

Mechanism of Cytotoxicity of Anticancer Platinum Drugs: Evidence That *cis*-Diamminedichloroplatinum(II) and *cis*-Diammine-(1,1-cyclobutanedicarboxylato)platinum(II) Differ Only in the Kinetics of Their Interaction with DNA¹

Richard J. Knox, Frank Friedlos, David A. Lydall, and John J. Roberts

Department of Molecular Pharmacology, Institute of Cancer Research, Sutton, Surrey SM2 5PX, United Kingdom

ABSTRACT

The kinetics of the aquation reactions of cisplatin and carboplatin and their subsequent reactions with DNA, both *in vitro* and *in vivo*, have been measured. The results have been extrapolated to indicate the expected cytotoxicity of these compounds in cells obtained from human cancer patients.

Rate constants for the aquation at 37°C of cisplatin and carboplatin of 8×10^{-5} and $7.2 \times 10^{-7} \text{ s}^{-1}$, respectively, were calculated from the half-life of these compounds in phosphate buffer, pH 7. This difference in their rate of activation was matched by their rates of binding to DNA. By use of a ¹⁴C-labeled ligand, carboplatin was shown to bind monofunctionally to DNA, after which there was a time-dependent formation of difunctional interstrand cross-links, formed from some of these initially monofunctional adducts. A similar, although faster, accumulation of cross-links was seen when cisplatin was bound to DNA. The loss of the ¹⁴C-CBDCA ligand of carboplatin was calculated to occur with a rate constant of $1.3 \times 10^{-5} \text{ s}^{-1}$ which was similar to that for the rate of formation of interstrand cross-links and faster than that for the monofunctional reaction with DNA. It was concluded therefore that the CBDCA ligand becomes a more labile leaving group once carboplatin has been mono-aquated. In contrast, both chloro-ligands of cisplatin were shown to leave at similar rates. The fact that other difunctional lesions were formed to the same extent, by equal bound doses of cisplatin or carboplatin, was indicated by the unwinding of supercoiled plasmid DNA. The effects of cisplatin and carboplatin on this DNA were the same once bound to the same extent. About a 100-fold larger dose of carboplatin was, as predicted by their rates of aquation, required to produce equivalent binding to plasmid DNA.

In vivo, equal binding of the two drugs to DNA of various cell systems resulted in equal cytotoxicity. Again a much larger dose (20- to 40-fold) of carboplatin was required to produce this equal binding. In general a DNA bound platinum level of about 20 nmol/g reduced cell survival by 90%, although certain cell lines were shown to be much more sensitive to DNA bound platinum.

Similar binding values, to those above, were obtained in the DNA extracted from cells of human cancer patients treated with cisplatin. It was inferred that the cytotoxic effect of this level of platinum on DNA would be (unless the cells were of a sensitive phenotype) about 90%.

This work has shown that once bound to DNA in equal amounts both cisplatin and carboplatin cause equal difunctional lesions, interstrand cross-links, and cytotoxicity. The large differences in dose required to produce these effects were accounted for by the much faster rate of aquation of cisplatin.

INTRODUCTION

Cisplatin [*cis*-diamminedichloroplatinum(II)] has become established as an antitumor agent of major clinical importance, since its introduction in the early 1970s (1, 2). However, the severe toxicity associated with this compound led to develop-

ment of second generation compounds (3, 4). Of these, carboplatin [*cis*-diammine(1,1-cyclobutanedicarboxylato)platinum(II)] has gone into clinical trials (5, 6).

The toxic side effects, in humans, of cisplatin are different from those of carboplatin. While patients who received cisplatin suffered emesis, ototoxicity, nephrotoxicity, and other side effects (7), carboplatin treated patients showed reduced emesis and no oto- or nephrotoxicity, with the dose-limiting toxicity being myelosuppression (4, 5).

Evidence strongly suggests that the cytotoxicity of cisplatin is due to its reaction with DNA or chromatin (8-10). Moreover this cytotoxicity has been correlated with the formation of DNA interstrand cross-links (11-14). Cisplatin can react with DNA or other nucleophilic sites only after at least one chloride ligand is replaced by water, generating, at neutral pH, a positively charged reactive electrophilic mono-aquo species. The mono-aquo species may undergo another aquation to the diaquo form and/or react with nucleophilic sites (15, 16).

The major product of the reaction of cisplatin with DNA was found to be the intrastrand cross-link between two adjacent guanine residues on the same strand of DNA (17, 18). The interstrand cross-link, between residues on opposite DNA strands, only accounted for a small percentage of the total DNA lesions (17, 19-21). Replacement of the two independent chloro-ligands of cisplatin by the bidentate CBDCA² ligand produced carboplatin, a molecule more resistant to aquation (4, 22). The presence of the less labile CBDCA ligand rather than the readily reactive chloro ligands of cisplatin should make carboplatin less reactive toward DNA than cisplatin, and this has been demonstrated on isolated supercoiled bacteriophage PM-2 DNA (23). In view of its biological activity and chemical inertness, it has been suggested that carboplatin may be activated enzymatically *in vivo* (4, 22).

Cisplatin is particularly effective in the therapy of testicular and ovarian cancer (1, 2). Clinical trials suggest that carboplatin may be as effective as cisplatin in the treatment of these tumors, and it also seems to be active in cancers which do not respond well to cisplatin (24) and tumors which have become "resistant" to cisplatin (5, 6). These clinical data suggest that cisplatin and carboplatin, although chemically very similar, may have different mechanisms of cytotoxicity. We have therefore undertaken a comparative study of cisplatin and carboplatin and measured their *in vitro* rates of aquation, their reactions with DNA, and their capacity to form mono- and difunctional adducts in DNA. In various cell systems we have measured the interaction with DNA and DNA binding of cisplatin or carboplatin, at doses producing known levels of cell kill. These results are discussed in terms of the *in vivo* kinetics of the two drugs and are related

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² The abbreviations used are: CBDCA, cyclobutane dicarboxylic acid; WS, sensitive strain of the Walker 256 tumor; WR, resistant strain of the Walker 256 tumor; PBS, phosphate-buffered saline; SSC, saline sodium citrate; TNE, 10 mM Tris-HCl, pH 8.0:10 mM NaCl:1 mM EDTA; HPLC, high-pressure liquid chromatography; XP, Xeroderma pigmentosum.

to the binding of platinum to DNA obtained from cisplatin or carboplatin treated human cancer patients.

