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# Inhibition of DNA synthesis by cross-linking the template to platinum-thiol derivatives of complementary oligodeoxynucleotides

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## ABSTRACT

Unsubstituted oligodeoxynucleotides, or oligodeoxynucleotides linked to poly-(L)-lysine, when hybridized to a 322 base long template, did not inhibit the production of full length DNA copies by the Klenow fragment of *E. coli* DNA polymerase I. However, synthesis was inhibited if the cysteamine derivative of the same oligomer was cross-linked to the template via $Pt^{II}$. Truncated products were formed by termination of DNA synthesis a small number of bases upstream from the 5'-end of the cross-linked oligomer. AMV reverse transcriptase behaved similarly but was also slightly inhibited by the hybridized oligomer or its poly-lysine derivative.

## INTRODUCTION

The use of negative-strand oligodeoxynucleotides to block the biological activity of viral RNAs is an exciting approach to the treatment of a variety of viral infections. While encouraging results have been reported in a number of model systems (1, 2), the precise mode of inhibition by the negative-strand oligomers is often unclear. In some case it seems likely that protein synthesis or nucleic acid replication is blocked (3-5), while in others the complementary oligomer appears to activate the destruction of the target RNA by ribonuclease H (6, 7).

Inhibition is reversed if the negative-strand oligomer dissociates from its target RNA spontaneously or due to the action of an advancing ribosome or polymerase molecule. Thus anything that strengthens the interaction of the oligomer with its target might enhance the effectiveness of an oligomer as an inhibitor. The attachment of poly-[L]-lysine or an intercalating acridine derivative to the end of an oligodeoxynucleotide, for example, has been shown to potentiate the inhibitory activity of some oligomers (8, 9).

The most certain way of effecting an irreversible attachment of an oligomer to its target is the formation of a covalent cross-link. Miller and his colleagues have shown that psoralen derivatized oligonucleoside methylphosphonates are cross-linked to complementary m-RNA by photoactivation. The cross-linking inhibited translation more effectively than hybridization to unmodified oligonucleoside methylphosphonates (10, 11). Thus it is important to develop modified oligonucleotides that will cross-link to their targets in the intracellular environment. The range of potential modifying agents is very broad (12-16), but

it seems reasonable to start with reagents closely related to chemotherapeutic alkylating agents, since many of the latter are known to attack DNA fairly specifically in the presence of proteins and other components of the intracellular medium.

In this paper we describe preliminary studies of cross-linking using cysteamine adducts of oligodeoxynucleotides and platinum compounds related to cis-platinum diamine dichloride. We show that platinum-thiol complexes are formed readily and cross-link to a complementary template. The covalently attached oligomer, unlike poly-lysine adducts that are hybridized to the template, effectively blocks elongation by the Klenow fragment of DNA polymerase I of *E. coli* or by AMV reverse transcriptase.

## MATERIALS AND METHODS

### Materials

The following were obtained from commercial sources: sulfur-free EDTA treated cellulose dialysis tubing, 1000 M.W. cutoff, Spectrum; poly-(L)-lysine (chloride salt; M.W. ~3000) (PLL), and trans-platinum$^{(II)}$diammine dichloride (TransPt$^{II}$), Sigma; hexalysine (acetate salt), Chemical Dynamics; potassium platinous chloride ($K_2PtCl_4$), Pfaltz and Bauer; dideoxy-ATP (ddATP), Boehringer; restriction enzyme Pvu II and terminal deoxynucleotidyl transferase (TdT), BRL; AMV reverse transcriptase, U.S. Biochemical Corp.; DNA polymerase I (Klenow fragment), ds M13mp18 DNA, and Msp1 digest of pBR322 used as molecular weight standards, New England Biolabs; $\gamma$-[$^{32}$P]ATP (3 $\mu$Ci/pmole), Amersham; 2,2'dithiobis(ethylamine dihydrochloride) (cystamine), CTC Organics. The deoxy-oligomers 5'-TCA TGG TCS TAG CTG TT (17mer/stopper) complementary to bases 6209-6225 of M13mp18 (+) strand; 5'-GTT TTC CCA GTC ACG AC (17mer/primer) complementary to bases 6310-6326 of the M13mp18 (+) strand; 5'-CAC AAT TCC ACA CAA C (16mer) complementary to bases 6169-6184 of M13mp18 (+) strand; the 19-mer 5'-AGA GAG AGA CAA GGA AGG A, and the 37mer 5'-TCG TAT GTT GTG TGG AAT TGT GAG CGG ATA ACA ATT were synthesized on an automated DNA synthesizer. The 17mer/stopper is available commercially from BRL, and the 17mer/primer and 16mer are available from New England Biolabs.

The purification of oligonucleotides, their conversion to 5'-phosphates or 5'-[$^{32}$P]-phosphates, and the synthesis of 5'-cystamine-[$^{32}$P]-derivatives has been described previously (17).

### Methods

High performance liquid chromatography (HPLC) of oligonucleotides was performed on RPC-5 at pH 12, using a perchlorate gradient as previously described (18). Electrophoresis was carried out on 0.5 mm - 1 mm thick 5%, 6% or 20% polyacrylamide gels, cast and run in 90 mM Tris borate buffer (pH 8.0), 1 mM EDTA with or without 7 M urea. Non-denaturing gels were run on 7.5 cm long, 1 mm thick, 6% polyacrylamide at 25 v. (7 ma) for ~4 hours. Autoradiographs of gels were obtained by exposure to Kodak X-Omat AR film at -80°C with

or without a Du Pont Cronex Lightning Plus intensifying screen. Oligonucleotide adducts were extracted from gels with 10 mM Tris (pH 7.4) and 0.1 mM EDTA, and purified on a Du Pont Nensorb nucleic acid purification cartridge. Radioactivity on gels was determined by slicing the appropriate zones and counting in a Beckman scintillation counter.

