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A Comparative Study of the Cytotoxicity and DNA-damaging Effects of *cis*-(Diammino)(1,1-cyclobutanedicarboxylato)-platinum(II) and *cis*-Diamminedichloroplatinum(II) on L1210 Cells

Kenneth C. Micetich,[1] Diane Barnes, and Leonard C. Erickson

Laboratory of Cell Biology and Pharmacology, Section of Hematology-Oncology, Department of Medicine, Loyola University of Chicago, Stritch School of Medicine, Maywood, Illinois 60153

ABSTRACT

Utilizing the DNA alkaline elution technique we have compared qualitatively and quantitatively the DNA lesions produced in L1210 cells after a 2 h exposure to the antitumor agents, *cis*-(diammino) (1,1-cyclobutanedicarboxylato)-platinum(II) (CBDCA) and *cis*-diamminedichloroplatinum(II) (DDP). DNA-protein and DNA interstrand cross-links are formed in cells exposed to either CBDCA or DDP. However, in comparison to DDP peak levels of these lesions occur 6 to 12 h later in CBDCA treated cells. Cytotoxicity studies reveal that CBDCA is 45 times less potent than DDP to L1210 cells when compared on a molar basis. The decreased cytotoxicity of CBDCA and the 12 h delay in peak cross-linking when compared to DDP is interpreted as a decreased reactivity of the intact CBDCA towards the DNA. This decreased reactivity may be due in part to the presence of a stable bidentate dicarboxylate chelate ring structure of CBDCA resulting in a much slower rate of hydrolysis to the active form of the drug.

INTRODUCTION

CBDCA[2] is a second generation platinum coordination compound in which a bidentate dicarboxylate chelate ligand replaces the two chlorides of the parent compound DDP (see Chart 1 for structure). These structural changes lead to significantly different pharmacological properties and toxicities from the parent compound. *In vivo* CBDCA has a longer initial half-life and is less extensively protein bound in the serum than is DDP (1).

In humans the toxicities of DDP and CBDCA are very different. Patients receiving DDP usually experience severe nausea and vomiting and require vigorous hydration to protect the kidneys from the damaging effects of the heavy metal compound. Myelosuppression is seen in less than 25% of patients. In contrast CBDCA administered to patients produces very little emesis and no renal toxicity. However, thrombocytopenia makes it dose limiting (2).

The available clinical data suggests that CBDCA has a spectrum of antineoplastic activity similar to DDP. In a Phase II trial of CBDCA administered as a continuous infusion at our institution we have noted antitumor activity in non-small cell lung cancer, bladder, and head and neck cancers (3). These tumors are also known to be responsive to DDP administration. Furthermore CBDCA is as active as DDP in untreated ovarian cancer patients and CBDCA is better tolerated by the patients (4, 5). Thus CBDCA may be an alternative antineoplastic agent to DDP in patients with significant cardiovascular or renal compromise or in patients who are unable to tolerate the significant constitutional symptomatology associated with DDP administration. While toxicity considerations suggest that CBDCA may be a useful alternative drug to DDP, it is not known if CBDCA will have the further advantage of being non-cross-resistant to DDP.

The antitumor effect of platinum compounds in general is thought to be due to reaction of the platinum molecule with nucleophilic sites on the DNA (6). The substitution of a dicarboxylate bidentate ligand for the two chlorides of DDP would decrease its reactivity with DNA due to the stability of the chelate ring structure. Previous work has demonstrated that the reactivity of CBDCA towards isolated PM-2 DNA is decreased when compared to that of DDP (7).

The biological activity of DDP is due to its hydrolysis in solution to form a monoaquated species which reacts readily with the DNA (8). Cells treated with DDP form DNA intrastrand and interstrand cross-links as well as DNA-protein cross-links (9–12). The DNA intrastrand cross-link is the predominant DNA adduct formed (9). The DNA interstrand cross-link accounts for less than 1% of the platination sites on the DNA (9, 13). Studies correlating cytotoxicity with DDP induced DNA interstrand cross-link formation have yielded conflicting results (12, 14, 15). Thus at the present time the DNA-platinum adduct(s) responsible for cytotoxicity is unknown. While DNA intrastrand cross-links are not measurable by any convenient technique, DNA interstrand and DNA-protein cross-links are easily measurable using filter elution methodology (11).

