Photocrosslinking in Ruthenium-Labelled Duplex Oligonucleotides

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The formation of a photoadduct between a [Ru(1,4,5,8-tetraazaphenanthrene)$_2$4,7-diphenylphenanthroline]$^{2+}$ complex chemically attached to a synthetic oligonucleotide, and a guanine moiety in a complementary targeted single-stranded DNA molecule was studied for ten 17-mer duplexes by denaturing gel electrophoresis. This photoadduct formation leads to photocrosslinking of the two strands. The percentage quenching of luminescence of the complex by electron transfer was compared to the resulting yield of photocrosslinked product. This yield does not only depend on the ionisation potential of the guanine bases, which are electron donors, but also on other factors, such as the position of the guanine bases as compared to the site of attachment of the complex. The photocrosslinking yield is higher when the guanine moieties are towards the 3′ end on the complementary strand as compared to the tethering site. Computer modelling results are in agreement with this preference for the 3′ side for the photoreaction. Interestingly, the photocrosslink is not alkali labile. Moreover, a type III exonuclease enzyme is blocked at the position of photocrosslinking.

KEYWORDS:

conjugates · DNA recognition · electron transfer · photocrosslinking · ruthenium

Introduction

The use of oligonucleotides (ODNs) for gene expression inhibition represents an attractive therapeutic approach. Oligonucleotides can target mRNA (antisense strategy)[1] or double-stranded DNA by triple helix formation (antigene strategy).[2] Numerous in vitro and in vivo studies have shown the efficacy of antisense oligonucleotides.[3] Several clinical trials directed at various targets that play a role in cancer are underway.[4] However, some major problems remain: low stability of oligonucleotides in biological media, poor delivery into cells and insufficient affinity of the probe for the target sequence (especially for the triple helix strategy). To circumvent these problems, a number of chemically modified oligonucleotides have been prepared. These modifications include changes in the backbone (for example, phosphorothioate analogues, etc.) and the sugar units (for example, 2′-O alkylated sugar, α-nucleosides, etc.) and attachment of a variety of reporter groups at the 3′ or 5′ extremities of the oligonucleotides.[5] In this context, the tethering of transition metal complexes to oligonucleotides has received considerable attention. Artificial sequence-specific nucleases have thus been designed by anchoring metal complexes such as Fe^{II}-EDTA (EDTA = ethylenediaminetetraacetate),[6] Cu^{II}-phenanthroline,[7] Fe^{II}-bleomycin[8] and metalloporphyrins on oligonucleotides.[9] Conjugation of ruthenium complexes to synthetic DNA has found widespread interest as a useful process for the preparation of luminescent DNA probes,[10] DNA photocleavage reagents,[11] triple-helix photoprobes[11d] or for studies of long-distance electron transfer.[12] Some transition metal complexes have also been shown to be highly efficient as chemotherapeutic agents targeting the nucleic acid. Indeed, some Pt(II) complexes are employed for the treatment of certain human cancers.[13] The anticancer properties of these agents have been shown to derive from the formation of adducts with DNA guanine or adenine bases and formation of intra- and interstrand cross-links.[14]

We recently demonstrated that Ru(II) complexes containing highly π-deficient polyazaaromatic ligands such as tap (1,4,5,8-tetraazaphenanthrene) are efficient DNA photoprobes. These

