# Induction of DNA-Protein Cross-Links by Platinum Compounds

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The differences between *cis*- and *trans*-diamminedichloroplatinum II (DDP) in forming DNA-protein cross-links in isolated human lymphocytes were investigated. Both *cis*- and *trans*-DDP can induce DNA-protein cross-links. We show that *cis*-DDP forms complexes between DNA and proteins faster than *trans*-DDP. This results from an increase in the quantity of DNA and platinum together with an increase in drug concentration. Under the same conditions *trans*-DDP causes a decrease in DNA-forming complexes with proteins. After a 12 h incubation of lymphocytes we observe a similar level of DNA in DNA-protein cross-links induced by DDP isomers, but more platinum appears in complexes induced by *trans*-DDP. The results obtained demonstrate that the antitumor drug – *cis*-DDP and the clinically ineffective *trans*-DDP induce links between DNA and proteins in a different manner. We suggest that the therapeutic activity of *cis*-DDP can in part arise from rapidly forming DNA-protein complexes which can destroy the most important cellular processes, such as replication and transcription.

## Introduction

*Cis*-diamminedichloroplatinum(II) (*cis*-DDP) is one of the most often used anticancer drugs. It shows high efficiency in the presence of testicular and ovarian cancers particularly. It is generally accepted that the anticancer activity of *cis*-DDP results from interactions with DNA. The drug binds co-ordinately with N(7) of purine bases forming monoadducts, which transform into inter- and intrastrand cross-links. Finally, *cis*-DDP forms mainly 1,2-d(GpG) (65%) and 1,2-d(ApG) (25%) intrastrand cross-links and to a lesser degree 1,3-d(GpXpG) intrastrand cross-links and interstrand cross-links (Eastman, 1986). The *trans* isomer of diamminedichloroplatinum – *trans*-DDP is a clinically ineffective compound. *Trans*-DDP with DNA forms mainly monoadducts, interstrand cross-links and 1,3-d(GpXpG) intrastrand cross-links (Eastman and Barry, 1987; Boudvillain *et al.*, 1995).

Adducts formed by *cis*- and *trans*-DDP change DNA structure in a different way. Poklar *et al.* (1996) showed that intrastrand link between guanines changes the B conformation of the oligonucleotide into the A conformation and reduces the thermal stability of the DNA duplex. The 1,2-d(GpG) and 1,2-d(ApG) cross-links induced by *cis*-DDP bend double helical DNA by 32–35° in the direction of the major groove and cause its unwinding by 13° (Bellon *et al.*, 1991). Recent studies demonstrate the bending of the helix axle by 47° in double-stranded DNA decamer containing a single intrastrand cross-link induced by *cis*-DDP (Coste *et al.*, 1999). The intrastrand 1,3-d(GpXpG) cross-link induced by *trans*-DDP bends the DNA helix in more than one direction, forming a hinge joint (Bellon *et al.*, 1991). Alterations in DNA conformation induced by *cis*-DDP are recognized by cytoplasmic (Oliński *et al.*, 1987; Miller *et al.*, 1991) and nuclear proteins (Ferraro *et al.*, 1992; Yaneva *et al.*, 1997). Recent years have yielded many reports concerning DNA-protein cross-links participation in the biological activity of platinum compounds. HMG box proteins in this respect are investigated intensively. These proteins specifically recognize the intrastrand 1,2-d(GpG) and 1,2-d(ApG) cross-links induced by *cis*-DDP, but do not recognize links induced by clinically inactive *trans*-DDP (Pil and Lippard, 1992; Farid *et al.*, 1996; Ohndorf *et al.*, 1997; Trimmer *et al.*, 1998). HMG box proteins also specifically inhibit repair of 1,2-d(GpG) and 1,2-d(ApG) cross-links induced by *cis*-DDP (Zamble *et al.*, 1996). Probably, characteristic alterations in DNA conforma-

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tion arising after *cis*-DDP and resulting here from DNA-protein interactions are responsible for the anticancer activity of this compound.

The objective of this work was the analysis of the differences between *cis*- and *trans*-DDP in the induction of DNA-protein cross-links. In order to detect DNA-protein cross-links we used the method described by Costa *et al.* (1996). The assay is based upon the precipitation of free proteins and proteins bound with DNA in the presence of SDS and $K^+$ ions. It accepts that the percentage content of protein-precipitated DNA in relation to total DNA in the cell is a measure of the links induced between DNA and proteins. In DNA fractions, which were precipitated with proteins we measured platinum quantity by atomic absorption spectrometry (AAS).

