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Detection of minor adducts in cisplatin-modified DNA by transcription footprinting

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Two DNA restriction fragments containing either a $d(GC)_5$ or a d(TTGCTTGATTAGTTGTGTT) insert were subjected to reaction with *cis*-diamminedichloroplatinum(II) and were then used as templates for RNA synthesis by T7 RNA polymerase. Within the $d(GC)_5$ insert, interstrand cross-links are preferentially formed. Within the second insert, the reactivity order of the potential binding sites is d(ApG) > d(GpC/GpC) = d(GpA) > d(GpTpG). In the presence of cyanide ions, the adducts are much less stable at the d(GpA) sites than at the d(GpCpG) sites, in double-stranded DNA.

cis-Diamminedichloroplatinum(II); Adduct; T7 RNA polymerase; Sodium cyanide

1. INTRODUCTION

DNA is generally accepted as the critical target for the antitumor drug *cis*-diamminedichloroplatinum(II) (*cis*-DDP). Most of the lesions produced in the in vivo and in vitro reaction with DNA have been characterized but it is not yet known which lesion(s) is (are) responsible for selective destruction of tumor cells [1–3]. The two major lesions result from intrastrand cross-links at d(GpG) and d(ApG) sites, representing respectively 65% and 25% of the total bound platinum. Interestingly, *cis*-DDP does not seem to react at d(GpA) sites. The minor adducts result mainly from cross-links between two guanine residues which are either on the same strand but separated by at least one nucleotide residue (d(GpNpG) sites) or on the opposite strands [4,5].

Recently, three reports showed that the interstrand cross-links represent 5–10% of the total bound platinum which suggested that the intrastrand cross-links at d(GpNpG) sites were hardly formed [6–8]. Initially, the purpose of our work was to determine the relative proportions of intra- and interstrand cross-links formed by *cis*-DDP at d(GpCpG) sites. In the current paper we show, by using transcription footprinting to probe the platination of DNA, that at low levels of platination interstrand cross-links are preferentially formed. Moreover, we find that among the minor lesions, the interstrand cross-links at d(GpA) sites are present in a non-negligible proportion.

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2. MATERIALS AND METHODS

2.1. *Oligonucleotides. DNA*

The oligonucleotides synthesized on an Applied Biosystems solid-phase synthesizer were purified by ion-exchange FPLC. Plasmid pSPKB(I) was obtained by cloning in plasmid pSP73KB [9] the oligonucleotide d(TTGCTTGATTAGTTGTGTT) with compatible ends (*Bam*HI and *Eco*RI). Plasmid $pSP(GC)_5$ was obtained by cloning in plasmid pSP73 the oligonucleotide d(GCGCGCGCGC) with compatible ends (*Bam*HI) After transformation of *Escherichia coli* HB101, the clonings were confirmed by DNA sequencing.

2.2. *Reaction of platination*

The reactions between *cis*-DDP and linear DNAs were done in 10 mM $NaClO_4$ during 24 h and at 37°C as described [10]. The oligonucleotide d(CTCCTCGCGCTTCTC) containing a single d(GpCpG) site was reacted with *cis*-DDP in 5 mM acetate buffer at pH 4 in conditions previously described [11]. The platinated oligonucleotide was purified by ion-exchange FPLC. The sites of platination (at the N7 position of guanine residues in the d(GpCpG) sequence) were verified by reverse-phase HPLC analysis of the digests after incubation of the oligonucleotide with nuclease P1 and then with alkaline phosphatase [12,13]. They were also confirmed by the reaction between chemical probes (dimethyl sulfate, hydroxylamine) and the platinated oligonucleotide [11]. To prepare a double-stranded oligonucleotide containing a single cross-link at the d(GpA) site, the procedure recently described [14,15] was used. Briefly, the oligonucleotide d(CTCCTCTCTCGATCTCCTCT) containing a unique G residue was first reacted with the platinum complex *cis*-$[Pt(NH_3)_2(N7\text{-}N\text{-}methyl\text{-}2,7\text{-}diazapyrenium)Cl]^{2+}$. After purification, the modified oligonucleotide was mixed with its complementary strand and incubated in 1 M NaCl during 24 h and at 37°C. After precipitation, the double-stranded oligonucleotide was incubated in 50 mM $NaClO_4$ overnight at 37°C. The oligonucleotide containing the intrastrand cross-link at the d(GpA) site was purified by gel electrophoresis under denaturing conditions. The nature and the location of the cross-link was verified by the reaction between chemical probes and the platinated oligonucleotide and by HPLC after complete enzymatic digestion of the oligonucleotide.

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2.3. *Transcription*

Transcription with T7 RNA polymerase was performed according to the protocol recommended by Promega. For nucleotide sequence analysis, the reaction mixtures were supplemented with 3'-deoxynucleoside triphosphates according to [16]. A Camag microdensitometer was used to quantitate the intensity of the bands on the gel. Only the area containing the bands of the cloned sequence was analysed. The control lane (unplatinated DNA) was used to fix the background. The relative intensity of the bands was calculated taking into account the radioactivity incorporated in each RNA.

