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# Antibodies against cisplatin-modified DNA and cisplatin-modified (di)nucleotides

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**Summary.** Cytotoxic effects of *cis*-diamminedichloroplatinum-(II) (*cis*-DDP) are thought to be mediated by binding to DNA. Studies on binding of *cis*-DDP to cellular DNA rely heavily on the availability of specific antibodies. We therefore raised and characterized four rabbit antisera: one against *cis*-DDP-modified DNA (antiserum NKI-A59) and three others against the *cis*-DDP-modified (di)nucleotides *cis*-$Pt(NH_3)_2$d(pApG) (NKI-A68), *cis*-$Pt(NH_3)_2$d$(GMP)_2$ (NKI-A10), and $Pt(NH_3)_3$dGMP (NKI-A39). Reactivities to platinum compounds were determined in an enzyme-linked immunosorbent assay (ELISA) and in a quantitative immunocytochemical assay. In the ELISA, NKI-A59 showed a high affinity for DNA heavily substituted with either *cis*-DDP or CBDCA [*cis*-diammine(1,1-cyclobutanedicarboxylato)platinum(II)]; amounts of platinum per well giving 50% inhibition ($IA_{50}$) were as low as 15 and 76 fmol, respectively. NKI-A59 also showed affinity to *cis*-DDP-modified poly[d(G-C)].poly[d(G-C)], poly(dC), and poly(dG). No affinity was found for *trans*-DDP [*trans*-diamminedichloro-platinum(II)]-modified DNA, enzymatically digested *cis*-DDP-DNA, or *cis*-DDP-modified poly(dA).poly(dT), oligo(dA)$\overline{15}$.oligo(dT)$\overline{15}$, oligo$(dG)_{21}$, oligo$(dG)_{42}$, or oligo$(dAAAG)_{10}$. The efficiency of binding to *cis*-DDP-DNA decreased with decreasing DNA modification levels. Although other *cis*-DDP-DNA- and *cis*-DDP-(di)nucleotide-specific antisera have been identified, NKI-A59 is the first antiserum described that is suitable for the in situ detection of *cis*-DDP-DNA adducts at clinically relevant platinum levels. Adduct-specific immunostaining signals in cultured RIF-1 cells or rat liver paralleled platinum-DNA binding as measured by atomic absorption spectroscopy. The antisera NKI-A68, NKI-A10, and NKI-A39 showed high affinity for their corresponding haptens and varying affinity for non-hapten *cis*-DDP-DNA adducts. Their affinity for digested *cis*-DDP-modified DNA was up to 30 times that for intact *cis*-DDP-DNA. Neither NKI-A68 nor NKI-A10 resulted in specific immunocytochemical staining of *cis*-DDP-DNA adducts. We conclude that NKI-A68, NKI-A10, and NKI-A39 are suitable for platinum-DNA adduct analysis of digested DNA in ELISA and that NKI-A59 is suitable for platinum-DNA adduct detection at the single-cell level using immunocytochemical methods.

## Introduction

*cis*-Diamminedichloroplatinum(II) (*cis*-DDP) is thought to be active through its covalent binding to nuclear DNA, which results in the inhibition of DNA replication [29, 44]. Interaction products of *cis*-DDP with DNA include a monofunctional adduct to guanine; bifunctional intrastrand adducts to either a pGpG, a pGpXpG (whereby X can represent any nucleoside), or a pApG sequence; bifunctional interstrand adducts between guanines on complementary strands; and cross-links between DNA and a protein or glutathione [3, 6, 17, 31, 44]. In vitro, *cis*-DDP can also react with cytosine or, to a much lower extent, with thymine, although these interactions do not occur in DNA [9, 12]. Although >90% of all adducts in DNA are intrastrand adducts, it is as yet unclear which adducts are responsible for the cytotoxic action of *cis*-DDP [28].

Recently, polyclonal and monoclonal antibodies raised against *cis*-DDP-modified DNA [19, 24, 33, 34] or *cis*-DDP-modified (di)nucleotides [5, 7] have been introduced

*Abbreviations:* *cis*-DDP, *cis*-diamminedichloroplatinum(II); *trans*-DDP, *trans*-diamminedichloroplatinum(II); CBDCA, *cis*-diammine (1,1-cyclobutanedicarboxylato)platinum(II); DACH-PT, [*sp*-4-2-(1R,2R)]-(1,2-diaminecyclohexane)dichloroplatinum(II); Pt-GG, *cis*-$Pt(NH_3)_2$[d(pGpG)-$N_7$(1),$N_7$(2)]; Pt-AG, *cis*-$Pt(NH_3)_2$[d(pApG)-$N_7$(1),$N_7$(2)]; G-Pt-G, *cis*-$Pt(NH_3)_2$ [5′-dGMP-$N_7$]$_2$; Pt-G, $Pt(NH_3)_3$-[5′-dGMP-$N_7$]; CT-DNA, calf thymus DNA; $R_b$, platinum-nucleotide ratio; bp, base pairs; $IA_{50}$, amount of inhibitor per microtiter well giving 50% inhibition in a competitive ELISA; BSA, bovine serum albumin; ELISA, enzyme-linked immunosorbent assay; HPLC, high-pressure liquid chromatography