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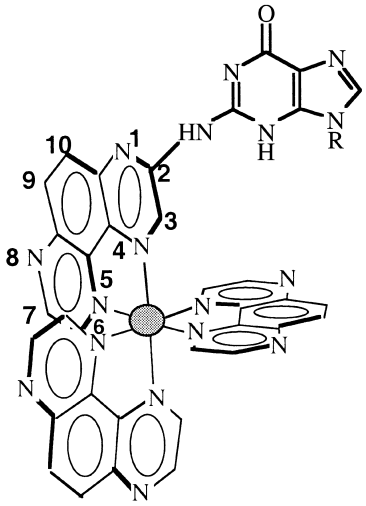
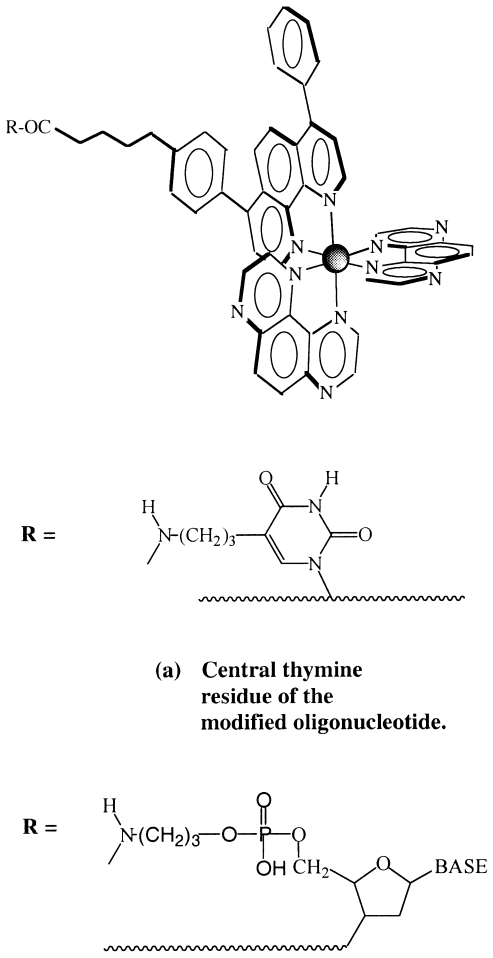
complexes are able to undergo photoadduct formation and can cause photocleavage of DNA.[15] A photoinduced electron transfer process is the first step in this DNA photodamage.[15a] The structure of the photoadduct has been determined in the case of the photoreaction of the $[Ru(tap)_3]^{2+}$ complex with guanosine-5′-monophosphate and calf thymus DNA (Scheme 1).[16] In order to target this photoreaction towards a guanine residue of a specific sequence, $[Ru(tap)_2dip]^{2+}$ (dip = 4,7-diphenylphenanthroline) was anchored on a series of oligonucleotides.[17] Significant luminescence quenching was observed upon hybridisation of the Ru-labelled oligonucleotides, provided that the complementary strand contained a guanine residue accessible to the complex. However, this extinction takes place only when guanine residues are present in the complementary strand. The percentage quenching has been correlated to the calculated ionisation potential of the guanine residues in the different sequences.[18] Therefore, the formation of a photoadduct that generates an irreversible photocrosslinking of the two strands has been attributed to an initial charge transfer process.[17] This photoreaction could offer an interesting opportunity to design new anticancer agents by combining the sequence specificity of the antisense oligonucleotide with the irreversible formation of a photocrosslink.



Scheme 1. *Structure of the photoadduct formed between the $[Ru(tap)_3]^{2+}$ complex and the guanine residue after acid hydrolysis.*

With the aim of studying the parameters controlling the electron transfer and photoadduct formation (that is, photocrosslinking), we prepared ten different duplexes containing various numbers of guanine residues located at particular positions of the complementary strand. The $[Ru(tap)_2dip]^{2+}$ complex was tethered to the oligonucleotides in two different ways, either in the middle of the sequence by derivatisation at position 5 of an internal thymine base, or at the 5′ terminal phosphate group of the sequence (Schemes 2 and 3).

