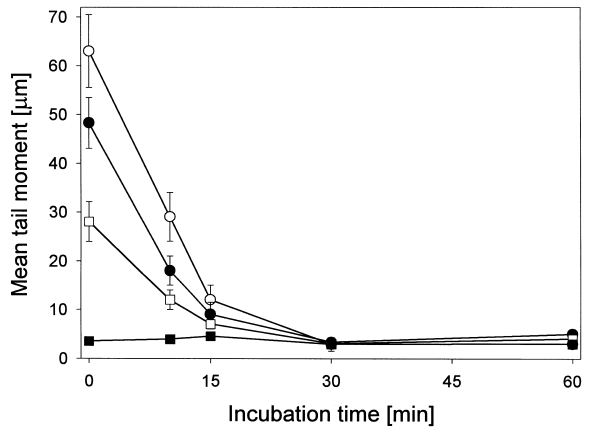

Figure 3. Time course of the repair of DNA damage in human lymphocytes exposed to $(NH_3)_2Pt(SeO_3)$ at 30 μM in the absence (○) or in the presence of vitamin C at 10 (●) and 50 μM (□) as compared with unexposed cells (■).

C did not contribute to the mechanism of protective action of vitamin C.

Endonuclease III treatment. Figure 6 presents the mean tail moment of the lymphocytes exposed for 1 h to $(NH_3)_2Pt(SeO_3)$ at 10 or 30 μM concentration followed by treatment with endonuclease III, compared with the cells untreated with the enzyme. It can be seen that lymphocytes treated with endonuclease III showed a greater tail moment than the untreated ones. This also concerned the control cells, but the difference between tail moments of the enzyme-treated

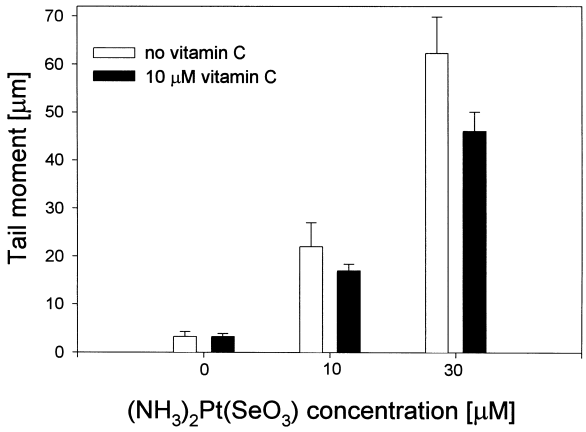

Figure 4. Mean comet tail moment of human lymphocytes exposed for 1 h at 37°C to the conjugate $(NH_3)_2Pt(SeO_3)$.

The cells were preincubated for 30 min with buffer alone (empty bars) or with buffer containing 10 μM vitamin C, which was washed out before incubation of the cells with the conjugate (filled bars).

and untreated cells increased with the increase of the conjugate concentration.

DISCUSSION

The results obtained indicate that the comet assay is a highly sensitive technique for analysis of DNA damage induced by platinum compounds used in chemotherapy. We have shown that it is suitable method for the detection of strand-breaking activity of the drugs. There is no evidence of biological action of the novel platinum antitumor drug, conjugate $(NH_3)_2Pt(SeO_3)$, except a report describing changes of blood platelet activation

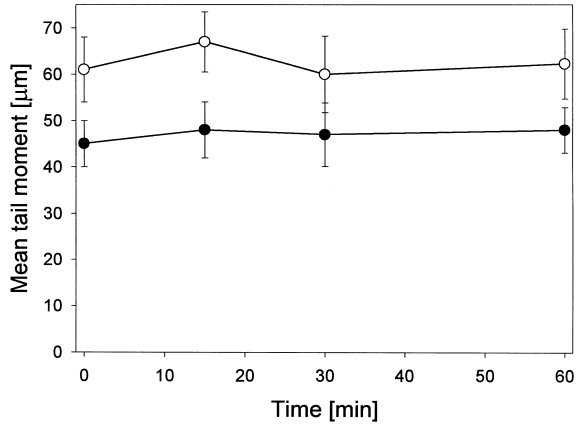

Figure 5. Mean tail moment of human lymphocytes exposed for 1 h at 37°C to the conjugate $(NH_3)_2Pt(SeO_3)$ at 30 μM after preincubation of the conjugate with buffer alone (○) or with buffer supplemented with 10 μM vitamin C (●) for the indicated lengths of time.

evoked by this compound [8] and our results on DNA damage [9, 10].