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to assess *cis*-DDP-DNA interaction products by radioimmunoassay [19], enzyme-linked immunosorbent assay (ELISA) [5, 7, 24, 33] and immunocytochemistry [34–39]. The combination of chromatography and ELISA discriminates individual *cis*-DDP-DNA interaction products in DNA isolated from experimental samples as well as those from patients. However, repeat measurements in individual patients is hampered by the need for relatively large samples, e.g., 20–40 ml blood. Quantitative immunocytochemical analysis requires only a few thousand cells and enables the discrimination of different cell types but not of different DNA adducts. ELISA and immunocytochemistry are therefore complementary, and both assays might be helpful in elucidating the mechanisms of platinum resistance in experimental systems and in cancer patients.

Poirier et al. [25] and Reed and co-workers [27] have used an antiserum elicited to *cis*-DDP-modified DNA to study the extent of adduct formation in *cis*-DDP-treated cancer patients. These studies showed some correlation between the level of in vivo *cis*-DDP-DNA binding in leukocytes (as assessed by ELISA) and the reduction in tumour mass. We have previously reported on the formation and stability of *cis*-DDP- and CBDCA-DNA adducts in solution, cultured cells, animals, and cancer patients [34–39]. In these immunocytochemical studies we used the antiserum NKI-A59, raised against *cis*-DDP-modified DNA. Binding of NKI-A59 to DNA of *cis*-DDP-exposed cell lines correlated with in vitro cell killing [38]. Recently, we observed that mean carboplatin-DNA adduct levels in buccal cells were higher among responders to platinum-based therapy than in patients showing progressive disease [39]. These results illustrate the potential usefulness of *cis*-DDP-DNA adduct analysis in the clinic.

This paper describes the further characterization of anti-*cis*-DDP-DNA antiserum NKI-A59 and the development of new antisera against *cis*-DDP-(di)nucleotide adducts. NKI-A59 is the first antiserum described that can detect *cis*-DDP-modified DNA in single cells at clinically relevant levels. The antisera NKI-A10, NKI-A39, and NKI-A68, raised against (di)nucleotide adducts, enable the quantitative analysis of all four major *cis*-DDP-DNA adducts. The results are compared with data on previously described antibodies raised against *cis*-DDP-modified DNA or (di)nucleotides.

## Materials and methods

*Animals and treatment.* Inbred male Sprague-Dawley rats (125–130 g) from the breeding colony of the Netherlands Cancer Institute received a single dose of 5, 10, or 15 mg/kg *cis*-DDP (Bristol-Myers, Weesp, The Netherlands) by i.p. injection. One control rat was injected with solvent only (1 ml 0.14 M NaCl). After 4 h, rats were killed by decapitation. Livers were dissected, frozen on dry ice, and stored at −80° C. One part of the liver was used for DNA isolation and the other was taken for cryostat sections [34].

*Cultured cells.* Murine RIF-1 cells (provided by Dr. R. F. Kallman, Stanford University, Calif.) were grown and maintained according to Twentyman et al. [41]; cells were seeded into 25-cm$^2$ flasks and were treated 2–4 days later while in the logarithmic growth phase. After treatment for 1 h with 0–276 μM *cis*-DDP, cells were washed twice and cytospin preparations were made [36].

*Isolation of DNA and atomic absorption spectroscopy.* DNA was isolated as previously described [7, 13] and hydrolyzed in 1 *N* HCl for 1 h at 70° C. Platinum was determined in a Spectra Zeeman 30 flameless atomic absorption spectrometer (Varian, Zug, Switzerland).

*Synthesis of platinum (di)nucleotides and immunogens.* The formation of the immunogen for the NKI-A59 antiserum, including the reaction of *cis*-DDP with calf thymus (CT)-DNA (resulting in a drug/nucleotide ratio, $R_b$, of $8 \times 10^{-2}$), and the formation of a complex with methylated bovine serum albumin have been reported elsewhere [34]. The dinucleotides d(pApG) and d(pGpG) were synthesized via an improved phosphotriester method [42] and were used as disodium salts. *cis*-$PtCl_2(NH_3)_2$ and $[PtCl(NH_3)_3]$ Cl were prepared from $K_2PtCl_4$ [22, 43]. 5′-dGMP was obtained from Sigma (St. Louis, Mo.) and was used without further purification. *cis*-$Pt(NH_3)_2$[d(pGpG)-$N_7$(1),$N_7$(2)] (Pt-GG), *cis*-$Pt(NH_3)_2$[d(pApG)-$N_7$(1),$N_7$(2)] (Pt-AG), *cis*-$Pt(NH_3)_2$[5′-dGMP-$N_7$]$_2$ (G-Pt-G), and $Pt(NH_3)_3$[5′-dGMP-$N_7$] (Pt-G) were prepared according to previously described methods [2, 4, 10, 20]. The platinum compounds were characterized by $^1$H nuclear magnetic resonance (NMR). The spectra were recorded on a Bruker WM 300 spectrometer and proved to be identical to those reported previously [10]. Covalent coupling of the haptens Pt-AG, G-Pt-G, and Pt-G to the carrier bovine serum albumin (BSA; Sigma) by 1-ethyl-3-(3-dimethyl-2-aminopropyl) carbodiimide-HCl (Sigma) was essentially performed according to the method of Halioran and Parker [11].