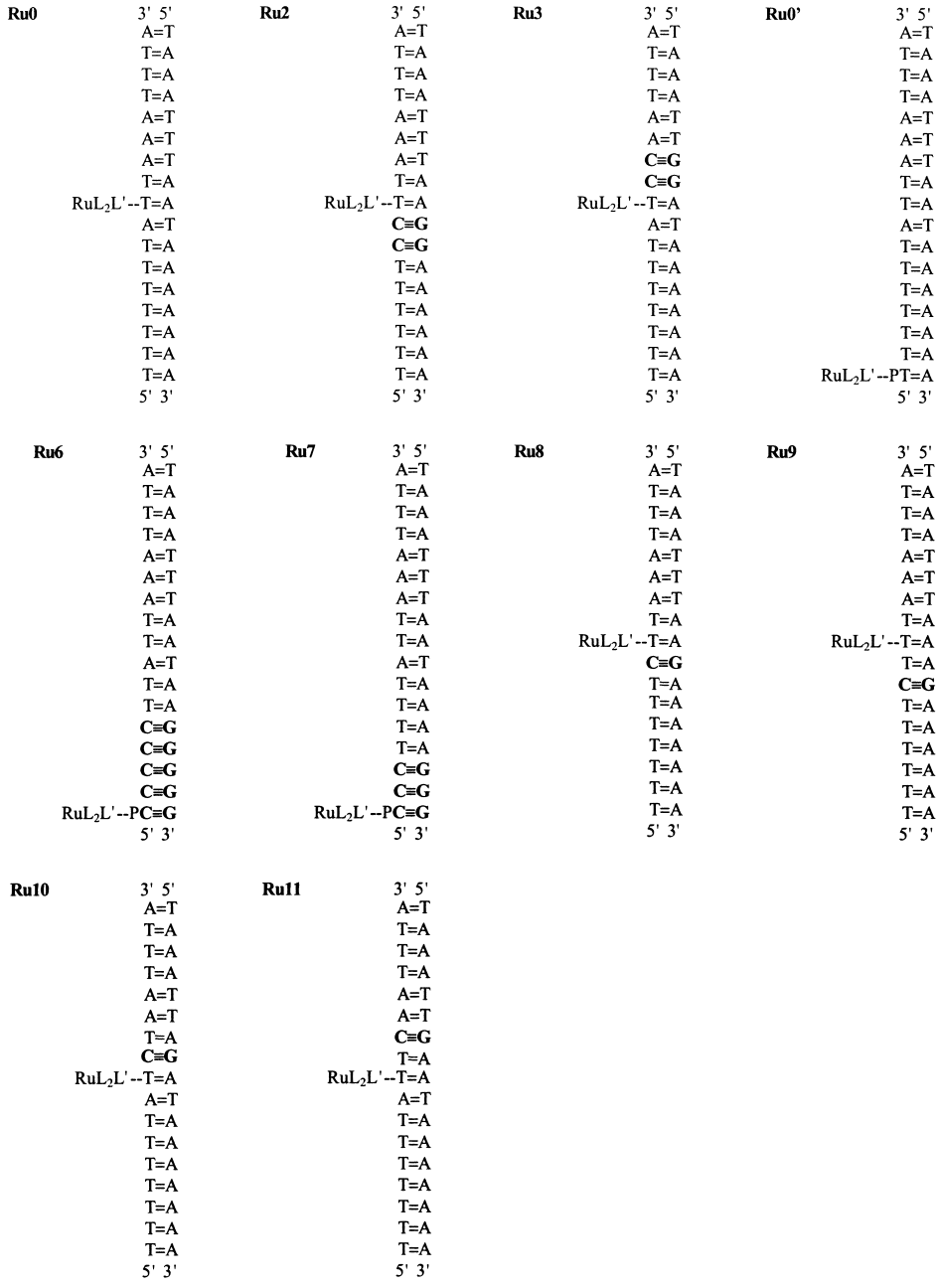


Scheme 2. *a) Anchoring of the $[Ru(tap)_2dip]^{2+}$ complex in the middle of the strand at position 5 of a modified thymine base; b) anchoring at the 5′-extremity.*

Results and Discussion

Percentage quenching in the Ru-labelled duplexes

As previously shown,[17–19] the presence of guanine residues in the complementary strands of the Ru-labelled oligonucleotides induces luminescence quenching (Table 1). Since steady-state luminescence data and lifetime measurements yielded the same percentage values, the quenching can be regarded as dynamic. The percentage quenching can be correlated to the ionisation potentials (I.P.) of the involved guanine residues upon first examination.[18] This is in agreement with quenching by electron transfer as previously demonstrated by flash photolysis of this complex with guanosine monophosphate.[15] Such a charge transfer process with a guanine residue generates a photoadduct[16] and therefore, the percentage quenching should also correlate with the percentage photoadduct produced. In other words, in the case of the oligonucleotide duplexes, a correlation should also be obtained between the ionisation potential and the percentage photocrosslinking of the two strands. In order to



Scheme 3. *The different sequences of duplex studied in this work. $RuL_2L' = [Ru(tap)_2dip]^{2+}$; P = terminal phosphate group.*

test this hypothesis, experiments were performed by gel electrophoresis to determine the percentage photocrosslinking.