**Addition of dideoxy A (ddA) to the 3' end of the 17mer/stopper**

ddA was added to the 3' end of the 17mer/stopper to give ddA-17mer/stopper using terminal deoxynucleotidyl transferase (TdT) (19). 2 O.D.'s of the oligonucleotide was treated with 30 U of TdT in 150 µl of buffer containing 100 mM potassium cacodylate (pH 7.2), 2 mM $CoCl_2$, 0.2 mM DTT and 0.6 mM ddATP at 37°C for 1 hour. 3'-ddA-5'-P-17mer/stopper or 3'-ddA-5'-[$^{32}$P]-17mer/stopper was purified by HPLC on RPC-5. ddA-17mer/stopper could be distinguished from starting material by gel electrophoresis (Fig. 1A).

**Digestion of ds M13mp18 DNA with Pvu II**

ds M13mp18 DNA was digested with the restriction enzyme Pvu II by incubation of 2 µg (0.4 pmoles) of ds M13mp18 DNA with 7 U of Pvu II in 30 µl of buffer containing 50 mM Tris, 6 mM $MgCl_2$, 50 mM KCl and 50 mM NaCl at pH 7.4 for 1 hour. The digested DNA, consisting of three DNA pieces 93, 322 and 6000+ base pairs long, was purified on a Nensorb column. The 322mer (+) strand is used as the template in our experiments.

**5'-[$^{32}$P]-phosphorylation of Pvu II cleaved ds M13mp18**

1.5 pmoles of Pvu II-cleaved ds M13mp18 was first dephosphorylated with 1 U of calf intestinal phosphatase in 50 µl of buffer containing 50 mM Tris (pH 8) and 0.1 mM EDTA as previously described (20). The dephosphorylated DNA was purified on a Nensorb column. ~1.5 pmoles of dephosphorylated DNA was labelled with [$^{32}$P] at its 5' terminus using a published procedure (20). The 5'-[$^{32}$P]-labelled DNA was separated from [$^{32}$P]-ATP on a Sephadex G-50 column and then further purified on a Nensorb column.

**Synthesis of poly-(L)-lysine and oligolysine adducts of ddA-5'-[$^{32}$P]-17mer/stopper**

0.2-0.3 nmoles of ddA-5'-[$^{32}$P]-17mer/stopper was reacted with 0.2 M poly-l-lysine (pH 7) and 0.15 M 1-ethyl-3,3-dimethylaminopropyl-carbodiimide (CDI) in 30 µl of 0.1 M 1-methyl-imidazole buffer at pH 7 for 1 hour at 50°C. 1 M phosphate buffer at pH 7 was then added to bring the final concentration of phosphate to 0.1 M. The reaction mixture was kept at 50°C for a further hour and then cooled. The product, ddA-5'-PLL-[$^{32}$P]-17mer/stopper, was separated from the starting materials by denaturing gel electrophoresis on 20% polyacrylamide (Fig. 1B). The product was extracted from the gel and purified on a Nensorb column.

ddA-5'-(hexalysine)-[$^{32}$P]-17mer/stopper was made via the phosphorimidazolide (ddA-5'-imidazole-[$^{32}$P]-17mer/stopper). ~0.1-0.2 nmoles of ddA-5'-[$^{32}$P]-17mer/stopper in 0.1 M imidazole buffer (pH 6.1) was treated with 0.15 M CDI at pH 6.1 for 1 hour at room temperature. 1 µl of 1 M NaOH was then added per 10 ml of reaction solution and the mixture incubated with 0.35 M hexalysine (acetate salt) at pH 8 for 1 hour at 50°C. The reaction mixture was then made up to 0.1 M with 1 M phosphate (pH 7) and then incubated

at 50°C for a further hour. It was purified by gel electrophoresis as described above for the PLL derivative.

**Formation of a platinum-linked cross product between 5'-thioethylamino-[$^{32}$P]-16mer and 5'-[$^{32}$P]-37mer**

5'-cystamine-[$^{32}$P]-16mer (s.a. 200-500 cpm/pmole) (10-20 pmole) was converted to the 5'-thioethylamino-[$^{32}$P]-16mer by treatment with 10 mM DTT for 1 hour in 10-15 µl of buffer containing 10 mM Tris (pH 7.2) and 0.1 mM EDTA (Fig. 1A). The solution was then dialyzed for 25 minutes against 1 l of buffer containing 1 mM phosphate (pH 7), 0.1 mM EDTA and 0.1 mM DTT, and twice for 25 minutes against buffer containing 1 mM phosphate (pH 7) and 0.1 mM EDTA. In some experiments the final dialysis buffers also contained 0.03 mM DTT. A solution of $K_2PtCl_4$ (0.7 mM) or TransPt$^{II}$ (0.5 mM) was made up freshly in buffer containing 10 mM phosphate (pH 7) and 30 mM NaCl. 1 µl of the $K_2PtCl_4$ or TransPt$^{II}$ solution was added for each 10 µl of the 5'-thioethylamino-[$^{32}$P]-16mer solution, and the resulting solution was incubated for 60 minutes at room temperature. An aliquot of this mixture containing 0.2-0.4 pmole of oligonucleotide (about 1 µl) was then added to 0.01-0.025 pmole of 5'-[$^{32}$P]-37mer (s.a. 0.5- 1.6 x $10^6$ cpm/pmole) in 15 µl of buffer containing 7 mM phosphate (pH 7 or 8) and 30 mM $NaClO_4$. The resulting solution (containing ~3-5 µM $K_2PtCl_4$ or TransPt$^{II}$) was left overnight at room temperature. The cross product was separated from 5'-[$^{32}$P]-37mer by denaturing gel electrophoresis on 20% polyacrylamide (Fig. 3).