*Antisera to* cis-*DDP-DNA or* cis-*DDP-(di)nucleotides.* The preparation of the rabbit anti-*cis*-DDP-DNA antiserum (NKI-A59) has been reported elsewhere [34]. The same protocol was used to immunize rabbits with the BSA conjugates of Pt-AG, G-Pt-G, and Pt-G. Pt-GG was used only as an HPLC marker and inhibitor in the ELISA. Antisera were used without further purification.

*Quantitative immunocytochemical assay.* Procedures for this assay have previously been described [34, 36–38]. It is emphasized that the protocol for cryostat sections [34, 37] differs from that for single cells [36, 38]. NKI-A59 was used at a dilution of 1 : 1,800; NKI-A68, NKI-A10, and NKI-A39 were tested at dilutions between 0 and 2,000. The adduct-specific nuclear staining density, i.e., the integrated optical density per nucleus, was assessed by microdensitometry scanning of 30–40 nuclei/sample and was expressed in arbitrary units [32]. All samples were stained in two independent tests.

*Reaction with oligo- and polynucleotides.* The following oligo- and polynucleotides were reacted with platinum drugs: highly polymerized CT-DNA type I (Boehringer); poly[d(G-C)].poly[d(G-C)] (500–1,500 bp; Boehringer); poly(dG).poly(dC) ($5 \times 10^4$ bp; Boehringer); poly(dG) (average $n = 750$; Pharmacia); poly(dC) (average $n = 350$; Pharmacia); oligo(dA)$_{\overline{15}}$·oligo(dT)$_{\overline{15}}$ (PL Biochemicals, Milwaukee, Wis.); poly(dA).poly(dT) ($10^5$ bp; Boehringer); oligo(dG)$_{21}$, oligo(dG)$_{42}$, and [d(AAAG)]$_{10}$. CT-DNA was reacted with the following platinum drugs: *cis*-DDP (Ventron, Karlsruhe, FRG), *trans*-DDP (provided by Dr. J. Reedijk, State University of Leiden, The Netherlands), and CBDCA (Bristol-Myers; 150 mg CBDCA and 150 mg mannitol/vial). CT-DNA was pretreated with deoxyribonuclease S1 (Sigma) for 3 h at 37° C, precipitated with an equal volume of 100% ethanol, washed twice with 75% ethanol containing 2% NaOAc, washed once with 95% ethanol, and dried under vacuum.

The final concentration of platinum drugs [dissolved in phosphate buffer (0.2 mM $NaH_2PO_4$, 0.8 mM$Na_2HPO_4$, 3 mM NaCl; pH 7.4)], CT-DNA [dissolved in phosphate-EDTA buffer (0.2 mM $NaH_2PO_4$, 0.8 mM $Na_2HPO_4$, 5 mM NaCl, 0.1 mM EDTA; pH 7.4)], and poly- or oligonucleotides [dissolved in TRIS-EDTA buffer (25 mM TRIS-HCl, 0.1 mM EDTA; pH 7.8)] in the reaction mixture (total volume, 1 ml) depended on the desired modification level. For example, a 100-fold molar excess of DNA-P relative to platinum was required to obtain an $R_b$ of $8 \times 10^{-3}$. The mixture was incubated for 18 h at 37° C in a dark environment and then dialyzed in a dark environment for 4 days at 4° C against phosphate-

**Table 1.** Recognition of platinum-modified poly- and oligonucleotides by antiserum NKI-A59 as illustrated by the $IA_{50}$ value

