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ENHANCED FORMATION OF REACTIVE SPECIES FROM *CIS*-DIAMMINE-(1,1-CYCLOBUTANEDICARBOXYLATO)-PLATINUM(II) (CARBOPLATIN) IN THE PRESENCE OF OXYGEN FREE RADICALS

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Abstract—Experiments were designed to investigate the influence of oxygen free radicals on the rate of conversion of the anticancer drug *cis*-diammine-(1,1-cyclobutanedicarboxylato)platinum(II) (CBDCA) to reactive species able to bind to DNA. A system containing the Fe–EDTA chelate and ascorbate was used to generate free radicals. The rate of drug conversion to by-products, during incubation in chloride-free phosphate buffer at 37°, was determined by HPLC analysis and found to be approximately 10 times faster in the presence of the free radical generating system, compared to CBDCA alone. The hydroxyl radical scavenger, mannitol, was able to reduce the rate of CBDCA conversion significantly, while an enhancing effect was observed in the presence of superoxide dismutase. The platinum containing species, which are formed in the presence of free radicals, were demonstrated to react with isolated salmon sperm DNA. The rate of platinum binding to DNA during incubation of CBDCA in the presence of the Fe–EDTA/ascorbate system was markedly enhanced. No effect on platinum binding to DNA during incubation with *cis*-diamminedichloroplatinum(II) (CDDP) in the same experimental conditions was observed, thus excluding an increased susceptibility of DNA itself to binding of platinum, due to DNA damage induced by free radicals. These findings support the hypothesis that the increased conversion of CBDCA, previously observed in our laboratory, which occurs in the presence of hemoglobin could be mediated by a Fenton-like reaction resulting in oxygen free radical production, thus providing potential clues to improvements in the clinical use of this drug.

Platinum coordination complexes are potent anticancer chemotherapeutic agents, which exhibit a broad spectrum of activity against solid tumors [1, 2]. Carboplatin (CBDCA†) has recently been introduced in the clinical practice to overcome some of the toxic effects of cisplatin (CDDP) [3]. Preclinical and clinical studies have shown that CBDCA, while possessing an antineoplastic activity similar to that observed for CDDP, is less nephrotoxic and emetogenic, with myelosuppression being the dose limiting toxicity [4, 5].

Cytotoxic effects of CBDCA, as well as of the other platinum coordination complexes, are mostly related to interaction with cellular DNA, since these compounds form inter- and intra-strand crosslinks and DNA-protein crosslinks [6–12].

It has been shown that these effects, for CDDP and CBDCA, are mediated by the formation of common intermediate species, i.e. the aquation products, in which the leaving ligands, such as the two chlorides and the cyclobutanedicarboxylatochelate, respectively, are substituted by water [8]. Bidentate ligands of platinum are quite stable as leaving ligands (the half-life of displacement is 168 hr for CBDCA, compared to 2.4 hr for CDDP) [8], thus reducing the rate of aquation of the molecules and their rate of reaction with DNA *in vitro* [8, 11]. In spite of the chemically unreactive nature of CBDCA and other platinum coordination complexes containing bidentate leaving groups [8, 13], these drugs exhibit significant cytotoxic effects, that have been postulated to involve an enzymatic cleavage *in vivo* [14, 15], or alternatively, as yet unknown modification processes.

Whatever the underlying mechanisms may be, conversion of the parent CBDCA molecule to aquation species *in vivo* seems to be the triggering event resulting in cytotoxicity. Presently, there is no evidence of possible factors that could affect the rate of bidentate ligands substitution inside the cells, thus limiting our knowledge of cytotoxicity mechanisms. We have previously reported that the presence of hemoglobin was able to increase remarkably the rate of intracellular activation of CBDCA to its reactive by-products, both in erythrocytes and in erythroleukemic cells induced to hemoglobin production [16, 17]. The enhancing effect of hemoglobin could be inhibited by its conversion to methemoglobin or carbomonoxyhemoglobin, thus suggesting an important role of the divalent heme iron and of its redox cycling in this process [16].