In intact cells it is not known whether the presence of the very stable ring structure of CBDCA allows reaction with DNA to the same extent as noted with DDP. We have therefore undertaken a comparative study of DNA lesions produced by CBDCA and DDP in L1210 cells. Specifically does CBDCA form DNA interstrand and DNA-protein cross-links? Also if these lesions are formed are the absolute frequencies and kinetics of formation of these DNA adducts the same as or different from those produced by DDP?

MATERIALS AND METHODS

Drug Treatment. CBDCA (M_r 371; solubility, >5 mg/ml in aqueous solution) was provided by the Investigational Drug Branch, National Cancer Institute. Stock solutions of drug (5 mM; 1.855 mg/ml of solvent) were prepared by vigorously stirring the drug in distilled water for 30 min at 37°C. The resulting solution was sterile filtered and used immediately. DDP (M_r 301; solubility, 1 mg/ml in aqueous solution) was obtained from Sigma Biochemicals (St. Louis, MO). Stock solutions of drug (0.5 mM;

[1] To whom requests for reprints should be addressed, at 2160 South First Avenue, Maywood, IL 60153.

[2] The abbreviations used are: CBDCA, *cis*-(diammino)(1,1-cyclobutanedicarboxylato)-platinum(II); DDP, *cis*-diamminedichloroplatinum(II); ISC, interstrand cross-link; DPC, DNA-protein cross-link.

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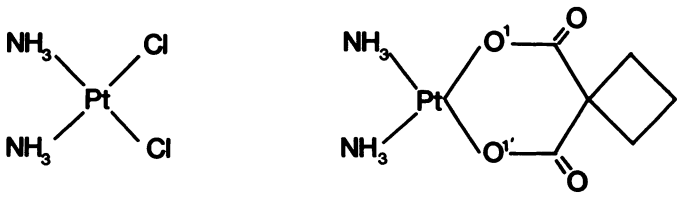

CIS-DDP **CBDCA**

Chart 1. The chemical structures of DDP and CBDCA.

1.0 mg/6.67 ml of solvent) were prepared as described above and used immediately. Exponentially growing L1210 cells were resuspended in fresh RPMI 1630 medium (supplemented with penicillin, streptomycin, 1 mM L-glutamine, and 15% heat inactivated fetal calf serum) and the cell density was adjusted to 3–5 × 10^5 cells/ml. The drug treatments were carried out in 25 cm^2 sterile flasks. To 4.5 or 9.0 ml of cells were added to the appropriate amount of drug solution and drug diluent to yield the final desired concentration of drug in 5 or 10 ml reaction volumes. The addition of a constant volume of drug solvent to all reaction mixtures ensures constancy of the extracellular milieu among drug treatments of differing concentrations. Cells were exposed to CBDCA or DDP for 2 h. The cells were then centrifuged at 800 × *g* and resuspended in RPMI without fetal calf serum, recentrifuged, and washed into RPMI 1630 supplemented with 15% heat inactivated fetal calf serum.

Survival Assays. Exponentially growing L1210 cells were treated and washed as above and immediately seeded into culture tubes as described as above and immediately seeded into culture tubes as described by Chu and Fisher (16). Surviving colonies were counted after 14 days and the log survival fraction was calculated. Colony forming efficiency of untreated cell growth varied between 70 and 90%.

Alkaline Elution Assays. Alkaline elution was performed as described by Kohn *et al.* (17). Exponentially growing L1210 cells were labeled 24 h prior to drug treatment with [^{14}C]thymidine, 0.02 μCi/ml (>56 mCi/mmol; New England Nuclear, Boston, MA). L1210 cells which were used as internal standards were labeled with [^{3}H]thymidine, 0.05 μCi/ml (20 mCi/mmol; New England Nuclear).

DNA Interstrand Cross-Links. Drug treated cells were washed and suspended in ice cold fully supplemented medium and subjected to 300 rads γ-irradiation (Gammacell 1000; Atomic Energy of Canada, Ltd., Ottawa, Canada). Cells, 6 × 10^5 (50% ^{14}C-labeled drug treated cells and 50% ^{3}H-labeled untreated cells) were deposited on a 0.8 μm pore size polycarbonate filter (Nucleopore Corp., Pleasanton, CA) and lysed with 5 ml of a solution containing 0.1% sodium dodecyl sulfate, 0.1 M glycine, and 0.025 M disodium EDTA, pH 10.1. Two ml of a solution of proteinase K dissolved in lysis (0.5 mg/ml; EM Biochemicals, Cincinnati, OH) was added to the upper chamber of a Swinnex filter holder and 40 ml of a solution of tetrapropylammonium hydroxide containing 0.1% sodium dodecyl sulfate and 0.02 M EDTA, pH 12.1, was overlayered and pumped through the filter at a rate of 0.035 ml/min. Five fractions were collected at 3 h intervals. The [^{14}C]DNA and the [^{3}H]DNA radioactivity remaining on the filter and in the eluted fractions was determined by counting in a Beckman LS 5800 liquid scintillation counter and the retention of label on the filter as a function of time of elution was calculated. DNA ISC frequency in rad equivalents was calculated by