| Poly- or oligonucleotide | Size[a] | $R_b$ value | $IA_{50}$ (fmol DNA-P/well) |
|---|---|---|---|
| DNA | Intact | 0 | >>3,000 |
| DNA[b] | Intact | 0 | >>3,000 |
| *cis*-DDP-DNA | Digest | $8.9 \times 10^{-2}$ | >>1,000 |
| *trans*-DDP-DNA | Intact | $3.7 \times 10^{-2}$ | >>2,000 |
| CBDCA-DNA | Intact | $4.6 \times 10^{-3}$ | 76 (3; 39–147)[c] |
| *cis*-DDP-DNA[b] | Intact | $8.9 \times 10^{-2}$ | 26 (1) |
| *cis*-DDP-DNA | Intact | $8.9 \times 10^{-2}$ | 13 (21; 6–30)[d] |
| *cis*-DDP-DNA | Intact | $8.0 \times 10^{-3}$ | 22 (4; 8–46) |
| *cis*-DDP-DNA | Intact | $8.0 \times 10^{-4}$ | 106 (4; 80–130) |
| *cis*-DDP-DNA | Intact | $1.6 \times 10^{-5}$ | 2,700 (1) |
| *cis*-DDP-DNA | Intact | $4.0 \times 10^{-7}$ | $10^5$ (1) |
| *cis*-DDP-poly[d(G-C)].poly[d(G-C)][b] | 500–1,500 | $6.5 \times 10^{-2}$ | 43 (3; 20–63) |
| *cis*-DDP-poly[d(G-C)].poly[d(G-C)] | 500–1,500 | $6.5 \times 10^{-2}$ | 5 (1) |
| *cis*-DDP-poly(dG).poly(dC)[b] | $5 \times 10^4$ | $3.7 \times 10^{-2}$ | 174 (3; 63–215) |
| *cis*-DDP-poly(dG).poly(dC) | $5 \times 10^4$ | $3.7 \times 10^{-2}$ | 63 (1) |
| *cis*-DDP-poly(dG) | 750 | $4.9 \times 10^{-2}$ | 320 (3; 180–646) |
| *cis*-DDP-poly(dC) | 150 | $3.6 \times 10^{-4}$ | 46 (2; 18–73) |
| *cis*-DDP-oligo(dA)$_{\overline{15}}$.oligo(dT)$_{\overline{15}}$ | 12–18 | $6.7 \times 10^{-3}$ | >>3,000 (2) |
| *cis*-DDP-poly(dA).poly(dT) | $10^5$ | $3.4 \times 10^{-3}$ | >>6,000 (1) |
| *cis*-DDP-oligo$(dG)_{21}$ | 21 | $6.0 \times 10^{-2}$ | >>1,000 (1) |
| *cis*-DDP-oligo$(dG)_{42}$ | 42 | $5.4 \times 10^{-2}$ | >>1,000 (1) |
| *cis*-DDP-$[d(AAAG)]_{10}$ | 40 | $4.3 \times 10^{-2}$ | >>1,000 (1) |

[a] Number of base pairs; for single-stranded DNA, number of nucleotides

[b] Native DNA; all other double-strand DNA was denatured

[c] Number of assays; range

[d] SD ± 6

$R_b$, Platinum-nucleotide ratio; >>, no inhibition observed at the highest amount tested; figures in parentheses represent the number of assays and range

EDTA buffer to remove unreacted drug and mannitol. All products were analyzed without enzymatic breakdown to (di)nucleotides unless stated otherwise. Enzymatic digestion and HPLC chromatography on Mono Q HR5/5 (Pharmacia) were performed as described elsewhere [6].

*Competitive ELISA.* Irradiated (5 Mrad) 96-well flat-bottom microtiter plates (Greiner, FRG) were coated with a fixed amount of *cis*-DDP-modified, heat-denatured CT-DNA ($R_b$, $8.9 \times 10^{-2}$; 7.3 pmol DNA-P, 0.65 pmol platinum/well; control wells contained unmodified CT-DNA only) by overnight evaporation at 40° C of 50 µl DNA solution in phosphate buffer (10 mM $KH_2PO_4$, 140 mM NaCl; pH 7.4). Plates were washed five times with wash buffer [10 mM $KH_2PO_4$, 140 mM NaCl, 0.05% Tween-20 (Serva, Heidelberg, FRG), 0.1% gelatin (BDH; pH 7.4)]. Double-strand DNA samples were denatured (10 min at 100° C) and quickly cooled on ice unless stated otherwise. For calculation of inhibition curves, 250 µl solution containing 0.75–750 fmol platinum and 250 µl diluted antiserum [NKI-A59, 80,000x; NKI-A68 and NKI-A10, 10,000x; NKI-A39, 1,000x; all diluted in 10 mM $KH_2PO_4$, 140 mM NaCl, 0.05% Tween-20, 2% BSA (Sigma); pH 7.4] were mixed and incubated for 90 min at 37° C. Microtiter wells were preincubated with 200 µl 1:200 (v/v) non-immune sheep IgG (National Institute of Public Health and Environmental Hygiene, The Netherlands; 14 µg IgG/well) for 1 h at 37° C and then washed five times with wash buffer.

Preincubated samples (100 µl/well, in quadruplicate) were added in a randomized way to the microtiter wells, incubated for 90 min on ice, and washed five times with 300 µl wash buffer. Then, 100 µl goat anti-rabbit Ig-alkaline phosphatase conjugate (Sigma; 1:4,000 dilution in wash buffer containing 1% BSA) was added to each well, incubated for 1 h at 37° C, and washed five times with 300 µl wash buffer and three times with 300 µl 100 mM diethanolamine (pH 9.8). A 100 µl solution containing 0.02 mM 4-methylumbelliferyl phosphate dilithium salt (Boehringer) in 10 mM diethanolamine, 0.1 mM $MgCl_2$, and 0.3 mM $NaN_3$ (pH 9.8) was added to each well. After incubation for 16–20 h at 20° C in a dark environment, fluorescence values were measured using a microfluor reader (Dynatech, Alexandria, Va.).