A fraction of hemoglobin-bound oxygen is known to be released as a superoxide anion (about 10^3 to 10^6 O_2^- molecules per erythrocyte per second) [18], that is readily converted to H_2O_2 by superoxide dismutase (SOD). Highly reactive hydroxyl radicals ($OH^\cdot$) can be generated through the interaction

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† Abbreviations: CBDCA, *cis*-diammine-(1,1-cyclobutanedicarboxylato)platinum(II); CDDP, *cis*-diamminedichloroplatinum(II); SOD, superoxide dismutase.

between O_2^- and H_2O_2, according to the iron-catalysed Haber–Weiss reaction or via the Fenton reaction [19]. Hemoglobin has been previously shown in our laboratory to decompose H_2O_2, according to a Fenton-like reaction in which ascorbate recycles methemoglobin to hemoglobin [20]. On the basis of these observations, we developed the working hypothesis that the enhancing effect of CBDCA activation by hemoglobin could be mediated by release of highly reactive oxygen species.

The aim of this study was to investigate CBDCA stability and its ability to bind to DNA *in vitro* (as a potential indicator of *in vivo* cytotoxicity) in the presence of oxygen free radicals. Since purified hemoglobin proved, in preliminary experiments, to be inadequate as a potential source of free radicals, because of binding of activated platinum species to amino acids and of their rapid quenching, we used an iron–EDTA chelate and ascorbate as a free radical generating system [21–28].

This system has been described to produce significant amounts of $OH^\cdot$, also in the absence of added H_2O_2, via an O_2^- dependent pathway, involving H_2O_2 as the intermediate [24]. Ascorbate acts in this system as a reducing agent for the chelate-complexed Fe^{3+} that is generated in step 1 (see Scheme). Therefore, a continuous Fe^{2+} auto-oxidation takes place, which leads to O_2^- production. On the other hand, $OH^\cdot$ radicals are generated from H_2O_2 through a Fenton-like reaction, according to the following steps [25, 27]:

$$Fe^{2+}\text{–EDTA} + O_2 \rightarrow Fe^{3+}\text{–EDTA} + O_2^- \quad (1)$$

$$O_2^- + O_2^- \xrightarrow{2H^+} H_2O_2 + O_2 \quad (2)$$

$$Fe^{2+}\text{–EDTA} + H_2O_2 \rightarrow Fe^{3+}\text{–EDTA} + OH^\cdot + OH^- \quad (3)$$

$$2\,Fe^{3+}\text{–EDTA} + \text{ascorbate} \rightarrow 2\,Fe^{2+}\text{–EDTA} + \text{dehydroascorbate.} \quad (4)$$

Reaction 1 is made more favorable by chelation with EDTA, which shifts the Fe^{2+}/Fe^{3+} potential to −0.12 V [19]. Furthermore, ascorbate itself has been suggested to react with O_2^- to form H_2O_2 and dehydroascorbate [28].

This paper presents evidence for an increase of CBDCA conversion to by-products, able to react with isolated DNA, in the presence of oxygen free radicals.

MATERIALS AND METHODS

Materials. CBDCA was kindly supplied by Bristol-Myers-Squibb (Evansville, IN, U.S.A.). CDDP, salmon sperm DNA, ascorbic acid, mannitol, catalase (from bovine liver, EC 1.11.1.6), SOD (from bovine erythrocytes, EC 1.15.1.1), deoxyribonuclease I (from bovine pancreas, EC 3.1.21.1) and micrococcal nuclease (EC 3.1.31.1) were purchased from the Sigma Chemical Co. (St Louis, MO, U.S.A.). Sodium EDTA was from Merck (Darmstadt, Germany).

