The Human Telomeric Sequence $(T_2AG_3)_n$ is Efficiently Cross-Linked by AN1 Binding to the Platinum of a *trans*-$Pt(NH_3)_2$ Chelate of an Antisense Oligo-2′-*O*-Methylribonucleotide

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In order to irreversibly cross-link an antisense oligonucleotide to the human telomeric sequence $(T_2AG_3)_n$, the inter-strand bridging rearrangement described by Leng et al. was investigated on (21-mer antisense) (20-mer target) duplexes: (G*NG*)(TA) → (G*NG)(TA*), G*NG* being a *trans*-[$Pt(NH_3)_2$\{1,3-(GNG)-*N7*-G,*N7*-G\}] chelate with N = U or T. Three different duplexes were studied, confirming first that for a DNA target strand the rearrangement was faster with a platinated oligo-2′-*O*-methylribonucleotide than with a deoxy oligonucleotide as antisense strand. Second, a telomeric DNA target strand was cross-linked faster than an all-purine oligonucleotide containing a single TA doublet. Accordingly, the half time of formation of the cross-linked hybrid duplex [2′-OMe-r(CCCUAACCCG*UGACCCUAACCC)]·[$d(T_2AG_3)_2(TTA*G_3)(T_2AG_3)$] is one minute, compared with the nine days required for the complete formation of the bridged d(CTCCTG*TGTCTC)·d(GAGATA*AGGAG) duplex. The NMR analysis of the latter showed that the platinum cross-link involves the A*N1 moiety as ligand. The timescale difference between the two reactions raised the question of an eventual kinetic cross-link which might isomerize to the final one observed. This question was addressed using 1-deazaadenine in the TA doublet of the three target strands of this work. No cross-linking reaction was observed, proving that AN1 is the bridging atom in all the cases studied so far.

Introduction

Transplatin *trans*-diamminedichloroplatinum(II) is clinically ineffective, as opposed to its stereoisomer cisplatin *cis*-diamminedichloroplatinum(II).[1−4] Nevertheless it reacts with both single- and double-stranded DNA. On the former, it gives an intra-strand *trans*-[$Pt(NH_3)_2$\{1,3-d(GNG)-*N7*-G,*N7*-G\}] platinum chelate (named G*NG*) with the sequences d(GNG), N being any nucleotide but G.[5] Such adducts were found to be stable in single-stranded oligonucleotides, except that formed on the d(CG*NG*) sequence, which rearranges into the 1,4-d(C*GNG*)-*N3*-C,*N7*-G chelate.[6,7] Upon pairing with the complementary strands, the G*NG* chelates rearrange into inter-strand adducts. In DNA/DNA duplexes and DNA/RNA hybrids, inter-strand cross-links are formed slowly and involve mainly the N7 atom of the 5′ guanine of the initial chelate and the N3 atom of its complementary cytosine.[8−10]

The discovery of this reaction allowed the conception of a new tool for antisense strategy, although therapeutic applications would require cross-linking reactions with short half times compared to most mRNA half-lives, which are of the order of minutes.[11] An investigation of the influence of the series and of the bases of the antisense and target strands led to the selection of hybrid duplexes between a platinated oligo-2′-*O*-methylribonucleotide and a target RNA containing a 5′-pyrimidine/adenine doublet in place of the triplet complementary to the GNG sequence. In these cases, the inter-strand cross-link is formed between the N7 atom of the 5′-G* of the intra-strand chelate and the facing A on the opposite strand after a few minutes.[12,13] The antisense platinated oligo-2′-*O*-methylribonucleotides can be relatively resistant to nucleases. They can enter the cells where they bind specifically and irreversibly to mRNA. The platinum complex bridging both oligonucleotides resists translating ribosomes and leads to inhibition of mRNA expression.[12,14,15] All these results were obtained with purine-rich target strands, suggesting that the platinated oligonucleotides could also bind irreversibly to the human telomeric sequence $(T_2AG_3)_n$ and therefore become potential inhibitors of telomerase activity.[16−18]

