Biochemical Pharmacology, Vol. 23, pp. 1345–1357. Pergamon Press 1974. Printed in Great Britain.

INTERACTIONS BETWEEN MAMMALIAN CELL DNA AND INORGANIC PLATINUM COMPOUNDS—I

DNA INTERSTRAND CROSS-LINKING AND CYTOTOXIC PROPERTIES OF PLATINUM(II) COMPOUNDS

JANET M. PASCOE and JOHN J. ROBERTS

Chester Beatty Research Institute, Institute of Cancer Research, Royal Cancer Hospital, Fulham Road, London SW3 6JB, England

(*Received* 4 *June* 1973; *accepted* 15 *September* 1973)

Abstract—The effects of *cis* and *trans* Pt(II)diammine dichloride on the colony forming ability of HeLa cells have been measured. Higher doses of *trans* Pt(II)diammine dichloride than the *cis* isomer had to be administered to cells to produce equal effects on cell survival. Determination of the amount of drug bound to cellular macromolecules showed that more of the *trans* compound was bound to DNA than *cis* when cell survival was reduced by the same amount, suggesting there was a real difference in the mechanisms of action of the two isomers. The levels of reaction of *cis* and *trans* Pt(II)diammine dichloride at known toxic concentrations with DNA, RNA and protein supported the hypothesis that DNA is the primary target of the platinum(II) compounds. The ability of equal doses of *cis* and *trans* Pt(II)-diammine dichloride to form DNA interstrand cross-links differed less when isolated DNA was used than when whole cells were treated with these agents. The difference in cross-linking ability of the two compounds in whole cells could explain the difference in toxicity between the *cis* and *trans* isomers. The importance of DNA interstrand cross-linking as a cytotoxic event in its own right, and as a possible indicator of other cytotoxic lesions is discussed.

Cis AND *trans* Pt(II)diammine dichloride have been shown to possess different cytotoxic properties in all the various systems in which they have been examined. These vary from bacteriophage[1] and bacteria[2] through cultured mammalian cells[3] to whole animals.[4,5] *Cis* Pt(II)diammine dichloride is a potentially useful antitumour agent having been found to be effective against certain animal tumours including sarcoma 180 and leukaemia L1210,[6] the ADJ/PC6A plasma cell tumour[7] and virus-induced reticulum cell sarcoma[8] in mice, and the Dunning ascitic leukaemia, Walker 256 carcinosarcoma[9] and DMBA-induced mammary carcinoma[10] in rats. It has also proved useful in experimental combination therapy with cytoxan against sarcoma 180[11] and leukaemia L1210[12] and with isophosphamide and ICRF 159 against leukaemia L1210.[12] By contrast the *trans* isomer is biologically much less active and does not possess antitumour properties.[7]

In this study we have sought an explanation in molecular terms for this difference in activity between the *cis* and *trans* platinum(II) compounds. Previous investigations had indicated the possible importance of interactions with DNA leading to an inhibition in the rate of DNA synthesis[3] and have provided evidence that cross-linking of DNA strands could occur when whole cells were treated with platinum compounds.[13]

We have therefore compared the effects of *cis* and *trans* Pt(II)diammine dichloride on the survival of HeLa cells (as measured by colony-forming ability) with their ability to bind to cellular macromolecules and to form DNA interstrand cross-links. An assessment has been made of the importance of DNA interstrand cross-linking as a cytotoxic event and of the importance of DNA, RNA and protein as targets for the platinum drugs.

The results provide some support for the hypothesis that the greater toxic action of *cis* Pt(II)diammine dichloride is due to its superior ability to its *trans* isomer to produce cross-links in cellular DNA. However, calculation of the proportion of platinum reactions producing DNA cross-links indicates it is initially at least, a rare event and therefore possibly of little importance in determining the toxic effects of platinum(II) compounds.

MATERIALS AND METHODS

Inorganic platinum compounds

Cis and *trans* Pt(II)diammine dichloride were provided by Professor M. L. Tobe.

Cells

HeLa cells were maintained in stirred suspension culture in Eagle's medium (MEM, Cat. No. F14, Grand Island Biological Co., New York, U.S.A.) supplemented with 7% foetal calf serum (Flow Laboratories Ltd., Irvine, Scotland) and containing 2×10^5 IU/l. benzyl-penicillin and 0·2 g/l. streptomycin sulphate B.P. (Glaxo Laboratories, Greenford, Middlesex, England). Under these conditions HeLa cells grew exponentially up to a concentration of at least 10^6 cells/ml with a doubling time of approximately 24 hr. Monolayer cultures of the cells on glass or plastic were maintained in Eagle's medium (MEM, Cat. No. F11, Grand Island Biological Co., New York, U.S.A.) supplemented with 15% foetal calf serum with the same concentrations of antibiotics as used in F14 medium.