Analysis of CBDCA stability. Quantitation of CBDCA, in order to determine the rate of its conversion to by-products in the different experimental conditions, was performed by HPLC analysis. Samples (100 μL) were injected onto a reverse phase C18 chromatographic column (3.9 × 300 mm, 10 μm particle size, Waters Associates, Inc., Milford, MA, U.S.A.), using milliQ H_2O, pH 5.0, as eluant at a flow rate of 0.5 mL/min. The eluate was monitored both by UV absorbance at 230 nm and by collection of 250-μL fractions, followed by atomic absorption analysis, as described below.

The rate of CBDCA conversion, either alone or in the presence of a free radical generating system, was determined by loss of peak area of the parent compound at 230 nm and by measuring platinum concentration in the different peaks.

Reaction mixtures contained the following reagents; 1–4 mM CBDCA, 1 mM sodium EDTA, 1 mM Fe–EDTA and 10 mM ascorbate. Incubations were performed in 100 mM sodium phosphate buffer, pH 7.2 at 37°. $FeSO_4$, EDTA and ascorbic acid solutions were prepared immediately prior to use. Ascorbic acid was neutralized and $FeSO_4$ and EDTA were pre-mixed at equimolar concentration prior to addition to the reaction mixture. Free radical scavengers were added to the samples at the following final concentrations; mannitol, 200 mM, catalase, 500 U/mL and SOD, 500 U/mL.

To characterize further the by-products formed by CBDCA in the presence of the Fe–EDTA/ascorbate system, two different HPLC analyses were used. Ion-pair chromatography, which allowed the separation of CDDP and its mono-aquated and di-aquated species, was performed using a C18 reverse phase column (3.9 × 300 mm, Waters Associates, Inc.), with a non-linear gradient, at a flow rate of 1 mL/min. The following mobile phases were used: buffer A consisted of 10 mM phosphate buffer, pH 3.0, with 2.5 mM 1-heptanesulfonate and buffer B was a 40% solution of acetonitrile in 10 mM phosphate buffer, pH 3.0, with 2.5 mM heptanesulfonate. The gradient consisted of 100% buffer A from 0 to 5 min, then a linear gradient to 100% buffer B from 5 to 10 min, followed by 100% buffer B from 10 to 30 min. CDDP eluted at 6.5 min, the mono-aquated species at 11.5 min, the di-aquated species at 23.2 min and CBDCA at 9.0 min. The mono-aquated form was obtained upon incubation of CDDP with $AgNO_3$ [29]. A second type of chromatography (gel permeation) consisted of a TSK-GEL SW column (TosoHaas, Montgomeryville, PA, U.S.A.) using H_2O, pH 5.0, as eluant at a flow rate of 0.5 mL/min. The eluates were monitored both by UV absorption at 230 nm and by determining platinum concentration in 0.5-mL fractions of the eluate.

Platinum binding to DNA. Salmon sperm DNA was dissolved in 10 mM sodium phosphate buffer, pH 7.2, to a final concentration of 1 mM (determined by UV absorbance at 260 nm, using $E_{260} = 6650$ [10]) and it was then incubated at 37° with either 4 mM CBDCA or 0.5 mM CDDP, alone or in the presence of the oxygen radical generating system described above. At each time interval, 1-mL aliquots of the samples were collected and DNA was precipitated by ethanol for three times to remove the unbound

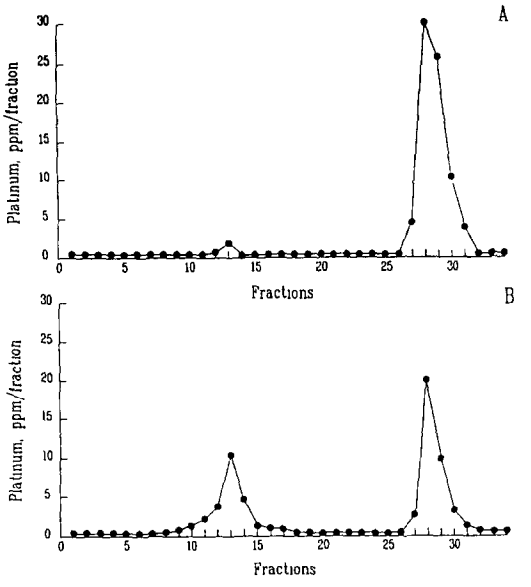

Fig. 1. HPLC analysis of CBDCA incubated in phosphate buffer, pH 7.2, at 37° for 24 hr either alone (A) or in the presence of Fe–EDTA/ascorbate (B). Chromatograms were obtained by plotting platinum concentration, measured in 250-μL fractions of the eluate. Dotted lines represent the solvent front.