## Materials and Methods

### *Chemicals*

*Cis*-DDP (*cis*-diamminedichloroplatinum), *trans*-DDP (*trans*-diamminedichloroplatinum), sodium dodecyl sulfate (SDS), potassium chloride, sodium chloride, sodium perchlorate, tris[hydroxymethyl]-aminomethane (Tris), diphenylamine, proteinase K, ribonuclease A, bovine serum albumin (BSA) and platinum atomic absorption standard solution were obtained from Sigma Chemical Co (St. Louis, MO, USA). All other chemicals were of the highest purity available.

### *Cells*

Lymphocytes were isolated from peripheral human blood according to the modified method of Böyum (1964). The blood was overlaid (1:1) on the mixture of 6% dextran dissolved in 0.9% NaCl and 75% uropolinum (82:18). The mixture was centrifuged at 1000×*g* for 1 h at 18 °C. The lymphocyte pellet was washed several times with 0.9% NaCl and then the pellet was suspended in sterile CPD solution (0.88 M trisodium citrate, 0.015 M citric acid, 0.139 M glucose, 0.158 M sodium phosphate, pH 5.63) with the addition of penicillin (100 units/ml) and streptomycin (100 μg/ml). Cells were counted in a Bürker chamber. The viability of the cells was measured by trypan blue exclusion assay.

### *Incubation conditions of lymphocytes with cis-DDP and trans-DDP*

Lymphocytes were incubated separately with *cis*- and *trans*-DDP for 2 h and 12 h at 37 °C. In this experiment an aqueous solution of platinum compounds at a basic concentration of 2 mg/ml was used. Cells were incubated with 0.1 mM, 0.5 mM and 1 mM *cis*-DDP or *trans*-DDP. Control experiments were performed by incubation of the cells without platinum compounds for 2 h and 12 h at 37 °C. All incubations were performed on a shaker in a dark place.

### *Preparation of DNA-protein cross-links*

DNA which participated in DNA-protein cross-links was isolated according to the method of Costa *et al.* (1996). Small modifications were introduced to the method. The lymphocyte pellet was washed twice with 0.9% NaCl after incubation with *cis*- and *trans*-DDP. Subsequently, cell samples were lysed in 0.5% SDS solution. DNA was sheared by passing of the cell lysates four times through a sterile aluminum needle fastened on a syringe. 0.5 ml of 100 mM KCl, 20 mM Tris-HCl, pH 7.5 was then added. The content was mixed by vortexing and the samples were then heated for 10 min at 65 °C. Samples were inverted and then placed on ice for 5 min to form potassium dodecyl sulfate precipitates. The precipitates were collected by centrifugation at 6000×*g* for 5 min at 4 °C. The supernatants were removed and the pellets resuspended in 100 mM KCl, 20 mM Tris-HCl, pH 7.5. After mixing the samples were again heated at 65°C for 10 min and the washing steps as well as the heating steps were repeated twice. Proteins bound with DNA were removed by proteinase K (0.2 mg/ml) in 100 mM KCl, 10 mM EDTA, 20 mM Tris-HCl, pH 7.5. Subsequently, in order to precisely remove proteins, a ¼ volume of 5 M sodium perchlorate and an equal volume of chloroform:isoamyl alcohol mixture (24:1) (v/v) were added (Johns and Paulus-Thomas, 1989). The samples were shaken for 30 min at room temperature and were then centrifuged at 10000×*g* for 10 min at 4 °C. An aqueous phase as added into 2 volumes of 97% ethanol. The DNA pellet was dissolved in TE buffer (1 mM EDTA, 10 mM Tris-HCl, pH 7.8). The concentration of DNA was measured by the colorimetric method with diphe-

nylamine (Burton, 1956) according to the modification of Gendimenico *et al.* (1988).

*Preparation of genomic DNA*

The total DNA of lymphocytes was isolated according to the method of Johns and Paulus-Thomas (1989) with small modifications. The lymphocyte pellet was washed twice with 0.9% NaCl after incubation with *cis*- and *trans*-DDP. Subsequently, cell samples were lysed in 0.5% SDS solution. An equal volume of 10 mM EDTA, 20 mM Tris-HCl, pH 7.5 was added to the cell lysate. Ribonuclease A was also added at a final concentration of 1 mg/ml. The mixture was incubated for 30 min at 37 °C and then for 10 min at 60 °C. Subsequently, proteinase K was added at a final concentration of 0.2 mg/ml. The mixture was again incubated at 37 °C for 1 h. In order to precisely remove the proteins, a ¼ volume of 5 M sodium perchlorate and an equal volume of chloroform: isoamyl alcohol mixture (24:1) (v/v) were added. The samples were shaken for 30 min at room temperature and were then centrifuged at $10000\times g$ for 10 min at 4 °C. An aqueous phase was added into 2 volumes of 97% ethanol. The DNA pellet was collected by centrifugation at $15000\times g$ for 20 min at 4 °C. DNA was dissolved in TE buffer (1 mM EDTA, 10 mM Tris-HCl, pH 7.8). The concentration of DNA was measured by a colorimetric method with diphenylamine (Burton, 1956) according to the modification of Gendimenico *et al.* (1988).