MATERIALS AND METHODS

All tissue culture media and sera were supplied by Gibco Ltd. (Paisley, U.K.). All other chemicals and enzymes were supplied by Sigma Ltd. (Poole, U.K.) unless otherwise stated.

Platinum Compounds. Cisplatin and carboplatin were kindly supplied by the Johnson Matthey Research Centre (Reading, U.K.) and *cis*-diammine(1,1-[^{14}C]cyclobutanedicarboxylato)platinum(II) (^{14}C]carboplatin) was a gift from Prof. K. Harrap, Institute of Cancer Research, Sutton, U.K. This labeled compound was supplied at a specific activity of 8.9 mCi/mmol and was used without further dilution. All drug solutions were prepared immediately prior to use.

Cell Growth and Radioactive Labeling Conditions. Cells of the Walker 256 tumor (WS) and a resistant substrain (WR) originally derived by continuous exposure to chlorambucil (25) were grown as a loosely aggregated (non-anchored) unstirred suspension in Dulbecco's modified Eagle's medium, supplemented with 1 mM additional glutamine and 10% horse serum, in a 10% CO_2 atmosphere. Under these conditions, cells grew with a population doubling time of 17 h and to a density limit of 2×10^6 cells/ml. Colony formation was determined in a 0.12% agar gel in McCoy's 5a medium, supplemented with 1 mM additional glutamine and 20% fetal calf serum.

Cellular DNA was radiolabeled by cell growth for 24 h in the presence of [^3H]thymidine (Amersham, U.K.) followed by a 2-h label-free chase period. A mixture containing 0.5 ml tritiated thymidine, as supplied (20–26 Ci/mmol, 1 mCi/ml), plus 2.4 ml non-radioactive thymidine solution (1 mg/ml in PBS), was prepared. This mixture was then used by adding 0.29 ml/100 ml of cell culture, giving final labeling conditions of 0.5 $\mu\text{Ci/ml}$ (50 mCi/mmol).

Under these conditions, DNA was labeled to a specific activity of about 5×10^6 dpm/mg.

Preparation of DNA. Walker cells, treated as above, were washed once in ice-cold PBS and flash frozen at -80°C . The human tumor cells contained in the ascitic fluids of patients who had been treated with platinum compounds were centrifugally collected into a single cell pellet, washed in PBS, and similarly frozen.

Frozen cell pellets were thawed into a *p*-aminosalicylic acid lysing solution and extracted with an equal volume of phenol solution, and the DNA was precipitated by 2 volumes of 2-ethoxyethanol as described previously (20).

The DNA was redissolved in water at about 1 mg/ml and (for DNA platinum binding determination only) treated with RNase (10 $\mu\text{g/ml}$; 37°C ; 10 min), before being deproteinated by digestion with Proteinase K (5 $\mu\text{g/ml}$; 37°C ; 30 min), reextracted with phenol solution, adjusted to 1% sodium acetate, reprecipitated by addition of ethanol to 70%, and washed extensively in 70% ethanol, prior to experimental usage.

Determination of the Time Course of DNA Interstrand Cross-Linkage. The tritium-labeled DNA, prepared as above, was dissolved in SSC at 0.3 mg/ml and reacted with the two compounds by addition of either 0.5 ml SSC containing 10 mM carboplatin (5 mM final concentration) or by addition of 0.5 ml SSC followed by 10 μl of a 100 \times solution of cisplatin in dimethyl sulfoxide (70 μM final concentration). The solutions were divided into six 1-ml aliquots (leaving a remainder) and after a 2-h reaction period all were precipitated individually with ethanol and washed exhaustively with 70% aqueous ethanol to remove unreacted drug. DNA pellets were stored as precipitates in 70% ethanol at -20°C prior to redissolving in SSC (1 ml) and incubation at 37°C for the times as indicated, before sedimentation analysis. Estimation of DNA interstrand cross-linking from the alkaline sucrose gradient profiles was as detailed previously (20). The DNA remaining after cross-link determination was precipitated, and specific binding of platinum to DNA determined by atomic absorption spectrophotometry as described previously (20, 26).

Preparation and Purification of Plasmid pSV2gpt. The plasmid, pSV2gpt, was purified from *Escherichia coli* (strain HB101) by a modification of the method described by Maniatis *et al.* (27). Chlor-

amphenicol (170 $\mu\text{g/ml}$) was added to a 1-liter culture of *E. coli* in late log phase, and the culture was incubated for a further 16 h prior to harvesting, in order to amplify the plasmid copy number. The plasmid was harvested from the pelleted bacteria by detergent lysis. The pellet was resuspended in 10 mM NaCl (2 ml) and treated successively with the following solutions at 4°C : (a) sucrose solution, 20 ml (25% sucrose in 50 mM Tris-HCl, pH 8.0); (b) lysozyme solution, 1 ml, 5 min (10 mg/ml in 100 mM Tris-HCl, pH 8.0); (c) EDTA, 2 ml, 10 min (250 mM); and (d) detergent, 2 ml, 15 min (0.5 ml Triton X-100:12.5 ml 250 mM EDTA:2.5 ml 100 mM Tris-HCl, pH 8.0:1.2 ml water).

The cellular debris was removed by centrifugation (30,000 rpm, 25 min, 4°C). The crude lysate was treated with Proteinase K (250 $\mu\text{g/ml}$, 60 min, 45°C), and the DNA was precipitated by ethanol at -20°C for 1 h and dissolved in TNE. The DNA solution was treated with RNase (50 $\mu\text{g/ml}$, 30 min, 37°C), and the RNase activity was removed by a further treatment with Proteinase K (100 $\mu\text{g/ml}$, 30 min, 37°C). The DNA was then reprecipitated by ethanol, redissolved in TNE, and subjected to caesium chloride/ethidium bromide density gradient centrifugation (40,000 rpm, 64 h, 20°C) (28).

The band containing the supercoiled DNA was collected, and after the ethidium bromide had been removed by butanol extraction, it was extensively dialyzed against TNE. The dialyzed DNA was further purified by microconcentration (Centricon 30 microconcentrators, Amicon Ltd., Glos., U.K.). The DNA solution was centrifuged (4500 \times g; 30 min), the retentate (approximately 25 μl) was diluted with 10 mM NaH_2PO_4 , pH 7 (2 ml), and the procedure was repeated. Sample purity (>95%) was determined by HPLC and agarose gel electrophoresis, and the sample yield (500 μg) was determined by absorbance at 260 nm. The purified plasmid solution was stored at -20°C .