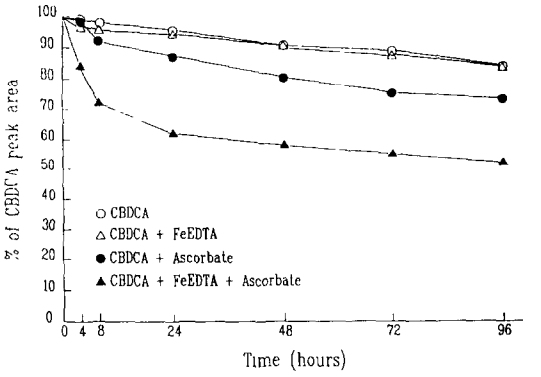

Fig. 2. Conversion of CBDCA to by-products in the different experimental conditions, determined by HPLC analysis as disappearance of peak area of the parent compound with time. Incubations were performed in phosphate buffer, pH 7.2, at 37°. CBDCA concentration was 4 mM. For other experimental details see Materials and Methods. Data are expressed as per cent of peak area of CBDCA at zero time. N = 3, SD values were less than 10%.

drug [30]. Dialysis of samples for 24 hr at 4° was also performed in parallel, to exclude the possibility that unbound platinum could persist after ethanol precipitation; the results obtained after determination of platinum bound to DNA were comparable to those observed after ethanol precipitation.

DNA bound platinum was determined by atomic absorption, following digestion of the samples with 65% HNO_3 for 2 hr at 160°. DNA concentration in each ethanol precipitated sample was determined by UV absorption at 260 nm. To avoid differences in absorption due to DNA fragmentation, it was resuspended in 10 mM Tris–HCl buffer, pH 7.1, 4 mM $MgCl_2$, 0.02% NaN_3 and incubated for 18 hr with 10 U/mL of deoxyribonuclease I and 10 μg/mL of micrococcal nuclease. The concentration of the resulting nucleotides was determined using a value of $E_{260} = 11.000$ [10].

Atomic absorption analysis. Samples were analysed using a Varian atomic absorption spectrometer AA100, with an air/acetylene flame, at a wavelength of 265.9 nm. Samples were treated with 1 N HCl and 1% lanthanum to overcome interference [16].

RESULTS

CBDCA stability

Figure 1 shows the chromatograms obtained by determining the platinum concentration in each fraction of the eluates after 24 hr of incubation at 37° of CBDCA either alone (Fig. 1A) or in the presence of the oxygen free radical generating system (Fig. 1B). CBDCA exhibited a retention time of 14.0 min corresponding to fractions 26–30. Another platinum containing peak was detected at approximately 6.5 min, which is likely to represent the aquation products. Total platinum recovery in the fractions collected from the eluates for CBDCA alone, or in the presence of Fe–EDTA, or of ascorbate was comparable in the three systems (93.0 ± 1.4% of the injected platinum). It was a little lower in the presence of the Fe–EDTA/ascorbate system (82% of injected platinum), thus indicating the possibility of an irreversible binding to the column of a small fraction of the platinum-containing species formed by this system.