*Measurement of platinum quantity*

The platinum quantity was assessed by atomic absorption spectrometry in DNA fractions, which were precipitated with proteins. Measurements were made in graphite tubs by Varian Spectra A 300/400. DNA samples were mineralised with $HNO_3$ for 1 h at 100 °C. The calibration curves at a range of 25–400 ng/ml were made using platinum atomic absorption standard solution.

*Statistical analysis*

Each value shown in the figures represents the mean ± SD for three determinations of DNA content and for five determinations of platinum quantity. Results were analysed using Fisher-Snedecor, *t*-Student and Cochran-Cox tests. The values from treated cells that differ by at least $p<0.05$ from the mean of untreated values were accepted to be statistically significant.

## Results

The viability of lymphocytes decreases after incubation with *cis*-DDP and *trans*-DDP (data not shown). The lowest viability of lymphocytes – about 75% ($p<0.05$) we observed after incubation with 1 mM *cis*-DDP for 12 h.

After incubation of lymphocytes for 2 h with *cis*-DDP at concentrations of 0.5 and 1 mM we observed statistically significant increases ($p<0.05$ and $p<0.02$ respectively) of DNA content in fractions precipitated with proteins (Fig. 1A). The participation of platinum in these complexes increases drug-dose dependently reaching a maximum of 2.45 ± 0.33 ng Pt/μg DNA (Fig. 1A). This result shows that *cis*-DDP can induce DNA-protein cross-links. Under the same conditions *trans*-DDP causes a decrease of DNA complexes with proteins (Fig. 1B). The DNA content amounts to about 2.5% after cell incubation for 2 h with *trans*-DDP at all used concentrations. In control cells this content is equal to 4.33 ± 0.89%. The quantity of platinum in links induced by *trans*-DDP is lower than with *cis*-DDP and it reaches a maximum of 1.43 ± 0.51 ng Pt/μg DNA for 1 mM *trans*-DDP (Fig. 1B).

After lymphocyte incubation for 12 h with *cis*- and *trans*-DDP we observed statistically significant ($p<0.05$) increases of DNA complexes with proteins only in the samples in which we used the highest concentration, 1 mM (Fig. 1C and D). Under these conditions *cis*- and *trans*-DDP induce DNA-protein cross-links at a similar level. 4% of DNA participates in these complexes. We showed an increase of platinum quantity bound to DNA after incubation of lymphocytes with *cis* and *trans*-DDP for 12 h in comparison with shorter incubation periods (Fig. 1C and D). The highest platinum quantity equalled 16.75 ± 0.14 ng Pt/μg DNA we observed in lymphocytes which were incubated with anticancer ineffective DDP isomer (0.5 mM *trans*-DDP) (Fig. 1D).

## Discussion

DNA-protein cross-links form under the influence of many different agents, for example: metals

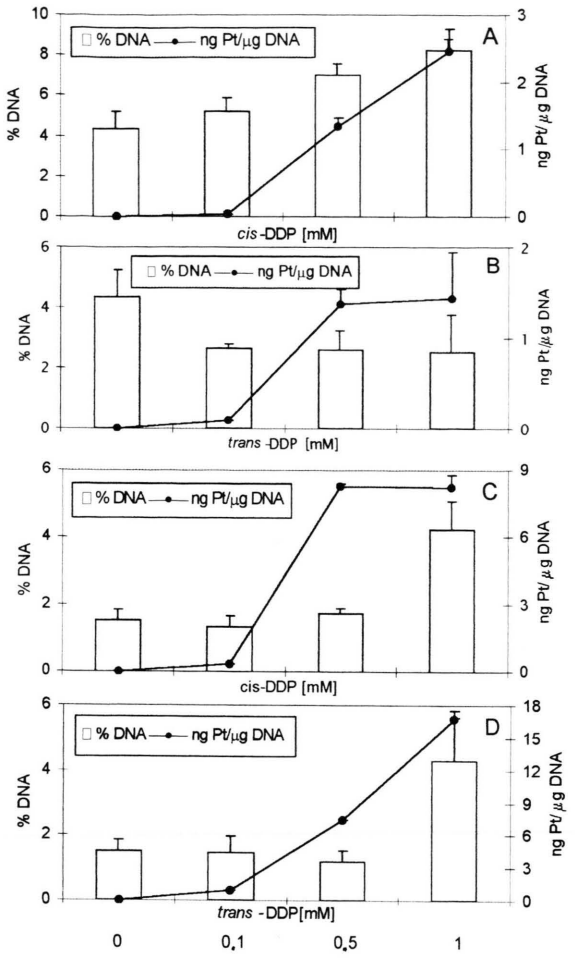


Fig. 1. The participation of platinum and DNA in DNA-protein cross-links induced by *cis*- and *trans*-diammine-dichloroplatinum II (DDP).
A. After incubation for 2 h.
B. After incubation for 2 h.
C. After incubation for 12 h.
D. After incubation for 12 h.

