# Transplatin-Modified Oligo(2′-*O*-methyl ribonucleotide)s: A New Tool for Selective Modulation of Gene Expression†

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*Received October 28, 1996; Revised Manuscript Received December 19, 1996*⊗

ABSTRACT: In the reaction between *trans*-diamminedichloroplatinum(II) and single-stranded oligo(2′-*O*-methyl ribonucleotide)s containing the sequence GNG (N being a nucleotide residue), the 1,3-*trans*-{Pt-$(NH_3)_2$[GNG]} cross-links are formed. The 1,3-intrastrand cross-links are inert within the single-stranded oligonucleotides. By contrast, they rearrange into interstrand cross-links when the platinated oligonucleotides are paired with their complementary RNA strands. The rate of the interstrand cross-linking reaction depends upon the sequence facing the intrastrand cross-links. When the complementary sequences are 5′-CN′C (N′ being a nucleotide), the rates are rather slow ($\tau_{1/2} \geq 3$ h at 37 °C). The rearrangement of the intrastrand cross-links into interstrand cross-links can be achieved in a few minutes when the triplets facing the intrastrand cross-links are replaced by doublet 5′-UA or 5′-CA. *In vitro*, the specificity of the cross-linking reaction between a platinated oligo(2′-*O*-methyl ribonucleotide) and its target sequence (containing the 5′-CA doublet) located within the coding region of Ha-ras mRNA is demonstrated by steric blocking of reverse transcriptase and translation machinery. Within the HBL100ras1 cells, this platinated oligonucleotide binds specifically and irreversibly to the cognate Ha-ras mRNA. It also inhibits the proliferation of the HBL100ras1 cells in a dose-dependent manner. The fast and specific interstrand cross-linking reaction triggered by the formation of a double helix between platinated oligo(2′-*O*-methyl ribonucleotide)s and RNA enhances the potential of the oligonucleotides which do not induce mRNA cleavage by RNase H, to modulate gene expression by steric blocking of the translation machinery.

During recent years, many studies have been devoted to the design of molecules capable of binding to DNA or RNA in a sequence-specific manner with the view of application in medecine and biotechnology. Oligonucleotides are potential candidates because of their ability to form specific duplexes with mRNA through Watson−Crick hybridization or triplexes with DNA through Hoogsteen hybridization (Stein & Cohen, 1988; Hélène & Toulmé, 1990; Uhlmann & Peyman, 1990; Thuong & Hélène, 1993; Pantopoulos et al., 1994; Wagner, 1994). There are already promising results showing that the oligonucleotides are effective against viral and cancer targets both *in vitro* and *in vivo* (Stein & Cheng, 1993; Gura, 1995), and even human clinical trials are in progress. The binding of oligonucleotides (the so-called antisense oligonucleotides) to mRNA leads to inhibition of translation or RNA metabolism by two general mechanisms: the degradation of the targeted mRNA by RNase H or the steric blocking of the cellular machinery (Hélène & Toulmé, 1990; Uhlmann & Peyman, 1990; Pantopoulos et al., 1994; Woolf, 1995). Among others, an advantage of the steric blocking is that oligonucleotides with chemically modified backbones can be used which improves their uptake by the cells and their resistance to nucleases (Miller, 1996). A major constraint is that the oligonucleotide·RNA hybrids have to be stable enough to avoid dissociation cause by the cellular machinery. When directed to the coding region, antisense oligonucleotides were dissociated from their target by the translating ribosomes (Melton, 1985; Maher & Dolnick, 1988; Liebhaber et al., 1992; Johansson et al., 1994; Bonham et al., 1995). The displacement of the oligonucleotides can be prevented by attaching to the oligonucleotides reagents which can react spontaneously or after light activation with RNA (Thuong & Hélène, 1993; Pantopoulos et al., 1994; Miller, 1996). However, the rates of the cross-linking reactions are generally slow; non-sequence-specific reactions have been often observed for chemically induced cross-links, and photochemical activation is difficult to realize in the *in vivo* experiments.

In this paper, we present an approach to linking covalently antisense oligonucleotides to their targets. It is based on the rearrangement of transplatin[1] 1,3-intrastrand cross-links into interstrand cross-links triggered by the formation of a double helix between the platinated oligonucleotides and their complementary strands (Dalbiès et al., 1994). We found conditions under which the cross-linking reaction, rather slow in the platinated DNA·DNA duplexes and even slower in the platinated DNA·RNA hybrids (Dalbiès et al., 1994), is complete in a few minutes. This is achieved by means of platinated oligo(2′-*O*-methyl ribonucleotide)s having an

† This work was supported in part by l'Agence Nationale de Recherche sur le Sida, l'Association pour la Recherche sur le Cancer, and la Ligue contre le Cancer.

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⊗ Abstract published in *Advance ACS Abstracts,* February 15, 1997.

[1] Abbreviations: 2′-OMe-RNA, oligo(2′-*O*-methyl ribonucleotide); RT, reverse transcription; transplatin, *trans*-diamminedichloroplatinum-(II).