Conversion of CBDCA, at a 4 mM concentration, to its by-products in different experimental conditions, is illustrated in Fig. 2. The conversion of the parent molecule in phosphate buffer obeys pseudo first-order kinetics, with a rate constant of $3.2 \pm 0.52 \times 10^{-7}\,sec^{-1}$, which is lower than those previously reported by Knox *et al.* [8], corresponding to $7.2 \times 10^{-7}\,sec^{-1}$, and by Allsopp *et al.* [31], corresponding to $5.7 \times 10^{-7}\,sec^{-1}$, respectively. When the Fe–EDTA/ascorbate system was added, the initial rate of conversion, calculated for the first 8 hr, was almost 10 times faster with a constant of $5.0 \times 10^{-6}\,sec^{-1}$. When the free radical production decreased, due to ascorbate consumption, the rate of conversion returned almost comparable to the control (Fig. 2). Increasing ascorbate concentration in the incubation medium led to an increase in the percentage of conversion of CBDCA to by-products (data not shown). No effects were observed following addition of the Fe–EDTA chelate alone, whose ability to generate free radicals in phosphate buffer, estimated by ethylene production from methional, was very low (Tonetti M, unpublished data). The slight increase of conversion due to the presence of ascorbate alone could be related to H_2O_2 production

Table 1. Effects of scavengers on the rate of CBDCA conversion, determined by HPLC analysis, in the presence of an oxygen free radical generating system

	% of Peak area*	
	6 hr	24 hr
Control	99.0	96.8
Fe–EDTA/ascorbate	76.6	41.8
Fe–EDTA/ascorbate + mannitol	91.2	82.8
Fe–EDTA/ascorbate + SOD	61.3	32.7
Fe–EDTA + catalase	72.9	44.3

* Expressed as per cent of CBDCA peak area at zero time.

CBDCA concentration was 1 mM in each sample. For further explanations see Materials and Methods.

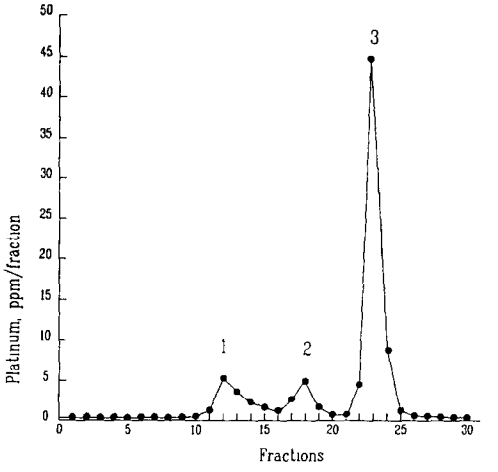

Fig. 3. HPLC gel-permeation analysis of CBDCA incubated in phosphate buffer (see Materials and Methods) in the presence of Fe–EDTA/ascorbate after 18 hr at 37°. The chromatogram was obtained by plotting platinum concentration, measured in a 0.5-mL fraction of the eluate. Peak 1 corresponds to the elution time of phosphate, peak 2 to Fe–EDTA/ascorbate and peak 3 to the parent CBDCA.

by ascorbate auto-oxidation and to the presence of contaminant iron.

To confirm further the effect of free radicals on the increase of CBDCA conversion, scavengers were added to the reaction mixtures containing the free radical generating system; in these sets of experiments a CBDCA concentration of 1 mM was used. The results obtained are reported in Table 1. The most relevant effect was observed after addition of 200 mM mannitol, a known scavenger of $OH^{\cdot}$ radicals, which was able to decrease the rate of conversion remarkably (the corresponding rate constant was $8.6 \times 10^{-7} sec^{-1}$, compared to $5.0 \times 10^{-6} sec^{-1}$ for the Fe–EDTA/ascorbate system alone). SOD, most probably through an increase of $O_2^{\cdot-}$ dismutation to H_2O_2, which then generates $OH^{\cdot}$ radicals, enhanced CBDCA conversion significantly, while no appreciable effect on this process was observed when catalase was added.

CDDP stability, determined by ion-pair chromatography, was not detectably affected by the presence of the oxygen free radical generating system or by Fe–EDTA or ascorbate alone. Since the rate of substitution of the leaving ligand chloride is a fast process *per se*, this could explain the lack of further increase.