Photoadduct formation

Continuous illumination of Ru-labelled duplexes

The different oligonucleotides were illuminated for one hour and the reaction mixtures were analysed by gel electrophoresis under denaturing conditions. Figure 1 shows the gel electrophoresis analysis of the illuminated sequences **Ru3**, **Ru7**, **Ru8** and **Ru10**, which are representative of the different behaviours exhibited by the duplexes. The presence of a retarded band that migrates like a duplex (i.e. a 34-mer) is attributed to the formation of a photoadduct, which involves photocrosslinking between the two strands. Illumination of the two reference sequences **Ru0** and **Ru0'** (containing no guanine) under the same conditions does not lead to the formation of this new species. Photoadduct formation that leads to crosslinking of the two oligonucleotide strands has been detected for all guanine-containing duplexes. The yield of photoadduct varied between 16 and 56% depending on the number and position of the guanine residues (Table 1).

Correlation between I.P., percentage quenching and photoadduct formation

Although a correlation can, upon first examination, be found between the I.P. of the guanine residues in the different sequences and the percentage quenching, careful examination of the data in Table 1 shows that the percentage quenching and photoadduct formation are also controlled by factors other than the I.P. This conclusion is more striking for photoadduct formation. Among other factors, the distance between the quencher and the excited complex should clearly play a role. This is evidenced by a comparison of sequence **Ru8** with **Ru9**, for which, even though the I.P. is the same, the percentage quenching and photoadduct formation is higher when the guanine residue is closer to the site of anchoring. The same conclusion can be drawn by comparing sequences **Ru10** and **Ru11**, although in this case, there is no difference in the percentage of photoadduct formed, probably because the amount of photoadduct formed is rather low for both sequences.

In addition to the I.P. and distance between quencher and complex, a third factor affects the quenching of luminescence and photoadduct formation: the position of the guanine residue (3′ or 5′ on the complementary strand) as compared to the anchoring site. A guanine residue 3′ from the anchoring site quenches luminescence more efficiently and gives a higher percentage photocrosslinking than a guanine residue positioned

Table 1. *Percentage quenching[a] and ODN adduct formation[b] for $[Ru(tap)_2dip]^{2+}$-labelled duplex oligonucleotides.[c]*

Duplex	Quenching [%][a]	Calcd I.P. [eV] HF/6 31G(d)[d]	ODN adduct formation [%][b]	Relative position of G[e]
Ru0′	-	–	0	–
Ru6	87 ± 2	6.17	56 ± 5	5′
Ru7	81 ± 2	6.26	50 ± 5	5′
Ru0	-	–	0	–
Ru2	59 ± 2	6.32	54 ± 5	3′
Ru3	49 ± 2	6.42	17 ± 4	5′
Ru8	38 ± 2	6.55	44 ± 4	3′
Ru9	30 ± 3	6.55	41 ± 4	3′
Ru10	31 ± 2	6.60	16 ± 4	5′
Ru11	23 ± 3	6.65	20 ± 4	5′

[a] Percentage quenching (Q) of luminescence of the complex: $Q = 1 - (I/I_0) = 1 - (\tau_M/\tau_{M0})$.[17, 18] τ_M is the pre-exponential weighted average lifetime [$\tau_M = (\Sigma\alpha_i\tau_i)/(\Sigma\alpha_i)$] and τ_{M0} the corresponding value for the reference sequence (**Ru0′** for **Ru6** and **Ru7**, and **Ru0** for the other sequences). Luminescence decay was monitored at 650 nm ($\lambda_{exc} = 379$ nm) and analysed according to a sum of exponential functions: $I(t) = \Sigma[\alpha_i \exp(-t/\tau_i)]$. α_i are the corresponding pre-exponential factors and τ_i the discrete lifetime components. [b] Yields were recorded after 1 h continuous illumination of a solution containing 2 pmol 5′-^{32}P-labelled duplex oligonucleotide. The intensity of the spot corresponding to the ODN adduct (photocrosslinked) was compared to the total radioactivity observed in the experiment. [c] All the measurements were performed at 25 °C (air-saturated solutions, 50 mM NaCl, 10 mM Tris – HCl, pH 7). [d] Calculated ionisation potentials of the guanine residues present in the different sequences.[18] [e] Position of the guanine residue on the complementary strand as compared to the anchoring site of the complex.

