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Reactivity of monofunctional *cis*-platinum adducts as a function of DNA sequence

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ABSTRACT

The purpose of this work was to study the chemical reactivity of monofunctional cis-platinum-nucleic acid adducts as a function of nucleic acid sequence. The first part of the paper deals with the formation of these adducts. It is shown that the ternary nucleic acid-cis-platinum-ethidium bromide complexes in which ethidium bromide and nucleotide residues are cross-linked by cis-platinum, are relatively unstable at 37°C. In the presence of acridine, ethidium bromide (but not cis-platinum) is slowly released which leads to the formation of monofunctional cis-platinum-nucleic acid adducts. After removal of acridine, the monofunctional adducts react further to become bifunctional. The second part of the paper deals with the kinetics of disappearance of the monofunctional adducts in several polynucleotides but not in poly(dG).poly(dC). When the adducts possess a chloride ligand, the limiting step in the cross-linking is the rate of aquation reaction of the chloride ligand. The rate constants are an order of magnitude larger when the monofunctional adducts do not possess a chloride ligand. In both the cases, the rate constants are apparently independent of the nucleic acid sequence.

INTRODUCTION

Numerous studies have shown that in vitro and in vivo cis-diamminedichloroplatinum(II) (cis-DDP) binds to DNA and it is generally thought that this binding is related to the antitumor activity of cis-DDP. The major adducts arise from intrastrand cross-links between two adjacent guanine residues and between an adenine residue adjacent to a guanine residue, respectively. Minor adducts arise from intrastrand cross-links between two guanine residues separated by at least one base residue and from interstrand cross-links between guanine residues (general reviews 1-4 and references therein). In vivo and in vitro, it seems that the lesions are made in two steps, an initial monofunctional adduct being converted with time in a bifunctional cross-link(5-10). The monofunctional adducts are not biologically active. Thiourea which can block monofunctional adducts inhibits the cytotoxic and mutagenic effects of the drug when added to mammalian cells pretreated with cis-DDP. On the other hand, after several hours of incubation of

cis-DDP and cells, the inhibition by thiourea is no longer observed and increasing amounts of bifunctional adducts are detected (9,10). The understanding of the chemistry of the cross-links is important to explain the biological effect of cis-DDP.

We were interested to know the importance of DNA sequence upon the rates of formation of the bifunctional adducts. To answer this question, we took advantage of the facts that 1) the reaction of cis-DDP and poly(dG-dC). poly(dG-dC) in the presence of ethidium bromide (EtBr) leads to the formation of ternary complexes in which platinum cross-links a nucleotide residue and an EtBr residue, 2) incubation of the ternary complex poly(dG-dC)-cisPt-EtBr in given conditions leads to the formation of monofunctional platinum-poly(dG-dC) adducts (11,12). We here show that more generally, this procedure can be used to prepare monofunctional platinum-nucleic acid adducts which allows the study of the kinetics of formation of bidentate adducts as a function of the nucleic acid sequence.

MATERIALS AND METHODS

Double-stranded polynucleotides purchased from Boehringer Mannheim and from Pharmacia, poly(dA-dG).poly(dC-dT) synthesized according to the procedure of Morgan et al. (13), Micrococcus luteus DNA prepared as previously described (14), were treated twice with phenol and then precipitated with ethanol. Stock solutions were made in 10 mM $NaClO_4$, 1 mM phosphate buffer, pH 7.5. [^{14}C]-thiourea (2.1 GBq/mmole) from New England Nuclear was deionized over AG 501 X8 (Bio-Rad) column before use. [^{14}C]-ethidium bromide (0.7 GBq/mmole) was from Centre de l'Energie Atomique. Cis-DDP was kindly provided by Dr. J.L. Butour (Toulouse). Deoxyribonuclease I, P1 nuclease and alkaline phosphatase were purchased from Boehringer Mannheim.