Determination of cell survival following treatment with platinum drugs

Cells were treated in suspension at an approximate concentration of $2{\cdot}5 \times 10^5$ cells/ml with platinum compound dissolved either in dimethyl sulphoxide (the final concentration of solvent being not greater than 1 per cent and the solution being made up and added to the cells immediately) or in sterile 0·1 M, pH 7·2 phosphate buffer (the final concentration of buffer being not greater than 5 per cent of the culture medium). After the desired treatment time (usually 2 hr) the cells were collected by centrifugation and resuspended in an equal volume of fresh warmed medium to ensure that any unreacted chemical was removed. Appropriate serial dilutions into suspension medium were immediately carried out in order to obtain not more than 200 colonies per dish when 1 ml of the final cell suspension was plated into a 50 mm Petri dish (Nunclon, Sterilin Ltd.) containing 5 ml F11 medium. Plates were maintained in an atmosphere of 5% CO_2: 95% air at 37° for 6 days. The medium was then replaced with 6 ml of fresh F11 medium and incubation continued for a further 5 days. Under these conditions the plating efficiency of control cells was between 70 and 80 per cent. Cell colonies were fixed with methanol and stained with a dilute aqueous solution of methylene blue before counting.

Binding of platinum compounds to cell constituents

Mass stirred suspension cultures were grown for this purpose and in a typical experiment aliquots containing 5×10^7 cells were treated with the agent under the same conditions used to obtain the survival curves. After 2 hr, treatment was terminated by collecting the cells by centrifugation, washing them with saline and then freezing the pellet at $-20°$.

Isolation of nucleic acids and protein

The phenol method of Kirby and Cook[14] was adapted as follows for isolation of DNA, RNA and cytoplasmic and nuclear protein for estimation of the amount of platinum bound to each fraction.

(i) *RNA.* Each cell pellet was resuspended in an ice-cold solution of 0·5% (w/v) sodium naphthalene disulphonate (1 ml/10^7 cells). An equal volume of a solution of phenol (phenol:water:8-hydroxyquinoline = 500 g:55 ml:0·6 g) was added, the mixture shaken vigorously and centrifuged at 3000 rev/min for 20 min. The supernatant was removed, washed with half a volume of phenol solution and 3 vol of absolute ethanol were added. RNA was left to precipitate at 4 overnight. The precipitate was collected by centrifugation, washed with a small volume of 75% ethanol, then with absolute ethanol and ether and was finally dried in a vacuum dessicator.

(ii) *DNA.* The interface pellet obtained after the 3000 rev/min centrifugation in (i) was suspended by vigorous shaking with a solution of 6% (w/v) *p*-aminosalicylate (sodium salt) (1 ml/10^7 cells). An equal volume of phenol was added and the solution shaken again. It was then centrifuged at 3000 rev/min for 20 min.

The supernatant was removed and washed with 0·5 vol of phenol. DNA was precipitated by the addition of 2 vol of 2-ethoxyethanol. The fibrous precipitate was twice washed with 75% ethanol and was then dissolved in 2·0% (w/v) sodium acetate/1·5% (w/v) sodium chloride buffer. Solution was effected by incubation at 4° for 8 hr. This solution was warmed to 37° and treated with ribonuclease (20 μl of a 1 mg/ml solution) for 30 min. Half a volume of phenol was added to stop the reaction, and the mixture was shaken prior to centrifugation at 3000 rev/min for 10 min. DNA was precipitated from the supernatant by the addition of 2 vol of 2-ethoxyethanol. The precipitate was washed with 75% ethanol, absolute ethanol and ether and dried in a vacuum dessicator.

(iii) *Cytoplasmic protein.* The phenol layer obtained from the 3000 rev/min centrifugation in (i) was washed with 0·5 vol of sodium naphthalene disulphonate solution, and cytoplasmic protein precipitated from it by the addition of 5 vol of a solution of 3 parts diethyl ether and 1 part methanol (v/v). The protein was collected by centrifugation and washed three times with the ether/methanol solution, then with ether before drying in a vacuum dessicator.

(iv) *Nuclear protein.* This was extracted from the phenol layer obtained from the 20 min, 3000 rev/min centrifugation in (ii) exactly as described in (iii).

DNA whose ultimate fate was analysis by caesium chloride density centrifugation was obtained by the following procedure which was also based on the phenol method of Kirby and Cook.[14] Each cell pellet was resuspended in an ice-cold solution of 6% (w/v) *p*-aminosalicyclic acid (sodium salt) plus 1% (w/v) triisopropyl naphthalene sulphonate (sodium salt) and 6% (v/v) *sec*-butanol (1 ml/10^7 cells) by vigorous shaking.

An equal volume of phenol solution (as described in (i) above) was added and the mixture shaken again. After centrifugation at 3000 rev/min for 20 min the upper aqueous layer was taken and washed with 0·5 vol of the phenol solution. DNA was precipitated from it by the addition of 2 vol of 2-ethoxyethanol, and the precipitate was washed twice with 75% ethanol. It was then dissolved in the appropriate buffer.

Assay of platinum bound to cellular macromolecules

Pure, dried cellular constituents, prepared as described, were dissolved to give solutions of 1 mg/ml. DNA and RNA were dissolved in 1 N HCl, and samples were heated for 1 hr at 100° to effect solution. The purity of nucleic acid solutions was checked by comparing their concentrations calculated from optical density readings at 260 nm (assuming an extinction coefficient of 20.000 at this wavelength) with those calculated on a weight basis. Proteins were dissolved in 5 N HCl by heating to 160° for 1 hr, and their concentrations were calculated on a weight basis only. 10, 20 or 50 μl aliquots of these solutions were assayed for platinum in a Perkin–Elmer atomic absorption spectrophotometer, model 306, fitted with a heated graphite atomiser, model HGA-70.