## Results

### *Anti-cis-DDP-DNA antiserum NKI-A59*

*Recognition of free platinum compounds and modified DNA.* The free drugs *cis*-DDP (2 pmol/well), *trans*-DDP (3 pmol/well), and CBDCA (10 pmol/well) as well as both native (2 pmol DNA-P/well) and denatured (3 pmol DNA-P/well) unmodified DNA could not be recognized by NKI-A59 in a competitive ELISA. In contrast, NKI-A59 showed a high affinity for highly *cis*-DDP-modified, undigested DNA, as indicated by the low $IA_{50}$ values obtained (Table 1). NKI-A59 showed no affinity for undigested *trans*-DDP-modified DNA or for *cis*-DDP-modified DNA digested to nucleotides (Table 1), indicating that this antiserum is unsuitable for testing hydrolyzed DNA in the ELISA. The lack of affinity for *trans*-DDP-DNA indicates that NKI-A59 is specific for adducts of *cis*-isomers.

*Modification-dependent affinity.* The binding efficiency of NKI-A59 for competitor *cis*-DDP-modified CT-DNA in the ELISA decreased with decreasing $R_b$ values for the competitor, as shown by the increasing $IA_{50}$ values achieved (Table 1; Fig. 1).

*Recognition of cis-DDP-modified poly- and oligonucleotides.* To define more closely the antigenic determinants of the NKI-A59 antiserum, a series of platinum-modified CT-DNAs as well as poly- and oligonucleotides were tested in the competitive ELISA. NKI-A59 showed affinity for *cis*-DDP-modified poly[d(G-C)].poly[d(G-C)], poly(dC), poly(dG).poly(dC), and poly(dG) (Table 1).

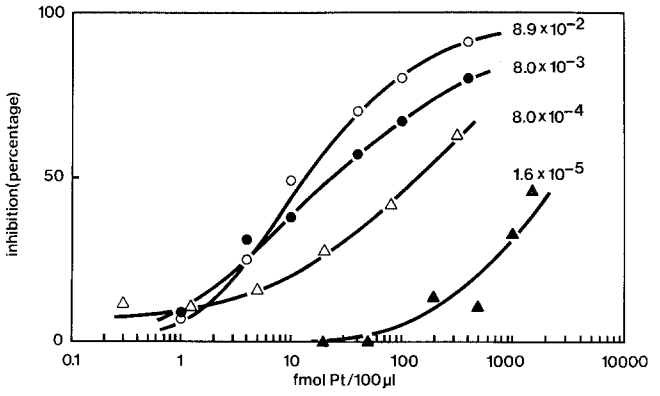


**Fig. 1.** Competitive inhibition in an ELISA of NKI-A59 binding to coated *cis*-DDP-modified DNA ($R_b$, $8.9 \times 10^{-2}$) by competitor *cis*-DDP-DNAs with different modification levels. Inhibition is expressed as the percentage of antibody binding in the absence of competitor

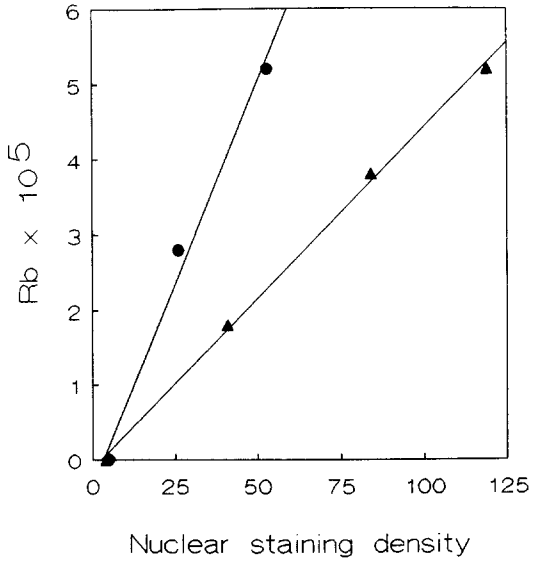


**Fig. 2.** *cis*-DDP-DNA adduction in rat liver (*triangles*) and cultured RIF-1 cells (*circles*) as measured by quantitative immunocytochemistry, atomic absorption spectroscopy and UV absorbance. The immunosignal was expressed as the mean nuclear staining density/cell (arbitrary units, fields of 30–60 cells measured). Platinum-DNA binding as measured by UV absorbance and atomic absorption spectroscopy was expressed as $R_b$. Rats were treated with 0, 5, 10 or 15 mg/kg *cis*-DDP and killed after 4 h; RIF-1 cells were incubated for 1 h with 0, 138 and 276 µM *cis*-DDP (single experiments). SE values amounted to 15%–20% of the mean nuclear staining densities. Correlation coefficient for each curve: $r > 0.99$

$IA_{50}$ values were higher for native than for denatured *cis*-DDP-modified poly[d(G-C)].poly[d(G-C)] or poly(dG).poly(dC), suggesting that denaturation increases accessibility to the antigenic sites of the DNA adducts. No inhibition was observed by *cis*-DDP-modified oligo(dA).oligo(dT), poly(dA).poly(dT), oligo(dG), or $[d(AAAG)]_{10}$ (Table 1). This indicates that NKI-A59 does not recognize Pt-GG, Pt-AG, or other adenosine-containing adducts in short DNA fragments, i.e., polymers comprising $\leq 42$ nucleotides.