**Formation of a platinum-linked cross product between ddA-5'-thioethylamino-[$^{32}$P]-17mer/stopper and the 322mer (+) strand of Pvu II-cleaved ds M13mp18**

10-15 pmoles of ddA-5'-cystamine-[$^{32}$P]-17mer/stopper (of known specific activity ~25,000-40,000 cpm/pmole) was reduced to ddA-5'-thioethylamino-[$^{32}$P]-17mer/stopper as described above for 5'-cystamine-[$^{32}$P]-16mer. Solutions of $K_2PtCl_4$ (0.7 mM) or TransPt$^{II}$ (0.5 mM) were made up freshly in buffer containing 10 mM phosphate (pH 7) and 30 mM NaCl. 1 µl of the $K_2PtCl_4$ or TransPt$^{II}$ solution was added for each 10 µl of the ddA-5'-thioethylamino-[$^{32}$P]-17mer/stopper solution and the resulting mixture was incubated for 60 minutes at room temperature.

An aliquot of this reaction mixture containing 0.2-0.4 pmole of the oligonucleotide (about 1 µl) was then added to 15 µl of buffer containing 7 mM phosphate (pH 7 or 8), 30 mM $NaClO_4$ and a known amount of Pvu II-cleaved ds M13mp18 (0.01-0.025 pmole) that had previously been heated at 95°-100° for 1 minute and then cooled on ice. The resulting solution (containing ~3-5 µM $K_2PtCl_4$ or TransPt$^{II}$) was left overnight at room temperature. In control experiments ddA-5'-[$^{32}$P]-17mer/stopper and 5'-thioethylamino-[$^{32}$P]-19mer (not complementary to the 322mer (+) strand) were treated with $K_2PtCl_4$ in the same way as described above for ddA-5'-cystamine-[$^{32}$P]-17mer/stopper and then reacted with the denatured Pvu II-cleaved ds M13mp18 overnight. In another set of experiments a mixture of

ddA-5'-thioethylamino-[$^{32}$P]-17mer/stopper and denatured Pvu II-cleaved ds M13mp18, after hybridization, was incubated overnight in the presence or absence of 5 μM $K_2PtCl_4$.

The platinum cross product formed between ddA-5'-thioethylamino-[$^{32}$P]-17mer/stopper and the 322mer was separated from ddA-5'-thioethylamino-[$^{32}$P]-17mer/stopper by denaturing gel electrophoresis on 6% polyacrylamide at pH 8. The yield was estimated from the radioactivity of the cross-product band and the known amount of Pvu II-cleaved DNA in the reaction mixture. To separate the platinum cross product from 322mer (+) strand that had not been cross-linked, electrophoresis was carried out on 36 cm long, 5 mm thick 5% polyacrylamide at 900-1000 v. (800-1000 ma) for 9 hours (Fig. 4B). The labelled cross product and the region of the gel containing the uncross-linked 322mer (+) strand were extracted from the gel and purified on a Nensorb cartridge.

**Hybridization efficiency of ddA-5'-[$^{32}$P]-17mer/stopper and ddA-5'-PLL-[$^{32}$P]-17mer/stopper with the 322 (+) strand of Pvu II-cleaved ds M13mp18**

0.01 pmole of Pvu II-cleaved ds M13mp18 in 5 μl of buffer containing 0.02 M phosphate at pH 8 was placed in a boiling water bath for 1 minute. The mixture was then cooled on ice and 0.25 pmole of ddA-5'-[$^{32}$P]-17mer/stopper or ddA-5'-PLL-[$^{32}$P]-17mer/stopper (of equal and known specific activity, ~25,000-30,000 cpm/pmole) was added. Sufficient $NaClO_4$ was added to bring the final concentration to 30 mM. After overnight incubation at room temperature, hybrid formation between the 322mer (+) strand and the 17mer adducts was detected by gel electrophoresis on a 6% polyacrylamide non-denaturing gel (Fig. 2).

**Effect of hybridization of ddA-5'-[$^{32}$P]-17mer/stopper and ddA-5'-PLL-[$^{32}$P]-17mer/stopper to a 322mer template on DNA synthesis by the Klenow fragment or reverse transcriptase**

0.3-0.4 pmole of ddA-5'-PLL-[$^{32}$P]-17mer/stopper or ddA-5'-[$^{32}$P]-17mer/stopper (~20,000-30,000 cpm/pmole) was hybridized at room temperature overnight in 15 μl of buffer containing 7 mM phosphate (pH 8) and 30 mM $NaClO_4$ to 0.025 pmole of Pvu II-cleaved ds M13mp18 (previously heated at 95°-100°C for 1 minute, then cooled on ice). A 2 μl aliquot from these solutions (containing 0.003 pmole of the 322mer (+) strand) was then hybridized to ~0.05-0.08 pmole of 5'-[$^{32}$P]-17mer/primer (s.a. 0.5 x $10^6$ - 1.4 x $10^6$ cpm/pmole) in 8 μl of buffer containing 50 mM Tris (pH 7), 10mM $MgCl_2$ and 0.1 mM DTT at room temperature for 1 hour. The 4 deoxynucleoside triphosphates were then added in a volume of 1 μl to give a final concentration of 0.5 mM. Finally, 2.5 U of the Klenow fragment (1 μl) was added. After incubating for 45 minutes at room temperature, the reaction was stopped by the addition of 1 μl of 0.1 M EDTA. The reaction mixture was then boiled for 1 minute in the presence of 7 M urea and the products of the enzyme reaction were analyzed by denaturing gel electrophoresis on 6% polyacrylamide.

In experiments with reverse transcriptase the identical procedure was used except that the enzyme reaction was carried out in buffer containing 50 mM Tris (pH 8.3), 8 mM $MgCl_2$, 0.4 mM DTT and 40 mM KCl. 3 U of reverse transcriptase was used in each experiment.