Reaction of cis-DDP and nucleic acid, in the presence or absence of ethidium bromide, and determination of the amounts of cross-linked ethidium bromide by filter assay at acid pH have been previously described (11). The amounts of bound [^{14}C]-thiourea to platinated nucleic acids were determined by filter assay at acid pH.

The platinum contents of the samples were measured with an atomic absorption spectrophotometer by Dr. J.L. Butour (Toulouse).

Enzymatic digestion of the platinated samples was performed as described (7,8). The digests were analyzed by HPLC on a 250x4.6 mm C18 column (Lichrospher CH18 from Société Française Chromato colonne) attached to a Hitachi Model 655 apparatus with a 30 min linear gradient of 0-30 % buffer B (buffer

A, 2 % CH_3CN, $AcONH_4$ 0.1 M, pH 5.9 ; buffer B, 24 % CH_3CN, $AcONH_4$ 0.1 M, pH 5.9). The flow rate was 1 ml/min and the detection was monitored at 260 nm.

Synthesis of cis-$[PtCl(NH_3)_2(dGuo)]Cl$ was achieved by reacting first one equivalent of $AgNO_3$ with one equivalent of cis-DDP (10 mM) in dimethylformamide for 12 hours at 37°C, filtration of AgCl and subsequent reaction of the filtrate with one equivalent of d-guanosine (10 mM) for 30 min at 40°C. The product was precipitated at 4°C by addition of 5 volumes of ether. Cis-$[PtCl(NH_3)_2(dGuo)]Cl$ (1 mM) in water was incubated with two equivalents of thiourea for 30 min at room temperature. HPLC profile of the mixture presented a major peak (elution time 12.5 min in conditions just described) which was collected. Elemental analysis was consistent with the formula cis-$[Pt(NH_3)_2(dGuo)\ (thiourea)]\ (CH_3COO)_2.H_2O$.

RESULTS AND DISCUSSION

We have previously shown that in the ternary complexes formed by incubation of nucleic acids, EtBr and cis-DDP, some EtBr molecules cannot be removed by extraction with butanol or by filtration at acid pH. Ternary complexes are not formed if EtBr is replaced by acridine as acridine is completely removed by extraction with butanol. The ternary nucleic acid-cisPt-EtBr complexes are relatively unstable at 37°C as EtBr (but not platinum) is slowly released. A study of poly(dG-dC)-cisPt-EtBr suggests the formation of an intermediate species in which platinum is monofunctionally bound. This monofunctional adduct is stabilized by acridine (11-12).

We now show that monofunctional adducts are also formed with other nucleic acids.

1. Monofunctional platinum-nucleic acid adducts

The nucleic acids (poly(dG-dC).poly(dG-dC), poly(dA-dC).poly(dG-dT), poly(dA-dG).poly(dC-dT), poly(dG).poly(dC) and natural DNA) were first mixed with EtBr and then a given amount of cis-DDP was added. After 24 hours of incubation at 37°C, the reaction mixtures were extracted with cold butanol to remove all the non cross-linked EtBr. The cross-linked EtBr per nucleotide molar ratio r_b(EtBr) depends upon the sequence of the nucleic acid while the bound platinum residue per nucleotide molar ratio r_b(Pt) is the same in all the samples (fig. 1, time t = 0). Then, the ternary complexes were incubated at 37°C in the presence of acridine. As shown in fig. 1, r_b(EtBr) decreases and r_b(Pt) is constant. The rates of EtBr release are about the same for all the ternary complexes ($T_{\frac{1}{2}} \simeq 7$ hours).