*Visualization of* cis*-DDP-induced DNA modifications.* Rat liver sections and RIF-1 cells were investigated. Figure 2 shows the relationship between the immunostaining signal (expressed in arbitrary units) and the level of platinum-DNA binding as measured by UV absorbance and atomic absorption spectroscopy. A good correlation was found for both liver and RIF-1 cells ($r > 0.99$), indicating that immunostaining is a quantitative indicator of total *cis*-DDP-DNA binding in situ. It should be noted that Fig. 2 shows data obtained using two different immunostaining protocols (see Materials and methods). It is not yet clear why the immunostaining assay for cryostat sections is more sensitive than that for single cells.

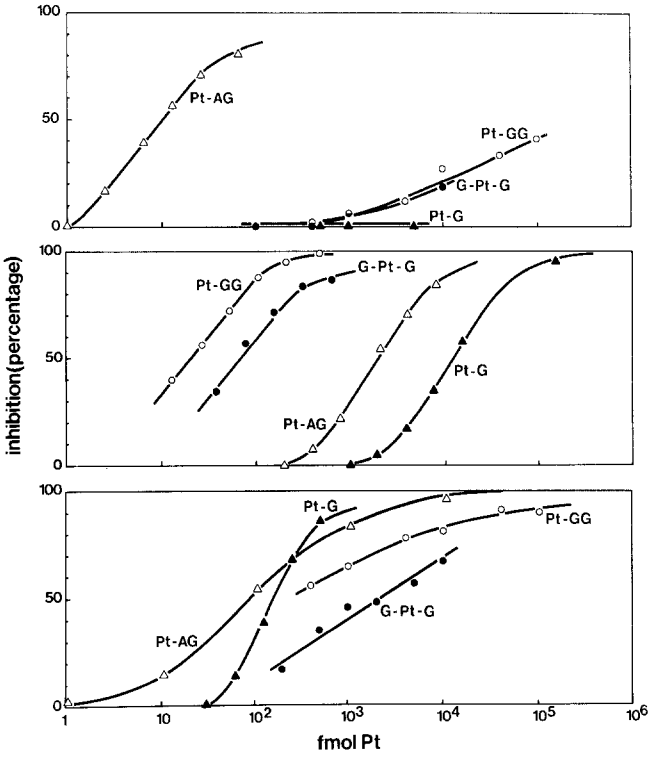


**Fig. 3.** Competitive inhibition in an ELISA of the binding of antiserum NKI-A68 (raised against Pt-AG; *top panel*), antiserum NKI-A10 (raised against G-Pt-G; *middle panel*), and antiserum NKI-A39 (raised against Pt-G; *bottom panel*) to *cis*-DDP-modified DNA by Pt-AG (*open triangles*), Pt-GG (*open circles*), G-Pt-G (*closed circles*), and Pt-G (*closed triangles*). Inhibition is expressed as the percentage of antibody binding in the absence of competitor

*Anti-cis-DDP-(di)nucleotide antisera*

*Reactivity to platinum compounds.* Figure 3 illustrates inhibition curves obtained from competitive ELISAs in which the antisera NKI-A68, NKI-A10, and NKI-A39 were combined with the major mono- and dinucleotide adducts. Table 2 summarizes the $IA_{50}$ values obtained in these experiments, although these values cannot easily be compared in the few instances in which the inhibition curves were not parallel.

NKI-A68, NKI-A10, and NKI-A39 all showed a high affinity for their corresponding hapten. The highest specificity was found for NKI-A68, since its cross-reactivity with the non-hapten adducts G-Pt-G, Pt-GG, and Pt-G was extremely low ($IA_{50}$ values, $>10^5$). NKI-A10 showed an

**Table 2.** Recognition of *cis*-DDP-modified (di)nucleotides by antisera NKI-A68, NKI-A10 and NKI-A39 as illustrated by the $IA_{50}$ value

| | $IA_{50}$ (fmol/well) | | |
|---|---|---|---|
| Antiserum | NKI-A68 | NKI-A10 | NKI-A39 |
| Hapten | Pt-AG | G-Pt-G | Pt-G |
| Inhibitor: | | | |
| G-Pt-G | $>10^5$ (1)[a] | 80 (19; 46–144)[b] | 1,500 (1) |
| G-Pt-G[c] | ND | 115 (3; 90–130) | ND |
| Pt-GG | $>10^5$ (1) | 25 (2; 22–28) | 200 (1) |
| Pt-AG | 10 (3; 10–11) | 1,300 (7; 500–2,050)[d] | 85 (2; 80–90) |
| Pt-G | $>10^5$ (1) | 10,000 (3; 4,400–13,000) | 155 (4; 60–250) |

[a] Number of assays; range
[b] SD ± 33
[c] From the Mono Q fraction of digested *cis*-DDP-DNA ($R_b$, $8,9 \times 10^{-2}$)
[d] SD ± 700
ND, Not determined

affinity for Pt-GG ($IA_{50}$, 20 fmol) that was higher than that for the hapten used for immunization (G-Pt-G; $IA_{50}$, 70 fmol). NKI-A10 displayed poor recognition of adducts in which only a single G was involved. The antiserum raised against the monoadduct (NKI-A39) recognized all four adducts, its affinity for the bivalent Pt-AG adduct being higher than even that for the hapten used for immunization.