S0006-2960(96)02695-5 CCC: $14.00 

additional nucleotide residue at the level of the adduct as compared to the RNA strands. In cell-free systems, the cross-linked oligonucleotides are able to stop specifically AMV reverse transcriptase and protein synthesis. In a cell culture system, a platinated oligo(2′-*O*-methyl ribonucleotide) complementary to a sequence of the coding region of Ha-ras mRNA induces a dose-dependent inhibition of HBL100-ras1 cell proliferation.

## MATERIALS AND METHODS

*Materials.* The oligodeoxyribonucleotides and oligo(2′-*O*-methyl ribonucleotide)s from Eurogentec (Belgium) were purified by strong anion exchange chromatography (MonoQ column, Pharmacia) as previously described (Dalbiès et al., 1994, 1995). The purified oligoribonucleotides were purchased from Genset (France). T4 polynucleotide kinase and ribonuclease T1 were from Boehringer Mannheim. AMV reverse transcriptase, RNase H, rabbit reticulocyte lysate, luciferase mRNA, rNTP, dNTP (N being a nucleotide), and amino acids were from Promega. The radioactive products were purchased from Amersham. All chemicals were from Merck, except *trans*-DDP, which was from Johnson-Mattey (U.K.).

*Platination of the Oligonucleotides.* The oligonucleotides were reacted with transplatin as previously described (Dalbiès et al., 1994, 1995) with minor modifications. The single-stranded oligonucleotides ($c = 30$ $\mu$M) containing a single (GNG) central sequence were incubated with 1.1 equiv of transplatin, in 10 mM $NaClO_4$, at pH 3.1 adjusted with $HNO_3$ (ultrapure), at 37 °C during 24 h. Then, the reaction mixtures were treated with thiourea (10 mM) during 10 min and at 37 °C to remove the monofunctionnal adducts (Eastman & Barry, 1987). The oligonucleotides containing a single 1,3-intrastrand cross-link were purified on a MonoQ column with a 0.2 to 0.7 M NaCl gradient.

*Linkage Isomerization Reaction.* The platinated oligonucleotides were mixed with the complementary strands at 0 °C and then incubated in various conditions. At several time intervals, aliquots were withdrawn and analyzed by polyacrylamide gel electrophoresis under denaturing conditions. The disappearance of the 1,3-intrastrand cross-links as a function of time was monitored by quantitation of the fraction of unreacted product with a Molecular Dynamics PhosphorImager (ImageQuant software version 3.3 for data processing). For the identification of the ribonucleotide involved in the interstrand cross-link, the hybrids containing an interstrand cross-link were purified by gel electrophoresis (20% polyacrylamide) under denaturing conditions, mixed with 2 $\mu$g of tRNA, and incubated in 10 mM NaOH for 10 min at 90 °C. Reaction products were resolved using a 24% polyacrylamide sequencing gel.

*Reverse Transcription.* The mixtures of capped Ha-ras mRNA (0.4 pmol), obtained by *in vitro* transcription of a plasmid construct containing the entire mutated Ha-*ras* sequence (Saison-Behmoaras et al., 1991), and of the oligonucleotides (3 and 20 molar equiv for the complementary and scrambled oligonucleotides, respectively) were incubated in 1x reverse transcription buffer during 10 min at 42 °C. After addition of 5′-end-labeled primer (3 pmol) complementary to the sequence between positions 209 and 226 (relative to the A of the AUG initiation codon) of the mRNA, the mixtures were further incubated during 10 min. Then, dNTPs (1.5 $\mu$mol each) and 2 units of AMV reverse transcriptase were added. The mixtures (10 $\mu$L final volume) were incubated for 70 min at 42 °C. The transcripts were precipitated twice with ethanol before loading on a denaturing 8% polyacrylamide gel. The sizes of the full-length and truncated transcripts were controlled by comparison of their migration with those of sequencing markers generated by the Sanger dideoxy method (not shown).

*In Vitro Translation.* The translation experiments were done in the presence of rabbit reticulocyte lysate as previously described (Gagnor et al., 1987; Walder & Walder, 1988) with minor modifications. The oligonucleotides were incubated for 10 min at 37 °C in 50 mM NaCl and 5 mM phosphate buffer at pH 7.5 with 0.4 pmol of capped Ha-ras mRNA (see above) and 0.1 pmol of luciferase control mRNA. Then, the amino acid mixture including $^{35}$S-labeled methionine (50 $\mu$M each, final concentration) and 17 $\mu$L of rabbit reticulocyte lysate were added. The volumes of the solutions were adjusted to 25 $\mu$L, and the mixtures were incubated during 30 min at 37 °C. Aliquots were withdrawn and heat-denatured in the presence of SDS and $\beta$-mercaptoethanol and loaded on a 0.1% SDS−10% polyacrylamide gel.

*HBL 100 Cell Proliferation Assays.* In 24-well plates, oligonucleotides were incubated with HBL100ras1 cells ($75 \times 10^3$ cells/well) in 500 $\mu$L of complete culture medium [Modified Eagle's Medium (ICN) supplemented with 7% heat-inactivated fetal calf serum (D. Dutscher, France), penicillin (50 u/mL), streptomycin (50 u/mL), and 4 mM glutamine] at 37 °C in a humidified atmosphere, 5% $CO_2$. After 24 h of incubation, the cells were trypsinized and counted. The percentages of inhibition were determined as $(n_t - n_i)/(n_t - n_0) \times 100$, where $n_t$ is the number of untreated cells after 24 h of incubation, $n_i$ is the number of treated cells, and $n_0$ is the number of cells counted at the beginning of the experiment.