Determination of DNA cross-linking

(i) *In vitro.* Cells were grown in the presence of a mixture of [^{3}H]thymidine and 5'-bromodeoxyuridine to introduce a heavy radioactively labelled DNA strand.[13,15] They were harvested and the DNA was isolated as described. It was dissolved in 0·1 M, pH 7·2 phosphate buffer at an approximate concentration of 0·1 mg/ml. 2 ml of this solution were used per treatment. Platinum compounds were either dissolved in 0·1 M pH 7·2 phosphate buffer or in dimethyl sulphoxide, the concentration of which did not exceed 5 per cent, and were added immediately. After the required time, treatment was stopped by precipitating the DNA with 2 vol of 2-ethoxyethanol. The precipitate was washed once with 75% ethanol and then dissolved in 0·6 ml 0·1 M, pH 12·5 phosphate buffer, and 0·5 ml of this solution was subjected to alkaline caesium chloride density gradient centrifugation as previously described.[16]

(ii) *In vivo.* Cellular DNA was labelled by growing HeLa cells in the presence of [^{3}H]thymidine and 5'-bromodeoxyuridine as previously described.[13,15] Treatment (usually of 10^7 cells) was carried out at a concentration of 6×10^5 cells/ml. Reagents were made up in dimethyl sulphoxide and added to the cells immediately. The solvent concentration did not exceed 3 per cent. Treatment was stopped by harvesting the cells by centrifugation and washing them in ice-cold saline. Cell pellets were stored at −20° prior to isolation of DNA by the method described. The DNA was dissolved in 0·6 ml, 0·1 M, pH 12·5 phosphate buffer and 0·5 ml of this solution was subjected to alkaline caesium chloride density gradient as previously described.[16]

Assay of radioactivity

Ten ml Triton-X scintillation fluid (70 g PPO, 3·5 g POPOP, 4 l. Triton-X and 6 l. toluene) was added to each sample and radioactivity assayed using a Packard liquid scintillation spectrometer, model 3375.

RESULTS

Effects on cell survival. The effects of a 2 hr treatment with either *cis* or *trans* Pt(II)-diammine dichloride on HeLa cell survival are shown in Fig. 1. Characterization of the curves according to the notation of Alper *et al.*[17] gave the results in Table 1. D_Q is the "quasi-threshold" or intercept dose and D_0 the dose increment required to bring about an average of 1 lethal event per cell once the exponential portion of the curve has been reached.

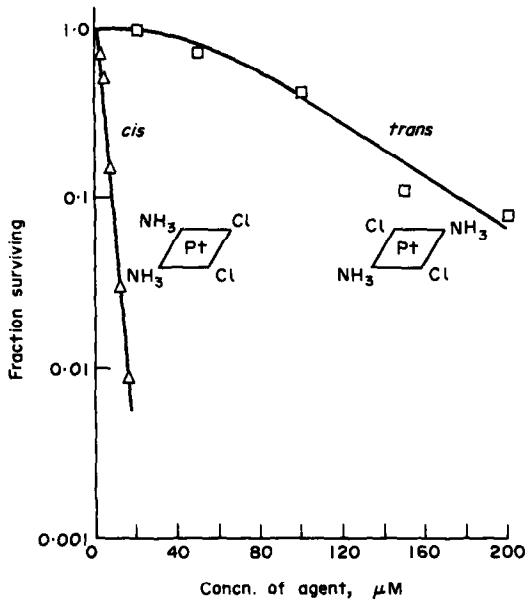

FIG. 1. Survival curves for treatment of HeLa cells in suspension culture with either *cis* Pt(II)diammine dichloride (△), or *trans* Pt(II)diammine dichloride (□), for 2 hr at 37°.

TABLE 1.

Compound	D_Q (μM) (shoulder width)	D_0 (μM) (slope of curve)
cis $Pt(II)(NH_3)_2Cl_2$	1	3
trans $Pt(II)(NH_3)_2Cl_2$	50	55

Calculation of the ratios of the D_0 values for *cis* and *trans* Pt(II)diammine dichloride show that, on a dose basis, the *cis* isomer is 18·5 times more toxic than the *trans* compound towards HeLa cells.

These results are consistent with the biological properties of *cis* and *trans* Pt(II)-diammine dichloride which have been observed in the wide variety of other systems investigated.[1,3,4,7]

Binding to cellular macromolecules. Determination of the amount of *cis* and *trans* Pt(II)diammine dichloride bound to DNA, RNA and protein yielded the results in Figs. 2 and 3. In Fig. 2 it can be seen that over the concentration range of 5–10 μM where cell killing was observed with the *cis* isomer only (*trans* Pt(II)diammine dichloride having no effect on cell survival), approximately twice as much of the *trans*