HPLC analysis of CBDCA samples containing Fe–EDTA/ascorbate, performed to obtain a preliminary characterization of the by-products generated in this system, indicated the formation of more than one different species. Incubation of CDDP or CBDCA alone in H_2O allowed the identification of the di-aquated species from both compounds. Conversely, upon incubation in phosphate buffer of CDDP and CBDCA, either alone or in the presence of the free radical generating system, the di-aquated species could not be virtually detected. Furthermore, a significantly decreased recovery of injected platinum from the eluate was observed in these conditions, indicating loss due to binding of some by-products to the column. This finding suggested that an interaction between species formed from CDDP and CBDCA, possibly the di-aquated forms, and phosphate could occur during incubation.

In order to obtain a complete recovery of platinum from the eluate of the phosphate containing samples, a different analysis, using a gel permeation column (see Materials and Methods) was carried out. Three major platinum containing peaks were identified from CBDCA incubated in the presence of the free radical generating system, which corresponded to the retention times of phosphate (approximately 25% of injected platinum), of Fe–EDTA and ascorbate (which co-eluted with 10% of injected platinum) and of the parent CBDCA molecule (approximately 60% of injected platinum), respectively (Fig. 3). Platinum associated with phosphate was also observed when CDDP or CBDCA alone were incubated in phosphate buffer (not shown). Fractions corresponding to the phosphate peak, obtained from CBDCA in the presence of Fe–EDTA/ascorbate, were collected, vacuum-concentrated and incubated with 100 mM NaCl or $CaCl_2$ (which caused partial precipitation of phosphate). In both cases, through HPLC analysis, a significant fraction of injected platinum (30% for NaCl and more than 50% for $CaCl_2$) was recovered in a peak which exhibited the same retention time as CDDP, while the remaining part still co-eluted with phosphate. Attempts to quench incubations of CBDCA with chloride directly were not successful because CBDCA and CDDP exhibited the same elution time in these chromatographic conditions.

Platinum binding to DNA

The amount of platinum bound to DNA was greatly enhanced by incubation of CBDCA in the presence of the Fe–EDTA/ascorbate system, as shown in Figs 4 and 5. The initial rate of interaction of platinum with DNA, calculated for the first 6 hr, was 2.0 nmol of platinum per μmol of DNA per hr for CBDCA alone, and 21.6 nmol per μmol of DNA per hr for CBDCA in the presence of the free

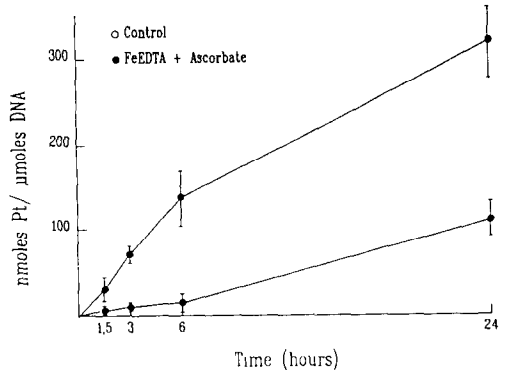

Fig. 4. Binding of 4 mM CBDCA, alone or in the presence of the Fe-EDTA/ascorbate system, to isolated salmon sperm DNA with time, during incubation in phosphate buffer at 37°. Platinum bound to DNA was determined by atomic absorption analysis of the ethanol-precipitated samples, as described in Materials and Methods.