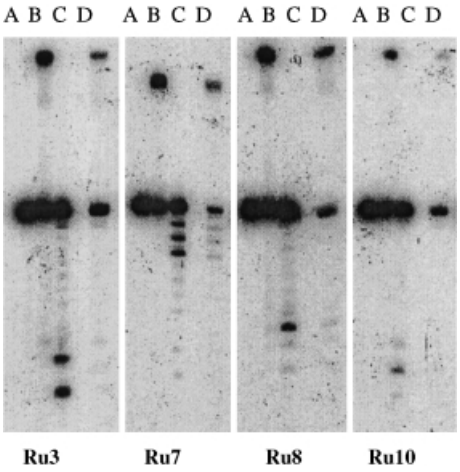


Figure 1. *Gel electrophoresis analysis of oligonucleotides **Ru3**, **Ru7**, **Ru8** and **Ru10** in 20% denaturing polyacrylamide gel. Lane A: non-illuminated duplex; lane B: duplex after one hour of illumination; lane C: G-specific treatment; lane D: piperidine treatment of the illuminated duplex.*

towards the 5′ end. This effect is evidenced by comparison of sequence **Ru8** with **Ru10** or **Ru9** with **Ru11**. As shown in Table 1, the difference is more pronounced for ODN adduct formation than for quenching. This result indicates that there is not a direct correlation between I.P. and ODN adduct formation. In Table 1, the order of presentation of the complexes corresponds to the decreasing percentage of quenching. However the order of decreasing percentage of ODN adduct formed is different: **Ru2** > **Ru8** > **Ru3** ≈ **Ru10** or **Ru11**. This lack of proportionality can be understood by consideration of the multistep character of the photoadduct production. Luminescence quenching by electron transfer generates a radical cation on the guanine residue and a radical anion on the tap ligand of the complex. These two radicals recombine to form a covalent bond between one of the tap ligands and the base.[16] The first step is clearly the electron transfer, which depends directly on the I.P., as determined from the correlation between the percentage quenching and the I.P. of the stack of guanine residues. However, the recombination of the radicals formed by the electron transfer may also depend on other constraints in the system. In particular, the formation of the covalent bond between the ligand and the guanine moiety is expected to depend tremendously on the geometry of the system. Thus, the results indicate that stretching of the complex linker along the duplex groove to form the photoadduct and produce photocrosslinking is favoured toward the 3′ side on the complementary strand. This geometric influence is also exemplified by sequences **Ru2**, **Ru8** and **Ru9**, which give a larger amount of ODN adduct than sequences **Ru3**, **Ru10** and **Ru11**, respectively. Moreover, it should be noted that the amounts of ODN adduct formed with sequences **Ru6** and **Ru7** are not very large, in contrast to the amounts expected from the corresponding low I.P. values of the stacks of three and five guanines. This observation stresses again the influence of the direction of stretching of the complex linker in the duplex groove (5′ direction disfavoured). These geometric factors play a much less important role for luminescence quenching.

It is also notable that for **Ru6** and **Ru7**, although the number of guanine residues is different, the total percentage of ODN adduct formed is similar. This result can be attributed to the fact that stacks of three and five G bases have similar low I.P. values. Moreover, as the fourth and fifth guanine residues in **Ru6** have a lower probability of being reached by the attached complex, the difference between the number of G residues in **Ru6** and **Ru7** does not influence the percentage of ODN adduct formed much.