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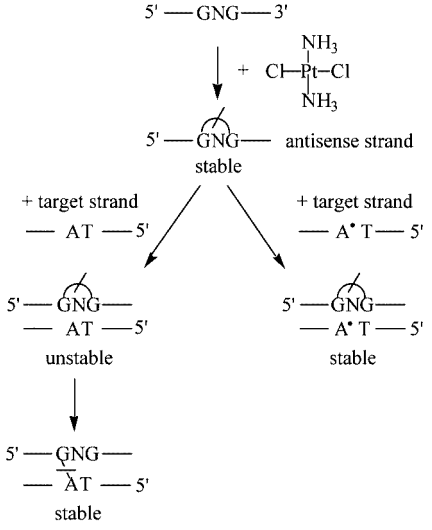
As already reported with target r-UA oligonucleotides,[12,13] we found that the most efficient platinated antisense strands belong to the 2′-*O*-methylribonucleotide series, with the G*NG* triplet facing a d-TA doublet. The fast cross-linking of the platinated ribonucleotide with the telomeric sequence (timescale of a few minutes) compared to that observed in the case of the bridged d(CTCCTG*TGTCTC)·d(GAGATA*AGGAG) duplex (timescale of a few days) raised the question of the possibility of two bridging pathways and/or the existence of a kinetic and a final products. For the cross-linked deoxy duplex, NMR spectroscopy has shown the presence of a *trans*-G*(*N7*)-Pt$(NH_3)_2$-A*(*N1*) bridge.[19] The presence of the 1,3-chelate, the base mismatch, the hybrid pairing and the missing nucleotide on the target strand could allow adenine N7 and/or N1 nucleophilic attack on platinum. This prompted us to check the AN1 involvement in our reactions. We have replaced adenine by 1-deazaadenine (A˙) in the (TA˙) target DNA strand of three duplexes where the G*NG* chelate belongs to DNA or 2′-OMe-RNA strands. No inter-strand cross-link was formed in any of the (G*NG*)(TA˙) duplexes containing 1-deazaadenine, showing the generality of the AN1 involvement (Scheme 1).



Scheme 1. Inter-strand cross-linking reaction, within a duplex structure, between the adenine of the TA target strand and the platinum atom of the G*NG* intra-strand chelate of the antisense strand; G*NG* = *trans*-[Pt$(NH_3)_2${1,3-(GNG)-*N7*-G,*N7*-G}], G* indicates an *N*7-bound guanine; the bridging reaction is not observed when A in the target strand is replaced by 1-deazaadenine A˙

Results and Discussion

The four antisense oligonucleotides used in this work were 21-mers − either DNA or 2′-OMe-RNA − with a G*NG* intra-strand *trans*-Pt$(NH_3)_2$ chelate in a central position (Figure 1). All of them were ^{32}P labelled at the 5′ position. The (G*NG*) strand was mixed with an equal amount of a (TA) or (TA˙) 20-mer target strand. In two cases, this target strand was the human telomeric $(T_2AG_3)_3$ sequence. Solid-phase synthesis of the oligonucleotides containing 1-deaza-2′-deoxyadenosine was performed in Dr. F. Seela's laboratory (Osnabrück University, Germany). The incorporation of the synthesized 1-deaza-2′-deoxyadenosine was checked by HPLC analysis of the nucleoside composition after enzymatic hydrolysis of the oligomers.[20,21] The duplex oligonucleotides (2 μM) were incubated at 37 °C for 165 minutes. Aliquots were withdrawn at regular time intervals and analyzed by polyacrylamide gel electrophoresis under denaturing conditions. As shown in Figure 2, when the G*NG* chelate is facing the 5′-TA doublet, a new band appears at the expense of that of the platinated single-stranded DNA or 2′-OMe-RNA. This new band migrates slower than that of the antisense strand. It corresponds to the antisense and target strands cross-linked by the *trans*-[Pt$(NH_3)_2${(*N7*-G),d(A)}] bridge as shown by Maxam−Gilbert footprinting, in agreement with previous results.[8,10,13] At the end of the incubation period (165 min), about 90% and 100% of the platinated antisense strand

1	5′ UCUCCUCUG*UG*UCUCCUUCUU AGAGGAGA A T AGAGGAAGAA 5′	2′-OMe-RNA DNA
2	5′ AACCCTAACCCG*TG*ACCCTAA TTGGGATTGGG A T TGGGATT 5′	DNA DNA
3	5′ AACCCUAACCCG*UG*ACCCUAA TTGGGATTGGG A T TGGGATT 5′	2′-OMe-RNA DNA
4	5′ CCCUAACCCG*UG*ACCCUAACCC GGGATTGGG A T TGGGATTGGGATT 5′	2′-OMe-RNA DNA

