An efficient, sequence-specific method for crosslinking complementary oligonucleotides using binuclear platinum complexes

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ABSTRACT

The binuclear Pt^{II} complexes, $[{\text{trans-Pt(NH}_3)_2\text{Cl}}]_2\{\text{NH}_2(\text{CH}_2)_n\text{NH}_2\}\text{Cl}_2$ ($n=4, 5$ or 6), crosslink oligodeoxynucleotide-5'-phosphorothioates rapidly, specifically and efficiently to complementary single-stranded oligodeoxynucleotide targets. In the case that we investigated in detail, the most abundant crosslink is formed to the G residue complementary to the 5'-terminal C residue of the phosphorothioate. Less efficient crosslinking occurs to many other residues of the target. The same Pt^{II} complexes also bring about crosslinking efficiently to the polypurine tract in triple-helical DNA.

INTRODUCTION

The use of antisense oligonucleotides to control the stability and function of messenger RNA is already an important research tool (1, 2). Once methods for delivery to cells in whole animals become available, the antisense oligonucleotides promise to find many clinical applications. Derivatives of oligonucleotides that can form covalent crosslinks to their targets should extend the range of those applications. We have shown (3–5) that monomeric Pt^{II} complexes, such as $\text{trans-[Pt(NH}_3)_2\text{Cl}_2]$, will specifically crosslink sulfur-containing oligonucleotide analogs, including phosphorothioates, to complementary targets. A major advantage of these crosslinking reagents is that they function within the physiological pH range and require no additional activators (for example, light).

Recently, Farrell and his co-workers (6) have developed a series of binuclear platinum complexes, $[{\text{trans-Pt(NH}_3)_2\text{Cl}}]_2\{\text{NH}_2(\text{CH}_2)_n\text{NH}_2\}\text{Cl}_2$ ($n = 4, 5$ or 6 ; Pt^4 , Pt^5 or Pt^6), that crosslink double-helical DNA (Figure 1). They have shown that the crossproducts contain both intrastrand and interstrand G-G and G-A crosslinks. Here we show that these same Pt^{II} complexes are much more efficient than $\text{trans-[Pt(NH}_3)_2\text{Cl}_2]$ in crosslinking 5'-phosphorothioates of oligodeoxynucleotides to their complements. No crosslinking occurs between mismatched strands, indicating a high degree of selectivity. We have also extended this method to crosslink triple-stranded DNA.

MATERIALS AND METHODS

Materials

The following were obtained from commercial sources: *cis*- and *trans*- $[\text{Pt(NH}_3)_2\text{Cl}_2]$ and snake venom phosphodiesterase (Type I), Sigma; K_2PtCl_4 , Pfaltz and Bauer; adenosine-5'- $[\gamma\text{-}^{35}\text{S}]$ -thiotriphosphate (650 mCi/ μmole) and adenosine-5'- $[\gamma\text{-}^{32}\text{P}]$ -triphosphate (3 Ci/ μmole), Amersham; adenosine-5'- γ -thiotriphosphate, Boehringer; T4 polynucleotide kinase, New England Biolabs; 1,4-diaminobutane, 1,5-diaminopentane and 1,6-diaminohexane, Aldrich.

Oligodeoxynucleotides

The oligodeoxynucleotide sequences listed in Table I were synthesized on an Applied Biosystems Model 391 automated DNA synthesizer and deprotected with NH_4OH overnight at 55°C . The deprotected oligonucleotides were purified by reverse-phase HPLC (RPC-5 column) at pH 12 using a perchlorate gradient (typically 30–70 mM over 30 minutes) containing 2 mM $\text{Tris}\cdot\text{HClO}_4$. After collection, the eluted oligonucleotides were desalted using a DuPont NENSORB 20 nucleic acid purification cartridge and stored at -20°C in 0.1 mM EDTA. The oligonucleotide C_1 containing an internal phosphorothioate group (the Sp isomer) was synthesized by a published procedure (5).

$[\gamma\text{-}^{32}\text{P}]$ -labelling was carried out by standard methods (7) using $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ and T4 polynucleotide kinase. The labelled oligonucleotides were separated from the starting materials by reverse-phase HPLC (perchlorate gradient) and were then desalted and stored as described above. $[\gamma\text{-}^{35}\text{S}]$ -labelled oligonucleotides were prepared in a similar fashion using $[\gamma\text{-}^{35}\text{S}]\text{ATP}$ (~ 1 mCi/ μmole) and were purified by reverse-phase HPLC at pH 8.5 using a perchlorate gradient. After desalting, the 5'-phosphorothioates were stored at -70°C in 0.1 mM EDTA.