$$\text{ISC} = \{[(1 - r_0)/(1 - r)]^{-½} - 1\} \times 300 \text{ rads}$$

where r and r_0 are the fractions of [^{14}C]DNA of treated and control cells remaining on the filter when 25% of [^{3}H]DNA is retained on the filter (17).

DNA-Protein Cross-Links. Drug treated and washed cells were suspended in ice cold medium and subjected to 3000 rads γ-irradiation. Cells were deposited on a 2.0 μm pore size polyvinylchloride filter (Millipore type BSWP; Millipore, Bedford, MA) and lysed as described above. The filter was washed with 5 ml of 0.02 M disodium EDTA, pH 10.1. The eluting solution used as tetraethylammonium hydroxide (Aldrich Chemical Co., Milwaukee, WI) containing 0.02 M EDTA, pH 12.1. Three fractions were collected at 3 h intervals. The retention of [^{14}C]DNA of treated cells and [^{3}H]DNA of untreated control cells was determined as described above. The DPC frequency (in rad equivalents) was calculated by

$$\text{DPC} = [(1 - r)^{-½} - (1 - r_0)^{-½}] \times 3000 \text{ rads}$$

where r and r_0 is the retention of [^{14}C]DNA and [^{3}H]DNA, respectively, after 6 h of elution (17).

RESULTS

The cytotoxicity of a 2 h exposure of CBDCA and DDP towards L1210 cells is presented in Chart 2, *A* and *B*, respectively. Qualitatively the curves are similar. D_0 values (obtained from the terminal straight portion of the cell survival curves) for CBDCA and DDP are 47 and 1.05 μM, respectively. Thus CBDCA is approximately 45 times less potent than DDP.

Alkaline elution profiles of DNA isolated from L1210 cells exposed to CBDCA for 2 h are shown in Chart 3. Chart 3*A* (elution performed with proteinase K) is a representative experiment performed 12 h following the addition of drug. With increasing the dose of drug we observed a dose dependent increase in the level of DNA interstrand cross-links. Chart 2*B* presents the elution profiles of drug treated L1210 cells exposed to 3000 rads of γ-irradiation 12 h following the addition of drug and assayed for DPC. With increasing dose of drug there was a dose dependent increase in the level of DNA-protein cross-links.

The kinetics of DDP and CBDCA induced ISC formation after a 2 h exposure of cells to drug is shown in Chart 4. DDP (3 or 6 μM) induced ISC levels reach a maximum between the sixth and 12th h following the addition of drug and are falling by the 18th hour. This is in agreement with work published previously by Zwelling *et al.* (11, 12). In contrast to DDP, CBDCA (200 or 300 μM) induced ISC levels do not peak until the 18th h and have only fallen slightly by the 24th h following drug exposure.

DDP and CBDCA induced DPC frequencies from three or more independent experiments are shown in Table 1. DPCs formed in DDP treated cells are detectable in significant quantity

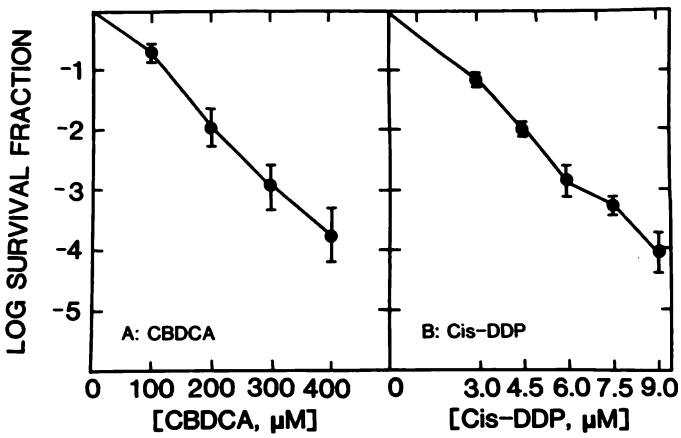

Chart 2. Survival of colony forming ability of L1210 cells exposed for 2 h to CBDCA (*A*) and DDP (*B*). *Points*, mean ± SD (*bars*) of at least three independent determinations.