3. RESULTS AND DISCUSSION

3.1. *Lesions in $d(GC)_5$ sequence*

To compare the formation of intrastrand cross-links versus interstrand cross-links, the $d(GC)_5$ sequence was cloned in the pSPKB plasmid. The resulting $pSP(GC)_5$ DNA was cleaved by the two endonucleases *Nde*I and *Hpa*I, which generated a 231 bp fragment with the T7 promoter. The fragment was platinated at $r_b = 0.025$, r_b being the molar ratio bound platinum per nucleotide. At this r_b, about 50% of the DNA fragments contained at least one interstrand cross-link [9]. Then, the sample was incubated in 0.2 M NaCN, pH 8.3 during 4 h and at 37°C to remove most of the major cross-links [17,18] but not the interstrand cross-links at d(GpC/GpC) sites [9] and the intrastrand cross-links at d(GpCpG) sites (vide infra). The DNA fragments with and without interstrand cross-links were separated by gel electrophoresis on a denaturing 3% agarose gel. After neutralization, the two samples were eluted and used as templates for RNA synthesis. No bands corresponding to premature termination were observed with the fragments without interstrand cross-links (Fig. 1, lane 1). In contrast, several bands were present at the level of the $d(GC)_5$ insert within the fragments containing the interstrand cross-links (Fig. 1, lane 2). The conclusion of this experiment is that in the reaction between *cis*-DDP and $d(GC)_5$ sequence, interstrand cross-links are preferentially formed.

As a complementary experiment, the frequency of interstrand cross-links as a function of total platinum was determined by studying the whole linear DNA pSPKB modified at several r_b. The fragments with and without interstrand cross-links were separated by electrophoresis on a denaturing 1.5% agarose gel and their relative amounts determined by scanning densitometry (not shown). We found that interstrand cross-links represented 5–10% of the total lesions which confirms previous results [6–8].

3.2. *Resistance to cyanide ions*

The resistance of the intrastrand cross-links at the d(GpCpG) sites to the reaction with cyanide ions was studied as follows. The 15-mer d(CTCCTCGCGCTTCTC) containing a single d(GpCpG) site was reacted with *cis*-DDP. The platinated oligonucleotide was ^{32}P end-labeled and then paired with its complementary oligonucleotide. The double-stranded oligonucleotide was incubated at 37°C in 0.2 M NaCN, pH 8.2. At various times, aliquots were withdrawn and analyzed by gel electrophoresis under denaturing conditions [18]. As a function of time, the intensity of the band corresponding to the platinated oligonucleotide decreased and a new band appeared which migrated as the unplatinated oligonucleotide (Fig. 2, right). The intrastrand cross-links were relatively resistant to cyanide ions. After 24 h of incubation less than 30% of the cross-links were removed.

A similar study was done on the double-stranded oligonucleotide d(CTCCTCTCTCGATCTCCTCT/AGAGGAGATCGAGAGAGGAG) containing a unique intrastrand cross-link at the d(GpA) site (pyrimidine-rich strand). The reaction was fast when compared to the previous one (Fig. 2), the half-life of the starting product being about 0.3 h. Another difference

Fig. 1. Autoradiogram of a denaturing 6% polyacrylamide gel, showing the termination products of T7 RNA polymerase using as template the *cis*-DDP-modified fragment (*Nde*I/*Hpa*I) including the $d(GC)_5$ sequence. Lanes 1 and 2 are relative to the DNA without interstrand cross-links and the DNA with interstrand cross-links, respectively. On the left, the sequence relative to the insertion in the DNA strand used as a template for RNA synthesis is indicated.

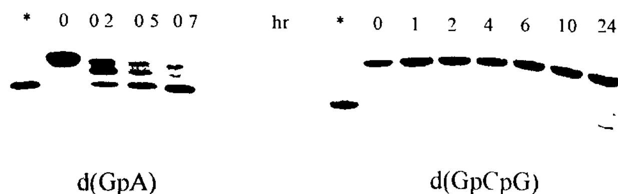

Fig. 2. Autoradiogram of a denaturing 24% polyacrylamide gel showing the products of the reaction between cyanide ions and a double stranded oligonucleotide containing either a single adduct at the d(GpCpG) site (right) or a single adduct at the d(GpA) site (left). The symbol (*) corresponds to the unplatinated double stranded oligonucleotide. The samples were incubated in 0.2 M NaCN at pH 8.2 and at 37°C during various times indicated on the figure. The cyanide ions were in large excess as compared to the oligonucleotide concentration.

in the behaviour of the two adducts was that two bands were detected on the autoradiogram relative to the d(GpCpG) adduct (the platinated and the unplatinated oligonucleotides) while three bands were present in the case of the d(GpA) adduct (the platinated oligonucleotide, the unplatinated oligonucleotide and an intermediate species).