After 20 hours of incubation at 37°C, acridine and EtBr were extracted

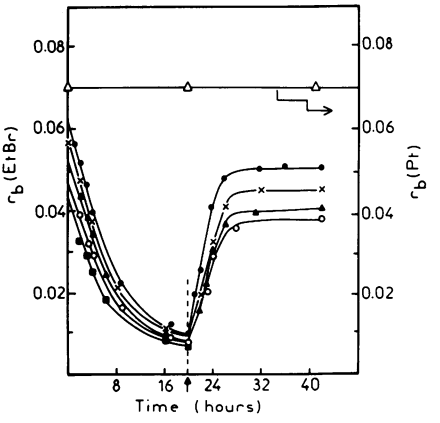

Figure 1 : Amount of cross-linked EtBr and of bound cis-platinum as a function of time. The ternary complexes poly(dG-dC)-cisPt-EtBr (●), poly(dA-dG).poly(dC-dT)-cisPt-EtBr (x), poly(dA-dC).poly(dG-dT)-cisPt-EtBr (▲), M. luteus DNA-cisPt-EtBr (o) and poly(dG).poly (dC)-cisPt-EtBr (■) were incubated at 37°C, in 10 mM $NaClO_4$, in the presence of acridine (acridine per nucleotide residue molar ratio = 0.3). After 20 hours of incubation, EtBr and acridine were removed and then EtBr was added. The values of r_b(EtBr) were determined by filter assay. (Δ) are relative to r_b(Pt).

with cold butanol. The new platinated species, termed platinated* nucleic acids, were then reacted either with thiourea or with EtBr to show the presence of monofunctional adducts.

Reaction with thiourea

Thiourea is known to bind irreversibly to monofunctional platinum-nucleic acid adducts thereby blocking the formation of bifunctional lesions (monoadduct quenching) (9,10,15). The platinated* nucleic acids were incubated at 23°C for 10 or 60 minutes in 10 mM thiourea (8). In both cases, the

Table I

EtBr bound to ternary complexes, EtBr or thiourea bound to platinated* nucleic acids. In all the cases, r_b(Pt) is equal to 0.07.

	Ternary complexes	Platinated* nucleic acids	
	r_b(EtBr)	r_b(thiourea)	r_b(EtBr)
Poly(dG-dC).poly(dG-dC)	0.062	0.055	0.05
Poly(dA-dG).poly(dC-dT)	0.057	0.053	0.045
Poly(dA-dC).poly(dG-dT)	0.052	0.047	0.041
DNA	0.047	0.045	0.038
Poly(dG).poly(dC)	0.035		

amounts of bound [^{14}C]-thiourea were the same and no platinum was removed. For all the platinated* nucleic acids except platinated* poly(dG).poly(dC), the amount of bound thiourea is about 10 % smaller than the amount of bound EtBr in the ternary complexes at time t = 0 (Table I).

After incubation of thiourea and platinated* poly(dG-dC), poly(dA-dC).poly(dG-dT), poly(dA-dG).poly(dC-dT) and poly(dG).poly(dC), the nucleic acids were precipitated twice with ethanol, digested enzymatically (7,8) and the products were analyzed by HPLC. The HPLC profiles were qualitatively same as expected for nucleic acids modified by cis-DDP (16) with an additional peak (elution time, 12.5 mm) which contained most of the radioactivity and coeluted with the product cis-[Pt(NH_3)$_2$(dGuo)(thiourea)](CH_3COO)$_2$ (results not shown).

In summary, these experiments show that in platinated* nucleic acids, both monofunctional and bifunctional adducts are present, the relative proportions of adducts depending upon the sequence of the nucleic acid (Table I). In all the cases, acridine stabilizes the monofunctional adducts but less efficiently in platinated* poly(dG).poly(dC) than in platinated* poly(dG-dC). The monofunctional platinum residues are mainly bound to guanine residues.

Reaction with EtBr

We wanted to know whether ternary complexes could be formed by reacting EtBr and platinated* nucleic acids. We recall two previous results (11,12) : 1) after incubation of the ternary complexes at 37°C without acridine and complete removal of EtBr, addition of EtBr to the solution does not lead to the formation of ternary complexes ; 2) if a nucleic acid is first reacted with cis-DDP (termed platinated nucleic acid) and then EtBr is added, a ternary complex is not formed.