Table 2 shows that the $IA_{50}$ value for NKI-A10 recognition of a G-Pt-G-containing fraction from a Mono Q chromatography of digested DNA ($R_b$, $8.9 \times 10^{-2}$) fell within the $IA_{50}$ range for synthetic G-Pt-G. This demonstrates that the present method is suitable for *cis*-DDP-adduct detection in DNA digests (see also [5]). The affinity of both NKI-A68 and NKI-A10 for *cis*-DDP-modified CT-DNA strongly depended on whether or not the latter had been enzymatically digested (Table 3); the same held true for intact vs digested poly(dG). However, the $IA_{50}$ value of 650 fmol/well for digested *cis*-DDP-poly(dG), whereby Pt-GG adducts are predominantly expected, was higher than the $IA_{50}$ value for synthetic Pt-GG (25 fmol/well; Table 2). This phenomenon could be explained by incomplete digestion of the heavily platinated *cis*-DDP-poly(dG).

*NKI-A68 and NKI-A10 and immunocytochemistry.* The suitability of NKI-A68 and NKI-A10 for the detection of *cis*-DDP-DNA adducts in situ was tested in tissue sections (NKI-A10) or cytospin slides (NKI-A68) at high levels of DNA adduction. Antisera were diluted 0–2,000 times. Specific nuclear staining, however, could not be visualized in liver sections from rats treated with 10 mg/kg *cis*-DDP or in cytospin slides from buccal cells treated for 1 h with 17 μM *cis*-DDP. In the same immunostaining experiment, NKI-A59 displayed high levels of nuclear staining. NKI-A39 was not tested due to its relatively low affinity for the hapten (Table 2) and to the relatively low level of monofunctional adducts in *cis*-DDP-modified DNA (see [7]).

**Table 3.** Recognition of *cis*-DDP-modified (digested) DNA and polynucleotides by antisera NKI-A68 and NKI-A10 as illustrated by the $IA_{50}$ value

| Inhibitor | $IA_{50}$ (fmol/well) | | |
|---|---|---|---|
| | $R_b$ value | NKI-A68[a] | NKI-A10 |
| *cis*-DDP-DNA (*digest*) | $1.0 \times 10^{-2}$ | 28 | 11 |
| *cis*-DDP-DNA (*digest*) | $6.4 \times 10^{-3}$ | 30 | 11 |
| *cis*-DDP-DNA | $8.9 \times 10^{-2}$ | 700 | 1,350 |
| *cis*-DDP-poly(dG) (*digest*) | $4.9 \times 10^{-2}$ | ND | 650 |
| *cis*-DDP-poly(dG) | $4.9 \times 10^{-2}$ | ND | 5,000 |
| *cis*-DDP-poly(dG).poly(dC) | $3.7 \times 10^{-2}$ | ND | 15,000 |

[a] All single determinations
$R_b$, Platinum-nucleotide ratio; ND, Not determined

## Discussion

We characterized an antiserum against *cis*-DDP-modified DNA (NKI-A59) that has previously been shown to be useful for the immunocytochemical visualization of *cis*-DDP-DNA adducts at clinically relevant levels [36, 39]. As yet, no other antibodies with comparable properties have been reported. Quantitative immunocytochemical analysis using NKI-A59 enables clinical studies on the relationship between DNA platination, tumor response, and drug resistance. As yet, one drawback has been that immunocytochemistry cannot discriminate between individual *cis*-DDP-DNA interaction products. For that reason, we also developed and characterized adduct-specific antisera. Unfortunately, these antisera are not suitable for single-cell analysis of platinated DNA; however, they can be used to measure absolute adduct levels in a competitive ELISA. The adduct-specific antisera were raised against the platinated (di)nucleotides Pt-AG (NKI-A68), G-Pt-G (NKI-A10), and Pt-G (NKI-A39). These three antisera appeared to have properties similar to those of adduct-specific antisera reported by other investigators (see below).

Antiserum NKI-A59 was shown to be specific for the interaction products of *cis*-DDP and CBDCA with DNA. A similar specificity has been reported for other anti-*cis*-DDP-DNA antisera [24, 33]. The recognition of CBDCA-DNA adducts by anti-*cis*-DDP-DNA antisera is in agreement with the previous suggestion that CBDCA-DNA adducts are identical to *cis*-DDP-DNA adducts [1, 14].

