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IN VITRO EVOLUTION OF CISPLATIN/DNA MONOADDUCTS INTO DIADDUCTS IS DEPENDENT UPON SUPERHELICAL DENSITY

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SUMMARY: DNA binding of antitumor platinum(II) compounds accounts for cellular toxicity. Binding of *cis*-dichlorodiammineplatinum(II) (*cis*-DDP) to DNA involves the transient presence of monoadducts which evolve in a second phase into difunctional lesions which are far more toxic than the monoadducts. Temporal control of the monoadducts half-live is at least dependent upon the chemical nature of the *cis*-platinum derivative and the secondary structure of DNA. The effect of the degree of DNA superhelicity on the binding of *cis*-platinum derivatives as well as on the evolution of monofunctional adducts has been addressed on plasmid DNA. The rate of platination was not affected by the degree of DNA superhelicity. Similarly, when the evolution of the lesions was complete, no variation of toxicity was found with different populations of topoisomers, as determined by bacterial transformation efficiency. In contrast, when the kinetic of difunctional lesions formation was controlled *in vitro*, we observed a higher rate of formation on a supercoiled plasmid by comparison with a relaxed one. This result suggests that platinum-DNA adduct toxicity could be modulated by the topology of the chromosome.

Cis-dichlorodiammineplatinum(II) (*cis*-DDP) is a widely used antitumor agent that exerts its biological activity mainly by covalent binding to DNA [1]. This compound initially binds monofunctionally to DNA (preferentially to the N7 of guanine) and monofunctional adducts react further to produce the difunctionally bound lesions including DNA-intrastrand cross-links, DNA-interstrand cross-links and DNA-protein cross-links [2, 3]. Because of the transient nature of monoadducts, their genotoxic potency have not been extensively studied. However, two approaches has been followed to determine toxicity of monoadducts, either by using a monofunctional derivative ([PtCl(dien)]Cl) [4, 5] or by blocking the evolution of monoadducts with thiourea which irreversibly binds to the monofunctional Pt-DNA adduct [6-8]. Moreover, Hoffmann et al. have recently set up an *in vitro* experimental protocol on a phage DNA in order to produce a majority of monofunctional lesions which did not evolve

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Abbreviations:
cis-DDP: *cis*-dichlorodiammineplatinum(II); $[PtCl_2(dach)]$: (d,l) *trans*-1,2-diamminecyclohexanedichloroplatinum(II); rb: ratio of platinum bound per nucleotide.

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further due to high salt concentration [9]. In a second step, by decreasing salt concentration in the reaction medium, these monofunctional lesions were allowed to evolve into difunctional adducts. Despite discrepencies between the results obtained with *cis*-DDP-monoadducts and Pt(dien)-adducts, monofunctional adducts were much less efficient than difunctional lesions in blocking DNA replication *in vitro* [5, 9-11] and in inducing cell toxicity and mutagenicity [6-8, 12]. Hence, the reaction of conversion from mono- to difunctional adducts is of importance in the determination of cellular toxicity.

Among various parameters involved in the variation of the rate of difunctional adducts formation, DNA conformation might play a role [13, 14]. We addressed this question by using a plasmid treated *in vitro* by *cis*-DDP in order to obtain a majority of monoadducts and the kinetic of evolution of these monoadducts into difunctional lesions was controlled *in vitro* [9]. Plasmids of various degrees of superhelicity, treated as described above, were used in a bacterial transforming assay. The degree of superhelicity was found to affect the kinetic of conversion of monoadducts into diadducts.

MATERIALS AND METHODS

Bacterial strains and plasmids.

E. coli K12 strains AB1157 (*uvr+, rec+*), AB1886 (*uvrA6*) and AB2463 (*recA13*) were obtained from Dr. B. J. Bachmann (*Escherichia coli* Genetic Stock Center, Yale University). pHM plasmid was a gift from Dr. R.D. Wood (London). This 3738 bp pBS (p-Bluescript, Stratagene) derived plasmid contains a fragment of human DNA cloned in the EcoR1 site, and carries ampicillin resistance [15]. pHM plasmid was isolated by alkaline lysis procedure and further purified by centrifugations on one cesium chloride and two neutral sucrose gradients as described [15].

Chemicals and enzyme .

^{3}H-labelled (d,l) *trans*-1,2-diamminecyclohexane-dichloro-platinum-(II) [$PtCl_2$(dach)] (sp. act. 402 mCi/mmol) was a generous gift of Dr.S.J. Chaney via Dr. A. Sancar (University of North Carolina) [16]. *Cis*-dichloro-diammineplatinum(II) (*cis*-DDP) was a gift from R. Bellon Company. Topoisomerase I was purified from HeLa cells as previously described [17].