EtBr and platinated* nucleic acids were incubated at 37°C and the amount of cross-linked EtBr was determined at various time points. As shown in fig. 1, r_b(EtBr) increases and then becomes constant after a few hours. Thus, ternary complexes are formed. For all the samples, r_b(EtBr) are about 15 % smaller at the plateau than in the ternary complexes at time t = 0 (Table I). In first approximation, the rates of formation of the cross-links are independent of the nucleic acid sequence ($t_{\frac{1}{2}} \simeq 3$ hours).

Results relative to platinated* poly(dG).poly(dC) are not given in fig. 1. Qualitatively, it can be said that ternary complexes were also formed. However, the amount of monofunctional adducts being small, the determination of r_b were inaccurate.

In another set of experiments, platinated* poly(dG-dC), poly(dA-dC).

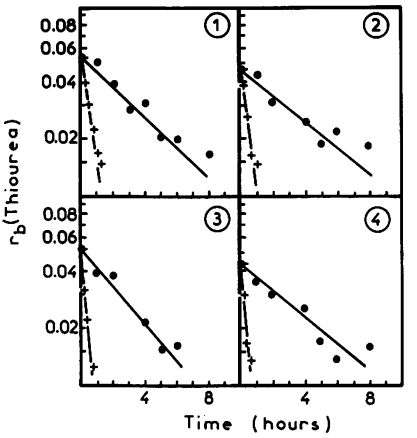

Figure 2 - Kinetics of disappearance of cis-platinum monofunctional adducts : (1) platinated* poly(dG-dC), (2) platinated* poly(dA-dC).poly(dG-dT), (3) platinated* poly(dA-dG).poly(dC-dT), (4) platinated* M. luteus DNA. The symbols (•) and (+) are relative to adducts which possess or do not possess a chloride ligand. The platinated* polymers were incubated at 37°C in 10 mM $NaClO_4$.

poly(dG-dT) and poly(dA-dG).poly(dC-dT) were incubated overnight at 37°C and then digested enzymatically. HPLC profiles of the digests look like those obtained with the corresponding platinated polymers (16) (results not shown).

In conclusion, all these results can be summarized as follows (DNA being either a natural or a synthetic double stranded nucleic acid) :

DNA + cis-DDP ⟶ DNA-cisPt (1)

DNA + cis-DDP + EtBr ⟶ DNA-cisPt-EtBr + DNA-cisPt (2)

DNA-cisPt-EtBr + acridine ⟶ DNA-cisPt*+DNA-cisPt + acridine + EtBr (3)

DNA-cisPt* + EtBr ⟶ DNA-cisPt-EtBr + DNA-cisPt (4)

DNA-cisPt* + thiourea ⟶ DNA-cisPt-thiourea (5)

DNA-cisPt* ⟶ DNA-cisPt (6)

In reactions (1) and (6), the main adducts arise from cross-links between nucleotide residues. In reaction (2), the adducts arise from cross-links between nucleotide residues and from cross-links between guanine residues and EtBr residues, the relative percentages depending upon the sequence of the nucleic acid. In reactions (3) and (4), the relative percentages of monofunctional and bifunctional adducts depend also upon the nucleic acid sequence (Table I).

Table II
Half-lives ($T_{\frac{1}{2}}$) and rate constants of cross-links formation in platinated* nucleic acids.