A peculiar property of NKI-A59 is that the recognition of *cis*-DDP-modified DNA depends on the level of modification of the DNA, i.e., the greater the platination, the greater the binding efficiency (expressed on a per adduct basis). A similar observation has been reported by other investigators for antibodies raised against *cis*-DDP-modified (di)nucleotides [5] and for antibodies raised against DNA modified with *cis*-DDP [8, 19, 33]. This effect has also been observed, albeit to a lesser extent, for antibodies against melphalan- [40], aminofluorene- [15], and benzopyrene-modified [16] DNA. This phenomenon results in

an underestimation of adducts at lower levels of DNA adduction [8, 16].

Despite these ELISA findings, the use of NKI-A59 antibodies for the quantitation of DNA adducts in an immunocytochemical assay revealed a linear dose-signal relationship for a broad range of experimental and clinical situations, resulting in $R_b$ values of around $10^{-6}-10^{-5}$ [34–39]. Apparently, *cis*-DDP-induced structural modifications in DNA in situ, which can be recognized by NKI-A59, are (partially) lost when DNA is isolated. Although the nature of this structural modification is unclear, it is known that binding of *cis*-DDP to DNA can cause large structural distortions, such as a 40°–70° kink in the DNA superhelix (see [29] for review).

The specificity of NKI-A59 for *cis*-adducts is demonstrated by the lack of binding of NKI-A59 to DNA adducts derived from the biologically inactive compound *trans*-DDP (cf. [18, 19, 24, 33]). Our results show that NKI-A59 preferentially binds to dG- and dC-containing intra- and/or interstrand cross-links in high-molecular-weight polymers. It remains to be determined whether NKI-A59 can recognize bivalent Pt-AG or monovalent Pt-G adducts in high-molecular-weight *cis*-DDP-modified DNA. NKI-A59 showed a low affinity, if any, for dA- and/or dT-containing adducts. Its affinity for *cis*-DDP-modified poly(dC), poly(dG).poly(dC), and poly[d(G-C)].poly[d(G-C)] were higher than that for *cis*-DDP-modified poly(dG), in which the major adduct will be the intrastrand cross-link Pt-GG. However, interaction products of *cis*-DDP involving dC do not exist in *cis*-DDP-DNA [29]. It is unclear whether the low affinity of NKI-A59 for poly(dG) is due to the small size of poly(dG) (i.e., 750 nucleotides) or indicates that antibody recognition is influenced by adduct flanking sequences.

The anti-*cis*-DDP-(di)nucleotide antisera NKI-A68, NKI-A10, and NKI-A39 were shown to have a high affinity for their corresponding haptens. In addition, NKI-A10 showed a substantial cross-reactivity to Pt-GG, whereas NKI-A39 also recognized Pt-GG and Pt-AG. These findings are similar to those of Fichtinger-Schepman et al. [5, 7]. These cross-reactivities can in fact be exploited to assay several HPLC-separated adducts in digested DNA using the same antiserum [5, 7]. As might be expected from their haptens, NKI-A68, NKI-A10, and NKI-A39 bind more effectively to *cis*-DDP-modified (di)nucleotides or to digested *cis*-DDP-modified DNA than to intact *cis*-DDP-DNA. These findings explain why NKI-A68 and NKI-A10 failed to bind to *cis*-DDP-DNA adducts in an immunocytochemical assay.

Antibodies against either *cis*-DDP- or [sp-4-2-(1R,2R)]-(1,2-diaminecyclohexane)dichloroplatinum(II) (DACH-Pt)-modified DNA [19, 23, 24, 30, 33, 34] or against *cis*-DDP-modified poly-, di-, or mononucleotides [5, 7, 26] have been reported by other authors. These antibodies showed a large variation in specificity, affinity, and suitability for immunological assays. The $IA_{50}$ values for the corresponding haptens of these antibodies ranged between 2.6 [5] and $10^3$ [30] fmol/well. No clear-cut relationship appeared to exist between the affinity of the antibodies for their haptens and the modification level of the DNA used for the immunization. Antibodies showing the highest affinity to their haptens ($IA_{50}$ values, <100 fmol) were invariably polyclonal antisera; IgM-class monoclonal antibodies showed higher $IA_{50}$ values (600–1,000 fmol hapten/well; [23, 33], R. A. Newman, personal communication).

Malfoy et al. [19] reported on an anti-*cis*-DDP-DNA antiserum that failed to detect in vivo *cis*-DDP-modified DNA in either ELISA or immunocytochemical assays. However, this antiserum did recognize in vitro modified DNA with a comparable $R_b$ value [19, 21]. The monoclonal antibodies to DACH-Pt described by Newman et al. [23] are the only antibodies that show substantial cross-reactivity with free platinum drugs. Our NKI-A59 antiserum recognizes *cis*-DDP-poly(dG) and *cis*-DDP-poly[d(G-C)].poly[d(G-C)], whereas other anti-*cis*-DDP-DNA antisera did not [18, 33]; this discrepancy is not attributable to differences in $R_b$ values.

Finally, the NKI-A59 antibody described herein has thus far proved to be the only antibody that binds to *cis*-DDP-DNA adducts in tissue sections or cells from cancer patients [34, 36]. Our recent observations [35, 38, 39] suggest that *cis*-DDP-DNA-adduct analysis may be used to predict tumor response in *cis*-DDP-treated cancer patients.

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