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Blood levels and serum protein binding of *cis*-Platinum(II) complexed to carboxymethyl-dextran

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Summary. Plasma levels and serum protein binding of *cis*-diamminedichloro-platinum(II) (cis-DDP) or *cis*-diamminediaquoplatinum(II) (cis-aq) complexed to carboxymethyl-dextran (CM-dex) with a molecular weight of 10,000 (T-10), 40,000 (T-40), and 250,000 (T-250) were investigated in BALB/c mice. Levels of active drug in the circulation after the i.v. or i.p. administration of the free or complexed drugs, as well as the loss of drug activity due to serum protein binding following incubation with mouse serum, were monitored by an antitumor in vitro assay using a drug-sensitive tumor cell line. Following i.v. injection of the complexes, active platinum(II) was maintained in the circulation at higher levels and for a longer period, whereas the free drug disappeared rapidly. The rate of disappearance of the complexed drug from the circulation was markedly influenced by the molecular size of the carrier CM-dex, since the retained amount of drug was considerably higher with the T-40 and T-250 complexes than with the T-10 complex. An i.p. injection resulted in a rapid and transient appearance of low levels of the free drugs in the blood, whereas in the case of the complexes, transport to the circulation was slower and their maintenance in the blood system was markedly higher. Serum protein binding was much slower with CM-dex-complexed drugs (regardless of the molecular size of the CM-dex carrier) than with the free drugs.

Introduction

cis-Diamminedichloroplatinum(II) (cis-DDP) is an antitumor agent exhibiting activity against a variety of human malignancies, mainly head and neck cancer and endometrial, ovarian, and testicular neoplasms [14]. Pharmacokinetic studies have shown a biphasic decline following i.v. administration [2, 3, 5, 10, 20] that is initiated by a rapid plasma clearance of the free platinum drug, which is determined by a combination of protein binding [3, 5, 7, 10, 12, 19, 20], urinary elimination [2, 3, 5], and transport to the tissues [10]. Within a short time (1–2 h) following administration, only 10%–20% of the drug can be detected in the circulation, in the form of irreversibly (and therefore inactive) protein-bound drug.

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The clinical use of cis-DDP is limited by its cytotoxic side effects [11]. This limitation has stimulated the development of macromolecular drug-carrier systems, in an attempt to decrease drug toxicity by altering its differential distribution and increasing the rate of its release. We have recently studied the antineoplastic activity of polymeric derivatives of cis-DDP and its structural analogue *cis*-diamminediaquoplatinum(II) (cis-aq) [15–17]. These platinum(II) compounds can form reversible and therefore pharmacologically active complexes with carboxylic groups on high-molecular-weight carriers. Complexes between cis-DDP or cis-aq and carboxymethyl-dextran (CM-dex) were found to be active against tumor cells both in vitro and in vivo. Their in vivo therapeutic activity against F9 embryonal carcinoma in BALB/c mice was influenced by the molecular size of the CM-dex carrier. Thus, when injected i.p., the CM-dex T-40 complexes were as active as the free drug but less toxic, whereas the T-10 derivatives were both less active and less toxic [16].

The purpose of the present study was to evaluate the blood levels and protein serum binding of the CM-dex-complexed platinum(II) (referred to as active drug equivalent) compared with those of the free drugs, focusing on the presence of pharmacologically active drug species.

Materials and methods

cis-DDP was kindly provided by Abic Co. (Ramat-Gan, Israel). *cis*-Diamminediaquo-platinous nitrate was prepared as previously described [15]. Dextrans T-10 (mol. wt., 10,000), T-40 (mol. wt., 40,000) and T-250 (mol. wt., 250,000) were purchased from Pharmacia Chemicals (Uppsala, Sweden). 0-Phenylenediamine (OPDA), obtained from Fluka AG (Buchs, Switzerland), was crystallized three times from ethyl acetate before use. *N,N*,-dimethylformamide (DMF) was obtained from Merck-Schuchardt (Munich, Germany) and was used after being kept for at least 2 weeks over an indicator-free molecular sieve. Culture media and reagents were from Biolab (Jerusalem, Israel). CM-dex was prepared as previously described [8]. The extent of carboxymethylation was determined by titration with sodium methoxide. The T-10, T-40, T-250 derivatives were found to contain 3–3.3 μmol carboxymethyl group/mg dextran.