*RT-PCR.* Total RNA was extracted from the cells as previously described (Schwab et al., 1994; Duroux et al., 1995). Briefly, the treated or untreated cells ($1-2 \times 10^6$) were incubated during 5 min at 4 °C in 500 $\mu$L of lysis buffer [0.15 M NaCl, 10 mM Tris-HCl (pH 7.5), 1.5 mM $MgCl_2$, and 0.65% NP-40]. After centrifugation, the supernatants were mixed with 500 $\mu$L of urea/SDS buffer [7 M urea, 10 mM Tris·HCl (pH 7.5), 10 mM EDTA, and 1% SDS]. The samples were centrifuged, and total RNA was precipitated with ethanol. The RNA (2 $\mu$g) was reverse-transcribed, and the resulting cDNAs were co-amplified by PCR according to the manufacturer's recommendations (Perkin-Elmer Cetus) using primer pairs specific for Ha-ras [d(CTGTTGGACATCCTGGATAC) and d(CCCGGTGCGCATGTACTG) for the 72 bp fragments and d(TGAGGAGCGATGACGGAATA) and d(GTATCCAGGATGTCCAACAG) for the 183 bp fragments] and for $\beta_2$-microglobulin [d(AAGATGAGTATGCCTGCCGT) and d(ATGCTGCTTACATGTCTCGAT)]. The number of cycles was 25 (94 °C for 50 s, 57 °C for 50 s, and 72 °C for 20 s). After precipitation with ethanol, reaction products were separated by 10% polyacrylamide gel electrophoresis and visualised by ethidium bromide staining. Quantitation of the band intensities was done with a Videocopy system (Bioprobe systems) using Densylab software (Microvision Instruments) for data processing.

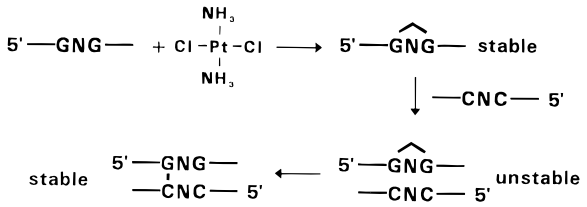


FIGURE 1: Scheme of the linkage isomerization reaction within DNA. The reaction between transplatin and the oligodesoxyribonucleotide (GNG) yields a (G1,G3)-intrastrand cross-link. The pairing of the platinated strand with its complementary strand promotes the rearrangement of the (G1,G3)-intrastrand cross-link into an interstrand cross-link.

## RESULTS AND DISCUSSION

*Interstrand Cross-Linking Reaction.* Recently, we have shown that hybridization of oligodeoxyribonucleotides containing a single transplatin (G1,G3)-intrastrand cross-link with their complementary DNA or RNA strands promotes the rearrangement of the intrastrand cross-links into interstrand cross-links. The experimental process which comprises two main steps, formation of intrastrand cross-links within single-stranded oligonucleotides and rearrangement of the intrastrand cross-links into interstrand cross-links in the duplexes, is schematically represented in Figure 1. The reaction between transplatin [*trans*-diamminedichloroplatinum(II)], the clinically ineffective stereoisomer of the antitumor drug cisplatin [*cis*-diamminedichloroplatinum(II)], and single-stranded oligonucleotides containing the sequence d(GNG) (N being a nucleotide residue) yields 1,3-*trans*-{Pt$(NH_3)_2$[d(GNG)]} cross-links (Lepre & Lippard, 1990). Under physiological conditions, these adducts are kinetically inert as long as the platinated oligonucleotides are single-stranded (Comess et al., 1990; Lepre & Lippard, 1990; Dalbiès et al., 1994, 1995). The pairing of the platinated oligonucleotides with their complementary strands promotes the rearrangement of the 1,3-intrastrand cross-links into interstrand cross-links (Dalbiès et al., 1994). The half-lives of the 1,3-intrastrand cross-links within the platinated DNA·RNA hybrids are large (14−50 h) as compared to most of the mRNA half-lives which limits the potential use of the promoted cross-linking reaction for therapeutic applications.

Our first results suggested a major effect of the conformation of the hybrids on the rate of the cross-linking reaction (Dalbiès et al., 1994). This was further investigated by acting on the nature of the backbone of the platinated strand (the deoxyribonucleotide residues were replaced by 2′-*O*-methyl ribonucleotide residues) and on the complementary RNA strand at the level of the adduct [the base triplet opposite the adduct was replaced by a mismatched triplet or by a doublet; the corresponding RNA strands were named RNA or RNA(−1), respectively].