Reaction of Cisplatin or Carboplatin with Plasmid DNA. Reaction was started by the addition of appropriate volumes of aqueous solutions of cisplatin or carboplatin to pSV2gpt (100 $\mu\text{g/ml}$) in 10 mM NaH_2PO_4 , pH 7. After twenty-four h in the dark at 37°C , the reaction was quenched by the addition of NaCl to 200 mM, and the unbound drug was removed by microconcentration. The retentate was diluted to 150 μl , of which 20 μl were loaded directly onto an agarose gel, and the remainder was stored at -20°C for subsequent determination of bound platinum and plasmid recovery (>70%).

Agarose Gel Electrophoresis. Samples (20 μl) were electrophoresed through a horizontal or vertical agarose gel (1.5%) for 20 h at a potential gradient of 1 V/cm in 2 mM EDTA:40 mM Tris:20 mM acetic acid, pH 7.5. The DNA was visualized under UV light after staining with ethidium bromide (1 $\mu\text{g/ml}$ for 20 min). Migration distance was measured relative to that of untreated Form I (supercoiled) DNA.

Measurement of Aquation Rates. One mM solutions of cisplatin or carboplatin in 0.1 M NaH_2PO_4 , pH 7, were passed through a 2- μm filter and incubated at 37°C . At various times thereafter samples (10 μl) were injected onto a Bio-Sil ODS-10 (250 \times 4 mm) reverse phase HPLC column and eluted with H_2O (0.5 ml/min), the eluate was continually monitored by UV absorbance at 230 nm. The cisplatin peak eluted at 4.6 min, carboplatin at 9.0 min, and aquation products (which were not retained by the column) at 2.7 min. Aquation was measured by loss of peak area of the parent compound with time.

Measurement of Kinetics of Binding to DNA by Cisplatin and Carboplatin. Calf-thymus DNA (2 mg/ml in 0.1 M NaH_2PO_4 , pH 7) was reacted with either carboplatin (in 0.1 M NaH_2PO_4 , pH 7) or cisplatin (in 0.1 M NaH_2PO_4 , pH 7, + 10% dimethyl sulfoxide) to a final concentration of 1 mM and was incubated at 37°C . At various times thereafter samples were removed, and the DNA was separated from unbound drug by HPLC. Samples (1 ml) were injected onto a TSK G5000 PW (600 \times 7.5 mm) GPC column and eluted with 0.1 M NaH_2PO_4 , pH 7 (1.0 ml/min); the eluate was continually monitored by absorbance at 260 nm. The DNA eluted between 9 and 17 min; the unbound drug eluted between 30 and 45 min. Specific platinum binding to DNA was determined by atomic absorption spectrophotometry. For experiments involving the reaction of [^{14}C]carboplatin with DNA this isolated DNA was further incubated at 37°C , and samples were re-separated, as above, at various times thereafter. The loss of the ^{14}C -ligand from DNA was followed by reduction in the ^{14}C -activity co-eluting with the DNA peak.

RESULTS

Aquation of Platinum Compounds. In chloride-free phosphate buffer, pH 7, cisplatin had a half-life of 2.4 h in contrast to a half-life of 268 h for carboplatin (Fig. 1), *i.e.*, there was a 112-fold difference in their rates of aquation. These values represent rate constants of $8 \times 10^{-5} \text{ s}^{-1}$, at 37°C , for cisplatin [published, $1.1 \times 10^{-4} \text{ s}^{-1}$ (29)] and $7.2 \times 10^{-7} \text{ s}^{-1}$, at 37°C , for carboplatin, assuming pseudo-first order kinetics to apply.

Reaction with DNA. Aquation of platinum II complexes generates products capable of reacting with DNA. Cisplatin bound to DNA at an initial rate of 6.3 mg Pt/g DNA/h in contrast to a rate of 81 μg Pt/g DNA/h for carboplatin (Fig. 2).

The reactive species may be either monoaquated and/or diaquated depending on the relative rate of the aquation reaction of the second arm ligand compared to the reactivity of the monoaquated species toward nucleophilic sites. Because the CBDCA ligand of carboplatin is bidentate, monoaquation would leave this ligand attached to the platinum, and subsequent reaction of this species with DNA will generate a CBDCA-platinum-DNA monoadduct. The presence of such adducts could be demonstrated by following the reaction of [^{14}C -CBDCA]carboplatin with DNA over several hours. At early times for every bound platinum atom (determined by atomic absorption spectroscopy) there was a corresponding CBDCA ligand (determined by radioactivity), but this CBDCA:platinum ratio decreased with time so that after a 50-h incubation with ^{14}C -carboplatin only 7% of the platinum atoms bound could be accounted for by radioactivity of the ligand. All the carboplatin which bound to DNA gave adducts which were initially monofunctional. Subsequent loss of ^{14}C -activity would indicate fur-

ther reaction of the second arm of those ligands already bound to DNA.

Stability of Monofunctional Adducts. Isolation and further incubation of DNA reacted with [^{14}C]carboplatin enabled determination of the half-life of the monofunctional lesion. The CBDCA ligand of carboplatin was released from the DNA with a half-life of 14.5 h (Fig. 3). No corresponding loss of total bound platinum accompanied the release of the ^{14}C -CBDCA. Loss of the ligand was therefore a result of reaction of the second arm of carboplatin and not loss of bound drug. Loss of the second arm of the ligand would enable difunctional DNA binding, and the time course of formation of a particular difunctional lesion, namely the interstrand cross-link, was followed using alkaline sucrose gradient sedimentation of tritium labeled DNA.

Kinetics of Interstrand Cross-Link Formation. Tritium-labeled DNA was reacted with cisplatin (70 μM) or carboplatin (5 mM) for 2 h, doses chosen to give equal levels of DNA binding. The actual binding values obtained were 3.8 and 3.6 μmol platinum/g DNA for cisplatin and carboplatin, respectively. The amounts of DNA interstrand cross-linking produced by these treatments during a subsequent 24- or 48-h incubation at 37°C were determined by alkaline sucrose gradient sedimentation analysis.