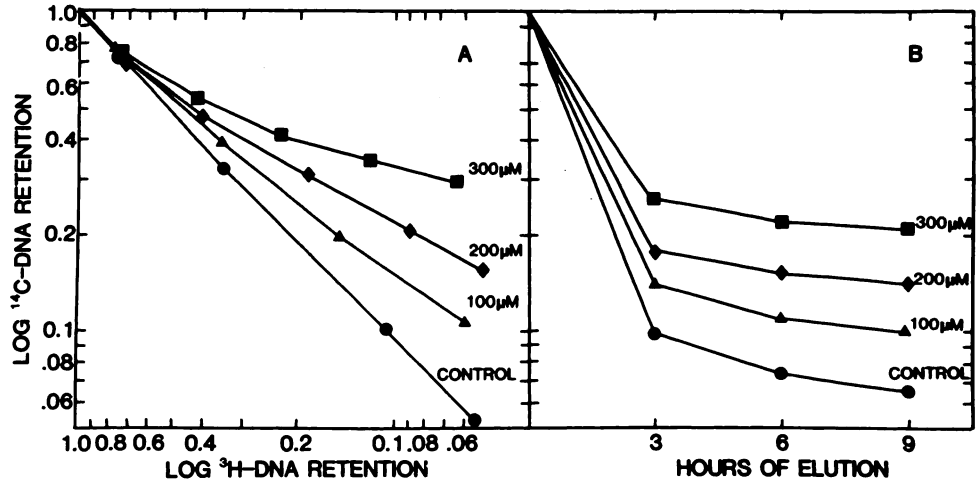

Chart 3. DNA alkaline elution profiles of L1210 cells exposed to CBDCA for 2 h and then incubated in drug free medium for 10 h. *A*, cells that received 300 rads of γ-irradiation and were analyzed for DNA interstrand cross-links with proteinase K; *B*, cells that received 3000 rads of γ-irradiation and were analyzed for DNA-protein cross-links without the addition of proteinase K.

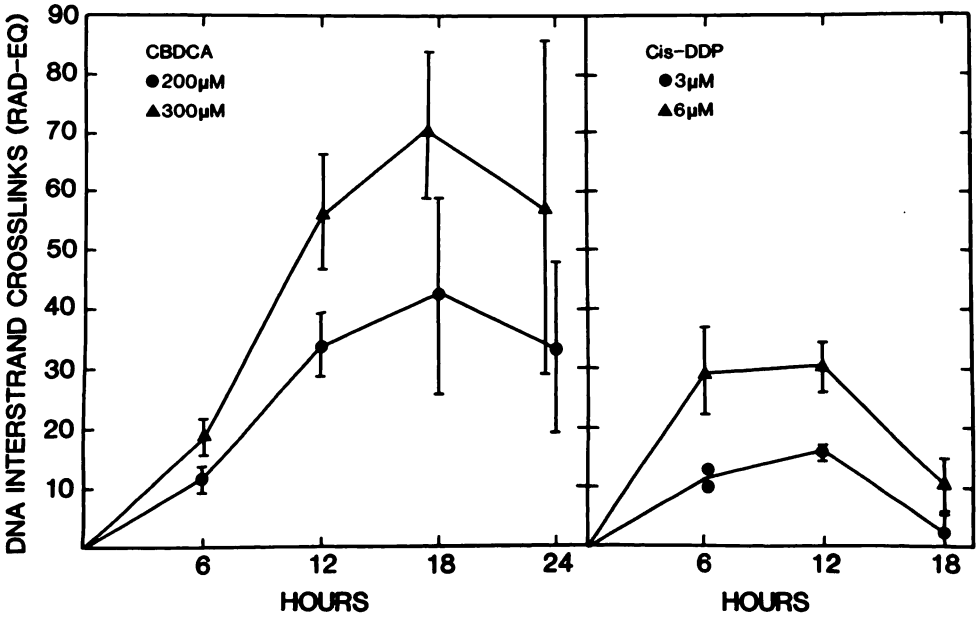

Chart 4. Kinetics of DNA interstrand cross-link formation of L1210 cells exposed to CBDCA or DDP. Alkaline elution was performed at the indicated times following the addition of drug. *Points*, mean ± SD (*bars*) of at least three independent determinations. Where necessary ▲ *points* slightly offset to the left when *error bars* would overlap. *RAD-EQ*, rad equivalents.