Preparation of DNA topoisomers.

Plasmid pHM RFI DNA (50µg) was incubated with 150 U of DNA topoisomerase I (1 U is the amount of enzyme required to relax 0.5 µg of plasmid DNA in 30 min. at 30°C) in 50 mM Tris-HCl pH 7.5, 120 mM KCl, 10 mM $MgCl_2$, 0.1 mM EDTA and 0.1 mM dithiotreitol in a final volume of 400 µl containing different amounts of ethidium bromide. The mixture was incubated for 30 min. at 30°C. The reaction was stopped by 10 mM EDTA followed by two phenol extractions. Samples were then dialyzed against 1 mM Tris-HCl pH 8, 0.1 mM EDTA (thereafter named TE buffer). The linking difference ($\Delta Lk = Lk - Lk^\circ$, where Lk is the mean linking number of the topoisomer distribution and Lk° is the mean linking number of the relaxed DNA) were estimated from ethidium bromide/DNA stœckiometric molar ratio during relaxation by DNA topoisomerase I, assuming quantitative ethidium bromide binding and using an unwinding angle of 26° per intercalated ethidium bromide molecule [18]. Degree of supercoiling of the substrate was controlled by electrophoresis on agarose gels containing various concentrations of chloroquine, and from the results, the reduction in Lk was calculated.

Agarose gel electrophoresis.

DNA samples with various superhelical densities were electrophoresed in 0.8% agarose gel with 90 mM Tris pH 8, 90 mM borate, 2 mM EDTA at 1,5 V/cm for 20 h (20°C) and with buffer recirculation. Gels were rinsed, stained with ethidium bromide and photographed under UV illumination.

Treatment of pHM with platinum compounds.

Stock solutions of [^{3}H][$PtCl_2$(dach)] and *cis*-DDP were prepared in 0.9% KCl. Plasmid DNA concentration was determined by UV absorption. [^{3}H][$PtCl_2$(dach)] was added to pHM DNA

with various superhelical densities (ΔLK = 0, ΔLK = -10, ΔLK = -30), to obtain final concentration of 33 μM for DNA and 7.5 μM for the drug in a reaction mixture that contained TE buffer and 12 mM KCl. The mixture was incubated at 37°C, aliquot samples were withdrawn at various time intervals, and the reaction was immediatly stopped on ice with 0.5 M NaCl (final concentration). In order to remove the unreacted drug, platinated template was dialyzed twice at 4°C for two hours against TE buffer, 0.5 M NaCl. Subsequently an overnight dialysis was performed in TE to allow complete evolution of monoadducts. The molar ratio of drug bound per nucleotide (rb) was determined by measuring the radioactivity in each sample with a liquid scintillation counter.

In another series of experiments, monoadducts were preferentially generated by treating pHM DNA (ΔLK = 0 and ΔLK = -30) with *cis*-DDP for 5 min. at 37°C with an initial molar ratio of drug versus DNA equal to 1. The evolution of monoadducts to diadducts was controlled by post-treatment incubation in low salt buffer as already described [9]. The reaction was in TE buffer supplemented with 15 mM NaCl, and was stopped on ice by raising NaCl concentration to 0.5 M. The unreacted drug was removed by dialyzing as described above in experiments with [^{3}H][$PtCl_2$(dach)]. No loss of DNA nor of platinum was observed after dialysis. Platinum quantification was performed by atomic absorption spectrophotometry (Perkin Elmer) as previously reported [4].

Transforming assays.

Competent bacteria were obtained by $CaCl_2$ procedure as described [19]. Plasmid DNA (10 to 100 ng) either treated with platinum or not was added to 200 μl competent cells. The mixture was kept for 45 min. on ice, heat shocked at 42°C for 2 min. and plated without expression phase on Luria agar containing 100 μg/ml of ampicillin. Identical values of relative transformation efficiencies were found whether an expression time period of 3 hours was done or not. The transforming frequency varied linearly from 10^5 to $6x10^5$ transformants per μg of DNA in the range of DNA concentration used.

RESULTS

Reactivity of ^{3}H [$PtCl_2$(dach)]towards pHM plasmid with various superhelical densities.