The platinated strands (18 nucleotide residues long with a single 1,3-intrastrand cross-link located in the middle of the sequence) were mixed with the 5′-end-labeled complementary RNA or RNA(−1) strands (18- or 17-mers, respectively) and incubated at 37 °C. As a function of time, aliquots were withdrawn and analyzed by gel electrophoresis under denaturing conditions. As shown in Figure 2, for each of the four samples, the intensity of the band corresponding to the single-stranded RNA strand decreases as a new band appears. This new band migrates more slowly than the starting material and corresponds to a product containing an

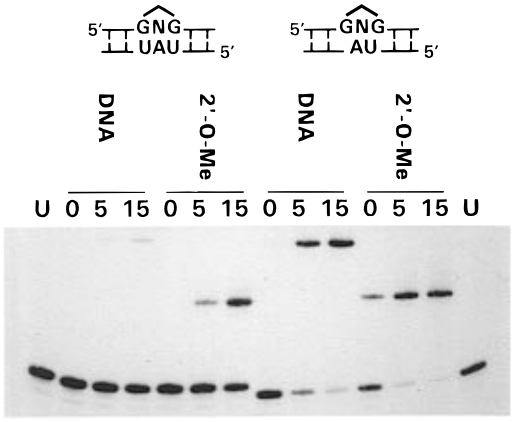


FIGURE 2: Rearrangement of the 1,3-intrastrand cross-links within platinated DNA·RNA and platinated 2′-OMe-RNA·RNA mismatched hybrids (left) and platinated DNA·RNA(−1) and platinated 2′-OMe-RNA·RNA(−1) hybrids (right). Autoradiogram of a denaturing 20% polyacrylamide gel of the 5′-end-labeled RNA strands 5′-AAGGAGA**UAU**AGAGGAGA-3′ (RNA) and 5′-AAGGAGA-**UA**AGAGGAGA-3′ [RNA(−1)] plus the platinated oligodeoxyribonucleotide 5′-TCTCCTCT**GTG**TCTCCTT-3′ (lanes DNA) or the platinated oligo(2′-*O*-methyl ribonucleotide) 5′-UCUCCUCU**GU-G**UCUCCUU-3′ (lanes 2′-*O*-Me). The samples (2 $\mu$M) were incubated at 37 °C in 150 mM $NaClO_4$, 5 mM phosphate buffer (pH 7.5), and 0.2 mM EDTA. Incubation times, in minutes, are indicated above the lanes. Lanes U refer to the 5′-end-labeled single-stranded RNA (left) or RNA(−1) (right) oligonucleotides, respectively.

interstrand cross-link. After 15 min of incubation, about 10% of intrastrand cross-links were transformed into interstrand cross-links within the mismatched platinated DNA·RNA hybrid, 50% within the mismatched platinated 2′-OMe-RNA·RNA hybrid, and close to 100% within the platinated DNA·RNA(−1) and the platinated 2′-OMe-RNA·RNA-(−1) hybrids. Under the same experimental conditions, the percentage of interstand cross-links in the platinated DNA·RNA hybrid without any mismatch [the central sequence was d(GTG)·r(CAC)] was less than 5% (not shown).

The $\tau_{1/2}$ values of the 1,3-intrastrand cross-links within several hybrids are given in Table 1. The three modifications (mismatch, doublet, and 2′-OMe strand) interfere differently with the formation of the interstrand cross-links. The fastest rearrangement occurs within the platinated 2′-OMe-RNA·RNA(−1) hybrids in which the RNA(−1) contains the doublet 5′-UA or 5′-CA. The replacement of the doublet 5′-UA or 5′-CA by the doublet 5′-AU or 5′-AC decreases largely the rate of the interstrand cross-linking reaction (Table 1), whereas the replacement by 5′-AA or 5′-GA has a less drastic effect ($\tau_{1/2} \simeq 0.5$ h). The inefficiency of the doublets 5′-AU and 5′-AC in inducing the rearrangement was not due to a partial dissociation of the hybrids as verified by gel retardation assays (not shown).

A study of some factors which could interfere with the cross-linking reaction in the platinated 2′-OMe-RNA·RNA-(−1) hybrids has been done. The rate was unchanged in NaCl or $NaClO_4$ over a large concentration range (50−500 mM) or by addition of $Mg^{2+}$ or $Mn^{2+}$ up to 10 mM. It was independent of the nature (A, U, or C) of the intervening N residue between the two chelated G residues and of the nature of the bases adjacent to the intrastrand cross-link. On the other hand, no cleavage of the interstrand cross-links was detected after incubation of the purified samples during 48 h at 37 °C, in 100 or 500 mM NaCl, in the presence (or not)

Table 1: Half-Lives (in Hours) of the 1,3-Intrastrand Cross-Links within Platinated DNA·RNA and 2′-OMe-RNA·RNA Hybrids[a]

| hybrid[b] | 5′-GNG· CCC-5′ | 5′-GNG· CAC-5′ | 5′-GNG· UAC-5′ | 5′-GNG· CAU-5′ | 5′-GNG· UAU-5′ | 5′-GNG· AU-5′ | 5′-GNG· UA-5′ | 5′-GNG· AC-5′ | 5′-GNG· CA-5′ |
|---|---|---|---|---|---|---|---|---|---|
| DNA·RNA | >24 | 20 | 18 | 1 | 1 | 0.06 | >24 | 1.5 | >24 |
| 2′-OMe-RNA·RNA | 6.5 | 3 | 4 | 0.25 | 0.25 | 0.02 | >24 | 0.02 | >24 |