Table 1

DNA-protein cross-link frequencies observed in L1210 cells exposed to CBDCA and DDP for 2 h

DNA-protein cross-links were measured in rad equivalents at indicated times following the addition of drug. Values are the mean ± SD of at least three independent determinations. Where SDs are not indicated the average of two independent determinations is reported.

Concentration (μM)	2 h	6 h	12 h	24 h
DDP				
3.0	75 ± 16	166 ± 14	233 ± 22	178 ± 77
4.5	119 ± 37	312 ± 72	329 ± 60	214 ± 78
6.0	180 ± 11	404 ± 68	501 ± 12	200 ± 73
CBDCA				
100	3.5	85 ± 3	111 ± 31	120
200	18.8 ± 5	156 ± 33	223 ± 69	287 ± 41
300	15.6 ± 13	212 ± 42	396 ± 67	487 ± 68

immediately following the removal of DDP from the cells at 2 h. By 6 h following the drug exposure they have reached at least 80% of the maximum value which occurs at 12 h. In contrast CBDCA induced DPCs are barely detectable immediately following the removal of drug and at 12 h have reached at least 80% of the 24 h value. DDP and CBDCA DNA-protein cross-link levels both show only a modest rise after the sixth and the 12th hours, respectively. Major differences between the two drugs are that CBDCA induced DPC formation lags behind that of DDP induced DNA-protein cross-links by about 6 h and that CBDCA DNA-protein cross-link frequencies are still rising 24 h after exposure of cells to drug. However, at equitoxic doses of DDP and CBDCA, DNA-protein cross-link levels formed in cells after exposure to either drug are quantitatively similar. Table 1 shows that DPC levels achieved between 6 to 12 h after L1210 cells are treated with 4.5 μM DDP and that those achieved between 12 and 24 h after cells are exposed to 200 μM CBDCA (an equitoxic dose) are quite similar.

The kinetics of platinum induced DNA ISC formation at equitoxic doses of DDP (4.5 μM) and CBDCA (200 μM) are compared in Chart 5. DDP reaches maximal ISC levels 6 to 12 h earlier than does CBDCA. CBDCA ISC are sustained after the 12th h whereas DDP induced cross-links fall much faster after the 12th h following drug removal. In the case of CBDCA treated cells this may be due to a prolonged formation of ISC. The ISC levels are, however, comparable to the two drug treatments 12 h following the removal of drug.

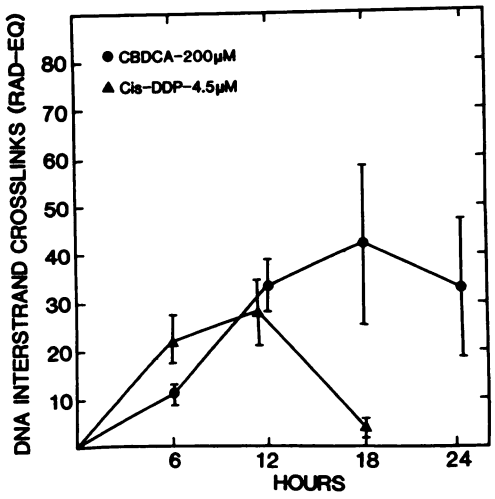

Chart 5. Comparison of the kinetics of DNA interstrand cross-link formation in L1210 cells treated with equitoxic doses of CBDCA and DDP. Alkaline elution was performed at the indicated times following the addition of drug. *Data points*, mean ± SD (*bars*) of at least three independent determinations. Where necessary ▲ *points* slightly offset to the left when *error bars* would overlap. *RAD-EQ*, rad equivalents.

DISCUSSION

The data presented comparing CBDCA to DDP, both platinum coordination compounds, indicate that CBDCA is similar to DDP in the type of DNA lesions it produces. However, CBDCA is dissimilar in the kinetics of lesion formation and lesion frequencies produced after a 2 h exposure of the drug to L1210 cells. As has been observed with DDP, CBDCA treatment of cells induces the formation of both DPC and ISC. Both of these lesions are characteristic of cells treated not only with DDP but also with other bifunctional alkylators (11, 12, 18, 19). However, our data shows that there are three major differences between the two drugs: (*a*) CBDCA is approximately 45 times less toxic than is DDP to L1210 cells when compared to a molar basis; (*b*) CBDCA induced DNA interstrand cross-links peak approximately 12 h later when compared to DDP induced kinetics of ISC formation; (*c*) CBDCA induced DPCs reach close to maximal levels about 6 h later than do DDP induced DPC.