Figure 1. The paired oligonucleotides **1**−**4** of this study; at the rearrangement site, the intra-strand cross-link G*NG* (G* denotes an *N*7 platinated guanine; N = T or U) faces the 5′-TA doublet; in the text the intra-strand platinated 21-mer is called the (G*NG*) antisense strand, and the target 20-mer either the (TA) or (TA˙) strand according to the presence of adenine or 1-deazaadenine

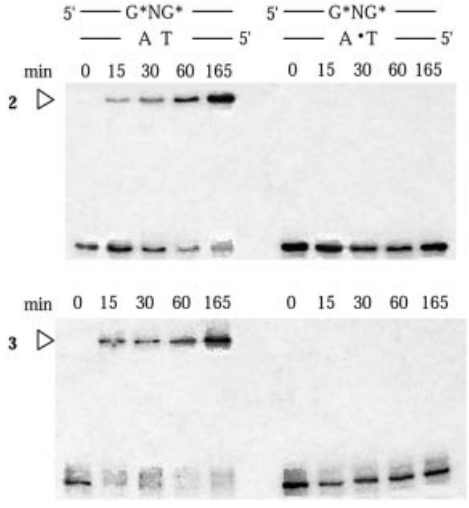


Figure 2. Cross-linking reactions within the DNA/DNA (**2**) and 2′-OMe-RNA/DNA (**3**) duplexes monitored by electrophoresis on a denaturing 20% polyacrylamide gel; the (G*NG*) strands were ^{32}P 5′-end labelled and paired with their complementary telomeric DNA target strand containing either TA or TA˙ (A˙ = 1-deazaadenine); the duplexes were incubated at 37 °C in 150 mM NaCl, 5 mM phosphate buffer, pH 7.5 and 0.2 mM EDTA; incubation times (in minutes) are given above the lanes; the inter-strand adduct is indicated by an arrowhead on the left

were cross-linked to the target strand within the platinated DNA/DNA duplex **2** and 2′-OMe-RNA/DNA hybrids **3** and **4**, respectively (Figure 2), with respective $t_{1/2}$ values of 40 minutes for the former and one minute for the latter two. The half reaction time for the platinated 2′-OMe-RNA/DNA hybrid **1** is 18 minutes. It is noteworthy that the rearrangement is faster when the platinated antisense strand is a 2′-OMe-ribonucleotide (see **2** vs. **3**), as already found.[12,13] Moreover, it is faster when the target deoxyoligonucleotide is the human telomeric sequence $(T_2AG_3)_n$ (see **1** vs. **3** and **4** in Figure 3).

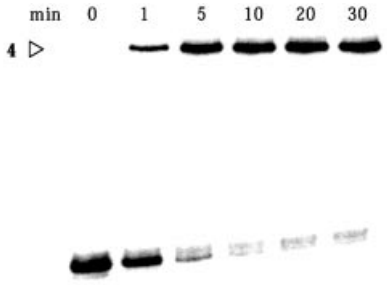


Figure 3. Cross-linking reaction within the 2′-OMe-RNA-(CCCUAACCCG*UG*ACCCUAACCC)/DNA$(T_2AG_3)_4$ hybrid **4**; autoradiogram of a denaturing 20% polyacrylamide gel; the (G*UG*) strand was ^{32}P 5′-end labelled; the hybrid was incubated at 37 °C in 0.2 M NaCl, 5 mM phosphate buffer, pH 7.5 and 0.2 mM EDTA; incubation times (in minutes) are given above the lanes; the inter-strand adduct is indicated by an arrowhead on the left

Replacing the adenine of the TA doublet by a 1-deazaadenine (TA$^{\cdot}$) suppresses the inter-strand cross-linking within any of the three paired sequences **1**−**3**. It must be noted that after 165 minutes, a product migrating like two cross-linked strands was present in a very small amount, accounting for ca. 1% of the total radioactivity for the systems **1** and **2**, and 4% for **3**. This product might result from a slow cross-linking reaction involving the A$^{\cdot}$N7 atom. To check further an eventual rearrangement of the 1,3-intra-strand chelate facing a 1-deazaadenine within the systems studied, the incubation was extended by 48 h for the platinated DNA/DNA duplex **2** (not shown). Some products appeared, with migration similar to that of as two cross-linked strands, and accounting for less than 2% of the total radioactivity. The amount of isolated material was too small to allow any further characterization. These data suggest that the N7 atom of the TA adenine, facing the G*NG* platinum chelate, is not competitive with the N1 atom of the same adenine when binding to the metal and cross-linking the antisense and target strands.