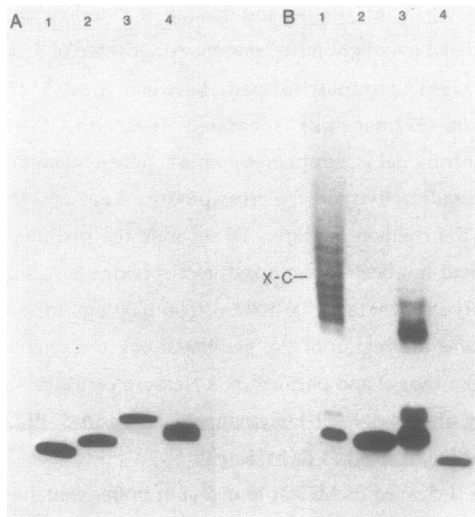


**Figure 1:** Autoradiogram of 20% denaturing gel showing:
A. Migration of: lane 1) 5'-[$^{32}$P]-17mer/stopper; lane 2) ddA-5'-[$^{32}$P]-17mer/stopper; lane 3) ddA-5'-cystamine-[$^{32}$P]-17mer/stopper; lane 4) ddA-5'-thioethylamino-[$^{32}$P]-17mer/stopper.
B. Lane 1) formation of ddA-5'-PLL-[$^{32}$P]-17mer/stopper from reaction of ddA-5'-[$^{32}$P]-17mer/stopper with 0.15 M CDI, and 0.2 M PLL in 0.1 M 1-methylimidazole buffer at pH 7 for 1 hour at 50°C; lane 2) ddA-5'-[$^{32}$P]-17mer/stopper; lane 3) formation of ddA-5'-hexalysine-[$^{32}$P]-17mer/stopper by treatment of ddA-5'-imidazole-[$^{32}$P]-17mer/stopper with 0.35 M hexalysine at pH 8 for 1 hour at 50°C; lane 4) 5'-[$^{32}$P]-17mer/stopper.

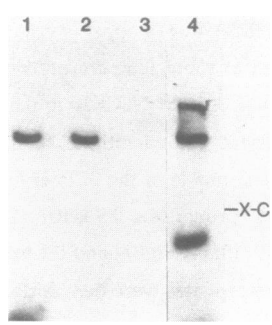


**Figure 2:** Autoradiogram of a 6% non-denaturing gel showing:
Lane 1) hybrid formation between a complementary ddA-5'-PLL-[$^{32}$P]-17mer/stopper and 322mer (+) strand of Pvu II-cleaved ds M13mp18; lane 2) hybrid formation between ddA-5'-[$^{32}$P]-17mer/stopper and 322mer; lane 3) attempted hybrid formation between a non-complementary 5'-[$^{32}$P]-19mer and 322mer; lane 4) 5'-[$^{32}$P]-6000+mer, 5'-[$^{32}$P]-322mer, and 5'-[$^{32}$P]-93mer markers.

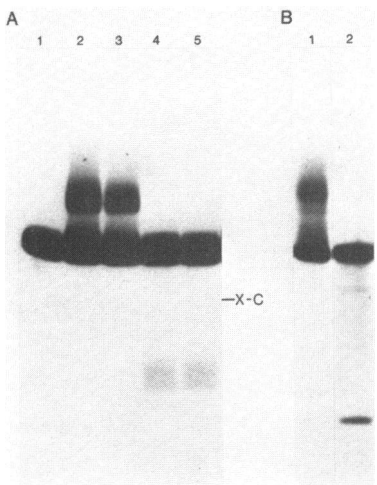


**Figure 3:** Autoradiogram of a 20% denaturing gel showing:

A. Lane 1) 5'-[$^{32}$P]-37mer; lane 2) formation of a $K_2PtCl_4$ cross product between 5'-[$^{32}$P]-37mer and a complementary 5'-thioethylamino-P-16mer, at pH 7; lane 3) same as lane 2, at pH 8; lane 4) attempted formation of a $K_2PtCl_4$ cross product between 5'-[$^{32}$P]-37mer and a non-complementary ddA-5'-thioethylamino-[$^{32}$P]-17mer, at pH 7; lane 5) same as lane 4, at pH 8.

B. Lane 1) formation of a TransPt$^{II}$ cross product between 5'-[$^{32}$P]-37mer and a complementary 5'-thioethylamino-P-16mer, at pH 7; lane 2) same as in lane 1 except a non-complementary ddA-5'-thioethylamino-P-17mer was used in place of the complementary 5'-thioethylamino-P-16mer.

**Effect of cross-linking the $K_2PtCl_4$ adduct of ddA-5'-thioethylamino-P-17mer/stopper to the 322mer template on DNA synthesis by the Klenow fragment or reverse transcriptase**

5-10 pmoles of ddA-5'-thioethylamino-[$^{32}$P]-17mer/stopper or ddA-5'-[$^{32}$P]-17mer/stopper (s.a. 20,000-30,000 cpm/pmole) was reacted with 0.07 mM $K_2PtCl_4$ for 1 hour at room temperature in buffer containing 1 mM phosphate (pH 7) and 3 mM NaCl. An aliquot of this mixture (about 1 µl) containing 0.3-0.4 pmole of oligonucleotide was then added to 0.025 pmole of denatured ds M13mp18 in 15 µl of buffer containing 7 mM phosphate (pH 8), and 30 mM $NaClO_4$. The resulting solution was left overnight at room temperature.

A 2 µl aliquot from these reaction mixtures (containing 0.003 pmole of the 322mer template) was hybridized with ~0.05-0.08 pmole of 5'-[$^{32}$P]-17mer primer (~0.5-1.5x10$^6$ cpm/pmole), and primer extension was carried out as described above for the PLL adduct.