When DNA enters the alkali of the sucrose gradients, the helix separates, and the resulting single strands sediment independently as a single peak with an average molecular weight characteristic of the individual preparation, usually in the region of 10^7 Daltons. If however there is one or more cross-links present in the original double-stranded DNA molecule, then the single strands generated by the alkali treatment remain joined together and sediment to a position corresponding to their combined molecular weights. Thus, the effect of introducing cross-links into the starting material is to generate a new "cross-linked" peak at twice the molecular weight of the un-cross-linked material. The numbers of cross-links in any particular starting size that would generate a given proportion of cross-linked material are calculated by a usage of the Poisson distribution as described previously (20). By division with the starting size, the cross-link frequency per unit size of DNA (scaled by 10^9 for convenience) can be calculated.

Both cisplatin (Fig. 4A) and carboplatin (Fig. 4B) produced cross-links in a time-dependent manner. It appeared that, at any given time, there were more cross-links produced by the cisplatin than by the carboplatin, since the sucrose gradient profile of the cisplatin treated DNA clearly showed a greater reduction in the proportion of material sedimenting upon the position of control, untreated DNA, with the concomitant

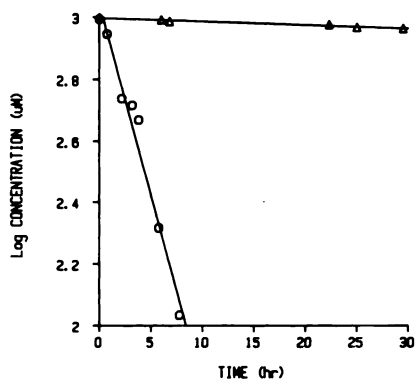


Fig. 1. Aquation of 1 mM cisplatin (O) or carboplatin (Δ) in chloride-free phosphate buffer, pH 7, at 37°C , with time. Drug concentrations were measured by HPLC as described in the text. Half-life for the reaction is 2.4 and 268 h for cisplatin and carboplatin, respectively.

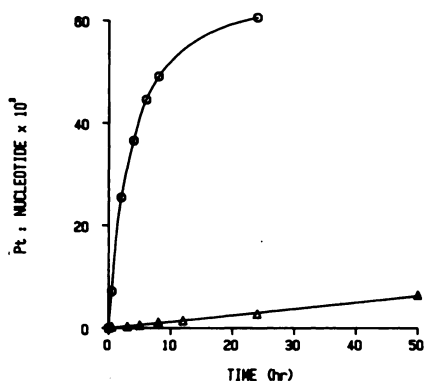


Fig. 2. Binding of 1 mM cisplatin (O) or carboplatin (Δ) to isolated calf-thymus DNA (2 mg/ml) in chloride-free phosphate buffer, pH 7, at 37°C , with time.

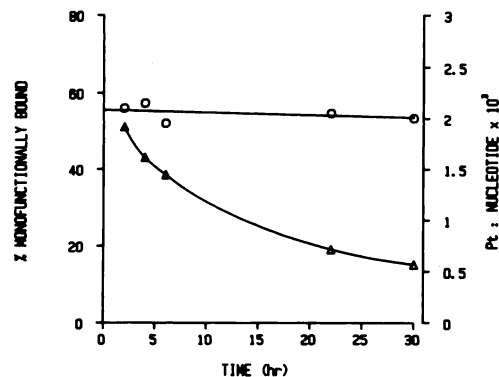


Fig. 3. Loss of ^{14}C -CBDCA ligand (Δ) or platinum metal (O) from DNA treated previously with carboplatin for 16 h. The times are for incubation at 37°C after removal of unbound drug and correspond to a half-life of 14.5 h for DNA bound CBDCA ligand.

increase in the amount sedimenting in the position of cross-linked DNA. However, the original (untreated control) average molecular weight of the carboplatin-treated DNA was only one-half that of the control of the cisplatin treated DNA (this degree of variation in size being within the normal range experienced using our DNA extraction technique). Thus when the specific frequency of cross-linking was calculated per standard size of untreated DNA (Table 1), then the extents of cross-linking produced by equal amounts of reaction with DNA were similar for both compounds. From a knowledge of the extents of reaction of cisplatin or carboplatin with DNA and the amounts of cross-linking produced, the frequency of cross-links as a proportion of the total bound platinum was calculated (Table 1). The frequency of cross-links per total DNA bound platinum adduct produced by cisplatin varied from approximately 0.5 to 1.4% over a 24-h period, and the frequency produced by carboplatin varied by a similar 0.7 to 1.9% but over a 48-h period.

Therefore at equal levels of reaction with DNA the frequency of interstrand cross-links, relative to total lesions, produced by cisplatin or carboplatin were very similar.