3.3. *Quantitation of minor adducts*

In order to determine the relative proportions of the minor *cis*-DDP adducts, the sequence d(TTGCTTGATTAGTTGTGTT) was cloned in the plasmid pSPKB. The resulting plasmid pSPKB(I) was linearized by the endonuclease *Nde*I and then platinated at three r_b (0.003, 0.006, 0.009). As shown in Fig. 3, RNA synthesis by T7 RNA polymerase using the platinated samples generated a population of RNA fragments of defined sizes. Bands were present at the level of the potential d(GpTpG), d(ApG), d(GpA) and d(GpC) binding sites but they were of different intensities. Scanning of the autoradiogram yielded the following percentages for the bands' intensities: 54% at the d(ApG) site, 22% at the d(GpC) site, 17% at the d(GpA) site and 7% at the d(GpTpG) site (precision 10%). Assuming that the intensity of each band is directly related to the frequency of adduct formation and that the d(ApG) adducts account for 25% of the total bound platinum, the adducts at the d(GpC), d(GpA) and d(GpTpG) sites represent 10, 8 and 3%, respectively, of the total bound platinum. It is also assumed that all the adducts are absolute blocks for the RNA polymerase. This has not yet been proved for T7 RNA polymerase but the bifunctional cross-links at d(GpG), d(ApG), d(GpTpG) and d(GpC) completely arrest *E. coli* and wheat germ RNA polymerases [19,20]. On the other hand, we do not have any obvious explanation for the presence of two bands at the level of each adduct.

Among the minor sites, d(GpTpG) is the least reactive with *cis*-DDP. In the competition experiments within $d(GC)_5$ sequences the intrastrand cross-links are hardly detected. Several studies have shown that the adducts are formed in two solvent-assisted reactions. The preferred site of initial binding of *cis*-DDP to DNA is the N7 atom of G residue and subsequently closure of the monofunctional adduct to bifunctional cross-link occurs [13,21]. The bases surrounding the potential binding sites interfere with the two reactions by modulating the reactivity of the G residues and the ability of the DNA double helix to be distorted [1–3]. Although several sequences have to be studied before drawing any firm conclusion, our results suggest that the formation of intrastrand cross-links at d(GpNpG) (N being T or C) sequences is a rare event.

It is generally accepted that *cis*-DDP binds to d(ApG) sites but not to d(GpA) sites within native DNA. However, a few reports mention the presence of cross-links at d(GpA) sites within DNA modified in vitro by *cis*-DDP [22–24]. Analysis of mutations induced in the SUP4-o gene of yeast by treatment with *cis*-DDP suggests that the majority of substitutions occurs at d(GpG) and d(GpA) sites [25]. Our results confirm that in vitro intrastrand cross-links are formed at d(GpA) sites and that they amount to about 8% of the total lesions.

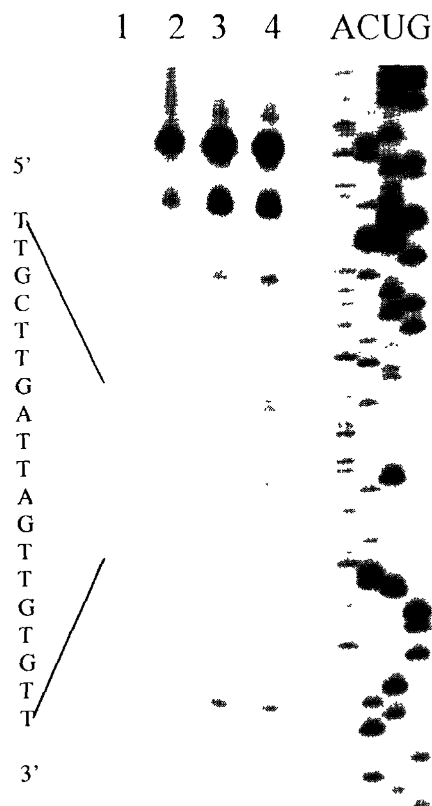

Fig. 3. Autoradiogram of a denaturing 6% polyacrylamide gel, showing the termination products of T7 RNA polymerase using as a template the *cis*-DDP-modified plasmid pspKB(I). Lane 1, untreated DNA; lanes 2–4, DNAs reacted with *cis*-DDP at r_b = 0.003, 0.006 and 0.009, respectively; lanes A, C, U and G are relative to chain-terminator marker RNAs. On the left, the sequence relative to the insert in the DNA strand used as a template for RNA synthesis is indicated.

With regard to the attack by the cyanide ions, the intrastrand cross-links at the d(GpA), d(ApG) and d(GpG) sites [18] behave similarly. In the presence of an excess of cyanide ions at pH 8.2 and at 37°C, the reactions are fast (the half-lives of the starting products are smaller than 0.5 h) and three products are detected (the platinated oligonucleotide, the unplatinated oligonucleotide and an intermediate species, probably *cis*-$[Pt(NH_3)_2(N7\text{-dGuo})CN]^+$ [18]). On the other hand, the reactions with the intrastrand cross-links at the d(GpCpG) and d(GpTpG) sites [13] are shown (the half-lives of the starting products are larger than 15 h) and two products are detected (the platinated and the unplatinated oligonucleotides). It is likely that a major parameter governing this reaction is the conformation of the platinated double helices, as previously suggested [18] to explain that the adducts at d(ApG) and d(GpG) sites are much more resistant to cyanide ions within platinated single-stranded DNA than within platinated double-stranded DNA.

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