cis-DDP and cis-aq complexes of CM-dex. Cis-DDP or cis-aq (20 μmol/ml), dissolved in double-distilled water

(DDW) by slight warming, was mixed with CM-dex (20 mg/0.5 ml DDW) and the reaction was allowed to proceed for 24 h at 37° C. The reaction mixtures were then dialyzed against DDW for 17 h to remove unbound drug. The platinum(II) content was determined by using the OPDA reagent as follows. Derivatives containing platinum(II) at a concentration range of 3–12 μg in 0.6 ml DDW were mixed with 0.6 ml OPDA in DMF (1.2 mg/ml). The reaction mixtures were placed in a 100° C water bath for 10 min and the absorbance was determined at 703 nm. The amount of platinum(II) in the complexes was determined in reference to standard solutions of the free platinum(II) compounds.

Evaluation of antitumor activity. The inhibition of DNA synthesis by free or complexed drug was determined as follows: 38C-13 lymphoma cells (10^4/0.1 ml) suspended in RPMI-1640 medium supplemented with 10% fetal calf serum, glutamine, antibiotics, and 5×10^{-5} *M* β-mercaptoethanol were distributed to wells of microtiter plates. The platinum(II) derivatives were added to the wells in 25-μl aliquots, and the plates were incubated for 21 h at 37° C in a humidified atmosphere of 5% CO_2 in air. At this time, 10 μl containing 0.5 μCi [H^3]-methylthymidine (Nuclear Research Center; Negev, Israel) was added to each well and incubation proceeded for 3 h. The reaction was terminated by means of a Titerteck cell harvester (Flow Laboratories), and the incorporated [H^3]-methylthymidine was measured using a β-scintillation counter.

Blood levels. Female BALB/c mice (3 months old) received i.v. or i.p. injections of the free drugs or drug complexes (as indicated) in 0.5 ml saline (NaCl was added to 0.15 *M* prior to injection). At set intervals, blood samples were drawn and centrifuged in an Eppendorf centrifuge. The serum was appropriately diluted in saline and tested (in the range of 2.5–3.75 μl serum) against 38C-13 lymphoma cells as described above (this procedure was completed within 40 min , during which the blood or serum samples were kept on ice). Serum samples from two or three untreated mice served as controls. The injected preparations were evaluated for their antitumor activity at the onset of each experiment. The maximal expected concentration of drug in the circulation was calculated from the amount of injected drug and the total blood volume of the mice, assuming that blood constitutes 8% of the mouse body weight [1]. Blood volume for the 15-min time point was corrected for the 0.5 ml solution injected, assuming that half of this volume was still retained in the blood [1].

Protein binding. Small volumes (20 μl) of aqueous solutions of cis-DDP, cis-aq, or their CM-dex complexes were added to aliquots of saline or a serum pool (280 μl) to obtain a concentration of 0.1 μmol/ml free or complexed drug. The solutions were incubated in a dark environment at 37° C for 24 h. At set intervals, samples drawn from the solutions were appropriately diluted in saline and tested for their cytotoxicity to 38C-13 cells as described above. Serum samples incubated with saline alone or drugs alone incubated in saline served as controls. The data presented in Table 1 and Figs. 1–3 are from duplicate samples of one of three to four experiments (one to two mice per group) that were highly reproducible.

Results

Activity of drug-CM-dex complexes

The in vitro antitumor activity of cis-DDP, cis-aq, and their complexes with CM-dex T-10, T-40, and T-250 is illustrated in Fig. 1. The inhibition of DNA synthesis in 38C-13 lymphoma cells following 24 h incubation with the various drug preparations was taken as a measure of drug activity. This incubation time was optimal for determining the activity of both free and slowly released, complexed drug (see [18]). As shown, the various complexes exhibited similar extents of activity that were not different from those of the free platinum(II) drugs. This indicates that under the experimental conditions used, the cis-DDP and cis-aq retained their pharmacological activity when complexed to the CM-dex carriers, irrespective of the molecular size of the carrier.