The level of cross-links appeared to approach their maximum

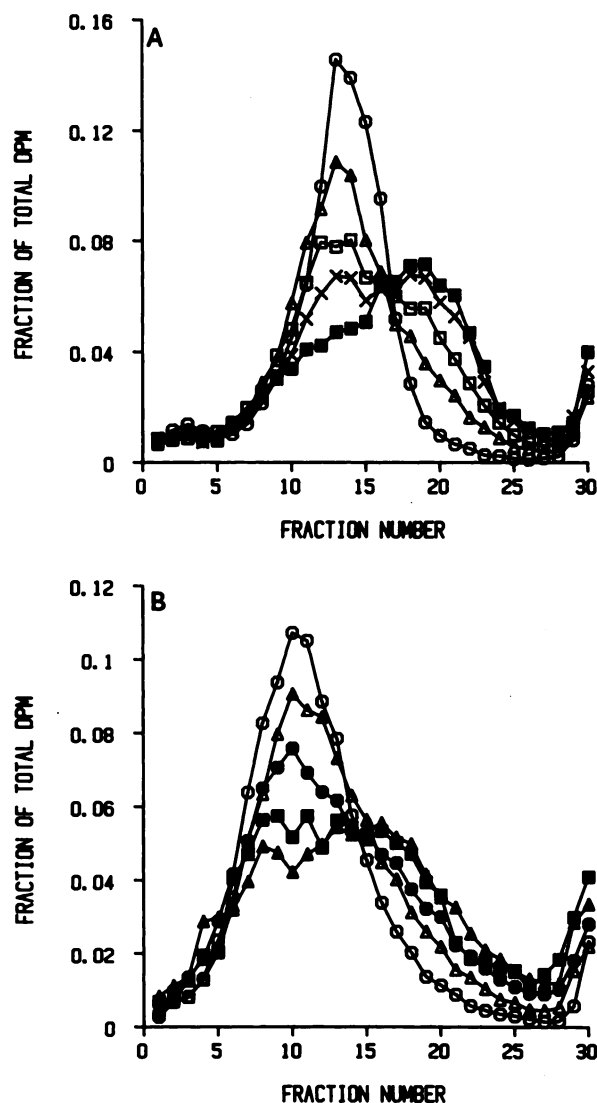


Fig. 4. Sucrose density gradient profiles showing the time dependent formation of cross-links on isolated ^3H -labeled DNA by (A) $70\ \mu\text{M}$ cisplatin or (B) $5\ \text{mM}$ carboplatin. Sedimentation, under the conditions described in the text, was toward the higher fraction numbers. Incubation times following an initial 2-h exposure of the DNA to drug are: \circ , untreated control; Δ , 0 h; \square , 2 h; \times , 4 h; \bullet , 6 h; \blacksquare , 24 h; and \blacktriangle , 48 h at 37°C .

Table 1 Specific DNA interstrand cross-linking frequency of cisplatin or carboplatin as determined by sedimentation analysis in sucrose gradients

H of incubation at 37°C after initial 2-h exposure to the platinum compound	Cross-link units per 10^9 Daltons	
	Cisplatin ($70\ \mu\text{M}$)	Carboplatin (5 mM)
0	9.2	13.2
2	14.2	14.2
4	19.2	19.5
6	20.2	21.3
8	20.9	
24	24.8	30.1
48		36.4

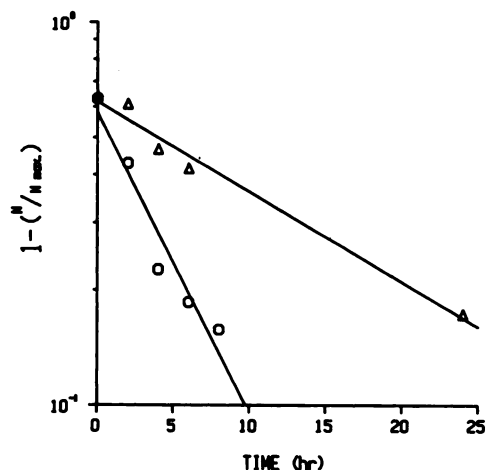


Fig. 5. Half-life of the cisplatin (\circ) or carboplatin (Δ) lesion that leads to a DNA interstrand cross-link. N_{max} is the maximum number of cross-links observed, and N is the number at a specific time. All data are derived from the sucrose density gradient profiles shown in Fig. 4. The half-life of the pre cross-link lesion, derived from this plot, was 3.5 h for cisplatin and 13.0 h for carboplatin at 37°C .

during the time course of the experiment. Therefore the half-life of the lesion that leads to the cross-link (the monofunctionally reacted platinum) may be plotted as $1 - N/N_{\text{max}}$ against time (where N is the number of cross-links, and N_{max} is the maximum number of cross-links). The half-life of the pre cross-link lesion was 3.5 h for cisplatin and 13.0 h for carboplatin (Fig. 5). This latter value for carboplatin compared well with a half-life of 14.5 h for the ^{14}C -ligand of carboplatin reacted monofunctionally on DNA. The similarity of these half-lives is consistent with a rapid reaction of the second arm of carboplatin with sites on DNA, once the CBDCA ligand has been lost.

The difference in the rate of formation of interstrand cross-links, by cisplatin or carboplatin, was about 4-fold. If first-order kinetics are assumed, the rate constants for the reaction of the second-arm of cisplatin or carboplatin are 5.5 and $1.3 \times 10^{-5}\ \text{s}^{-1}$ at 37°C , respectively.

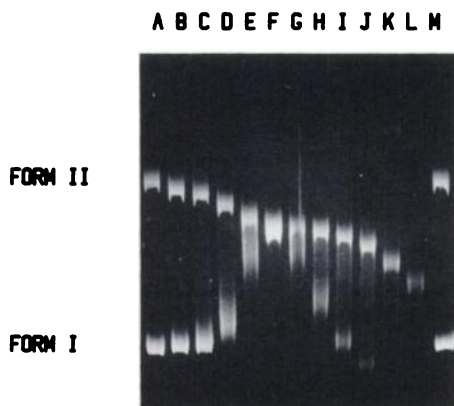
Effects on the Tertiary Structure of pSV2gpt. The interstrand cross-link only accounts for about 2% of the total DNA lesions produced by either drug, and therefore other difunctional lesions are probably formed. There is evidence that the major DNA lesion is the intrastrand cross-link between two adjacent guanine residues (17, 18), and these have been reported to account for about 60% of total lesions (17). Intrastrand lesions have been implicated in the "unwinding" of supercoiled DNA (30, 31), although other lesions may also be involved. The effects on supercoiled DNA, due to its modification by cisplatin or carboplatin, were qualitated using agarose gel electrophoresis. When cisplatin binds to Form I DNA this supercoiled structure is "unwound" (32-34), and its migration distance decreases. When fully unwound it comigrates with the Form II relaxed conformation of the plasmid. Further modification

rewinds the originally negatively supercoiled DNA to positive supercoils (23, 32–34). Both cisplatin and carboplatin produced this effect on pSV2gpt when it was treated with a range of concentrations (Fig. 6). Maximum unwinding of Form I (supercoiled) pSV2gpt occurred at a dose of 24 μM cisplatin or 2.4 mM carboplatin for 24 h (Fig. 7A). When this unwinding was related to the amount of bound platinum as opposed to dose (Fig. 7B), both cisplatin and carboplatin produced maximum plasmid unwinding at a bound platinum per nucleotide ratio of 0.05 (16 mmol platinum/g DNA). Hence equal binding of cisplatin or carboplatin produced equal unwinding of the plasmid and by implication equal formation of difunctional lesions.

Cytotoxicity of the Interaction of Cisplatin and Carboplatin with the DNA of Cells in Culture. Although there were large differences in dose *versus* effect for these drugs, we have shown that once bound to DNA in equal amounts both cisplatin and carboplatin cause equal amounts of interstrand and total cross-links. Were the reaction with DNA of these drugs to be the same *in vivo* as *in vitro* it would be expected that, although there would be differences in dose of these drugs required to cause equal binding, at equal binding of cisplatin or carboplatin there would be equal cytotoxicity. We have compared the DNA binding and the corresponding survival in various cell and tumor lines *in vitro* and *in vivo* when treated with cisplatin and carboplatin.