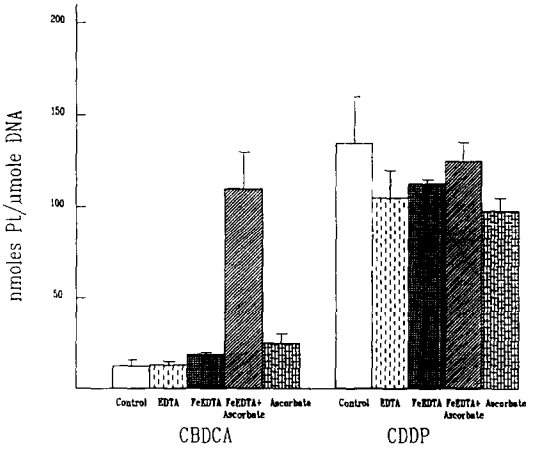

Fig. 5. Platinum bound to isolated salmon sperm DNA after 6 hr of incubation in phosphate buffer at 37°. CBDCA was 4 mM, CDDP 0.5 mM. For other experimental conditions see Materials and Methods. Data derived from three experiments.

radicals generating system, which corresponds approximately to a 10-fold increase. The presence of EDTA, Fe-EDTA or ascorbate alone had little or no effect (Fig. 5).

Furthermore, the extent of platinum binding was also determined using either heat denatured DNA or DNA pretreated with Fe-EDTA/ascorbate and subsequently purified by ethanol extraction. These experiments were designed to exclude the free radicals produced during incubation with Fe-EDTA/ascorbate, which cause DNA fragmentation or base modification [32], as this could make it more susceptible to platinum binding. In both cases the binding to DNA proved to be comparable to that observed after incubation of CBDCA with the native DNA (data not shown).

The reaction of CDDP with DNA was much faster than that of CBDCA, as shown in Fig. 5. In this case, however, the presence of Fe-EDTA/ascorbate, Fe-EDTA or ascorbate alone did not cause differences in binding to DNA. Accordingly, the increase in binding to DNA, observed after incubation of CBDCA with the free radical generating systems, seems to be due to an enhanced activation of the drug and not to other factors related to DNA modification, which would affect binding of both drugs to a comparable extent.

DISCUSSION

Aquation of both CDDP and CBDCA is believed to represent the rate limiting step for their reaction with cellular DNA and, accordingly, for their cytotoxic action [8]. The rate of hydrolysis of CDDP, which leads to the formation of aqua- or hydroxo-complexes, is strictly related to chloride concentration in the aqueous solutions [33]. When chloride concentration is lowered, as happens in the intracellular environment or in urine, aquation occurs rapidly with the consequent formation of more reactive platinum species.

On the contrary, the rate of ligand substitution for CBDCA is very slow and this probably accounts for its lower rate of reaction with isolated DNA [8], the differences in cytotoxicity [6] and the slower kinetics of crosslink formation observed in cell culture [6], compared to CDDP. It is not clear at present if, contrary to CDDP, intracellular factors may influence the rate of the bidentate ligand substitution.

The finding that hemoglobin is able to accelerate the rate of formation of reactive species [16] that can be related to the aquation products, is the first evidence that intracellular components can, presumably, play a role in the activation process of CBDCA. The precise mechanism of hemoglobin action is not known, but the requirement of heme iron in its divalent form suggests an involvement of oxygen free radicals, which can be generated by auto-oxidation of hemoglobin, through a Fenton-like reaction [20].

The incubation of CBDCA in phosphate buffer in the presence of free radicals leads to a faster rate of CBDCA conversion to by-products than observed without free radicals, as demonstrated by HPLC analysis. At present there is no conclusive evidence about the detailed molecular structure of these by-products, but they certainly include several different species. Consequently, the formation of different by-products could affect the rate and the patterns of platinum binding with DNA. A fraction of platinum was found to be associated with Fe-EDTA and ascorbate. Platinum complexes with ascorbate have been described [34], but their reactivity with DNA, as compared with CBDCA, is not clear.

The presence of the di-aquated products, demonstrated to be generated from both CDDP and CBDCA incubated in H_2O, could not be directly detected either in the presence of the free radical generating system, or when CDDP or CBDCA alone were incubated in phosphate buffer. On the contrary, a significant fraction of platinum derived from

CBDCA or CDDP conversion, either alone or in the presence of Fe–EDTA/ascorbate, was found to be associated with phosphate by HPLC analysis. These findings strongly suggest an interaction between aquated species and phosphate. Moreover, this association can be reversed by the subsequent addition of nucleophiles, such as chlorides, as demonstrated by the formation of CDDP upon addition of NaCl or, still to a greater extent, of $CaCl_2$, to the purified platinum-phosphate fraction.