Drug concentration in blood following i.v. administration

Blood levels of drug-carrier complexes as compared with those of the free drugs were evaluated by determining active drug levels in blood samples obtained at various intervals after the i.v. administration of 6.8 mg/kg free or complexed drug. Drug activity was assessed by monitoring the antitumor activity in the blood samples using the in vitro assay described above, namely, inhibition of DNA synthesis in 38C-13 cells. As shown in Fig. 2, drug levels declined rapidly following the administration of the free platinum(II) compounds, whereas the decrease in active drug after the injection of the complexes was slower and was influenced by the molecular size of the CM-dex carrier. Thus, levels of CM-dex T-10 complexed drug were generally only somewhat higher than those of the free platinum(II) compounds, whereas both the T-40 and T-250 CM-dex complexes were maintained at considerably higher levels and for longer periods.

Serum concentrations of active drug were determined by comparing the serum volumes required for 50% inhibition of DNA synthesis to drug concentrations in the original preparations at the 50% inhibition point (Table 1). Figure 3 illustrates the relative decline of drug in the circulation as a function of time following administration. As shown, after 15 min the free drugs were down to 2%–6% of their expected initial concentration. The T-10 complexes

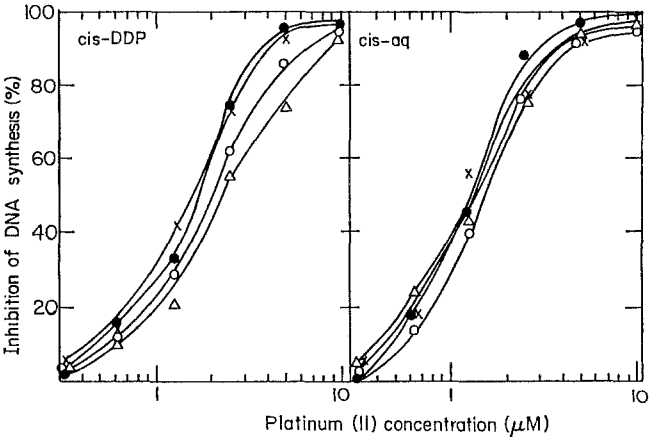

Fig. 1. Inhibition of DNA synthesis in 38C-13 cells by free drug (●) or drug complexed to CM-dex T-10 (○), T-40 (△), and T-250 (x)