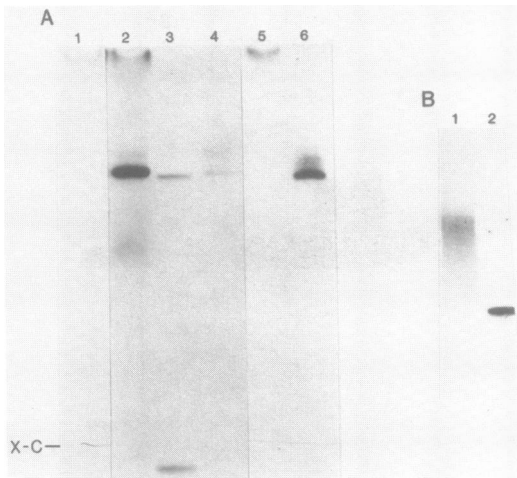


**Figure 4:** A. Autoradiogram of a 6% non-denaturing gel showing formation of platinum-linked cross products, at pH 7, between the 322mer (+) strand of Pvu II-cleaved ds M13mp18 and: lane 1) a non-complementary 5'-thioethylamino-[$^{32}$P]-19mer treated with $K_2PtCl_4$; lane 2) ddA-5'-thioethylamino-[$^{32}$P]-17mer/stopper treated with $K_2PtCl_4$; lane 4) ddA-5'-[$^{32}$P]-17mer/stopper treated with $K_2PtCl_4$; lane 5) a non-complementary 5'-thioethylamino-[$^{32}$P]-19mer treated with $TransPt^{II}$; lane 6) ddA-5'-thioethylamino-[$^{32}$P]-17mer/stopper treated with $TransPt^{II}$; lane 3) unmodified 5'-[$^{32}$P]-322mer and 5'-[$^{32}$P]-93mer markers.

B. Autoradiogram of a 5 mM thick 5% denaturing gel carried out at 800-1000 v. for 9 hours showing: lane 1) separation of platinum 322mer cross product from unlabelled, uncross-linked 322mer; lane 2) 5'-[$^{32}$P]-322mer marker.

**Primer extension on gel-purified platinum cross product by the Klenow fragment and reverse transcriptase**

~0.002 pmole of the gel-purified 322mer cross product (s.a. 20,000-30,000 cpm/pmole) (Fig. 4B) or an aliquot of the uncross-linked 322mer (+) strand (isolated from the same gel) was hybridized to 0.05-0.06 pmole of 5'-[$^{32}$P]-17mer/primer (s.a. 0.5 - 1.4 x $10^6$ cpm/pmole) for 1 hour at room temperature. Primer extension with the Klenow fragment (2.5 U) or reverse transcriptase (3 U) was carried out in 10 µl volumes and analyzed as described above.

## RESULTS

### The synthesis of 5'-poly-(L)-lysine-[$^{32}$P]-oligomers

The method that we used to prepare the PLL derivative of ddA-5-[$^{32}$P]-17mer/stopper was originally developed for the synthesis of amine derivatives of oligodeoxynucleotides (21). However, since PLL forms more stable ionic complexes with oligonucleotides, we had to incubate the reaction products with 0.1 M phosphate to dissociate non-covalent adducts prior to isolation of the covalent adduct by electrophoresis. The results of a typical separation

are illustrated by the autoradiogram shown in Fig. 1B. ddA-5'-PLL-[$^{32}$P]-17mer/stopper can be seen as a series of bands with significantly lower mobilities than the starting ddA-5'-[$^{32}$P]-17mer/stopper. In lane 3 the corresponding autoradiogram for a preparation of ddA-5'-hexalysine 6-[$^{32}$P]-17mer/stopper is shown. Comparison of lanes 1 and 3 indicates that the preparation of 5'-PLL-[$^{32}$P]-17mer/stopper contained a mixture of adducts most of which involve peptides substantially longer than the hexapeptide.

The ddA-5'-PLL-[$^{32}$P]-17mer hybridyzed as efficiently as unmodified ddA-5'-[$^{32}$P]-17mer with a complementary 322mer as shown in Fig.2.

**Formation of platinum-linked cross product between 5'-thioethylamino-P-16mer and 5'-[$^{32}$P]-37mer** (see Fig. 5(a) for structure of the hybrid)

When 5'-thioethylamino-P-16mer was treated with either $K_2PtCl_4$ or TransPt$^{II}$ and an aliquot of the resulting reaction mixture containing the platinum oligonucleotide adduct incubated with a complementary 5'-[$^{32}$P]-37mer overnight at pH 7, a cross product was formed between the two oligomers in ~30-55% yield. Yields were slightly lower at pH 8. Figure 3 shows an autoradiogram of a 20% denaturing gel in which bands due to cross-linked [$^{32}$P]-labelled products are visible (for $K_2PtCl_4$ in Fig. 3A, lanes 2 and 3, or for TransPt$^{II}$ in Fig. 3B, lane 1). When ddA-5'-thioethylamino-P-17mer, which is not complementary to the 37mer, was treated with $K_2PtCl_4$ or TransPt$^{II}$ in the same way, no cross product between the two oligomers was observed (Fig. 3A, lanes 4 and 5, and Fig. 3B, lane 2). These results indicate that one or more cross products are formed only when a platinum-containing oligomer is hybridized to its complement.

**Formation of a platinum-linked cross product between ddA-5'-thioethylamino-[$^{32}$P]-17mer/stopper and the 322 base (+) strand of Pvu II-cleaved ds M13mp18**

M13mp18 RF DNA (7150 bp) is cleaved by Pvu II to give 3 DNA pieces - 93, 322 and 6735 bp long. The 322 base pair fragment (+) strand is used as the template in our experiments. It contains the Messing polylinker cloning site and part of the lac i and lac Z genes (22). When ddA-5'-thioethylamino-[$^{32}$P]-17mer/stopper was treated with $K_2PtCl_4$ and an aliquot of the reaction mixture containing the platinum oligonucleotide adduct was incubated with unlabelled denatured Pvu II-cleaved ds M13mp18 overnight at room temperature and pH 7 or 8, [$^{32}$P]-labelled cross product was formed which had almost the same mobility on a 6% denaturing gel as the [$^{32}$P]-322mer (Fig. 4A, lane 2). The highest yield of cross product (50-60%) was obtained when ddA-5'-thioethylamino-[$^{32}$P]-17mer/stopper was isolated and reacted with $K_2PtCl_4$ in the presence of 0.03 mM DTT. In the absence of DTT, 30-50% yields of cross product were obtained at pH 7. At pH 8 the yield was 20-40%. In a control reaction in which a non-complementary 5'-platinum-thioethylamino-[$^{32}$P]-19mer was used in place of ddA-5'-platinum-thioethylamino-[$^{32}$P]-17mer/stopper, no cross product with the 322mer could be detected (Fig. 4A, lane 1), indicating that the formation of cross product is