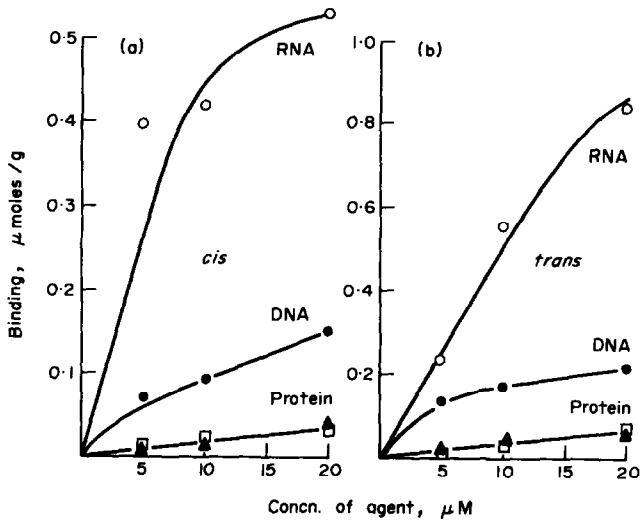

FIG. 2. Extents of binding of (a) *cis* Pt(II)diammine dichloride and (b) *trans* Pt(II)diammine dichloride to RNA (○), DNA (●), cytoplasmic protein (▲) and nuclear protein (□) following treatment of HeLa cells in suspension culture for 2 hr at 37°.

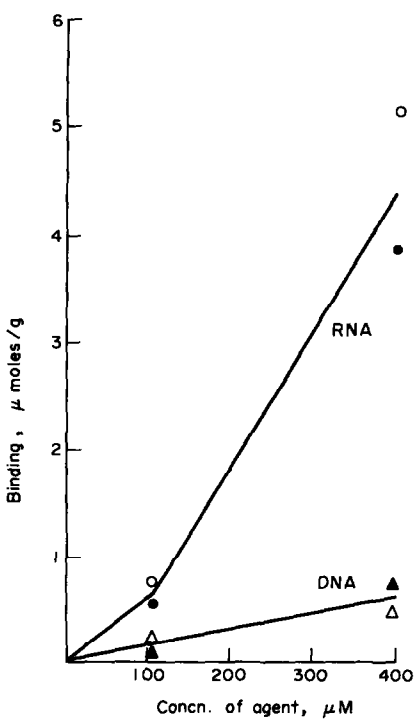

FIG. 3. Extents of binding of *cis* Pt(II)diammine dichloride (○, △) and *trans* Pt(II)diammine dichloride (●, ▲) to RNA (○, ●) and DNA (△, ▲) following treatment of HeLa cells in suspension culture for 2 hr at 37°.

isomer was bound to DNA, RNA or protein as the *cis* isomer. In Fig. 3 where the doses were increased up to 400 μM, and *trans* Pt(II)diammine dichloride caused a significant decrease in the observed cell survival, macromolecular binding was of the same order for both isomers. It is clear from Figs. 2 and 3 that for both *cis* and *trans* Pt(II)diammine dichloride there was more binding to RNA than to either DNA or protein over the entire dose range studied.

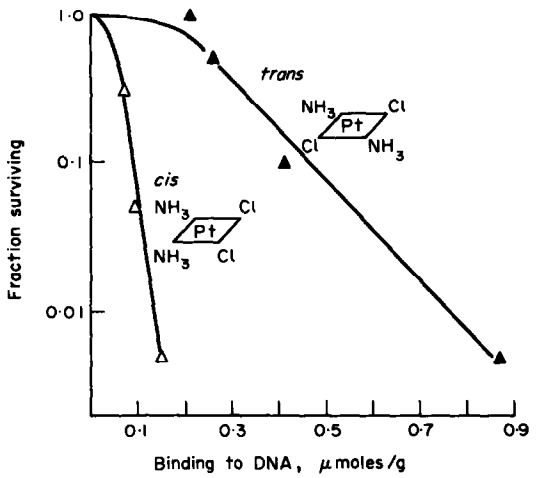

FIG. 4. Relationship between the survival of HeLa cells and the extents of the binding of *cis* Pt(II)diammine dichloride (△) and *trans* Pt(II)diammine dichloride (▲) to their DNA. The levels of DNA platination resulting from treatment with particular concentrations of *cis* or *trans* Pt(II)diammine dichloride were obtained from Figs. 2 and 3, and the effect of these same concentrations on the colony forming ability of similarly treated cells was obtained from Fig. 1.

These results show that the lack of effect of *trans* Pt(II)diammine dichloride on HeLa cell survival is in no way due to a relative inability to penetrate the cell membrane and react with macromolecules therein. Also they are consistent with the finding by Gale *et al.** that *cis* Pt(II)diammine dichloride is not actively transported into either lymphocytes or Ehrlich ascites tumour cells, but most probably enters by passive diffusion.

Relationship between macromolecular binding and cell survival. Differences in concentration of *cis* and *trans* Pt(II)diammine dichloride required to produce equivalent effects on HeLa cell survival need not necessarily be indicative of the true amount of reaction occurring with cell constituents, but could (as has been shown in the case of some monofunctional alkylating agents[18]) merely reflect differences in the relative ease of penetration of the two isomers into cells. It would therefore be expected that a comparison between the amount of reaction with cell macromolecules and the effect on cell survival would be more useful than one between concentration of drug administered to cells and observed cell killing.