Platinum compounds

The binuclear platinum complexes, Pt^4 , Pt^5 and Pt^6 , were prepared using the method of Farrell and co-workers (6). ^1H NMR and IR spectra were satisfactory for all compounds.

Gel electrophoresis

Electrophoresis was carried out using 0.75 or 1.00 mm thick polyacrylamide gels (19% w/v acrylamide and 1%

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bis(acrylamide)) containing TBE buffer, and run in 0.89 M Tris/boric acid and 2 mM EDTA. Denaturing gels contained 7 M urea and were run at 850 V (~15 mA) for 3–3.5 hours. The loading buffer contained TBE buffer, urea and the tracking dyes, xylene cyanol FF and bromophenol blue. Non-denaturing gels contained TBE buffer, glycerol and dyes, and were run at ~150 V (7 mA) for 3–4 hours. The gels were autoradiographed with Kodak XAR-5 film at –70°C (denaturing gels) or 4°C (non-denaturing gels) with or without a Du Pont Cronex Lightning Plus intensifying screen. The crosslinked bands were quantitated by excising and counting the appropriate gel slices.

Formation of platinum-linked crossproducts between oligonucleotide 5'-phosphorothioates and complementary targets

In a typical experiment, the 5'-phosphorothioate 17mer, SpA (1.0 μ l; 0.10 pmole), and 0.5 μ l of 0.1 mM KBH₄ were added to 2 μ l of 1 mM phosphate/0.1 mM EDTA buffer at pH 7.4. To this mixture was added 0.5 μ l of a 10 μ M solution of the platinum reagent. The final concentration of the platinum reagent was 1.0 μ M. Lower final concentrations of platinum reagents were obtained by adding 0.5 μ l of less concentrated solutions of the platinum complexes. The resulting solution was preincubated at 37°C for 90 minutes. The [³²P]-labelled 27mer target, [³²P]pA' (0.5 μ l; 0.01 pmole), was heated in a separate centrifuge tube to 60°C for 3 minutes and then immediately added to the platinum-phosphorothioate mixture. Finally, 0.5 μ l of 0.5 M NaClO₄ was added to the reaction mixture, and the solution was left at room temperature for 15 minutes to allow the two oligonucleotides to hybridize. The total reaction volume was 5.0 μ l. The reaction mixture was then left at 37°C for 60 minutes. Finally, the crosslinking reaction was terminated by addition of the dye/urea/buffer mixture. The products were analyzed on 20% denaturing polyacrylamide gels. The effect of the concentration of the platinum complex, the reaction times, the pH and the temperature were studied using obvious modifications of the same general procedure. The methods described above were also used to study crosslinking of the phosphorothioate 16mers, SpC₂ and SpC₅, to the complementary target, [³²P]pC'.

In other experiments, mixtures containing SpA and the Pt^{II} complex were preincubated for one hour at 37°C and then dialyzed for one hour against distilled water at 4°C before the target was added. This procedure was used to remove any excess unbound platinum complex and so to ensure that the platinated phosphorothioate oligomer was the reactive species.

Formation of platinum-linked crossproducts between a 16mer C₁ containing an internal phosphorothioate group and a complementary 37mer target [³²P]pC'

Crosslinking of the oligonucleotide C₁, containing an internal phosphorothioate group to the complementary target [³²P]pC' was carried out as described above for 5'-terminal phosphorothioates.

Formation of platinum-linked crossproducts in a triple-stranded helix

In our experiments to study crosslinking in triple-stranded structures we used the phosphorothioate-containing oligomer, SpA^{Me}. This oligonucleotide contains 5-methylcytidine (5-Me-C) residues in place of cytidines, as the replacement of C residues

Table I. Structures of the oligodeoxynucleotides used in this work.