[a] The half-lives ($\tau_{1/2}$) of the 1,3-intrastrand cross-links were determined at 37 °C in 150 mM $NaClO_4$, 5 mM phosphate buffer, and 0.2 mM EDTA at pH 7.5. The error when $\tau_{1/2} \geq 0.25$ h is ~10% as determined from the variation of multiple measurements of each value. For the faster reactions, the error may be up to 50%. [b] The top strand is the platinated 5′-TCTCCTCT**G**T**G**TCTCCTT-3′ DNA strand (bold characters refer to the chelated residues) or the platinated 2′-OMe-RNA strand having the same sequence, except that all the T residues are replaced by U residues. The sequences of the bottom RNA strands are 5′-AAGGAGA[ ]AGAGGAGA-3′, where [ ] corresponds to a triplet or a doublet. DNA·RNA and 2′-OMe-RNA·RNA hybrids are referred to by their central sequences with N = T or U, respectively.

of 10 mM glutathione. Under the same experimental conditions, it was verified that the 1,3-intrastrand cross-links within the single-stranded 2′-OMe-RNA were stable.

A first conclusion of this work is that the 1,3-intrastrand cross-links within oligo(2′-*O*-methyl ribonucleotide)s are stable as long as the oligonucleotides are single-stranded. The 1,3-intrastrand cross-links rearrange into interstrand cross-links when the platinated oligonucleotides are hybridized with the complementary RNA strands. The rearrangement is complete within a few minutes when the triplet facing the 1,3-intrastrand cross-link is replaced by doublet 5′-UA or 5′-CA. With the view of the antisense strategy, another advantage of the oligo(2′-*O*-methyl ribonucleotide)s is a better resistance to nucleases (Sproat et al., 1989; Morvan et al., 1993; Cummins et al., 1995).

*Identification of the Interstrand Cross-Link.* To identify the bases in the interstrand cross-links resulting from the fast rearrangement of the 1,3-intrastrand cross-links, two sets of experiments were done. The cross-linked hybrids, $^{32}$P-labeled either at the 5′-end of the upper strand (strand which bore the former 1,3-intrastrand adduct) or at the 5′-end of the lower strand, were eluted from the more slowly migrating bands (Figure 2).

In one set of experiments, the hybrids were subjected to a mild alkaline hydrolysis to obtain one cleavage of the RNA backbone per hybrid, and the resulting fragments were analyzed by gel electrophoresis under denaturing conditions. The results relative to the hybrid having for a central sequence d(GTG)·r(UA) are shown in Figure 3. The fragments up to the cross-linked residue are detected, and thus, we conclude that the cross-linked residue is the A residue opposite the initial intrastrand cross-link. The same results were obtained with the hybrids having for central sequences d(GTG)·r(UAU), 2′-OMe(GTG)·r(UAU), or 2′-OMe(GTG)·r(UA), respectively.

The cross-linked residues within the upper strand were deduced from Maxam-−Gilbert footprinting with dimethyl sulfate as previously described (Comess et al., 1990; Dalbiès et al., 1994, 1995). The experiments (not shown) were done on the two hybrids having for central sequences d(GTG)·r(UA) and d(GTG)·r(UAU). In both cases, the cross-linked residue was the 5′-G residue of the initial intrastrand cross-link.

*Mechanism of the rearrangement.* Although the mechanism of the linkage isomerization reaction is not yet completely elucidated, the fast rates of the cross-linking reaction in the platinated DNA·RNA(−1) and platinated 2′-OMe-RNA·RNA hybrids support a direct nucleophilic attack on the platinum residue by a base residue on the opposite strand (Dalbiès et al., 1994; Boudvillain et al., 1995) and argue against a reaction proceeding through a solvent-

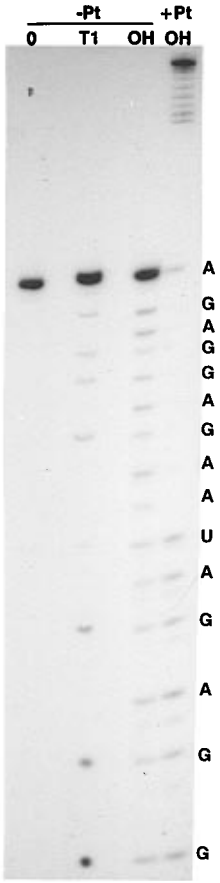


FIGURE 3: Identification of the cross-linked ribonucleotide within the DNA·RNA(−1) hybrid containing an interstrand cross-link. Autoradiogram of a denaturing 24% polyacrylamide gel of the products resulting from alkaline hydrolysis (lanes OH) of the 5′-end-labeled RNA(−1) free (lanes −Pt) or cross-linked to the DNA strand (lane +Pt). Lane 0 refer to the untreated 5′-end-labeled RNA-(−1) strand. In lane T1, the pattern of bands reflects cleavage at G residues induced by the partial digestion of the 5′-end-labeled RNA-(−1) strand by RNase T1 (Donis-Keller et al., 1977).

associated intermediate as is usually observed in the reaction between platinum(II) derivatives and DNA (Lepre & Lippard, 1990). A major difference between some of the results reported here and those on the platinated DNA·DNA duplexes is the nature of the base residue attacking the platinum residue. In the platinated DNA·DNA duplexes, the interstrand cross-links were essentially between the 5′-G of the former intrastrand adduct and the complementary C (Dalbiès et al., 1994). The present footprinting experiments inferred that, in the platinated DNA· or 2′-OMe-RNA·RNA hybrids, the interstrand cross-links are essentially between the N7 of the 5′-G (top strand) and the A residue within the UAU or UA sequence (RNA strand). Whether the platinum residue is bound to the N7 or N1 of the A residue is not yet