Mannitol, a scavenger of hydroxyl radicals [27], was able to inhibit the enhanced conversion of CBDCA in the presence of the oxygen free radical generating system, thus giving further support to a role of $OH^{\cdot}$ in the mechanism of CBDCA conversion to by-products. SOD, which accelerates the formation of H_2O_2 from O_2^- (with a rate constant of 2×10^9 M/sec, compared to 8×10^4 M/sec of the non enzymatic dismutation at neutral pH [19]), enhanced the rate of conversion of the drug, probably by increasing the amount of H_2O_2 available for the Fenton reaction. The lack of an inhibitory effect of catalase, which should conversely be expected, owing to the leading role of H_2O_2 in the generation of $OH^{\cdot}$ radicals [19], can be related to the reported inhibitory effect of ascorbate on this enzyme activity [35].

To confirm that the platinum containing species, which are formed from CBDCA in the presence of free radicals, are able to react with DNA, the amount of platinum bound to salmon sperm DNA was determined in the different experimental conditions. Based on the structural identity of the aquated compounds derived from CDDP and CBDCA, respectively, and of the type of adducts that are formed with DNA [6, 8], the amount of CDDP bound to DNA in the presence of the Fe–EDTA/ascorbate system was also investigated, in order to exclude that factors, other than free radicals, could interfere with the binding process.

The initial rate of DNA binding of CBDCA in the presence of Fe–EDTA/ascorbate proved to be approximately 10 times faster than with CBDCA alone, while no effect whatsoever was observed for CDDP. Therefore, it can reasonably be excluded that the increase in binding observed when CBDCA is in the presence of Fe–EDTA/ascorbate could be due to DNA fragmentation or to base modifications induced by the free radicals [32].

There are no available data about the mechanisms of action of free radicals on CBDCA. One possibility is a direct oxidation of the cyclobutanedicarboxylic acid, which can make the leaving group more labile. However, it is worth noticing that the production of free radicals triggered by the Fe–EDTA/ascorbate system is highly enhanced in the presence of CBDCA (Tonetti M, unpublished data). This suggests that more complex events, including a direct participation of platinum in the generation of free radicals (e.g., by redox cycling mechanisms) might take place. Specifically, the formation of octahedral platinum(IV) species through oxidation by H_2O_2, cannot be excluded. Platinum(IV) species could then be followed by reduction to platinum(II) by ascorbate [36].

The data reported in this study, besides giving possible clues to the mechanisms of hemoglobin action in promoting CBDCA bioconversion, can provide useful information for the clinical use of this drug. In particular, the increase in oxygen free radical production occurring in many physiological and pathological conditions, e.g. by activated granulocytes and, in particular, tumor-infiltrating macrophages, could lead to an increase in CBDCA activation at selective sites. Furthermore, some of the combination therapy protocols, currently used in the clinical setting, include the association of CBDCA with redox cycling drugs, e.g. anthracyclines, or with radiotherapy [37, 38]. If the oxygen free radicals can be confirmed to play a significant enhancing role for CBDCA activation also inside the cells *in vivo*, these schedules of combination could give an advantage, compared to the use of CDDP. The amounts of hydroxyl radicals generated in the Fe–EDTA/ascorbate system can hardly be compared with those produced intracellularly, e.g. in the hemoglobin-synthesizing erythroleukemic cells. Nevertheless a localized production of free radicals, e.g. by radiotherapy or by DNA-intercalated drugs, such as doxorubicin, could lead to an increase of drug interaction with DNA. Experiments to evaluate the potential therapeutic importance of free radical activation of CBDCA are currently in progress.

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