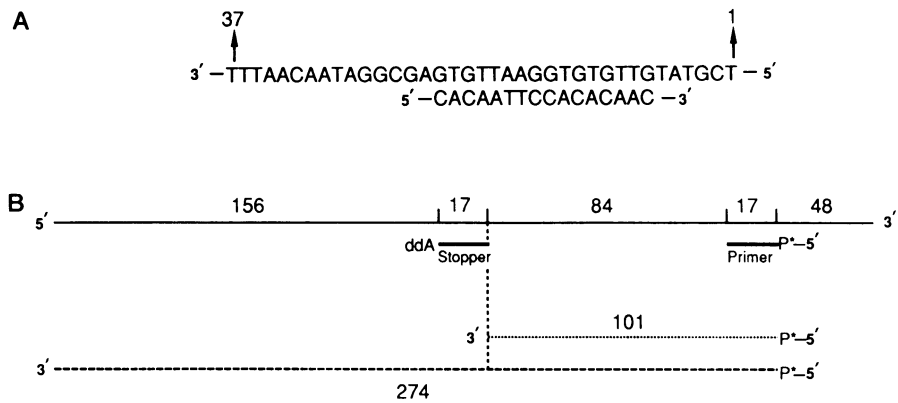


**Figure 5:** (a) Structure of the hybrid formed between 5′-P-37mer and 5′-P-16mer.
(b) Configuration of primer and stopper oligomers on the 322mer (+) strand template.
"$\dot{p}$" designates the [$^{32}$P]-label at the 5′-terminus of the primer. "ddA" indicates a dideoxynucleotide at the 3′ terminus of the stopper; the presence of ddA prevents the extension of the stopper. The full length product (274 bases) derived from extension of the primer by the Klenow fragment or reverse transcriptase is indicated by a dashed line; the truncated product (101 bases) formed by termination at the stopper is indicated by a dotted line.

hybridization-dependent. When ddA-5′-[$^{32}$P]-17mer/stopper was treated with DTT and $K_2PtCl_4$ in exactly the same way as ddA-5′-cystamine-[$^{32}$P]-17mer/stopper and then reacted with the 322mer, a very small amount (<5%) of cross product was obtained at pH 7 (Fig. 4A, lane 4). These control experiments indicate that substantial cross-linkage is achieved only when a thiol derivative of a complementary oligomer is used in the cross product reaction. Similar results were obtained when ddA-5′-thioethylamino-[$^{32}$P]-17mer/stopper was reacted with TransPt$^{II}$ instead of $K_2PtCl_4$ and then cross-linked to the 322mer (Fig. 4A, lane 6). The yield of cross product at pH 7 was 25-35%.

In most of the experiments described above we first reacted ddA-5′-thioethylamino-[$^{32}$P]-17mer/stopper with $K_2PtCl_4$ for 60 minutes and then used the platinum-thiol adduct that was formed, in the presence of excess $K_2PtCl_4$ (5-6 μM), to form the cross product with the 322mer (+) strand. In subsequent experiments we found that almost equivalent yields of cross-product were obtained when ddA-5′-thioethylamino-[$^{32}$P]-17mer/stopper was hybridyzed directly to the 322mer (+) strand and the solution incubated overnight in the presence of 5 μM $K_2PtCl_4$.

**Termination of DNA synthesis by ddA-17mer/stopper and its adducts**

Figure 5(b) shows the positions of the 17mer/primer and the 17mer/stopper on the 322mer (+) strand of Pvu II-cleaved ds M13mp18. A full primer extension by DNA I

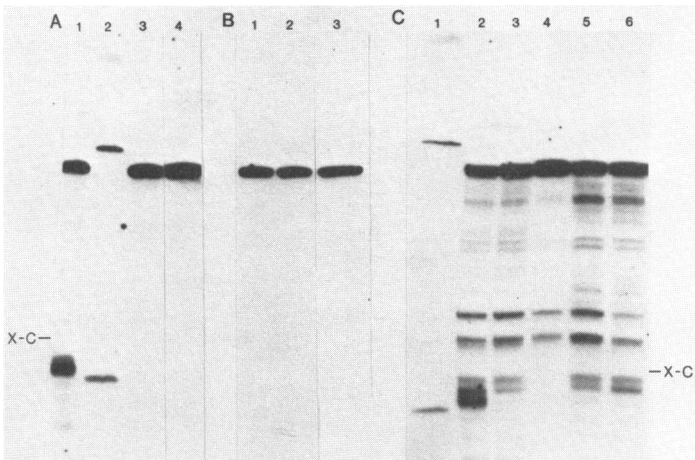


**Figure 6:** Autoradiogram of a 6% denaturing gel showing:
A. Primer extension by the Klenow fragment on 322mer (+) strand: lane 1) after formation of $K_2PtCl_4$ cross product with ddA-5'-thioethylamino-[$^{32}$P]-17mer/stopper; lane 3) after attempted formation of platinum cross product with $K_2PtCl_4$ treated ddA-5'-[$^{32}$P]-17mer/stopper; lane 4) in the absence of added oligomer; lane 2) 5'-[$^{32}$P]-322mer and 5'-[$^{32}$P]-93mer markers.
B. Primer extension by the Klenow fragment on 322mer (+) strand: lane 1) after hybridization to ddA-5'-PLL-P-17mer/stopper; lane 2) after hybridization to ddA-5'-P-17mer/stopper; lane 3) in the absence of added oligomers.
C. Primer extension by reverse transcriptase on 322mer (+) strand: lane 2) after formation of the $K_2PtCl_4$ cross product with ddA-5'-thioethylamino-[$^{32}$P]-17mer/stopper; lane 3) after attempted formation of a $K_2PtCl_4$ cross product with ddA-5'-P-17mer/stopper; lane 4) in the absence of added oligomers; lane 5) after hybridization to ddA-5'-PLL-P-17mer/stopper; lane 6) after hybridization to ddA-5'-P-17mer/stopper; lane 1) 5'-[$^{32}$P]-322mer and 5'-[$^{32}$P]-93mer markers.

polymerase or reverse transcriptase would give a product 274 bases long. The 5' end of the stopper oligonucleotide is located 101 bases from the 5' end of the primer. If the hybridized stopper oligonucleotide prevents the enzyme from completing the synthesis, an oligonucleotide no longer than 101 base pairs would be formed. In order to prevent primer extension of the stopper oligonucleotide, we routinely added ddA to the 3' end of the stopper.