Knowledge of the extents of reaction of *cis* and *trans* Pt(II)diammine dichloride with DNA, RNA and protein at concentrations which produced measurable effects on cell survival permitted the construction of curves of log. cell survival vs macromolecular binding. The curves obtained for DNA are shown in Fig. 4. They could be

* G. R. Gale, C. R. Morris, L. M. Atkins and A. B. Smith, personal communication.

characterized along the lines proposed by Alper *et al.*,[17] except that B (signifying binding) was substituted for D (signifying dose). The binding coefficients so obtained are presented in Table 2. B_Q is the shoulder width of the curve and B_0 the slope. Curves similar to those in Fig. 4 were constructed to relate the extents of reaction with RNA and protein to cell survival. The B_Q and B_0 values for RNA and protein are likewise given in Table 2.

Table 2.

	Binding to macromolecules					
	B_Q (μmoles/g) (shoulder width)			B_0 (μmoles/g) (slope of curve)		
	DNA	RNA	Protein	DNA	RNA	Protein
cis $Pt(II)(NH_3)_2Cl_2$	0·045	0·300	0·002	0·0225	0·030	0·00675
trans $Pt(II)(NH_3)_2Cl_2$	0·170	0·300		0·125	0·650	

In Table 3 the approximate molecular weights of DNA, RNA and protein are noted together with the binding coefficients measured for *cis* and *trans* Pt(II)diammine dichloride. By taking the molecular weights of these macromolecules into account it is possible to calculate the number of platinum molecules bound to each when the surviving fraction is reduced from f to $0{\cdot}37f$, i.e. when there has been an average of one lethal event per cell. There are many more platinum molecules bound per DNA molecule than per RNA or protein molecule because the molecular weight of DNA is much greater than that of RNA or protein. These results agree with the idea that DNA is probably the most important target for the platinum drugs.

The DNA B_Q values for *cis* and *trans* Pt(II)diammine dichloride can similarly be used to enable calculation of the number of platinum molecules bound to each DNA molecule before cell survival is affected. Thus it is found that approximately 45 *cis* Pt(II)diammine dichloride molecules and 170 *trans* Pt(II)diammine dichloride molecules are bound to each DNA molecule of 10^9 mol. wt before a measurable reduction in cell survival is observed.

The difference in the DNA B_Q and B_0 values for *cis* and *trans* Pt(II)diammine dichloride suggests that there is very probably a real difference in the reactions of these two compounds with DNA and justifies more detailed studies on the nature of the platinum–DNA interaction.

Table 3.

		cis $Pt(II)(NH_3)_2Cl_2$		*trans* $Pt(II)(NH_3)_2Cl_2$	
Molecule	Approx. mol. wt	B_0 (μmoles/g)	No. Pt mol. bound when surviving fraction reduced from f to $0{\cdot}37f$	B_0 (μmoles/g)	No. Pt mol. bound when surviving fraction reduced from f to $0{\cdot}37f$
DNA	1×10^9	0·0225	22 Pt/DNA mol.	0·125	125 Pt/DNA mol.
mRNA	4×10^6		1 Pt/8 mRNA mol.		2·5 Pt/mRNA mol.
rRNA	$0{\cdot}5$–1×10^6	0·030	1 Pt/30 rRNA mol.	0·650	1 Pt/2 rRNA mol.
tRNA	$2{\cdot}5 \times 10^4$		1 Pt/1500 tRNA mol.		1 Pt/70 tRNA mol.
Protein	1×10^5	0·00675	1 Pt/1500 protein mol.		

DNA cross-linking. In the case of the bifunctional alkylating agents available evidence indicates that their cytotoxic properties towards bacteriophage may well be connected with their ability to form inter- and intra-strand cross-links in DNA.[19,20] The observation that the Walker carcinoma with acquired resistance to melphalan was also resistant to *cis* Pt(II)diammine dichloride[21] suggested that DNA cross-linking could also contribute significantly to the cytotoxic properties of the inorganic platinum compounds. We have previously demonstrated that *cis* Pt(II)diammine dichloride is able to form interstrand cross-links in the DNA of cultured HeLa cells[13] and now wish to see whether the different cytotoxic properties of *cis* and *trans* Pt(II)-diammine dichloride are attributable to a variation in their abilities to cross-link DNA.

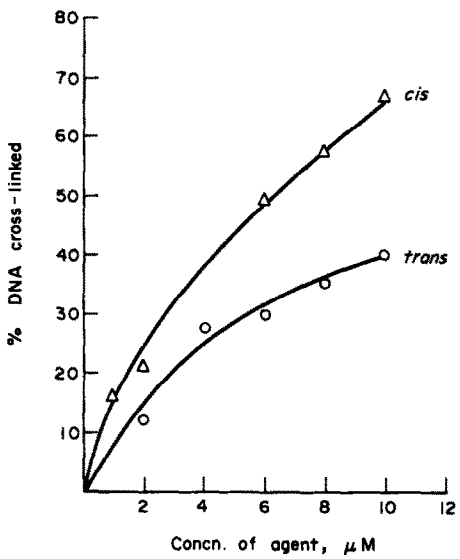

FIG. 5. Proportion of isolated HeLa cell DNA cross-linked by *cis* Pt(II)diammine dichloride (△) and *trans* Pt(II)diammine dichloride (○) following treatment in neutral phosphate buffer for 2 hr at 37°.