A	5' SpCCTTTTCCTTCCTTTT 3'
A'	5' TTTAAAAGGGAAGGAAAAGGTAAAGAC 3'
B	5' CAGAAATGGAAAAGGAAGGGAAAATT 3'
B'	5' AAATTTTCCTTCCTTTTCCATTCTG 3'
C ₁	5' CACAATTC(S)CACACAAC 3'
C'	5' TCGTATGTTGTGTGGAATTGTGAGCGGATAACAATT 3'
C ₂	5' SpCACAATTCACACAAC 3'
C ₅	5' SpGCTCACAAATTCACAC 3'

by 5-Me-C stabilizes triple-helical DNA (8). Initially, the appropriate Pt^{II} complex (0.5 μ l; 10.0 μ M) and 5'-phosphorothioate oligomer (0.1 pmole; 1.0 μ l) were preincubated together at 37°C for 90 minutes (in 0.5 μ l phosphate/EDTA buffer containing 0.5 μ l of 0.1 mM KBH₄). Next, the complementary 27mers, [³²P]pB (0.01 pmole; 1.0 μ l) and B' (0.10 pmole; 1.0 μ l), were heated together to 60°C for 3 minutes, cooled to room temperature, and then added to the Pt-phosphorothioate mixture. Finally, 0.5 μ l of 1.0 M NaClO₄ was added to the resulting mixture and the oligonucleotide/Pt^{II} mixture was left at room temperature for 15 minutes. The mixture was then left at either room temperature or 37°C for an additional two hours and then loaded on a 20% denaturing gel for analysis. This experiment was performed at pH 6.1, 6.8 and 7.4.

Identification of the sites of crosslinking on the 27mer target [³²P]pA'

We used the following procedure to identify the location of interstrand crosslinks on the 27mer target, [³²P]pA'. The crosslinking reaction was carried out as described above and when it was complete, 10 μ l of a solution of Type I snake venom phosphodiesterase (0.1 units/ml in 0.11 M Tris.HCl/NaCl (pH 8.8) containing 15 mM MgCl₂) was added to the reaction mixture. The resulting solution was incubated at 37°C for 1 hour, and the enzymatic reaction was then terminated by the addition of 5 μ l of 0.5 M EDTA. This procedure led to the digestion of the target from the 3'-end up to the site of the platinum crosslink.

The phosphodiesterase was extracted with 20 μ l of a phenol/CHCl₃/i-amyl alcohol mixture (25:24:1 v/v). Next, 40 μ l of 1 M NaCN was added, and the resulting solution was heated to 37°C overnight to remove the platinum complexes from the target. The product was then dialyzed for 2 hours against 2 liters of distilled water at 4°C.

We then prepared markers of known length for comparison to the truncated sequences obtained by the above procedure. First, we modified approximately 0.10 pmole of the target oligonucleotide, [³²P]pA', under the conditions described by Maxam and Gilbert (9) and lyophilized the resulting solutions to dryness. We then removed the 3'-terminal phosphate groups by incubating the oligonucleotides at 37°C for 90 minutes with 30 units of T4 polynucleotide kinase in 50 μ l of kinase buffer (10). The products from the crosslinking reaction and the dephosphorylated products from the Maxam–Gilbert reaction were then run on adjacent lanes of a 20% denaturing polyacrylamide gel.

We also performed a control experiment in which we treated the target, [³²P]pA', with phosphodiesterase under identical conditions to those described above and then performed Maxam–Gilbert sequencing. We analyzed the products after each procedure on a 20% denaturing gel.

Figure 1. Binuclear platinum (II) complexes used in this work.

RESULTS

Formation of platinum-linked crossproducts between the 5'-phosphorothioate 17mer SpA and the complementary 27mer target [^{32}P]pA'

Farrell and co-workers have demonstrated that the binuclear platinum compounds Pt^{4-6} (see Figure 1) react with double-stranded DNA to form intrastrand and interstrand crosslinks. Crosslinking presumably occurs between all suitably placed G or A residues (6). We have attached these same binuclear Pt^{II} complexes to 5'-terminal phosphorothioate groups introduced into oligonucleotides to confer sequence-specificity on the crosslinking reaction. The sequences of the complementary oligomers **A** and **A'** that were used in most of our crosslinking experiments are illustrated in Figure 2(a). The target consists of bases 2244–2270 from the DNA sequence of the HIV1 virus (11). It was chosen because it contains a 17-base homopurine tract. Non-denaturing gel electrophoresis indicates that [^{32}P]pA and **A'** hybridize completely under our reaction conditions (data not shown).