WS and WR strains of the Walker 256 tumor show markedly

A: CISPLATIN



B: CARBOPLATIN

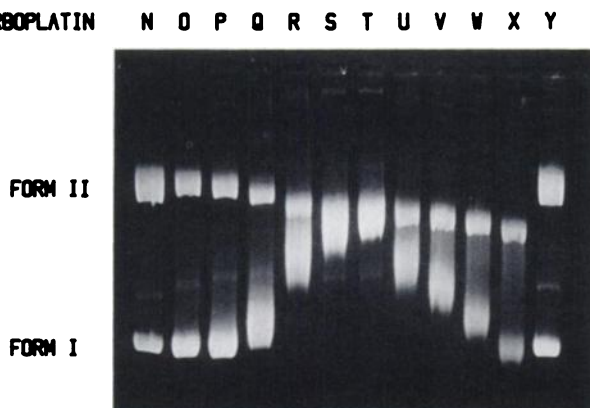


Fig. 6. Conformational changes induced by (A) cisplatin or (B) carboplatin on pSV2gpt DNA as shown by electrophoresis through (A) vertical or (B) horizontal agarose gels. Form I is the supercoiled covalently closed circular conformation of the DNA; Form II is the relaxed circular conformation. All drug treatments were for 24 h at 37°C under the conditions described in the text. The drug concentrations were: Lane A, 0 μM ; Lane B, 2 μM ; Lane C, 4 μM ; Lane D, 8 μM ; Lane E, 16 μM ; Lane F, 24 μM ; Lane G, 32 μM ; Lane H, 60 μM ; Lane I, 120 μM ; Lane J, 240 μM ; Lane K, 480 μM ; Lane L, 800 μM ; Lane M, 0 μM ; Lane N, 0 μM ; Lane O, 200 μM ; Lane P, 400 μM ; Lane Q, 800 μM ; Lane R, 1,600 μM ; Lane S, 2,000 μM ; Lane T, 2,400 μM ; Lane U, 4,800 μM ; Lane V, 7,200 μM ; Lane W, 9,600 μM ; Lane X, 15,000 μM ; and Lane Y, 0 μM .

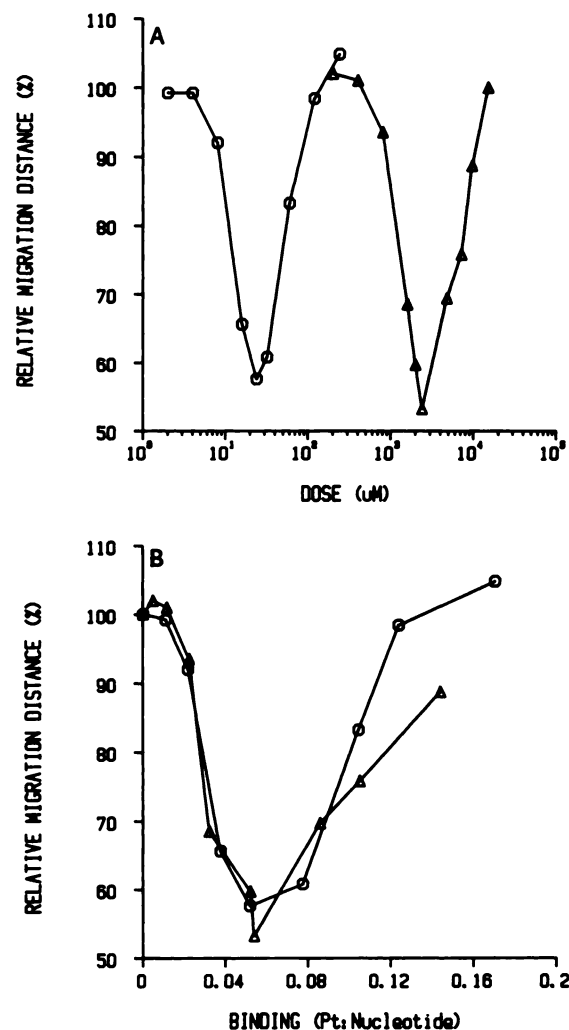


Fig. 7. Effect of cisplatin (O) or carboplatin (Δ) on the migration of supercoiled plasmid DNA through an agarose gel (A) as a function of dose and (B) as a function of the DNA bound platinum. All drug treatments were for 24 h at 37°C under the conditions described in the text. Migration distances were measured relative to that of untreated supercoiled DNA.

different survivals following exposure to cisplatin (Fig. 8A). The same differential cytotoxicity (30-fold) was seen in response to carboplatin (Fig. 7B), and for both Walker strains the dose of carboplatin required to produce the same toxicity as cisplatin was about 25-fold greater. Binding of platinum to DNA following exposure of WR and WS to equitoxic doses of cisplatin or carboplatin is shown in Table 2. Doses of cisplatin or carboplatin which gave comparable DNA binding produced about equal cytotoxicity in WR. Similar results were shown in WS, although for this sensitive cell line, the cytotoxicity produced at similar levels of DNA binding was far greater.

Table 3 is a compilation of new and previously published data relating cell survival to levels of cisplatin and carboplatin binding to DNA. It is apparent that in the majority of cases, a DNA binding of approximately 20 nmol platinum/g reduced survival to about 10%, thus resembling the pattern seen in WR. The only exception was in cells derived from a person suffering from XP, a defined DNA repair deficiency syndrome, in which survival was reduced to 0.2% at this binding level.