In the first instance therefore we examined the abilities of *cis* and *trans* Pt(II)diammine dichloride to form interstrand cross-links in isolated DNA. It can be seen from Fig. 5 that low doses of *cis* and *trans* Pt(II)diammine dichloride are capable of cross-linking isolated DNA over the dose range studied (2–10 μM), and that the *cis* isomer is between 1·5 and 2 times as effective as the *trans* compound under the conditions employed. This difference is not great enough to account for the 5·3-fold difference in DNA B_0 values and 4·5-fold difference in DNA B_Q values observed for *cis* and *trans* Pt(II)diammine dichloride (see Fig. 4). It would thus appear that the ability to cross-link isolated DNA is not directly related to the cytotoxic properties of the platinum(II) compounds.

Secondly we extended our previous studies concerning the ability of *cis* Pt(II)diammine dichloride to cross-link cellular DNA[13] to include the *trans* isomer. In Fig. 6 the amount of cross-linked DNA is plotted against the concentration of *cis* or *trans* Pt(II)diammine dichloride administered to whole HeLa cells. In this situation the difference in the abilities of the two isomers to cross-link DNA is of the order of 10-fold

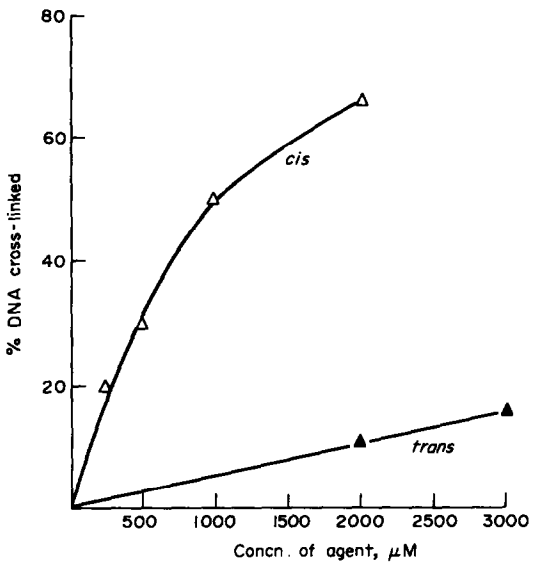

Fig. 6. Proportion of DNA cross-linked by *cis* Pt(II)diammine dichloride (△) and *trans* Pt(II)diammine dichloride (▲) following treatment of HeLa cells in suspension culture for 2 hr at 37°.

being much greater than the 1·5–2 fold difference in cross-linking abilities observed on treatment of isolated DNA (see Fig. 5). Furthermore very much higher concentrations of both platinum compounds had to be administered to whole cells to produce the same level of cross-linking as was observed in the case of isolated DNA. Thus it can be seen from Table 4 that whereas 10 per cent cross-linking was observed on treatment of isolated DNA with either 0·5 μM *cis* Pt(II)diammine dichloride or 1·0 μM *trans* Pt(II)diammine dichloride, HeLa cells had to be treated with 300 and 1900 times these doses respectively to produce the same amount of cross-linking in cellular DNA.

DISCUSSION

Macromolecular targets of the platinum compounds. Evidence supporting the hypothesis that the platinum drugs most probably exert their cytotoxic effect by direct reaction with DNA rather than by RNA or protein-mediated mechanisms is their observed effect on DNA, RNA and protein synthesis in mammalian cells. In the cases of Ehrlich ascites tumour cells,[22] human amnion AV_3 cells[3] and phytohaemagglutinin stimulated human lymphocytes[23] it has been shown that whilst RNA and protein synthesis were barely affected by treatment with low doses of antitumour

Table 4.

Compound	Concn required to produce 10% DNA cross-linking *in vitro* (μM)	Concn required to produce 10% DNA cross-linking *in vivo* (μM)
cis $Pt(II)(NH_3)_2Cl_2$	0·5	150
trans $Pt(II)(NH_3)_2Cl_2$	1·0	1900

platinum compounds allowing high cell survival, DNA synthesis was significantly inhibited. It has previously been noted that the inorganic platinum compounds resemble cytotoxic alkylating agents (in the case of which there is a large amount of evidence supporting the view that they owe their cytotoxic properties to their ability to react directly with DNA[24]) in this respect.[25] The observations that low doses of antitumour platinum compounds enhanced the levels of DNA polymerase in human amnion AV_3 cells in spite of the fact that they inhibited enzyme activity *in vitro*[26] and that millimolar concentrations of $Pt(En)Br_2$ were required to inhibit leucine aminopeptidase *in vitro*[27] suggest that inhibition of DNA synthesis results from its direct reaction with the platinum drugs, and give rise to the possibility that continuing turnover of RNA and protein could reduce such inhibitory effects of the platinum drugs on these molecules as do occur.

Our observations that many platinum molecules were bound to each DNA molecule whereas only a few of the total number of RNA or protein molecules were platinated when cell survival was reduced by a given amount (see Table 3) support the hypothesis that the platinum drugs exert their cytotoxic effects by reaction with DNA itself, and that the observed inhibition of DNA synthesis in mammalian cells following treatment with antitumour platinum compounds is unlikely to have arisen as a consequence of RNA or protein malfunction. Furthermore the levels of reaction of *cis* and *trans* Pt(II)diammine dichloride with protein would be too low to produce ennzyme inhibition unless considerable specificity of attack occurred.