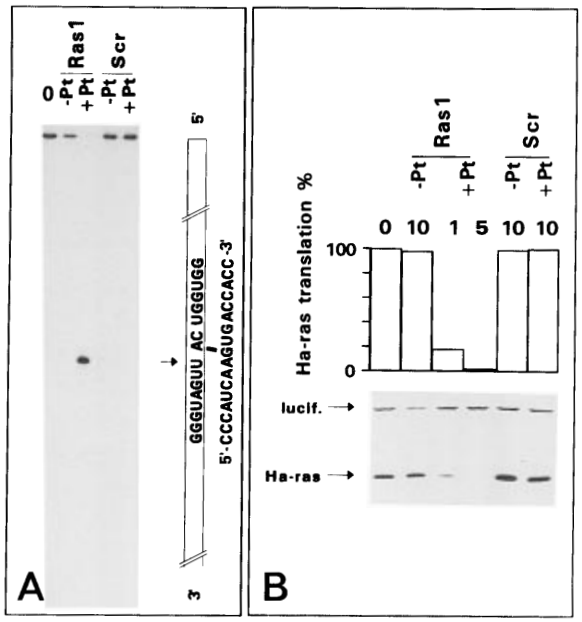


FIGURE 4: Sequence specificity of the cross-linking reaction. (A) *In vitro* reverse transcription in the presence of unplatinated (lanes −Pt) or platinated (lanes +Pt) oligonucleotides. Lanes Ras1 correspond to the oligo(2′-*O*-methyl ribonucleotide) 5′-CCCAUCAA**GUG**ACCACC-3′ complementary to the sequence 5′-GGUGGU**CA**UUGAUGGG-3′ between positions 130 and 145 (relative to the A of the AUG initiation codon) of the Ha-ras coding region, and lanes Scr correspond to the related scrambled oligo(2′-*O*-methyl ribonucleotide) 5′-CCACACU**GCG**AACCUAC-3′. (B) *In vitro* inhibition of capped Ha-ras mRNA translation by unplatinated (lanes −Pt) or platinated (lanes +Pt) oligo(2′-*O*-methyl ribonucleotide). Lanes Ras1 and Scr refer to the oligonucleotides tested in the reverse transcription experiments. The oligonucleotide/Ha-ras mRNA ratios are indicated on the top of the figure. The translational efficiency was expressed as the percentage of Ha-ras-translated protein in comparison to the luciferase one (internal control). The ratio of Ha-ras and luciferase band intensities was defined as 100% translation when no oligonucleotide was present (lane 0).

known [we favor the N1 because the replacement of the UA doublet by the UG doublet slowed the rate of the interstrand cross-linking reaction (not shown)]. As studies on nucleosides indicate a reactivity between platinum(II) complexes and guanosine, adenosine, or cytidine in the order G > A > C (Lepre & Lippard, 1990), the different rates of the cross-linking reaction in the platinated DNA·DNA, platinated DNA·RNA(−1), and platinated 2′-OMe-RNA·RNA(−1) hybrids are due to the relative positions of the attacking nucleotide residue and the platinum residue more than to the chemical nature of the attacking nucleotide residue. These relative positions are determined by the local conformation of the double helix which, in addition, can interfere with the rate of the reaction by both entropic and enthalpic contributions to ground-state destabilization and/or transition-state stabilization.

*Specificity of the Interstrand Cross-Linking Reaction.* To prove that the interstrand cross-linking reaction did not induce nonspecific binding, capped Ha-ras mRNA (820 nucleotide residues) was incubated for 10 min with the platinated oligo(2′-*O*-methyl ribonucleotide) 5′-CCCAUCAAGUGACCACC-3′ (a 17-mer named Ras1) complementary to the sequence between positions 130 and 145 relative to the A of the AUG initiation codon of Ha-ras mRNA (Figure 4A). This sequence (16 nucleotides) contains in its center the doublet 5′-CA, and the 14 nucleotide residues from the 3′-end are in a loop as suggested by a computer-generated model of Ha-ras mRNA secondary structure (Dessen et al., 1990). The location of the irreversibly linked oligonucleotide was revealed by primer extension with AMV reverse transcriptase. As shown in Figure 4A, the polymerization reaction is completely stopped by the cross-linked oligonucleotide. In fact, two stops are detected, a major one at the level of the cross-link and a minor one at the level of the 5′-end of the oligonucleotide. The specificity of the binding was further proved by competition experiments. The platinated oligonucleotide was incubated for 10 min with a mixture of Ha-ras mRNA and luciferase mRNA (30-fold molar excess), and then the reverse transcriptase was added. The results with or without luciferase mRNA were identical (not shown). On the other hand, the same oligonucleotide but unplatinated is a poor inhibitor of reverse transcription. As compared to other oligo(2′-*O*-allyl RNA) (Boiziau et al., 1995), the unplatinated oligonucleotide Ras1 is more easily displaced by the reverse transcriptase. The platinated and unplatinated noncomplementary (scrambled) oligonucleotides (named Scr) do not affect cDNA synthesis.