In preliminary experiments, we studied the effect on the crosslinking efficiency of the time for which 0.1 μM Pt^{4-6} and SpA were left together before the introduction of the target (the preincubation period). We initially varied the preincubation period from 15 minutes to 24 hours, while keeping the reaction time after hybridization constant at one hour. We found that crosslinking efficiency increased with longer preincubation times up to 90 minutes and then remained essentially unchanged. Next, we used a 90 minute preincubation period and varied the time for which the two hybridized strands were in contact in the presence of Pt^{II} (the reaction time) from 15 minutes to 24 hours. The crosslinking yield increased for reaction times up to 2 hours and then remained constant. In all further experiments we used a preincubation time of 90 minutes and a reaction time of 60 minutes to achieve a crosslinking efficiency that was within a few percent of the maximum attainable. We next studied the crosslinking yield as a function of platinum concentration at our standard conditions. The results are summarized in Table II.

Figure 2(b, c) presents an autoradiogram of a typical denaturing 20% polyacrylamide gel illustrating the crossproducts formed from SpA and [^{32}P]pA' in the presence of Pt^4 and Pt^6 , respectively. The intense, low mobility bands in lanes 5–8 of both autoradiograms indicate efficient formation of covalent crosslinks between SpA and the target [^{32}P]pA'. No corresponding crosslinks are formed when SpA or the Pt^{II} reagent is omitted from the reaction mixture. Similar results were obtained with Pt^5 (data not shown). If any of the Pt^{II} reagents was preincubated with the unmodified 17mer (containing a 5'-OH group) or if a mismatched 5'-phosphorothioate replaced SpA, virtually no interstrand crosslinking was observed, provided the platinum concentrations did not exceed 0.10 μM (Figure 2(b), lane 2, and Figure 2(c), lane 4).

Figure 2. (a) Configuration of the 5'-[^{32}P]-27mer target **A'** and the crosslinking complementary 5'-phosphorothioate SpA. (b) Autoradiogram of a 20% denaturing gel containing the products of crosslinking reactions between the 5'-[^{32}P]-27mer target **A'** and SpA (lanes 5–8) or unphosphorylated **A** (lanes 2–4). Pt^{II} concentrations are 0.01 μM , lane 5; 0.05 μM , lane 6; 0.10 μM , lanes 2 and 7; 0.50 μM , lanes 3 and 8; 1.00 μM , lane 4. For reaction conditions see text. Lane 1 shows the position of the band corresponding to the target **A'**. (c) Autoradiogram of a 20% denaturing gel showing: [^{32}P]pA' (lane 1) and products from the reaction of SpA and [^{32}P]pA' in the presence of various concentrations of Pt^6 [0.05 μM (lane 5), 0.10 μM (lane 6), 0.50 μM ($\lambda\alpha\nu\epsilon$ 7), 1.0 μM (lane 8)]. Lane 2 shows the results of a control experiment in which the target **A'** was treated with 0.05 μM Pt^6 . Lane 3 corresponds to a mixture of SpA and [^{32}P]pA' in the absence of Pt^6 . Lane 4 shows the results of the reaction of the unphosphorylated 17mer **A** and [^{32}P]pA' in the presence of 0.05 μM Pt^6 . Reaction conditions are described in the text.

Table II. Yields of crosslinked products formed in the reaction between the 5'-phosphorothioate 17mer SpA and the complementary 5'-[^{32}P]-27mer target **A'** in the presence of varying concentrations of Pt^{4-6} . The reaction conditions are described in the text.

[Pt] (μM)	Pt^4	Pt^5	Pt^6
0.01	8%	4%	14%
0.05	21	19	36
0.10	26	29	41
0.50	54	38	51
1.00	58	47	64

Two sets of products were observed when the unmodified 17mer was allowed to react with the [^{32}P]-labelled target in the presence of high concentrations of Pt^4 (lanes 3 and 4), Pt^5 and Pt^6 (data not shown). The first set, containing interstrand crosslinks, had mobilities very close to those of the products formed from SpA and [^{32}P]pA' in the presence of low concentrations of the Pt^{II} compounds. The other set had electrophoretic mobilities only slightly lower than that of the unmodified target. These products must consist of platinum adducts of the target. The platinum may be bound to a single base or may crosslink a pair of bases. These same products are formed when the target alone is incubated in the presence of a high concentration of Pt^{4-6} (data not shown).