These results agree with the very slow rate of isomerization observed when the UA doublet of a target strand is replaced by UG at the rearrangement site.[12] They also agree with the NMR analysis of the *trans*-Pt(NH_3)$_2$ cross-linked DNA/DNA duplex d(CTCCTG*TGTCTC)·d(GAGATA*AGGAG), which showed that the A*N1 atom is bound to platinum.[19] It is noteworthy that the much longer time needed for the bridging of this duplex (nine days for complete conversion) compared to the speed of our reactions (one minute), shows that the AN1 binding to the platinum is a common feature of all these rearrangements, whatever their efficiency, and can be compared to the (G*NG*)(CN′C) → (G*NG)(CN′C*) cross-linking involving the 3′-CN3 of the target strand. Within a B-DNA the AN1 and CN3 atoms occupy similar positions and this might be retained in the (G*NG*)(CN′C) and (G*NG*)-(TA) duplexes despite the mispairing and the missing nucleotide at the rearrangement core. This could be favored by the stabilizing effect of the 2′-OMe-ribonucleotide nature of the (G*NG*) strand.[13]

In conclusion, we have shown that a platinated 2′-OMe-RNA-antisense strand efficiently cross-links a $(T_2AG_3)_n$ telomeric target with a one minute half-reaction time. This irreversible binding to telomeric sequences might inhibit telomerase activity. Moreover, our results suggest that all the rearrangements involving the G*NG*/TA core involve the AN1 atom of the target strand acting as a bridging ligand to platinum, independently of the nature of the antisense strand and of the cross-linking rate.

Experimental Section

Materials and Methods: The oligodeoxyribonucleotides and oligo-2′-*O*-methyl ribonucleotides (from Eurogentec Belgium) were purified by ion-exchange chromatography (monoQ column, Pharmacia) as described previously.[12,13] The 1-deaza-2′-deoxyadenosine phosphoramidite was synthesized and employed in solid-phase oligonucleotide synthesis.[20−23] The purified oligonucleotides containing 1-deazaadenine were checked by hydrolysis with snake-venom phosphodiesterase followed by alkaline phosphatase yielding the monomeric nucleosides. The presence of the modified nucleotide was controlled by HPLC analysis of the nucleoside composition. The T4 polynucleotide kinase was purchased from Boehringer Mannheim and the radioactive products from Amersham. All other chemicals were purchased from Merck, except *trans*-diamminedichloroplatinum(II), which was a gift of Johnson Matthey (UK).

Platination: The single-stranded oligonucleotides containing a single GNG triplet were reacted with transplatin as described previously.[12,13] The platinated oligonucleotides were incubated in the presence of thiourea to remove the monofunctional adducts[6] and were purified on a monoQ column. The formation of the *trans*-[Pt(NH_3)$_2$\{1,3-(GNG)-*N7*-G,*N7*-G\}] intra-strand chelates was verified by the non-reactivity of the platinated G residues with dimethylsulfate.[10,13]

Linkage Isomerization Reaction: The 5′[^{32}P]-labelled platinated oligonucleotides (2 μM) were mixed at 0 °C with their complementary strands and then incubated at 37 °C in 5 mM phosphate buffer at pH 7.5, 150 mM NaCl and 0.2 mM EDTA. Aliquots were withdrawn at various times and analyzed on electrophoresis gel under denaturing conditions (20% polyacrylamide/7 M urea). The disappearance of the labelled strands containing the intra-strand chelate was monitored by quantitation of the gel bands with a Molecular Dynamics PhosphorImager using ImageQuant software version 3.3 for data processing. The location of the inter-strand cross-link was deduced from Maxam−Gilbert footprinting.[8]

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