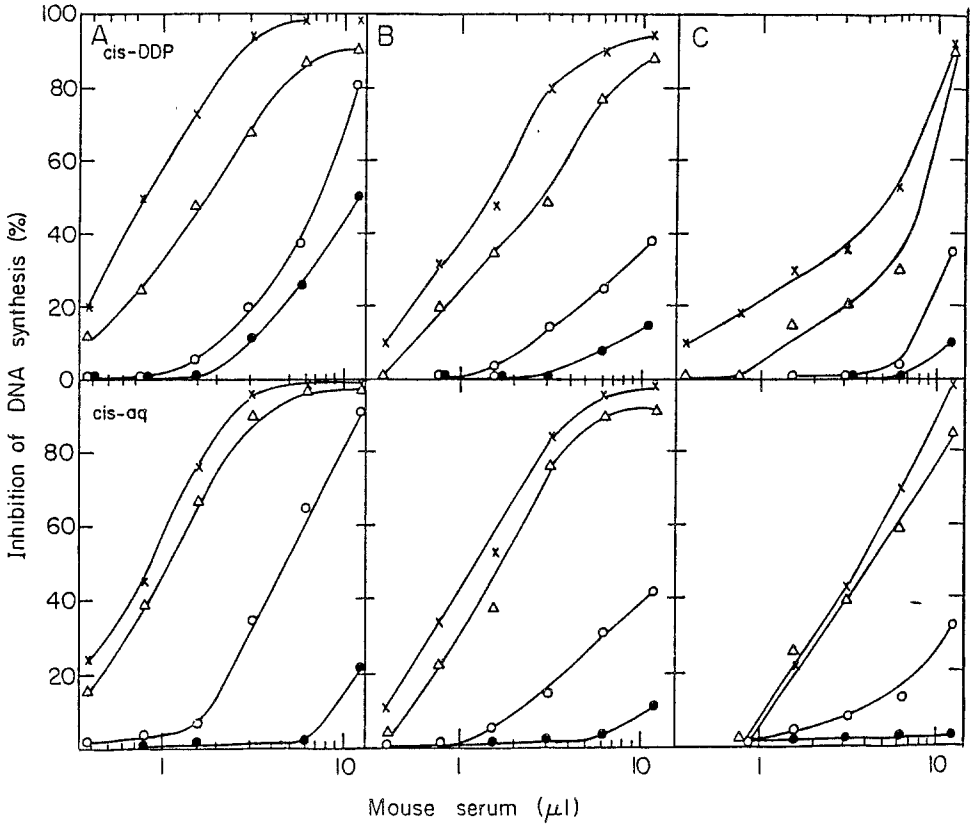

Fig. 2. Inhibition of DNA synthesis in 38C-13 cells by serum drawn from mice at 15 min (*A*), 90 min (*B*), and 6 h (*C*) following i.v. administration of 6.8 mg/kg free drug (●) or drug complexes of CM-dex T-10 (○), T-40 (△), and T-250 (x). (Identical inhibition curves were obtained when the various drug derivations were prepared in 90% mouse serum)

Table 1. Active drug in mouse serum following i.v. administration of free cis-DDP, cis-aq, and their respective complexes with CM-dex

Drug complexes with CM-dex	50% inhibition caused by				Drug in mouse serum (μg/ml)[c]		
	Drug (μg)[a]	Mouse serum (μl)[b]					
		15 min	100 min	6 h	15 min	100 min	6 h
cis-DDP:							
(free drug)	0.067	12.5	>50	>50	5.3	<1.3	<1.3
T-10	0.075	7.2	20	20	10.4	3.7	3.7
T-40	0.082	1.7	2.8	8.5	48	29	9.7
T-250	0.06	0.78	1.5	5.2	77	40	11.5
cis-aq:							
(free drug)	0.049	>25	>50	>50	<2.0	<1.0	<1.0
T-10	0.056	4.5	18	20	12.5	3.1	2.7
T-40	0.053	1.1	1.8	4.5	48	30	11.3
T-250	0.045	0.85	1.3	3.8	53	35	12

[a] The amounts of drug (free or complexed) causing 50% inhibition of DNA synthesis were extrapolated from the inhibition curves (see Fig. 1)
[b] The volumes of serum required for 50% inhibition were extrapolated from the inhibition curves (see Fig. 2)
[c] Equivalent to free cis-DDP or cis-aq

were also eliminated at a fast rate, although levels of the complexed drug in this case were somewhat higher (14%–17%). When the drugs were complexed to CM-dex T-40 or T-250, drug activity in the serum was significantly higher, amounting to 64%–100% after 15 min and 35%–47% after 100 min. Even after 6 h their levels were still higher (11%–14%) than those of the free drugs at 15 min post-injection.

To test whether the difference in drug maintenance in the blood is affected by the dose injected, free cis-DDP, cis-aq, and their complexes with CM-dex T-40 were given at doses that were 4-fold higher than in the previous experiment (i.e., 28 mg/kg). Evaluation of active drug at 90 min following injection (Fig. 4) showed a rapid elimination of the free drugs, with residual values that did not exceed 1%–2% of the initial concentration, namely, not signifi-

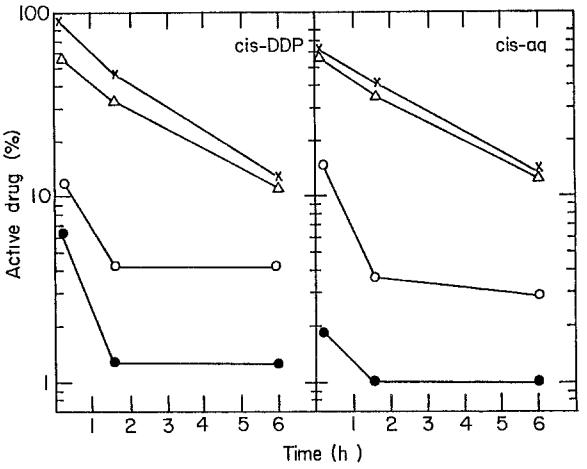

Fig. 3. Active drug in the circulation following i.v. administration of 6.8 mg/kg free drug (●) or drug complexed to CM-dex T-10 (○), T-40 (△), and T-250 (x)