In order to study the binding of platinum(II) compounds to DNA with different degrees of superhelicity as well as the toxicity of DNA-adducts present on these topoisomers, the ^{3}H labelled derivative [$PtCl_2$(dach)] was used. This agent has similar reactivity towards DNA as *cis*-DDP [1, 20] and its DNA binding was easily quantified. pHM plasmid populations of various degree of superhelicity were prepared by complete relaxation with DNA topoisomerase I in the presence of appropriate concentration of ethidium bromide (Fig.1A). The reactivity of [$PtCl_2$(dach)] towards various DNA topoisomer populations (ΔLk = 0; -10; -15; -20; -25; -30; -35) was independent from their degree of superhelicity under these experimental conditions (Fig. 1B and data not shown). The kinetic of platinum binding was linear on these topoisomers up to 25 min., with a rate of 0.2 adduct per min. per DNA plasmid molecule.

Adducts toxicity as determined by bacterial transforming frequency.

The degree of DNA superhelicity did not modify the yield of platinum binding to DNA but could modulate the type of adducts on plasmid DNA. Bacterial transformation experiments were performed to assess these effects with wild-type (AB1157), excision repair deficient mutant (AB1186) and mutant in recombination and SOS repair (AB2463). By lowering the superhelical density, the frequency of bacterial transformation was slightly decreased ([21] and data not shown). However, after standardization with the corresponding untreated topoisomer, the frequency of transformation of wild-type bacteria with platinated plasmids was independent from plasmid topology (ΔLk=0, -10, -20, -30) (Table 1). Either the lesions

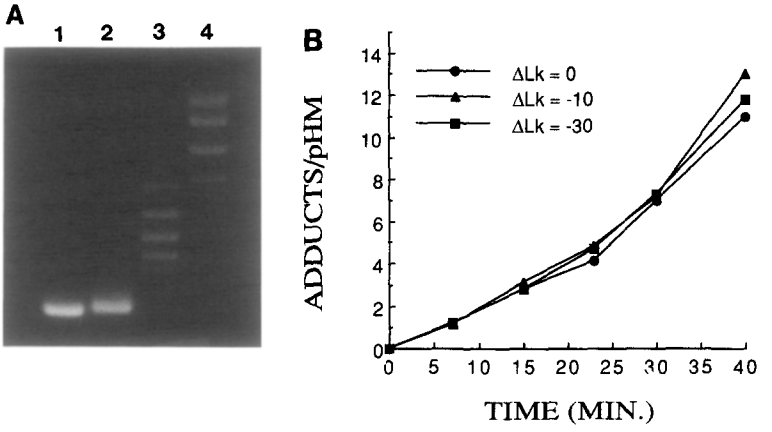

Figure 1 : Effect of supercoiling on cis-DDP DNA binding.

A : Electrophoresis in a 0,8% agarose gel of different populations of topoisomerases (1 : ΔLK = -30 ; 2 : ΔLK = -20 ; 3 : ΔLK = -10 ; 4 : ΔLK = -0) (see Materials and Methods). The direction of migration is from top to bottom.

B : Kinetics of incorporation of [$PtCl_2$(dach)] into pHM with different superhelical densities(● : ΔLK = 0 ; ▲ : ΔLK = -10; ■ : ΔLK = -30). The drug to nucleotide ratio in the reaction mixture was 0,25. The reaction was performed at 37°C ; samples were taken at time intervals and the adduct formation was measured as described in Materials and Methods.

were similar in term of toxicity or the difference was corrected by DNA repair processes. The transforming frequencies determined with repair defective mutants were also unchanged whatever the degree of DNA superhelicity (Table 1). DNA lesions were repaired mainly by the excision repair pathway since the values of transforming frequency were lowered as for instance by 7-8 and 2 fold with 5 adducts per plasmid in AB1886 (*uvr*$^-$) and AB2463 (*recA*$^-$) respectively ([22] and data not shown). Thus, variation of the degree of DNA superhelicity, although it might affect the repartition of the adducts on DNA, did modify neither the kinetic of platinum binding nor the global toxicity of the lesions.

Effect of DNA topology on the conversion of monoadducts into biadducts.

The experimental conditions used above were unable to shed light on the effect of supercoiling on the kinetic of conversion of monofunctional to difunctional adducts since the

Table 1 : Number of Pt/DNA adducts per plasmid lowering the transformation efficiency to 10% in *E. coli* with different repair capabilities

ΔLk	*rec*$^+$ *uvr*$^+$[a]	*rec*$^+$ *uvr*$^-$[a]	*rec*$^-$ *uvr*$^+$[b]
0	9,8 ± 0,1	5 ± 0,12	8,4
-10	10,1 ± 0,12	4,8 ± 0,15	8,3
-20	10 ± 0,09	5 ± 0,1	8,5
-30	9,9 ± 0,12	4,9 ± 0,14	8,2

[a] Mean of 3-4 independent experiments ± SD.
[b] Mean of two independent experiments.