The products of chain extension of the 5'-[$^{32}$P]-17mer/primer on 322mer by the Klenow fragment alone, or in the presence of ddA-17mer/stopper or its adducts were subjected to gel electrophoresis. An autoradiogram of the gel is illustrated in Fig. 6A and B. Inspection of lanes 1 and 2 (Fig. 6B) shows that neither the 17mer or its poly-lysine adduct, when hybridized to the template, interferes with DNA synthesis. In both cases the only product

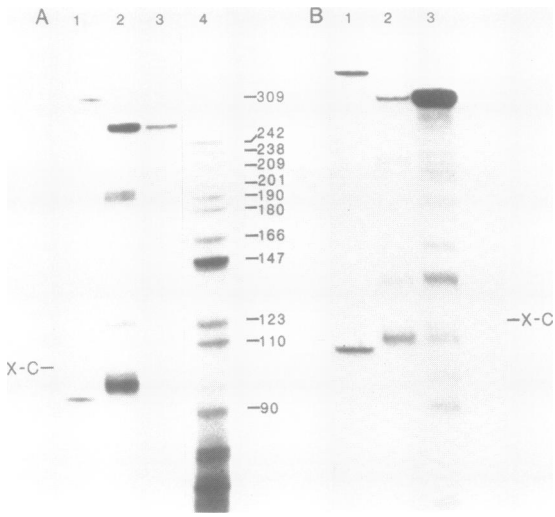


**Figure 7:** Autoradiogram of a 6% denaturing gel showing:
A. Lane 1) 5'-[$^{32}$P]-322mer and 5'-[$^{32}$P]-93mer markers; lane 2) primer extension by the Klenow fragment on gel-purified 322mer (+) strand crosslinked to $K_2PtCl_4$-treated ddA-5'-thioethylamino-[$^{32}$P]-17mer/stopper; lane 3) primer extension by the Klenow fragment of gel-separated uncrosslinked 322mer (+) strand from same reaction as in lane 2; lane 4) 5'-[$^{32}$P]-molecular weight standards from Pst I-cleaved pBR322.
B. Lane 1) 5'-[$^{32}$P]-322mer and 5'-[$^{32}$P]-93mer markers; lane 2) primer extension by reverse transcriptase on gel-purified 322mer (+) strand cross-linked to $K_2PtCl_4$-treated ddA-5'-thioethylamino-[$^{32}$P]-17mer/stopper; lane 3) primer extension by reverse transcriptase of gel-separated uncross-linked 322mer (+) strand from same reaction as lane 2.

visible is the full length 274 base oligomer; and it is formed as efficiently as in the absence of any complementary stopper (lane 3).

The products of chain extension in a reaction mixture containing the covalent $K_2PtCl_4$ adduct of ddA-5'-thioethylamino-P-17mer/stopper with 322mer are very different (Fig. 6A, lane 1). In this case a product with a mobility slightly less than that of a 5'-[$^{32}$P]-93mer marker can be seen in significant amounts. The length of this oligomer is somewhat shorter than the 101mer that would be expected if extension proceeded up to the 5'-end of the stopper. Presumably the cross-linkage occurs at one or more bases a few nucleotides upstream of the stopper (23, 24). The percentage of product that was truncated close to the stopper was similar to the percentage of template molecules that had formed cross-links, and the yield of full-length product was similarly reduced. In the control experiment using platinum treated ddA-P-17mer/stopper, significant amounts of only the full length 274mer could be seen (lane 3).

In experiments with reverse transcriptase (Fig. 6C), two shortened oligomers (~100 bases long) are visible when ddA-5'-P-17mer/stopper or ddA-5'-PLL-P-17mer/stopper is hybridyzed to the 322mer (+) strand (lanes 5 and 6). They make up ~10% of total oligomers

formed. These bands are not visible in a control experiment where no complementary oligomer is added to the reaction mixture. When ddA-5'-thioethylamino-P-17mer/stopper is cross-linked to the 322mer (+) strand via platinum, an additional more significant stoppage point is visible, corresponding to a product 96-98 bases long (lane 2). It makes up about 30% of total material synthesized, and is not visible when $K_2PtCl_4$-treated ddA-5'-P-17mer is used (lane 3) in place of ddA-5'-thioethylamino-P-17mer/stopper.

These experiments with the Klenow fragment or reverse transcriptase indicate that a complementary oligonucleotide, when it is cross-linked to the template, is a significantly more efficient inhibitor of primer extension than the unmodified oligomer or its poly-lysine derivative when they are hybridyzed to the template.

**Primer extension on purified platinum-linked 322mer cross product by DNA polymerase I and reverse transcriptase**

Figure 7 illustrates the results of an experiment in which the gel-purified platinum-linked 322mer cross product was used as a template for extension of the 5'-[$^{32}$P]-17mer/primer by the Klenow fragment or reverse transcriptase. The results (Fig. 7A, lane 2, and 7B, lane 2) were similar for the two enzymes. ~20% of the material synthesized consisted of the full-length 274mer. When the cross product was repurified a second time on 5% polyacrylamide, the percentage of full-length 274mer obtained dropped to ~5% (results not shown). We believe that this residual synthesis past the site of the covalent cross-link is due to the presence of small amounts of unmodified 322mer in the product. The principal product of the synthesis is an oligomer about 96-98 bases long. In addition two minor products corresponding to oligomers approximately 119 and 198 bases long are also obtained.