The specificity of the cross-linking reaction was further tested by looking at the efficiency of the cross-linked oligonucleotides in inhibiting protein synthesis in a cell-free system. The experiment was done on capped Ha-ras mRNA after reaction with the platinated oligo(2′-*O*-methyl ribonucleotide) Ras1. It has been first verified that the kinetics of the cross-linking reaction were unchanged by the addition of rabbit reticulocyte lysate and that there was no degradation of Ha-ras mRNA even after addition of RNase H (not shown). In a second experiment, the platinated oligonucleotide was mixed with Ha-ras and luciferase mRNAs and incubated for 10 min at 37 °C, and then amino acids and rabbit reticulocyte lysate were added. The products of translation were analyzed by SDS−PAGE (Figure 4B). The platinated oligonucleotide Ras1 completely inhibits Ha-ras mRNA translation but does not affect luciferase mRNA translation. The corresponding unplatinated oligonucleotide and the related platinated or unplatinated scrambled oligonucleotides have no effect on Ha-ras mRNA translation. This is the second example showing that the oligo(2′-*O*-alkyl ribonucleotide)s directed to the coding region of mRNAs do not stop the translating ribosomes unless they are covalently bound to RNA (Johansson et al., 1994). The translation and the reverse transcription experiments were repeated with another platinated oligonucleotide recognizing the coding region of Ha-ras mRNA between positions 4 and 19 (relative to the A of the AUG initiation codon). Similar results were obtained (not shown), and thus, we conclude that *in vitro* the platinated oligonucleotides bind specifically to their targets and are not displaced by the translating ribosomes.

*Inhibition of HBL100ras1 Cell Proliferation.* The ability of the platinated oligonucleotide Ras1 to inhibit translation was also tested by using the well-described cell system HBL100ras1. This clone was obtained from the human cell line HBL100 that was transformed with the Ha-ras gene mutated on the 12th codon (Lebeau et al., 1991). In the transfected cells, the oncogenic p21 Ras proteins trapped in their activated GTP-bound state for extended periods of time provide abundant growth stimulatory signals to the cells. Thus, selective depletion of Ha-ras proteins in HBL100ras1 cells leads to inhibition of cell division. Several experiments

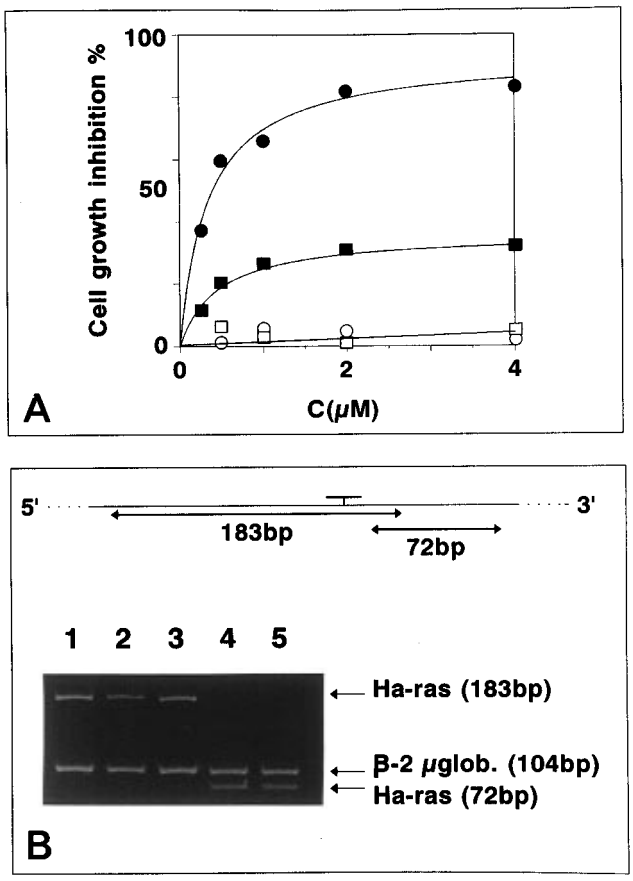


FIGURE 5: (A) Inhibition of HBL100ras1 cell proliferation as a function of oligonucleotide concentration [oligonucleotides, unplatinated (■) or platinated (●) Ras1 and unplatinated (□) or platinated (○) scrambled Scr]. Each point corresponds to the mean value (error < 15%) of at least two triplicate culture experiments. (B) Ha-ras and $\beta_2$-microglobulin mRNA expression in HBL100ras1 cells exposed for 24 h to 4 $\mu$M unplatinated (lane 3) or platinated (lanes 2 and 5) Ras1 oligonucleotide. Lanes 1 and 4 refer to untreated cells. The location of the 72 and 183 bp Ha-ras fragments obtained by RT-PCR, relative to the mRNA portion cross-linked with platinated Ras1, is schematically depicted above the lanes.

have shown that HBL100ras1 cell proliferation was specifically inhibited by antisense oligonucleotides targeted to the Ha-ras message, probably by a RNase H-dependent mechanism (Schwab et al., 1994; Duroux et al., 1995). As shown in Figure 5A, the treatment of HBL100ras1 cells with the platinated oligonucleotide Ras1 induces a dose-dependent inhibition of cell proliferation over the range of 0−4 $\mu$M (50% inhibition at a concentration of ~0.4 $\mu$M). The same oligonucleotide but unplatinated has a slight cytostatic effect on the cells. The growth inhibition does not exceed 30% at 4 $\mu$M, whereas the scrambled platinated or unplatinated oligonucleotides have no effect. In each assay, a high cell viability (>95%) was found by using the trypan blue exclusion method.