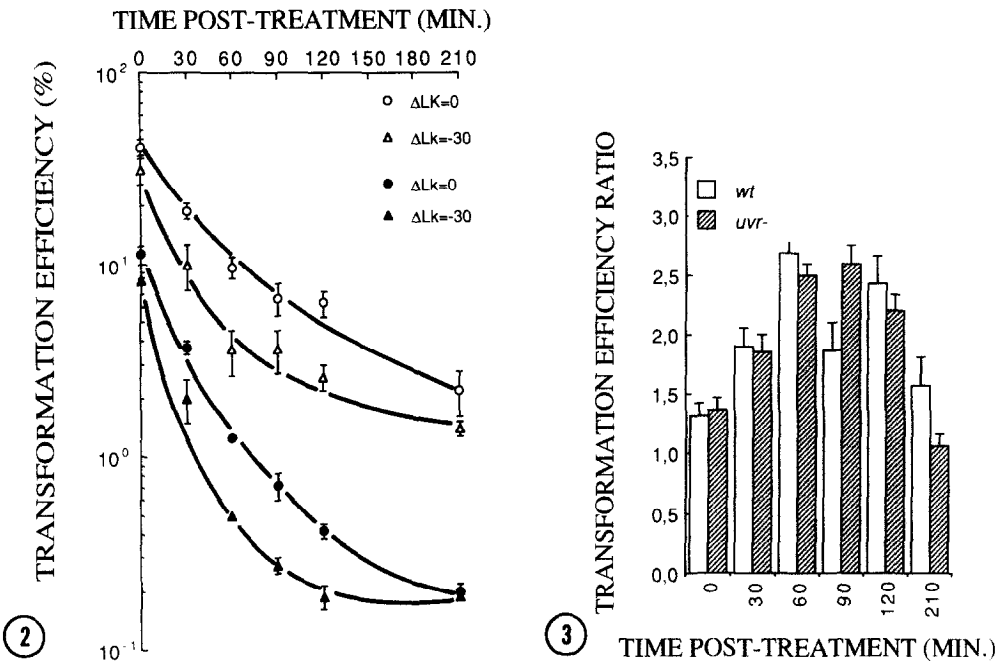

Figure 2 : Transformation efficiency of cis-DDP damaged pHM with ΔLK = 0 (● , ○) and ΔLK = -30 (▲ , △) as a function of post-treatment time. After the removal of the unincorporated drug (5 minutes treatment), plasmid substrates were post-incubated in low salt buffer to allow monoadducts to evolve in biadducts. Aliquots were withdrawn at time intervals to transform AB1157 (open symbols) and AB1886 (closed symbols). Values were the mean of 3-4 independent experiments ±S.E.

Figure 3: Ratio of transformation efficiency with relaxed versus supercoiled platinated plasmid. Data were from Fig. 2 and values were given ±S.E.

overnight dialysis step of platinated plasmids allowed complete evolution of adducts [13, 22]. Therefore, to test this parameter, a plasmid DNA either supercoiled (ΔLk = -30) or relaxed (ΔLk = 0), was treated with *cis*-DDP to produce about 70% of monofunctional adducts [9]. The DNA samples were then dialyzed against low salt buffer (10 mM $NaClO_4$), that allows the conversion of monofunctional into difunctional adducts during 4 hour dialysis. At various time, aliquot samples were withdrawn and used for bacterial transformation and platinum quantification. The extent of platination was identical for the two forms, (12-13 adducts per plasmid). Immediatly after drug binding (5 min. treatment), the transformation efficiency of AB1157 and AB1886 was inhibited by 60% and 90% respectively (Fig. 2), indicating the presence of toxic lesions within the 12-13 adducts present on the plasmid, and the involvement of excision repair pathway in bacterial survival. During post-treatment incubation, the evolution into diadducts took place as revealed by an increase in toxicity with each topoisomer population. However, this decrease of transformation efficiency was more pronounced with the supercoiled plasmid than with the relaxed one; this difference leveled up to about 3 fold after 1.5 hours of evolution and then decreased (Fig. 3) indicating the

completion of the reaction as previously reported [9]. This result was found both with AB1157 (wt) and AB1886 ($uvrA_{6}$) strains.

DISCUSSION

Pt-DNA monoadducts are transiently present on DNA both *in vivo* and *in vitro* after treatment with platinum(II) compounds. An indirect approach with thiourea which bocks the evolution of monoadducts has be undertaken and revealed a slow rate of conversion of monoadducts within several hours [6, 8, 23]. Hence, any factor allowing variation in this kinetic of evolution would play a role in the toxic potency of the compound tested. Among parameters such as the chemistry of the platinum derivative used, the rate of aquation of the labile ligand or the sequence context, we tested the involvement of the degree of DNA superhelicity.