When the platinum-phosphorothioate mixtures were dialyzed against distilled water for one hour after preincubation of SpA and the Pt^{II} complex, but before addition of the target, the crosslinking yield after a one hour reaction at 37°C dropped to approximately 20%. In this experiment no intrastrand crosslinks were formed, because excess crosslinker was removed before the [³²P]-labelled target strand was introduced.

Formation of platinum-linked crossproducts between the 5'-terminal phosphorothioate 16mers SpC₂ and SpC₅ and the complementary 37mer target [³²P]pC'

We repeated the crosslinking experiments described above, but using two 5'-terminal phosphorothioate 16mers (SpC₂ and SpC₅) and a complementary 37mer target, [³²P]pC'. Non-denaturing gel electrophoresis showed that both 16mers hybridized efficiently to the target. The autoradiogram (results not shown) of the 20% denaturing gel containing the crossproducts indicates that the 16mer phosphorothioates are crosslinked to the target oligonucleotide by Pt⁶. The crosslinking efficiencies using 1.0 μM Pt⁶ are 46% for SpC₂ and 41% for SpC₅. We also performed the crosslinking experiments using 0.1 μM Pt⁶ and obtained 28% and 31% crosslinking yields for SpC₂ and SpC₅, respectively.

Formation of platinum-linked crossproducts between a 16mer C₁ containing an internal phosphorothioate group and a complementary 37mer target [³²P]pC'

The oligonucleotide C₁ was preincubated with 0.10 μM Pt⁶ for 90 minutes at 37°C and subsequently hybridized to its complement, [³²P]pC', and left at 37°C for 60 minutes. The autoradiogram of a denaturing gel of the products (not shown) displayed a series of bands corresponding to interstrand crosslinks between the two oligonucleotides. These crossproducts had mobilities similar to those of the products observed in corresponding experiments with the 5'-terminal phosphorothioates. The crosslinking yield (37%) was slightly lower than was observed when the same experiment was performed using SpC₂ containing a 5'-terminal phosphorothioate (46%).

Formation of platinum-linked crossproducts in a triple-stranded helix

We synthesized a 5'-terminal phosphorothioate 17mer (SpA^{Me}) that contained 5-methylcytidine residues in place of cytidine, and studied crosslinking reactions in a triple-helix containing the [³²P]pB and B' sequences (Figure 3(a)). The crosslinking procedure was essentially the same as that described above, except that the final reaction was carried out at 25°C rather than 37°C. An autoradiogram of a gel illustrating the crossproducts is shown in Figure 3(b) (lanes 2–4). The maximum crosslinking yield obtained was ~68%. When the reaction was carried out at 37°C, the crosslinking efficiency decreased markedly (lanes 5–7). When SpA (with C residues replacing 5-Me-C) was used, the crosslinking yields were less than 5% (data not shown). The crosslinking yield in reactions between SpA^{Me} and [³²P]pB·B' did not vary appreciably over the pH range 6.1 to 7.4, but when SpA was used, crosslinking was observed only at low pH (6.1).

We performed a similar set of experiments in which the pyrimidine-rich strand B' was labelled rather than the purine-rich B strand. No crossproducts were observed under any conditions. The oligomer A contains the nearly-palindromic sequence, TTTTCCTTCCCTTTT, which can, in principle,

Figure 3. Configuration of the triple helix formed from the 5'-[³²P]-27mer target B, the complementary 27mer B' and the crosslinking 5'-phosphorothioate 17mer SpA^{Me}. (b) Autoradiogram of a 20% denaturing gel showing the formation of crossproducts between SpA^{Me} and [³²P]pB from the incubation of the DNA triplex in the presence of 1.0 μM Pt⁶ at 25°C (lanes 2–4) and 37°C (lanes 5–7) at various pHs [pH 6.1 (lanes 2 and 5), pH 6.8 (lanes 3 and 6), pH 7.4 (lanes 4 and 7)]. Lane 1 shows the position of the band due to the polypurine target.

hybridize to the target to form a DNA duplex with two mismatches. The two hybridized strands might then be crosslinked by the platinum complexes. To eliminate this alternative explanation of the crosslinking that we attributed to triple-strand formation, we allowed SpA^{Me} and [³²P]pB to react in the presence of Pt⁵ under the conditions used with triple-helical DNA. We found only a small amount of crosslinking (<5%), far too little to explain the amount of product obtained with the triple-helical system.