Binding of Platinum to DNA from Cells of Cancer Patients Treated with Cisplatin or Carboplatin. Cells were collected from biopsies from cancer patients who had received either cisplatin or carboplatin, and the specific binding of platinum to the DNA was measured (Table 4). The binding of platinum to the DNA

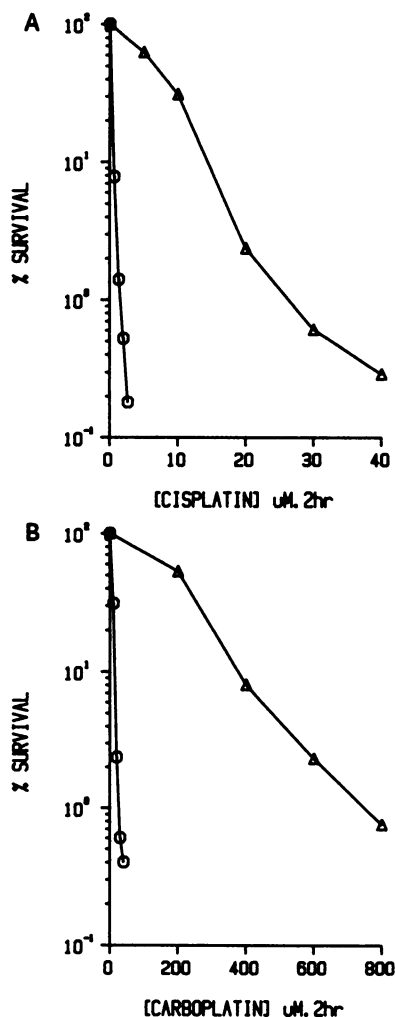


Fig. 8. Survival of the sensitive (O) and resistant (Δ) strains of the Walker 256 tumor following treatment by (A) cisplatin and (B) carboplatin. Cells (2×10^5 /ml) were treated for 2 h at 37°C and assayed by colony-forming ability as described in the text.

Table 2 Specific binding to DNA and the survival on exposure to cisplatin or carboplatin in the sensitive and resistant strains of the Walker 256 tumor

Cell type	Dose/compound (μM/2 h)	Cell survival (%)	Binding to DNA (nmol/g)
WR	10 μM cisplatin	41	19
	200 μM carboplatin	58	17
WS	5 μM cisplatin	0.1	10
	60 μM carboplatin	0.4	8

of cells harvested from the ascites fluids of ovarian carcinoma patients treated with cisplatin (Table 4) at 30 mg/m² was 10.3 nmol platinum/g, 24 h after administration of the cisplatin. Following the higher dose of 50 mg/m², a higher value of 28.8 nmol platinum/g was observed to the cells isolated from a pleural effusion. However, at the highest dose of 100 mg/m² or repeated doses of 4×20 mg/m², a further proportional increase was not observed, the binding levels being again approximately 10 nmol/g. This was possibly a result of the later times of biopsy after cisplatin treatment in these cases and may be a reflection of the presence of DNA repair in the ascites cells.

The DNA extracted from a metastatic nodule of an OAT cell carcinoma patient who had received 400 mg/m² carboplatin showed a DNA platinum binding of 2.2 nmol/g (Table 4). A higher dose of carboplatin was required to produce similar DNA binding in this case, reflecting the situation in Walker cells.

Table 3 Cell survival and cisplatin or carboplatin binding to DNA in various cell systems *in vivo* and *in vitro*

System	Dose/compound	Survival (%)	Binding to DNA (nmol/g)
Cisplatin			
Mouse bone marrow (38)	10 mg/kg	20 (spleen colonies)	10
Mouse B16 melanoma (38)	4 mg/kg	80 (lung colonies)	10
Mouse ADJ PC6 plasmacytoma (39)	1.6 mg/kg	ID ₉₀	4
Human pancreatic tumor xenograft (40)	10 mg/kg	10 (<i>in vitro</i>) ^a	6
HeLa (39)	10 μM/1 h	20	10
Chinese hamster V79 (39)	30 μM/1 h	20	15
Human fetal lung cells	10 μM/1 h	20	10
Xeroderma pigmentosum fibroblasts (26)	20 μM/2 h	0.2	10
Carboplatin			
ADJ/PC6 plasmacytoma (39)	14.5 mg/kg	ID ₉₀	1.8
Chinese hamster V79	120 μM/4 h	10	4
Human pancreatic tumor xenograft	100 mg/kg	30 (<i>in vitro</i>) ^a	15.5

^a Survival and DNA binding were determined *in vitro* 24 h after administration of drugs.

Table 4 Binding of platinum to the DNA obtained from the cells of human cancer patients treated with cisplatin or carboplatin

Cell type	Dose of drug (mg/m ²)	Time of biopsy (h)	Binding to DNA (nmol/g)
Cisplatin			
Ovarian carcinoma	100 mg/m ²	62	11.2
	4×20 mg/m ²	24 (after last dose)	10.4
	30 mg/m ²	24	10.3
Ovarian carcinoma pleural effusion cells	50 mg/m ²	28	28.8
Carboplatin			
OAT cell carcinoma metastatic nodule	400 mg/m ²		2.2

DISCUSSION

In Vitro Reactions of Cisplatin and Carboplatin. In chloride-free buffer, pH 7, there was a 112-fold difference in the rates of the aquation reactions of cisplatin and carboplatin. Similar differences were observed in the rates of their binding to DNA, and this finding strongly supports the proposition that aquation is the rate limiting step in the reaction of both of these compounds with DNA. Increasing the concentration of chloride in the reaction medium would drive the equilibrium position of the aquation of cisplatin (but not carboplatin) in favor of the unreactive unaquated form. Therefore chloride would reduce the concentration of the reactive aquo species of cisplatin, and the reaction with DNA would be decreased. Within a cell, therefore, where the chloride concentration is about 150 mM (35), the difference in the rates of reaction of cisplatin and carboplatin with DNA should be less marked, as compared with the difference seen in the chloride free reaction conditions described above.

Carboplatin reacted with DNA to give, as the first product of reaction, only monofunctional adducts, although these adducts, once formed, could react further to become difunctional. The fact that the primary reaction is monofunctional implicates

the monoquo form of carboplatin rather than the diaquo form as the principal reactive species. During subsequent incubation of the initially formed product, the amount of platinum bound to DNA remained constant, while a progressive loss of the labeled CBDCA ligand and (at a similar rate) formation of (difunctional) interstrand cross-links was observed. Cisplatin-treated DNA accumulated approximately equal numbers of interstrand cross-links to carboplatin-treated DNA during this post-treatment incubation (although cross-links accumulated faster in the cisplatin treated DNA). The fact that cisplatin-treated DNA also accumulated cross-links and that the initial overall extent of cross-linking was the same as that produced by carboplatin, when the level of reaction with DNA were the same for both compounds, suggests that cisplatin also binds to DNA in a predominantly monofunctional manner. This is supported by the work of Johnson *et al.* (16), which showed that the monoquated form of cisplatin is the prevalent reactive species.