Plasmid DNA topology was controlled by an *in vitro* reaction with topoisomerase I in the presence of ethidium bromide; then, these DNA substrates were incubated with *cis*-platin(II) derivatives and host cell plasmid reactivation was determined after bacterial transformation. No modification of binding and toxicity was found with different populations of topoisomers. However, the substrate used is a naked plasmid DNA and this result cannot simply be extended to a more complex structure such as the chromosome. For example, superhelical density has been shown to modify the affinity of many proteins for DNA [24], that in turn might also modulate the accessibility of target DNA to the drug. Moreover, *cis*-DDP binds preferentially to the DNA linker between nucleosomes [25]. On the other hand, the value of transforming efficiency was much dependent on *uvrA* gene and less on *recA* gene which implies that excision-repair is the main repair pathway of plasmid recovery. On the contrary, *recA* gene integrity has been shown to be essential for bacterial survival after *cis*-DDP treatment and *recA* mutants were much more sensitive than *uvrA* strains [4]. However, recombination was not expected to play a major role in plasmid reactivation because the plasmid concentration used in this assay was too low to allow multiple host transformations. DNA topology has been involved in UvrABC incision efficiency of several DNA lesions *in vitro* ; for instance, negative supercoiling stimulates greatly incision of psoralen cross-links whereas it had no effect on monoadducts incision [26]. In our conditions, although bacteria discriminated supercoiled and relaxed forms of pHM since their transformating efficiency were different, no difference in toxicity of platinum lesions on the two plasmid forms was found. It could be suggested that DNA topology effect on incision efficiency of platinum/DNA adducts, if any, is too low to be detected or that the main toxic lesions are poor substrates for UvrABC. Interestingly, platinum intrastrand diadducts are less recognised by the procaryotic excision repair complex than mono-adducts [27].

In order to test the role of DNA superhelicity on the rate of conversion of monofunctional into difunctional adducts, either relaxed or highly supercoiled plasmid were treated with *cis*-DDP and their transforming efficiencies compared. After treatment, which allows mainly the formation of monofunctional lesions, di-adducts were already present since the transforming efficiency was down to 40% and 10% in w.t. and *uvrA* strains respectively (Fig. 2). If plasmid DNA was treated by *cis*-DDP to obtain complete evolution of adducts, a similar value of

survival would be obtained for about 3 adducts per plasmid.([28] and data not shown). Therefore, taking into account the low toxic potency of mono-adducts, the percentage of di-adducts in our conditions (12-13 adducts per plasmid) can be estimated to about 25-30%, which corresponds to the value determined after the same experimental procedure on a single-stranded M13 DNA phage [9]. The evolution into di-adducts was almost complete after 210 min since the decrease in transforming efficiency tended to reach a plateau (Fig. 2). However, the bacterial survival was equal to 2 and 0.2% in w.t. and *uvrA* strains respectively, which correspond to survival after transformation by a plasmid damaged with 6-7 adducts ([28] and data not shown). Since a lower survival would be expected with 12-13 platinum lesions per plasmid, this result indicates that poorly toxic lesions such as mono-adducts were still present on the substrate. One can suggest that the binding of platinum in a reaction with a high initial ratio drug/DNA (Ri) (see Materials and Methods) leads to mono-adducts which could not evolve into toxic lesions, possibly due to the sequence context ; this biais would be lowered in the case of treatment with a low Ri for a long time period. The decrease in transformation efficiency was more pronounced with the supercoiled form of pHM plasmid than with the relaxed one when either AB1157 (wt) or AB1886 ($uvrA_6$) strains were transformed. This results on plasmid DNA *in vitro* points out a role of DNA superhelical density on the evolution kinetic of *cis*-DDP monofunctional adducts into toxic di-adducts. *In vivo*, DNA topology could similarly modulate the distribution of adducts into chromosomal domains ; psoralen cross-links have been found to be initially more abundant in transcriptionally active chromatin regions whereas nitrogen mustard cross-links were less abundant [29], and DNA torsional variations associated with transcription might contribute to this differential repartition of adducts. Moreover, in some chromosomal domains, superhelical density could favor interaction of proteins or glutathione with platinum monofunctional lesions by lowering their evolution kinetic into diadducts which could lead locally to decrease in toxic consequences on vital processes like DNA replication or transcription. However great efforts remain to establish experimentally these hypothesis on the more complex situation of chromosomal DNA *in vivo*.

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