Computer modelling for photoadduct formation

We tried to prove the presence of the geometric difference (3′ versus 5′) deduced from the percent ODN adduct formed by computer modelling. The lack of a well-developed force field for ruthenium complexes means that the computer modelling performed in this work is a coarse approach. Nevertheless, we

think this rough model is complementary to the experimental results and can help to interpret some of the data discussed above. A conformational search was performed on the metal complex attached to oligonucleotides **Ru8** and **Ru10** by rotating the bonds along the linker from 0 to 360° in fixed increments (see the Experimental Section), with the torsion angle values corresponding to torsion minima. All possible combinations of torsion angles were generated. This systematic search produces data that is impractical to analyse given the number of conformations generated. Moreover, some combinations of torsion values may be incompatible and lead to high energy structures because of atom clashes either within the linker or between the complex and the oligonucleotide. A selection was performed for each ensemble of conformations on the basis of two criteria: 1) As we are searching for conformations that could potentially lead to the formation of a photoadduct between the tap ligand and the only guanine residue in the sequences, we selected those conformations that feature a distance lower than 6 Å between the C2 and C7 atoms of each tap ligand (for numbering, see Scheme 1) and the six-membered ring of the guanine base. 2) Energetic conditions were also imposed (see the Experimental Section). This selection gave rise to a severe restriction in the number of conformations and produced 69 and 36 conformations for the two sequences **Ru8** and **Ru10**, respectively. Interestingly, these numbers correlate with the percentage photoadduct formation for a guanine residue in the 3′ direction (**Ru8**) and for one in the 5′ direction (**Ru10**) from the point of anchoring. This outcome suggests that the observed differences arise mainly from geometric requirements fulfilled by conformers selected on the basis of sampled torsion minima within the linker.

Stability of the ODN photoadduct

Piperidine treatment

Oxidative modifications to nucleic acids quite often take place on guanine nucleobases. A common method used to detect such damage is treatment with heat and alkali, which leads to strand scission. This method relies upon the removal of the nucleobase by *N*-glycosidic bond hydrolysis, followed by β-elimination at the resulting abasic site. In practice, piperidine is the amine most commonly used to catalyse β-elimination. The illuminated duplexes **Ru2**, **Ru8** and **Ru10** were treated with piperidine (1 M) at 90 °C for 1 hour. Very little cleavage (less than 3%) was detected at the level of the guanine residues of the complementary strand (Figures 1 and 2). This cleavage is attributed to natural thermal depurination because a control experiment consisting of the same treatment but applied to duplexes that had not been illuminated led to a comparable amount of cleavage.

Piperidine treatment of the duplexes after illumination leads to a decrease in the intensity of the spot corresponding to the ODN adduct and to an increase of that corresponding to the single-strand oligonucleotide. In order to clarify this behaviour upon treatment with alkali, we isolated the spots corresponding to the ODN adducts for the illuminated sequences **Ru2**, **Ru8** and **Ru10** on a preparative gel. Treatment of the extracted ODN adducts with piperidine leads to a negligible amount of specific cleavage but to the appearance of a new band that moves on the gel at the same rate as the initial single strand. Thus, the ODN photoadduct seems to be partially destroyed by piperidine treatment. This behaviour could be explained by a partial dechelation of the complex under basic conditions, which could generate a species similar to the starting material (Scheme 4). Indeed, although very stable in acidic conditions, complexes containing π-deficient ligands such as tap are unstable in basic conditions. This partial dechelation of the ruthenium moiety was confirmed by treatment of the free $[Ru(tap)_2dip]^{2+}$ complex with piperidine. This treatment induces a loss of emission, a decrease in intensity of the metal-to-ligand charge transfer band at 422 nm in the absorption spectrum of the complex and the occurrence of a new bathochromic band at 550 nm, characteristic of the bis-chelated complex. Dechelation was also detected by thin layer chromatography.