	Monofunctional platinum adduct			
	with a chloride ligand		without a chloride ligand	
	$T_{\frac{1}{2}}$ (mn)	k (s^{-1})	$T_{\frac{1}{2}}$ (mn)	k (s^{-1})
Platinated* poly(dG-dC)	228	5×10^{-5}	34	3.5×10^{-4}
Platinated* poly(dA-dC) .poly(dG-dT)	258	4.5×10^{-5}	39	3×10^{-4}
Platinated* poly(dA-dG) .poly(dC-dT)	240	4.8×10^{-5}	22	5×10^{-4}
Platinated* M. luteus DNA	252	4.5×10^{-5}	25	4.6×10^{-4}

2. Cross-link formation

The kinetics of disappearance of the monofunctional adducts in platinated* nucleic acids have been studied by monoadduct quenching. Two sets of experiments were performed, the monofunctional adducts either possessing or not possessing an inner shell chloride ligand. Platinated* nucleic acids, prepared as described in section 1, were used either directly or after incubation at 0°C for 15 minutes in 0.5 M NaCl and subsequent ethanol precipitation. They were incubated at 37°C, and at various times, aliquots were reacted with thiourea. The variations of r_b (thiourea) are shown in figure 2. In table II are given the half-lives of the monoadducts and the rate constants, calculated assuming first order kinetics. In both sets of experiments, the rate constants are, in first approximation, independent of the sequence of the platinated* nucleic acids. The rate constants relative to the adducts with a chloride ligand are an order of magnitude smaller.

A rate constant of 5.5×10^{-5} s^{-1} for the reaction of the second chloride ligand of cis-DDP with DNA has been deduced from the rate of formation of interstrand cross-links (17), a value in good agreement with those reported here. A rate constant of 4.2×10^{-5} s^{-1} for the aquation reaction of the second chloride ligand of cis-DDP free in solution has been also reported (18). Our

results confirm that the rate constants for the aquation of the second chloride ligand of cis-DDP free or in a monodentate adduct are identical.

The rate constants for the formation of the interstrand adducts (platinated* poly(dG-dC)) and intrastrand adducts (platinated* poly(dA-dC).poly(dG-dT)) are the same. In B-DNA, the distances between the two guanine residues in the sequences GCG/CGC, GC/CG and CG/GC are about 8.5, 6.5 and 9.1 A respectively (19). Thus, it does seem that the structural fluctuations, necessary for the formation of the cross-links, are a limiting step to the reaction.

The rate constant for the formation of the intrastrand cross-links between adjacent adenine and guanine residues and the rate constant for the formation of the other adducts are the same (Table II). This reflects two opposite effects. A favorable effect is that adenine residues are closer to the monofunctional platinum adduct than to the other residues and a less favorable effect is that adenine residues are less reactive than guanine residues (20).

In platinated DNA, the two main adducts are d(GpG)cisPt and d(ApG)cisPt (2-4). Since acridine is of weak efficiency in the stabilization of the monofunctional adducts in platinated* poly(dG).poly(dC), we assume that in platinated* DNA, we are mainly looking at the formation of d(ApG) adducts which explains the value of the rate constant. The relative low efficiency of acridine in the stabilization of platinated* poly(dG).poly(dC) reflects probably the larger reactivity of the monofunctional adduct with an adjacent guanine residue.

In the case of the monofunctional adducts which do not possess a chloride ligand, it is not known whether the second arm of platinum interacts weakly with nucleotide residues or is in aquated form (21,22). Nevertheless, since the rate constant of cross-link formation is about 8 times larger than that corresponding to the adducts with a chloride ligand, it can be concluded that the rate of aquation reaction of the second chloride ligand is the limiting step for cross-link formation in the reaction of cis-DDP and nucleic acids.

CONCLUSION

Intercalating dyes can alter the mode and the specificity of binding of cis-DDP to nucleic acids (11,12,23-26). The ternary complexes formed in the reaction of double-stranded nucleic acids, EtBr and cis-DDP are relatively unstable at 37°C. An intermediate species is formed in which platinum binds in a monofunctional manner. We have used this intermediate species to study

the kinetics of formation of the cross-links between two nucleotide residues. The limiting step in these kinetics is the aquation rate of the second chloride ligand. For several adducts except d(GpG), the kinetics are apparently independent of the distance between the nucleotide residues.

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