Identification of the sites of crosslinking on the 27mer target

We determined the locations of platinum crosslinks to the target by using a 3'-exonuclease, snake venom phosphodiesterase, to digest the target oligonucleotide ([³²P]pA') up to the crosslinked site. The platinum was then displaced with an excess of sodium cyanide (13). The truncated products were identified by comparison with the products in the G and G+A lanes of a Maxam–Gilbert sequencing gel. The sequence of the target is shown in Figure 4(a). The 3'-phosphate groups of the Maxam–Gilbert products were removed with T4 polynucleotide kinase prior to electrophoresis to facilitate the comparison.

The autoradiograms of the denaturing 20% polyacrylamide gels containing the truncated oligomers obtained by the above procedure are illustrated in Figure 4(b). Four or five major products were observed when high (1.00 μM) concentrations of Pt^{4–6} were used (lanes 2–4). At lower concentrations of Pt^{4–6} (lanes 7–9), two major and several minor products are visible. Comparison with the dephosphorylated Maxam–Gilbert products (lanes 5 and 6) enables us to identify the positions of crosslinking. (It should be noted that the enzyme-modified oligonucleotides appear to be one nucleotide 'above' the corresponding Maxam–Gilbert products since the phosphodiesterase stops on the 3'-side of the platinated base.)

The major products from reactions containing 0.05 μM Pt^{4–6} contain crosslinks to G₁₉ and G₂₀. Some minor bands are observed due to crosslinking to bases upstream of the phosphorothioate group, as are some very weak bands due to crosslinking to G_{13–14} and A_{15–18}. When 1.00 μM Pt^{II} is used

Figure 4. (a) Sequence of the 5'-[³²P]-27mer target A'. (b) Autoradiogram of a 20% denaturing gel establishing the position of crosslinks on the target 5'-[³²P]pA' formed during crosslinking with 0.05 μM Pt⁴⁻⁶ (lanes 2-4) or 1.0 μM Pt⁴⁻⁶ (lanes 7-9). Lanes 5 and 6 illustrate the 3'-dephosphorylated products of the Maxam-Gilbert G and G+A sequencing reactions, respectively. Lane 1 shows the position of the band due to the 5'-[³²P]-27mer target A'.

(lanes 2-4), additional products are detected in which crosslinking occurs to bases as far from the phosphorothioate group as G₈ (~12 nucleotides away from the phosphorothioate group). The yield of the latter products is greatest when Pt⁶ is used as a crosslinking reagent. Crosslinking is most efficient to G residues and least efficient to C.

When the unmodified target, [³²P]pA', was treated with phosphodiesterase, we did not observe any products on the gel. This shows that the target is completely digested by the enzyme.

DISCUSSION

It is well-known that mononuclear Pt^{II} complexes such as cis- and trans-[Pt(NH₃)₂Cl₂] react efficiently with double-stranded DNA (14). The cis-complex yields a large number of intrastrand crosslinks and a much smaller number of interstrand crosslinks (14). Recently, Farrell and his co-workers have shown that a group of binuclear Pt^{II} complexes, Pt⁴, Pt⁵ and Pt⁶, give rise to a much higher proportion of interstrand crosslinks (6). Earlier work from our laboratory indicates that cysteamine adducts of oligonucleotides (3) and oligonucleotide-5'-phosphorothioates (4, 5) react with Pt^{II} complexes, including cis- and trans-[Pt(NH₃)₂Cl₂], to form adducts that crosslink efficiently and specifically to complementary target oligonucleotides. We decided, therefore, to study the crosslinking of oligonucleotide-5'-phosphorothioates to complementary targets with Pt⁴, Pt⁵ and Pt⁶.

The binuclear Pt^{II} complexes used in this work are substantially more effective as crosslinking agents than the mononuclear complexes that we have used previously. They react rapidly with phosphorothioate groups. The reaction of SpA with the binuclear Pt^{II} reagents is almost complete in 90 minutes at 37°C and pH 7.4. Crosslinking of the adducts to the complementary targets [³²P]pA' is also rapid and efficient, typically giving yields of 58-75% crosslinked product in 60 minutes. Crosslinking to non-complementary oligonucleotides is

Figure 5. Accessible structures of target and 5'-phosphorothioate oligodeoxy-nucleotides in crosslinking by (a) Pt⁴⁻⁶; (b) monomeric Pt^{II}. The range of the crosslinker is greatly increased when Pt⁴⁻⁶ are used in place of monomeric Pt^{II} complexes.

negligible. A direct comparison of the binuclear complexes with trans-[Pt(NH₃)₂Cl₂] under the conditions used in our experiments (1 μM reagent for 1 hr at 37°C) shows that the binuclear reagents react approximately 6 times faster than trans-[Pt(NH₃)₂Cl₂] (data not shown).