Although it is supposed that cisplatin activity is based on its cytotoxicity resulting from the interaction with DNA, several lines of evidence suggest that it may have some long-term side effects. Rats and mice develop leukaemia, skin tumors and other carcinomas after exposure to cisplatin [20, 21]. Studies with human lymphocytes provide evidence of chromosome breakage and rearrangements [22]. Moreover, the appearance of secondary malignancies in cancer patients treated with cisplatin suggested that these tumors might have been induced by the drug [23]. Genotoxicity can be considered as the origin of the antitumor activity of platinum compounds, but the genotoxic effect exerted by the drugs on normal cells may not surpass their effect on cancer cells. In search for new platinum compounds characterized by less severe side effects than those evoked by cisplatin complexes of platinum with substances, which could exert anti-genotoxic effects in normal cells were created.

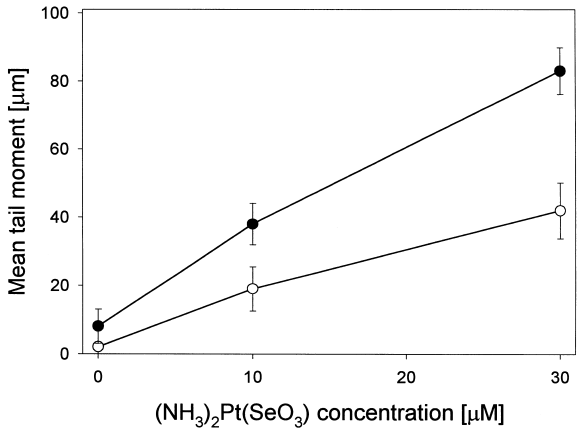

Figure 6. Mean tail moment of human lymphocytes exposed for 1 h at 37°C to conjugate $(NH_3)_2Pt(SeO_3)$ at indicated concentrations with (●) or without (○) subsequent treatment with endonuclease III at 1 μg/ml.

Selenium displays a protective action against a broad spectrum of toxic substances, including genotoxicants [5]. It was reported that selenite could reduce the nephrotoxicity of cisplatin without reducing its antitumor activity [24]. In the present work we have clearly shown that the conjugate $(NH_3)_2Pt(SeO_3)$ exerts a pronounced genotoxic effect on normal human peripheral blood lymphocytes. It seems therefore very doubtful whether it is advisable to use intravenously this drug in chemotherapy unless protective steps against its genotoxic action in normal cells are undertaken. The only reasonable direct application of this compound seems to be an injection to the tumor. We found that vitamin C at 10 and 50 μM partly protected DNA of normal human lymphocytes against strand breaks induced by $(NH_3)_2Pt(SeO_3)$ at high concentrations. We showed also that there was no direct chemical inactivation of the conjugate by the vitamin.

Protective action of vitamin C on normal cells raises a question whether the effect exerted by the vitamin would affect the genotoxic potential of the conjugate in cancer cells. This problem cannot be solved without performing experiments on cancer cells and their results may depend on the nature of particular cancer.

Oxidised bases in DNA can be probed by endonuclease III, as proposed by Collins *et al.* [25], because this enzyme nicks DNA at sites of oxidised pyrimidines [26]. Breaks can be detected by the alkaline comet assay. We showed that endonuclease III treatment of lymphocytes exposed to the conjugate evoked a pronounced increase in the tail moment of the cells. This increase might have been caused by the enzyme introducing DNA sin-

gle-strand breaks in the place of oxidised pyrimidines.

It was reported that the cisplatin-combination chemotherapy induced a fall in plasma antioxidant levels, that could reflect a failure of the antioxidant defense mechanism against oxidative damage induced by platinum-based anticancer drugs. This probably resulted from consumption of antioxidants caused by chemotherapy induced-oxidative stress as well as renal loss of water-soluble, small molecular mass antioxidants such as uric acid [27].

The presented results on the limited protective action of vitamin C should be taken into account when the conjugate is to be applied in chemotherapy, but further research is needed, especially with cancer cells, to clarify this point.

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