The data taken together suggest that CBDCA in comparison to DDP has decreased reactivity towards the DNA. This lessened reactivity can occur at several levels. The cells may take up less CBDCA. This would account for the decreased cytotoxicity of CBDCA but could not alone explain the 12 h difference between the two drugs in producing peak DNA effects following a 2 h drug exposure. Alternatively CBDCA and DDP may have comparable cellular uptake but CBDCA possessing the very stable dicarboxylate chelate ring structure must undergo opening of the chelate ring in order to react with DNA. This could explain not only the cytotoxicity difference between the two drugs but also the 12 h delay in producing peak DNA effects. Our data cannot address the question of differences in cellular uptake of the two drugs. However, it is likely that the stability of the bidentate chelating ligand of CBDCA in contrast to the excellent leaving capacity of the two chloride groups of DDP is an important factor in explaining the differences between the two platinum compounds.

To form an interstrand DNA cross-link DDP undergoes hydrolysis to form a DNA reactive monoaquated species and a monofunctional DNA platinum adduct forms (20). Over time the remaining platinum-chloride bond of the monofunctional adduct either undergoes hydrolysis and/or a nucleophilic attack by DNA and a bifunctional cross-link is formed with the DNA. Thus DDP induced ISC formation is delayed. Replacement of the chloride species with water is necessary for reaction with DNA and in fact the rate of reaction of DDP with DNA is proportional to the rates of the hydrolysis reactions (21). In a similar manner it is probable that CBDCA undergoes two successive hydrolysis reactions in order to form monofunctional and then bifunctional adducts with DNA. The rate and equilibrium constants of the hydrolysis reactions of CBDCA must be smaller than those of DDP in order to explain the differences in cytotoxicity and DNA damaging effects between CBDCA and DDP. In this context the delay of CBDCA induced ISC formation relative to DDP must be due to either a slower rate of monofunctional adduct formation and/or a slower rate of monofunctional to bifunctional adduct conversion.

Although the hydrolytic rates for CBDCA are not known Cleare *et al.* (22) have demonstrated that CBDCA dissolved in water shows no change in molar conductance over time. However, CBDCA dissolved in dilute sodium chloride solution does show about a 20% increase in molar conductance over time indicating substitution by chloride does occur (22). In contrast DDP shows a significant increase in the molar conductivity over time when dissolved in water indicating hydration. Thus CBDCA is relatively unreactive in aqueous solution when compared to DDP. It is probable that CBDCA undergoes opening of the chelate ring structure in the presence of chloride ion in the extracellular and intracellular environment. To explain the differences in the cytotoxicity and DNA damaging effects between the two drugs one must postulate that the ring opening of CBDCA to render the molecule DNA reactive is slow and the equilibrium position favors the intact molecule.

Because of the unreactive nature of CBDCA in solution compared to DDP Cleare (23) has suggested that enzymatic cleavage of the chelate ring structure occurs *in vivo* (23). At the present time there is no evidence for or against this occurrence. However, Mong *et al.* (7) have demonstrated an interaction between CBDCA and isolated PM-2 DNA indicating that CBDCA can react with DNA *in vitro*. Additionally recent nuclear magnetic resonance studies of CBDCA in aqueous solution indicate that rapid ring rotation occurs about the chelating oxygens (Chart 1, O^1 and $O^{1'}$) (24). Thus direct coordination of a water molecule or a chloride ion to platinum can occur and trap the bidentate chelate in a half-open position rendering CBDCA DNA reactive.

In conclusion L1210 cells treated with CBDCA accumulate DPC and ISC which are characteristically formed in cells treated with DDP. In CBDCA treated cells these DNA lesions form at a slower rate than in DDP treated cells. For CBDCA to form monofunctional and bifunctional DNA adducts successively, two hydrolysis reactions probably occur in a stepwise fashion. The rates of these reactions will probably be slower than similar reactions of DDP since there is a delay in formation of CBDCA induced ISC relative to DDP induced ISC formation. Since Cleare *et al.* (22) have demonstrated that there is an increase in molar conductance over time of CBDCA dissolved in chloride ion containing solution, work is currently underway to determine if the frequency of CBDCA induced DNA lesions and cytotoxicity can be altered by dissolving the drug in solutions of various

chloride ion concentrations and for varying times prior to exposing L1210 cells to the drug.

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