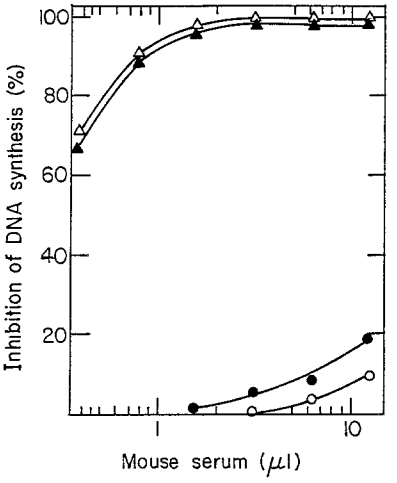

Fig. 4. Inhibition of DNA synthesis in 38C-13 cells by serum drawn from mice at 90 min following i.v. administration of 28 mg/kg free cis-DDP (○), free cis-aq (●), cis-DDP-CM-dex T-40 (△), and cis-aq-CM-dex T-40 (▲)

cantly higher than in the preceding experiment in which lower drug doses were used. On the other hand, the levels of the complexed drugs were 4-fold higher than after the injection of 6.8 mg/kg, amounting to 33% of the initial drug concentration in the blood.

Drug concentration in blood after i.p. administration

Figure 5 illustrates the pattern of drug levels in the blood following i.p. administration of the various drug derivatives. In contrast to the free drugs that were transiently detectable at low levels shortly after injection and were then rapidly eliminated, the complexes reached the circulation at a slower rate. However, once in the blood, they were maintained at higher levels and for longer periods. The pharmacokinetics of the free or complexed drugs in this case were influenced by the gradual transport of the drugs from the peritoneal cavity to the circulation and their concomitant blood clearance. Therefore, the overall transport-clearance process of the complexes seemed to be much slower than that of the free drugs.

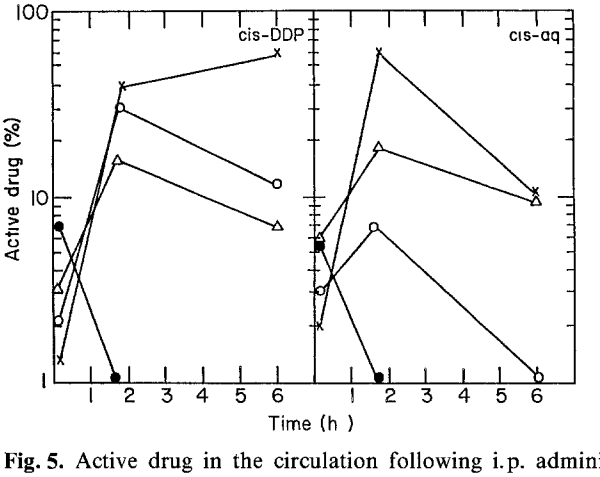

Fig. 5. Active drug in the circulation following i.p. administration of 6.8 mg/kg free drug (●) or drug complexed to CM-dex T-10 (○), T-40 (△), and T-250 (x)

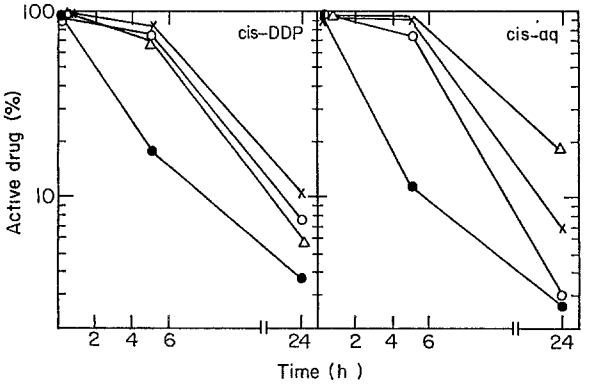

Fig. 6. Active drug in serum incubated with free drug (●) or drug complexed to CM-dex T-10 (○), T-40 (△), and T-250 (x). The activity of free or complexed drug incubated in saline did not change much during incubation and was identical to that of drug incubated with serum at time 0

Protein binding

In view of the documented capacity of cis-DDP to bind to serum proteins and thereby to lose its pharmacological activity, it was of interest to test the serum-binding properties of the CM-dex-complexed drugs in comparison with those of the free drugs. The various derivatives were incubated with mouse serum, and the antitumor activity in samples drawn following different incubation periods was evaluated. As shown in Fig. 6, active drug levels decreased with the time of incubation in all preparations tested. However, the rate of inactivation of the cis-DDP or cis-aq complexed to CM-dex was considerably slower than that of the free drugs. For example, after 5 h only 12%–18% of the free drugs were active, whereas the complexes retained 70%–100% of their initial activity. Even after 24 h, some of the complexes were more active than the free platinum(II) compounds. The rate of drug inactivation was not affected by the molecular size of the CM-dex carrier.