Cell survival and DNA binding. The existence of shoulders on the plots of log. cell survival against DNA binding for *cis* and *trans* Pt(II)diammine dichloride shown in Fig. 4 is notable in that it could indicate that not all the cellular DNA is essential for the retention of the ability to divide and form colonies, or could imply the existence of some kind of DNA repair mechanism within the cell.[28] DNA repair processes have been shown to modify the outcome of alkylation damage[29] and u.v.-induced lesions[30] in mammalian cells, and studies on *Escherichia coli* indicated that mutation in specific genes, which are known to be involved in DNA repair processes, greatly modified the response to these agents.[31] It would therefore be expected that DNA repair processes might also be capable of modifying the outcome of damage inflicted on mammalian cells by the platinum compounds. Although it is not the purpose of the present investigation to cover this field, the alteration of the outcome of platinum-inflicted damage by cellular repair processes must be borne in mind when assessing the outcome of drug treatment.

By relating DNA binding to cell survival we have eliminated possible artifacts caused by differences in the ease of penetration of drugs into cells. Thus the difference in the shoulder widths and slopes of the curves relating cell survival to DNA binding in Fig. 4 could either represent in whole or in part a difference in the mechanism of action of *cis* and *trans* Pt(II)diammine dichloride at the molecular level, or possibly a difference in the repairability of their reaction products.

In measuring the capabilities of *cis* and *trans* Pt(II)diammine dichloride to cross-link DNA we have therefore sought a difference in the mechanism of action of these two compounds which could account for the observed differences in their cytotoxic properties.

DNA cross-linking. Band sedimentation through alkaline sodium chloride showed that both *cis* and *trans* Pt(II)diammine dichloride were able to cross-link isolated

bacteriophage T7 DNA.[1] In agreement with these results renaturation studies have established that both isomers are able to cross-link isolated mammalian cell DNA.* Use of alkaline sucrose gradient centrifugation and hydroxyapatite column chromatography revealed that *trans* Pt(II)diammine dichloride was slightly less effective than its *cis* isomer in forming interstrand DNA cross-links.†

Our findings that low doses of both *cis* and *trans* Pt(II)diammine dichloride are capable of cross-linking isolated HeLa cell DNA are in agreement with the above observations. It is possible that the difference in cross-linking ability of the two isomers is either larger or smaller than examination of Fig. 5 would imply, because treatment with equal concentrations of *cis* or *trans* Pt(II)diammine dichloride may not necessarily give rise to equal binding to DNA. It is of interest in this context that it has been suggested the ineffectiveness of solutions of *trans* Pt(II)diammine dichloride made up in phosphate buffer to inactivate bacteriophage could be attributable to a greater tendency for it to aggregate than to react with nucleophilic centres.[1] Measurement of the extents of reaction of *cis* and *trans* Pt(II)diammine dichloride with isolated DNA would clarify this point and establish whether or not the different toxic properties of these compounds were related to their ability to cross-link DNA *in vitro*.

That the larger difference in abilities of *cis* and *trans* Pt(II)diammine dichloride to cross-link cellular DNA was great enough to account for their observed variance in toxicity towards HeLa cells indicates that cultured mammalian cells are potentially more valuable as a tool for elucidating mechanisms of antitumour drug action than entirely *in vitro* systems. It is possible that the different ratios of *cis*:*trans* cross-linking ability *in vitro* and *in vivo* may be due to the conformation and reactivity of DNA within the cell nucleus being altered by association with histone and other proteins. The fact that very much higher concentrations of *cis* and *trans* Pt(II)diammine dichloride are required to produce the same amount of cross-linking *in vivo* as *in vitro* is also notable. The data in Table 4 showing that for the *cis* isomer 300 times the *in vitro* dose, and for the *trans* compound 1900 times the *in vitro* dose, must be given to produce the same extent of cross-linking *in vivo*, is to be compared with the corresponding results for platinum(IV) compounds.[3,2] and chlorambucil‡ which have been found to cross-link relatively more efficiently *in vivo* compared to *in vitro* than the platinum(II) compounds. Thus only eight times the *in vitro* dose of chlorambucil and 8–12 times the *in vitro* dose of the platinum(IV) compounds need be administered to whole cells to produce the same level of DNA interstrand cross-linking therein.