(Altman *et al.*, 1995; Kuykendall *et al.*, 1996) pyrrolizidine alkaloids (Coulombe *et al.*, 1999), formaldehyde (Shaham *et al.*, 1996) and γ irradiation (Balasubramaniam and Oleinick, 1995). Numerous reports have shown that the anticancer drug – *cis*-DDP and clinically inactive compound – *trans*-DDP also induce DNA-protein complexes (Oliński *et al.*, 1987; Kašpárková and Brabec (1995). In this work we study the influence of DDP isomers on the formation of DNA-protein cross-links in lymphocytes isolated from human peripheral blood. We based identification of these complexes on the participation of DNA and platinum in them.

The results obtained in this work show that *cis*- and *trans*-DDP can induce DNA-protein cross-links in different manners. *Cis*-DDP has already produced links between DNA and proteins in 2 h incubation of lymphocytes. It results from increasing DNA and metal quantity together with increasing drug concentration (Fig. 1A). *Trans*-DDP causes a decrease of DNA-protein links after incubation of lymphocytes for 2 h. The quantity of DNA in control cells is less than in cells incubated with *trans*-DDP (Fig. 1B). Platinum quantity in links induced by *trans*-DDP is less than in links induced by *cis*-DDP. Perhaps it results from the fact that *trans*-DDP reaches the cell nucleus slower than *cis*-DDP because it easily reacts with proteins and other molecules (Uchida *et al.*, 1986). Interactions of the ineffective DDP isomer with molecules other than nuclear DNA can on the one hand prevent binding of *trans*-DDP with DNA. On the other hand interactions between *trans*-DDP and proteins in cytoplasm can lead to the destruction of natural links among nucleic acid and proteins.

The incubation of lymphocytes for 12 h causes the formation of DNA-protein cross-links at a similar level for both 1 mM *cis*-DDP and 1 mM *trans*-DDP (Fig. 1A and B). But more platinum appears in complexes induced by *trans*-DDP (Fig. 1D).

The results obtained in this work might suggest that the antitumor activity of *cis*-DDP in part can arise from interactions between DNA damaged by platinum and proteins. Adducts of *cis*-DDP are recognized by many different proteins. Among proteins bound with DNA after treatment of *cis*-DDP are cytoskeleton proteins: actin (Miller *et al.*, 1991), cytokeratins 39 kDa, 49 kDa and 56 kDa (Oliński *et al.*, 1987) and nuclear matrix proteins (Miller *et al.*, 1991; Ferraro *et al.*, 1992). Perhaps the structural and functional changes of these proteins and alterations of protein location shown after treatment with *cis*-DDP can be one of the reasons for the drug's cytotoxicity.

From the viewpoint of antitumor activity the most interesting finding are reports about specific interactions of *cis*-DDP adduct with nuclear proteins. It has been shown that some transcription factors containing HMG domains recognized DNA damage induced by *cis*-DDP. These are

mitochondrial transcription factor – mtTF (Huang *et al.*, 1994) and upstream binding factor of RNA polymerase I (Chao *et al.*, 1996; Jordan and Carmo-Fonseca, 1998). UBF changes position under the influence of *cis*-DDP. It penetrates from the nucleolus to nucleoplasm and reacts with *cis*-DDP adduct there (Chao *et al.*, 1996). Therefore the *cis*-DDP adducts compete for UBF binding with gene promotors of rRNA. Behind the change of UBF location in HeLa cells, observed alterations were made in the position of other proteins participating in transcription of rDNA, which were RNA polymerase I, TATA-binding protein – TBP and TBP-associate factors for RNA polymerase I – $TAF_{IS}$ (Jordan and Carmo-Fonseca, 1998). *Cis*-DDP inhibits RNA synthesis by RNA polymerase II (Vichi *et al.*, 1997). Inhibition of activity of RNA polymerase II results from selective binding of TBP with damaged DNA by the drug.

*Cis*-DDP changes DNA-protein interactions. The cytotoxic effects of this compound may be the consequence of the speed of formation of links between DNA and proteins. Intensive studies are necessary to explain the proteins which bind to DNA-Pt adducts. It is hoped that this research will contribute to the understanding of the mechanisms of *cis*-DDP activity.


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