There was a 4-fold difference in the rate of reaction leading to the formation of interstrand cross-links between the DNA treated with cisplatin or carboplatin. In contrast, there was an 80-fold difference in the rate of formation of the corresponding monofunctional DNA lesion. A rate constant of $5.5 \times 10^{-5} \text{ s}^{-1}$ at 37°C was calculated for the reaction of the second arm of cisplatin with DNA, using the rate of formation of interstrand cross-links on DNA. This value compares well with a published rate constant for the aquation reaction of the second chloride ligand of cisplatin in free solution of $4.2 \times 10^{-5} \text{ s}^{-1}$ (29). A value of $8 \times 10^{-5} \text{ s}^{-1}$ was calculated for the rate constant of the aquation reaction of the first-arm chloro ligand; therefore both chloro ligands of cisplatin reacted at similar rates, and the reactivity of the second chloro ligand seems to be the same whether the molecule is in free solution or bound by its first arm to DNA. This is consistent with cross-link formation proceeding by an aquated intermediate, not by a direct reaction with DNA. In contrast, the second arm of the CBDCA ligand of carboplatin reacted with DNA with a rate constant of $1.3 \times 10^{-5} \text{ s}^{-1}$, at 37°C , which is 18-fold faster than that measured for the aquation of the first-arm of $7.2 \times 10^{-7} \text{ s}^{-1}$. The fact that the rates of reaction of the arms of carboplatin are significantly different suggests that the CBDCA ligand becomes a more labile leaving group once carboplatin has been monoquated.

The presence of interstrand cross-links has been correlated with the cytotoxicity of cisplatin and its *trans*-configured analogue (11–13). However, the cross-links formed are not chemically identical. Because cisplatin and carboplatin differ only in their leaving ligand, the difunctional lesions produced on DNA should be chemically identical with respect to the platinum moiety. Therefore the same DNA lesions, if similarly produced by either drug within the cell, should be equally toxic. This view was supported by this study, which showed that there was equal formation of interstrand cross-links by cisplatin or carboplatin, once bound in equal amounts to DNA *in vitro*, and that equal binding of these two compounds to the DNA of cells in culture produced equal cytotoxicity.

These data do not unequivocally show the interstrand cross-link to be cytotoxic. Only about 2% of the total platinum lesions on DNA were involved in DNA interstrand cross-links, in agreement with previously published data (19–21). It is therefore possible that the formation of interstrand cross-links is merely a reflection of the accumulation of other difunctional lesions which may be more cytotoxic. The fact that other difunctional lesions are formed, in about equal amounts, by cisplatin or carboplatin was shown by their equal ability to

unwind supercoiled DNA. Although there was a large difference in the dose required to produce maximum unwinding of DNA by cisplatin or carboplatin, the 100-fold larger dose of carboplatin required produced DNA binding about equal to the smaller dose of cisplatin. Equal binding of cisplatin or carboplatin produced equal unwinding and by implication equal numbers and types of difunctional lesions.

In summary, once bound to DNA in equal amounts cisplatin and carboplatin cause about equal total cross-links, interstrand cross-links, and cytotoxicity. The large differences in dose required to produce these effects were accounted for by the very much faster aquation of cisplatin.

In Vivo Reactions of Cisplatin and Carboplatin. In a variety of cell systems, a dose of cisplatin or carboplatin which produced a DNA binding of 20 nmol/g resulted in about a 90% cell kill in most of the cell lines examined. However, exceptional sensitivity to DNA bound platinum was observed in XP cells (26) and in the sensitive strain of the Walker tumor (WS), a cell line sensitive to difunctional agents (36). There was the same differential cytotoxicity between this sensitive cell line (WS) and its derived resistant counterpart (WR) when treated with either cisplatin or carboplatin, although the dose of carboplatin required to produce equal cytotoxicity was about 25-fold greater in both the WS or WR cell lines. Therefore, the mechanism of action of cisplatin and carboplatin appears to be identical in the Walker carcinoma.

In general, between 20- and 40-fold more carboplatin than cisplatin was required in the cell systems investigated to produce equivalent binding and hence cytotoxicity. This 20–40-fold dose and modification factor, relative to cisplatin in these cell lines, is less than the 80-fold difference predicted from our *in vitro* results. This discrepancy may be explained by the effect of the prevailing intracellular chloride concentration reducing the concentration of the active monoquated form of cisplatin as described above. However it has been suggested that there is an enzymic cleavage of carboplatin which activates the compound *in vivo* (4, 22). Our work shows that a non-enzymic reaction between carboplatin and water occurs *in vitro* which allows carboplatin to react with DNA. The close agreement between the relative reactivity of cisplatin and carboplatin *in vitro* and *in vivo* suggests that such an enzymic activation of carboplatin does not occur.

Binding of cisplatin or carboplatin to the DNA in cells obtained from human cancer patients also followed the pattern of dose response as described above. Although the data for carboplatin were limited, as anticipated from their relative reaction rates, a single dose of 400 mg/m² was less effective at producing DNA bound platinum than was a single 30 mg/m² dose of cisplatin.

At a cellular and molecular level we have demonstrated that, once bound to DNA, the actions of cisplatin and carboplatin are the same. We would therefore predict that cells treated by these two compounds would display total cross-resistance (as for the sensitive and resistant Walker cells). Clinically there are examples in which carboplatin appears to be active toward cancers that normally do not respond well to cisplatin (24) and in tumors that have become "resistant" to cisplatin (4, 6). Our results suggest that this is not an effect at the cellular level but may reflect differences in the pharmacokinetics of the two compounds in these cases.

By inference from the cytotoxic effects produced at similar binding levels measured in our laboratory cell systems we would predict that the cell kill achieved in these human clinical samples would be approximately 90%, after these single treatments,

unless the tumor cells had the exceptional sensitivity of the WS or XP cell lines. The fact that cisplatin and carboplatin therapy is very effective against ovarian and particularly testicular carcinomas suggests that indeed these cancers may be composed of a sensitive cell type. On a cisplatin dose basis this may well be the case (37), and preliminary data suggest that a cell line derived from a human teratoma may indeed be intrinsically sensitive to cisplatin.³

The use of any anticancer agent in the treatment of human patients is limited by its general toxicity. In this respect carboplatin is superior to cisplatin, in that about 10-fold more carboplatin can be administered before the dose-limiting toxicity is reached. However our results indicate that an even larger dose of carboplatin than is now being used clinically may be required to produce equal binding to DNA and thus cytotoxicity equivalent to that of cisplatin.

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³ M. F. Pera, Jr., personal communication, May, 1985.