The rapid and efficient crosslinking brought about by the binuclear complexes allows them to be used at very low concentration. Thus Pt⁴, Pt⁵ and Pt⁶ bring about 27-48% crosslinkage when used at a concentration of 0.1 μM. The ability to crosslink messenger RNAs while using very small amounts of platinum will be important in application to medicine.

We chose to work with the phosphorothioate SpA, a polypyrimidine oligonucleotide, so that we could later compare crosslinking in double- and triple-helical complexes. However, this introduced the possibility that the efficient crosslinking that we observed was in some way connected with the absence of purine nucleotides in the phosphorothioate strand. The absence of purines would eliminate or at least greatly reduce the efficiency of 'suicide' by intramolecular crosslinking. We, therefore, examined the crosslinking reaction with two other oligonucleotide-5'-phosphorothioates. We found that under our standard conditions the phosphorothioates SpC₂ and SpC₅ crosslinked to the target [³²P]pC' with efficiencies of 46% and 41%, respectively. The difference between this value and the 75% efficiency achieved with SpA may reflect the effect of intrastrand crosslinking. Alternatively, it may reflect the different accessibilities of purine residues in the target strand. It is clear, however, that the crosslinking reaction is a general one.

The three binuclear reagents give similar patterns of crosslinked products. The most intense band on the gel corresponds to a product crosslinked to the G residue that is directly hydrogen-bonded to the 5'-terminal C residue. Less efficient crosslinking occurs to the adjacent G residue in the double helix and to all of the 7 residues of the single-stranded segment of the target. The residue closest to the phosphorothioate in the single-stranded region of the target is a T residue. Its proximity to the phosphorothioate probably explains why it is as susceptible a target as the adjacent A and G residues.

There is a major difference between the preferred positions of crosslinking of mononuclear and binuclear complexes. In the case of mononuclear complexes the major target residues are purines located 2–5 residues upstream of the phosphorothioate group (5). With the binuclear complexes crosslinking occurs over a region of 9 bases, including the two terminal residues of the base-paired region of the target. Presumably the length and flexibility of the linking diamine in the binuclear complexes permits attack at a much greater number of sites on the target (Figure 5).

Oligonucleotides containing internal phosphorothioate groups crosslink to complementary targets in the presence of mononuclear Pt^{II} complexes, but with low efficiency (5). We find that the yield is increased substantially to 37% when Pt^6 is used in place of a mononuclear complex. However, the oligonucleotide with an internal phosphorothioate is still crosslinked with lower efficiency than are those with 5'-terminal phosphorothioate groups (41–75%).

The triple helix formed by SpA^{Me} with **B** and **B'** (Figure 3(a)) is an excellent substrate for crosslinkage with Pt^6 at 25°C. Crosslinks are formed to the purine-rich strand **B** with an efficiency of 53–68% but, as would be expected, scarcely at all to the pyrimidine-rich strand **B'**. If the temperature was raised to 37°C or if C was substituted for 5-Me-C in SpA^{Me} , the crosslinking yield dropped dramatically. This is consistent with the known stabilization of triple-stranded structures by replacement of the standard base C by its 5-methyl derivative (8), and with the relatively low melting points of triple-stranded structures.

The results presented in Figure 3 show that crosslinking the triple-helical complex with Pt^6 produces a unique product. When double-helical complexes are crosslinked we always detect two or more distinct products. The detailed explanation of this difference is not obvious. In general, we believe that it is the rigidity of the double-helical segment beyond the terminal phosphorothioate group that limits the number of accessible residues in the target in the case of the triple helix. For a double-helix, the single-stranded region beyond the terminal phosphorothioate is highly flexible, so more residues are accessible to the crosslinking reagent.

We are currently developing chemically stable oligonucleotide derivatives that crosslink efficiently and specifically to messenger RNA and to polypurine tracts of genomic DNA. Molecules of this kind will be tested *in vivo* as antisense therapeutic reagents and as modifiers of genomic DNA.

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