To further prove the cross-linking of the platinated oligonucleotide Ras1 to Ha-ras mRNA, the level of intact Ha-ras message in the cells (normalized to the internal reference $\beta_2$-microglobulin mRNA) was determined by RT-PCR experiments on total RNA. The amplification of reverse-transcribed RNA in the presence of either a pair of primers that hybridized to sequences flanking the Ha-ras target or a pair that hybridized to sequences on the 3′-side of the target yielded 183 and 72 bp DNA fragments, respectively (scheme in Figure 5B). As the relative intensities of the bands (lanes 4 and 5 in Figure 5B) corresponding to Ha-ras (72 bp) fragments and $\beta_2$-microglobulin fragments (internal control) originated from the untreated cells and the cells treated with the platinated oligonucleotide Ras1, respectively, are identical, it can be stated that the platinated oligonucleotide Ras1 has no effect on the amount of Ha-ras mRNA. By contrast, the comparison of the relative intensities of the bands (lanes 1 and 2 in Figure 5B) corresponding to Ha-ras (183 bp) fragments and $\beta_2$-microglobulin fragments from the untreated cells and the cells treated with the platinated oligonucleotide Ras1, respectively, shows that the Ha-ras (183 bp) fragment synthesis is strongly decreased (70%). The specific inhibition of amplification in the RT-PCR experiments is due to a block of the RT step by the platinated oligonucleotide Ras1 cross-linked to its target. It was verified that the unplatinated oligonucleotide Ras1 (lane 3 in Figure 5B) or the platinated or the unplatinated scrambled oligonucleotides (not shown) did not affect the level of intact Ha-ras message. Thus, we conclude that, even in the complex medium of the cell, the rearrangement of the 1,3-intrastrand cross-links into interstrand cross-links occurs specifically.

*Concluding Remarks.* In the context of the antisense strategy, several works have demonstrated that the efficiency of oligonucleotides which do not function by causing RNase H-catalyzed cleavage of mRNA is greatly enhanced when the oligonucleotides bind irreversibly to the targeted RNA. The irreversible binding is obtained with chemical agents or photoreactive group-derivatized oligonucleotides [general reviews and references therein of Thuong and Hélène (1993), Pantopoulos et al. (1994), Tabone et al. (1994), and Miller (1996)]. Platinum(II) complexes tethered to oligonucleotides have also been used (Vlassov et al., 1983; Chu & Orgel, 1989, 1990; Gruff & Orgel, 1991). A major problem is that each platinum(II) residue bears one leaving group that may be substituted by nucleophiles present in the medium, which subsequently prevents the cross-linking reaction with the target. The rearrangement of the transplatin 1,3-intrastrand cross-links into interstrand cross-links within hybrids offers a new possibility of irreversibly linking the two strands of a double helix and appears to present several advantages over the known methods. The 1,3-intrastrand cross-links are stable within the single-stranded oligonucleotides, even in the presence of 10 mM glutathione. The rearrangement does not require any additional external factor. It is triggered by the formation of a double helix between the platinated oligonucleotides containing a single transplatin (G1,G3)-intrastrand cross-link and their complementary strands. The rearrangement can be fast and specific. The conformation of the hybrids and the nature of the base residues within the RNA strand facing the 1,3-intrastrand cross-link play a major role in the rate of the cross-linking reaction. Favorable conditions for a complete rearrangement in a few minutes are the use of platinated oligo(2′-*O*-methyl ribonucleotide)s and the choice of a sequence within the RNA target containing doublet 5′-UA or 5′-CA instead of the triplet complementary to the 1,3-intrastrand cross-link. Work is in progress to extend these results to other backbone-modified oligonucleotides. Preliminary results (not shown) indicate a fast rearrangement when the platinated oligo(2′-*O*-methyl ribonucleotide)s are replaced by platinated oligo(2′-*O*-allyl ribonucleotide)s or mixed oligo(2′-*O*-methyl ribonucleotide/nucleoside methylphosphonate)s. In cell-free and cell culture

systems, the results support the finding that the fast rearrangement of the 1,3-intrastrand cross-links into interstrand cross-links does not induce nonspecific cross-links. They also support an arrest of translation by a steric blocking and not by a RNase H activation, the oligo(2′-*O*-methyl ribonucleotide)s being cross-linked to a sequence of the mRNA coding region far away from the initiation codon. *In vitro* experiments show that the platinated oligonucleotides are able to block specifically AMV reverse transcriptase. All these points suggest that the platinated oligonucleotides are of interest in the context of the antisense strategy. In addition, the platinated oligonucleotides can serve as a molecular tool with various applications such as the trapping of intermediate states during the folding of large RNAs or during DNA transcription.

## ACKNOWLEDGMENT

We are deeply indebted to Pr. C. Hélène, Dr. R. Rahmouni, and A. Schwartz for helpful discussions.

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BI962695F