Discussion

This study demonstrates several parameters that may reflect the pharmacokinetic behavior of cis-DDP or cis-aq complexed to CM-dex of different molecular sizes, in

comparison with that of the free drugs. The results obtained with free cis-DDP are in accord with previous studies showing an extensive elimination of active drug from the circulation after i.v. administration. This elimination involves two stages: an initial rapid clearance from the blood, followed by a slower process of drug binding to serum proteins that results in drug inactivation [3, 5, 10, 20].

In contrast to the free drug, the administration of cis-DDP or cis-aq complexes of CM-dex T-40 or T-250 resulted in a marked prolongation of active drug levels in the circulation; this was due to both slower initial rates of clearance of these high-molecular-weight substances and their reduced susceptibility to drug inactivation due to serum protein binding. Consequently, not only was a higher fraction of the complexed drug maintained in the circulation, but its activity was retained for a longer period since their interaction with serum proteins was a very slow process. Similar results were reported for another cis-DDP analogue, *cis*-diammine-1,1-cyclobutane dicarboxylate platinum(II) (CBDCA), the carboxyl groups of which are involved in the coordination of Pt [6].

The results of i.p. administration indicate that in comparison with that of the free drugs, the retention of the complexes in the peritoneal cavity was prolonged and their transport to the circulation was delayed. Once in the circulation, the complexes were at least potentially available for further pharmalogical effects due to their increased maintenance in an active drug form. These results emphasize the advantage of the complexes over the free drug for intracavitary chemotherapy [11]. This treatment modality has been shown to have the potential of markedly increasing total drug delivery for certain types of human malignancies, such as ovarian cancer [8], since total tumor exposure to the drug is considerably increased. The results of the present study suggest that macromolecularization of the drug may further increase its intracavitary rention.

Pharmacological studies analyzing the kinetics of free and total cis-DDP generally show a biphasic clearance pattern, with a rapid phase half-life of considerably less than 1 h, followed by a slow phase in which the remaining drug in the circulation was associated with plasma proteins, thus persisting for several days [2, 3, 5, 7–9, 19, 20]. Sulfhydryl groups in proteins that appear to react very effectively with cis-DDP due to nucleophilic displacement of the chlorides appear to be responsible for most of the stable drug-protein bonds [13]. The protein-bound species of the drugs were shown to be inactive due to their irreversible association: protein-bound cis-DDP prepared in vitro was inactive both in vitro and in vivo [10, 19] and, following the administration of cis-DDP, only the free circulating platinum species were active [2]. Thus, 80%–90% of the active drug disappeared from the circulation within a short time after administration, some of it being slowly excreted through the urine [2, 3, 5]. The rest of the drug was distributed between the plasma and deeper compartments, such as the tissues [10], where it became protein- or tissue-bound. The antineoplastic effect of cis-DDP might therefore be attributed to the initial distribution of drug to the tissues and its tendency to remain somewhat elevated in certain organs such as the ovary, uterus, and kidney [10]. This phenomenon correlates with the therapeutic activity of the drug but is also consistent with its well-established renal toxicity [4].

Once cis-DDP is associated with the high-molecular-weight proteins in the plasma, it can be maintained in the circulation for several days in an inactive form. This protein-bound fraction could theoretically serve as a pool for the supply of free cis-DDP to the tissues if the binding between the drug and the proteins were reversible, but this is not the case. The results of the present study suggest that the administration of platinum(II) drugs that are complexed to an adequate high-molecular-weight carrier in a reversible manner may provide a pool for the release of active drug. The CM-dex-complexed drugs are eventually inactivated due to their irreversible interaction with serum proteins, but since this is a slow process, the drugs have the opportunity to exert their pharmacological effect beforehand. The reversible linkage between platinum(II) and CM-dex is due to the feasibility of substitution or exchange reactions either with ligands exerting higher affinity towards platinum(II), such as DNA [15], or proteins under conditions of large-protein abundance. The mechanism of action effected by the complexes seems to involve the prolonged maintenance and sustained release of the active ingredient, which is eventually delivered to the DNA target molecule in the tumor cell. This type of drug delivery may also lead to reduced toxicity of the drug due to its slower initial distribution to the tissues. The results presented here provide some indications for the potential benefit of cis-DDP or cis-aq complexes of CM-dex for clinical use.

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