## DISCUSSION

A number of authors have emphasized the potential importance of modified oligonucleotides that can form cross-links with complementary sequences (10, 12, 14, 16). Vlassov and his colleagues have pioneered the use of $Pt^{II}$ complexes for cross-linking (12). A simpler, alternative approach to the synthesis of platinum adducts that are suitable for cross-linking makes use of oligonucleotide derivatives that contain a free sulfhydryl group. Reaction with a $Pt^{II}$ halide yields a co-ordination compound that can react further to form a covalent cross-link.

$$\text{Oligonucleotide-linker-SH} + \left[Pt^{II}Cl_4\right]^{2-} \longrightarrow \text{oligonucleotide-linker-S-}PtCl_3$$

$$\xrightarrow{\text{hybridize}} \text{covalently cross-linked product}$$

Adducts in which cysteamine is attached to the terminal 5'-phosphate of an oligonucleotide are readily synthesized from unprotected oligonucleotides or nucleic acids. They

hybridize normally to a complementary oligomer and, in the presence of $Pt^{II}$ complexes such as $[PtCl_4]^{2-}$, cross-link directly to it. This provides a very simple route to cross-linked oligomers.

We could not determine directly the yields of the $Pt^{II}$ complexes formed by the thioethylamine adducts of oligonucleotides because these complexes do not give sharp bands on electrophoresis. However, we found that after treatment of 5'-thioethylamino-[$^{32}$P]-16mer for 2 hrs with $10^{-4}$ M $K_2PtCl_4$ at room temperature, more than 50% of the starting oligomer was converted to material that moves more slowly than the starting material on the gel. When 5'-[$^{32}$P]-16mer was treated with $K_2PtCl_4$ in the same way, less than 5% of the starting material was affected.

The cysteamine adduct of the 16mer reacted readily with the complementary 37mer in the presence of 5 μM $K_2PtCl_4$ to form a stable covalent adduct in greater than 50% yield. With 0.5 μM $K_2PtCl_4$ the yield was 15-20%. When we attempted to cross-link the same 16mer to a complementary 322mer, little cross-linking was achieved unless the 16mer was used at a very high concentration. We found (experimental data not shown) that this is due to the inefficient hybridization of the 16mer with the 322mer, presumably because the latter forms a self-structure involving the sequence complementary to the 16mer. When we replaced the 16mer by a 17mer sequence complementary to a different region of the 322mer, cross-linking occurred readily.

Under the conditions of our experiment, the Klenow fragment of *E. coli* DNA polymerase I efficiently displaces complementary oligonucleotides from the template strand and continues synthesis beyond them. Poly-lysine adducts of the stopper oligonucleotide, which should form more stable double-helical complexes (8), are also displaced. However, covalent cross-linking of a complementary oligomer to the template blocks synthesis beyond the cross-link. AMV reverse transcriptase behaves similarly on a covalently cross-linked template, but is slightly inhibited by the unmodified complementary oligomer or by its poly-lysine adduct.

When the mixture of the cross-linked 322mer product and uncross-linked 322mer that is obtained in a cross-linking reaction is used as template, the proportion of truncated product obtained is roughly equal to the proportion of template that is cross-linked. This suggests that the blockage is almost complete on a cross-linked template. Truncated chains have a length of 96-98 base-pairs, slightly shorter than the 101 base product anticipated if synthesis continued up to the blocker. When gel-purified cross-linked template is used, blockage is extensive and, after two gel-purifications, almost complete (>95%). However, after gel-purification, the template directs the synthesis of small amounts of two products that are longer than the major stoppage product. The proportion of these products is very similar in experiments using DNA polymerase and in those using reverse transcriptase. We believe that the original reaction mixture contains small amounts of products that are cross-

linked further from the primer than the hybridization site, perhaps because the self-structure of the template brings nucleotides downstream from the blocker close to the 5'-terminus of the blocker. The gel-purification procedure increases the proportion of these products in the material that is finally isolated.

An important paper by Pinto and Lippard is relevant to some of our results (25). They showed that $Pt^{II}$ halide complexes react directly with DNA templates to produce adducts that block primer extension. Cis[$(NH_3)_2PtCl_2$] reacted most specifically with the template, so that subsequent primer extension gave substantial amounts of specific length sequences; trans[$(NH_3)_2PtCl_2$] reacted less specifically, leading to the formation of many truncated different sequences in smaller amounts. In agreement with these results, we found that cis[$(NH_3)_2PtCl_2$] was unsuitable as a cross-linking agent because it led to a specific pattern of termination, even when there was no possibility of cross-linking. The use of $K_2PtCl_4$ as the cross-linking agent reduced the level of non-specific termination to acceptable levels (Fig. 6A, lane 1).

The principal objective of these experiments was to show that covalent cross-links formed by agents that are effective in the intracellular environment can be very efficient blockers of primer extension by DNA polymerases and reverse transcriptases. We made no attempt to maximize the efficiency of forming cross-links. It seems almost certain that a systematic study of the sequence-dependence of cross-linking would uncover rules that would permit much more efficient cross-linking. The failure of the 16mer to cross-link effectively with the 322mer, although it does cross-link to the 37mer (a subsequence of the 322mer) indicates that the self-structure of the target plays an important part in determining the efficiency of cross-linking.

Antisense oligonucleotides have been used successfully to prevent the expression of viral genes in cell culture (2, 8, 26-28). Thus, although the therapeutic use of oligonucleotides and their analogues and derivatives may be far away, it is likely to be successful in the long run. Oligonucleotides that block by reversible hybridization may be useful in many circumstances. Our results suggest that, in some contexts, reagents that form irreversible cross-links in the intracellular environment may be more effective.

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