From the DNA binding data and the *in vivo* cross-linking studies it is possible to calculate the number of interactions with DNA required to produce an interstrand cross-link. In the case of *cis* Pt(II)diammine dichloride the results of such a calculation show that about 1 in 400 reactions results in a DNA interstrand cross-link after a 2 hr treatment. For the *trans* isomer this figure is 1 in 4000. However it must be remembered that every platinum(II) molecule is theoretically capable of forming a cross-link if given sufficient time because the aquo species is at least as reactive as the chloro species. Therefore cross-linking could ultimately be a more frequent event than these calculations imply, perhaps even approaching the frequency of one in

* H. C. Harder, personal communication.
† M. Shimizu and B. Rosenberg, personal communication.
‡ J. J. Roberts, unpublished results.

eight reactions calculated for mustard gas.[13,18] It is noteworthy that the number of DNA interstrand cross-links which must be introduced into HeLa cells by the two inorganic platinum(II) compounds considered here, to reduce the surviving fraction from f to $0{\cdot}37f$ is at least of the same order. Thus 350 cross-links must be introduced into every HeLa cell by *cis* Pt(II)diammine dichloride and 200 by its *trans* isomer. Therefore, DNA interstrand cross-linking, if not itself the most important cytotoxic event appears to run approximately in parallel to the major cytotoxic lesion. This could well be another type of cross-link—perhaps a DNA intrastrand cross-link, as proposed in the case of bacteriophages,[1] or one between DNA and protein.

Acknowledgements This work was supported by a grant from Rustenburg Platinum Mines Ltd. We wish to thank Perkin Elmer for the provision of technical facilities, and Mr. J. Woodward for skilled assistance in the platinum determinations. We are grateful to Professor M. L. Tobe for providing the platinum compounds.

REFERENCES

1. K. V. Shooter, R. Howse, R. K. Merrifield and A. B. Robins, *Chem.-Biol. Interactions* **5**, 289 (1972).
2. S. Reslova, *Chem.-Biol. Interactions* **4**, 66 (1971/72).
3. H. C. Harder and B. Rosenberg, *Int. J. Cancer* **6**, 207 (1970).
4. B. J. Leonard, E. Eccleston, D. Jones, P. Todd and A. Walpole, *Nature, Lond.* **234**, 43 (1971).
5. R. J. Kociba and S. D. Sleight, *Cancer Chemother. Rep.* **55**, 1 (1971).
6. B. Rosenberg and L. van Camp, *Cancer Res.* **30**, 1799 (1970).
7. T. A. Connors, M. Jones, W. C. J. Ross, P. D. Braddock, A. R. Khokhar and M. L. Tobe, *Chem.-Biol. Interactions* **5**, 415 (1972).
8. R. W. Talley, *Proc. Am. assoc. Cancer Res. Abs.* **11**, 78 (1970).
9. R. J. Kociba, S. D. Sleight and B. Rosenberg, *Cancer Chemother. Rep.* **54**, 325 (1970).
10. C. W. Welsch, *J. natn. Cancer Inst.* **47**, 1071 (1971).
11. L. van Camp and B. Rosenberg, *Proc. VII International Chemotherapy Congress*, Prague, August 1971, Avicenum, in press.
12. J. M. Venditti, *Proc. VII International Chemotherapy Congress*, Prague, August 1971, Avicenum, in press.
13. J. J. Roberts and J. M. Pascoe, *Nature, Lond.* **235**, 282 (1972).
14. K. S. Kirby and E. A. Cook, *Biochem. J.* **104**, 254 (1967).
15. C. R. Ball and J. J. Roberts, *Chem.-Biol. Interactions* **4**, 297 (1971).
16. C. R. Ball and J. J. Roberts, *Chem.-Biol. Interactions* **2**, 321 (1970).
17. T. Alper, J. F. Fowler, R. L. Morgan, D. D. Vonberg, F. Ellis and R. Oliver, *Int. J. Radiobiol.* **35**, 722 (1962).
18. J. J. Roberts, J. M. Pascoe, J. E. Plant, J. F. Sturrock and A. R. Crathorn, *Chem.-Biol. Interactions* **3**, 29 (1971).
19. A. Loveless and J. C. Stock, *Proc. roy. Soc.* **B150**, 423 (1959).
20. A Loveless and G. Shields, *Br. Emp. Cancer Campaign Ann. Rep.* **39**, 52 (1961).
21. T. A. Connors, *Proc. VII International Chemotherapy Congress*, Prague, August 1971, Avicenum, in press.
22. J. A. Howle and G. R. Gall, *Biochem. Pharmac.* **19**, 2757 (1970).
23. J. A. Howle, H. S. Thompson, A. E. Stone and G. R. Gale, *Proc. Soc. exp. Biol. Med.* **137**, 820 (1971).
24. J. J. Roberts, T. P. Brent and A. R. Crathorn, *Eur. J. Cancer* **7**, 515 (1971).
25. J. J. Roberts and J. M. Pascoe, *Proc. VII International Chemotherapy Congress*, Prague, August 1971, Avicenum, in press.
26. H. C. Harder and B. Rosenberg, *3rd International Biophysics Congress of I.U. for Pure and Applied Biophysics*, Abs. 1969, p. 31, I.D.13.
27. R. W. Guthrie, P. Melius and J. E. Teggins, *J. med. Chem.* **14**, 75 (1971).
28. R. H. Haynes, *Photochem. Photobiol.* **3**, 429 (1964).
29. J. E. Plant and J. J. Roberts, *Chem.-Biol. Interactions* **3**, 343 (1971).
30. J. E. Cleaver, *Nature, Lond.* **218**, 652 (1968).
31. J. Drobnik, M. Urbankova and A. Krekulova, *Mutation Res.* **17**, 13 (1973).
32. J. M. Pascoe and J. J. Roberts, *Biochem. Pharmac.* **23**, 1359 (1974).