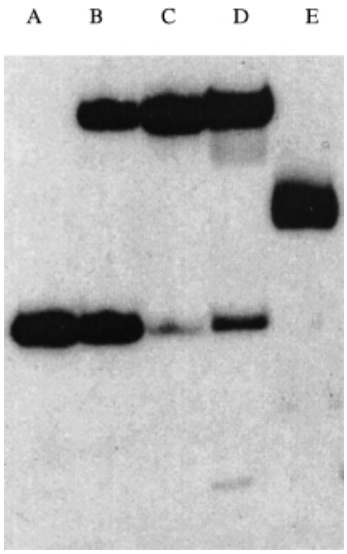
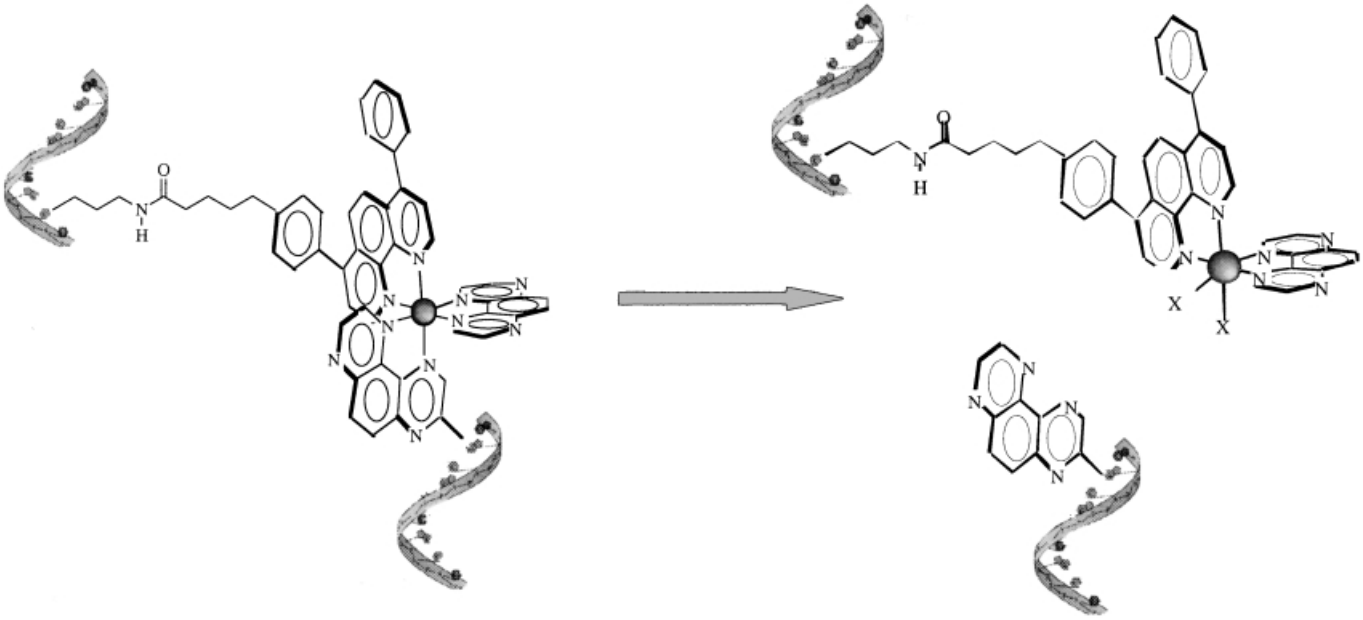


Figure 2. *Gel electrophoresis analysis of oligonucleotide* ***Ru8*** *in 20% denaturing polyacrylamide gel. Lane A: non-illuminated duplex; lane B: duplex after one hour of illumination; lane C: isolated ODN adduct; lane D: piperidine treatment of the isolated ODN adduct; lane E: enzymatic treatment of the isolated ODN adduct.*

Enzymatic treatment

In plasma and tissues, oligonucleotides are quickly degraded by nucleases. The major pathway is an exonucleolytic degradation that proceeds through successive removal of nucleobases from the 3′ end of the oligonucleotide.[20] The photocrosslinked duplexes **Ru2**, **Ru8** and **Ru10** were purified by gel electrophoresis and incubated in the presence of exonuclease III from *Escherichia coli* to examine their resistance to such a 3′-exonucleolytic activity. The digested DNA samples were analysed by electrophoresis on a denaturing polyacrylamide gel (PAGE) and visualised by autoradiography (Figure 2). The appearance of a single band that moves faster than the adduct but slower than the 17-mer target strand strongly suggests that one or both 3′



Scheme 4. *Schematic representation of dechelation of the complex in the ODN adduct under basic conditions. X = a monodentate ligand.*

end(s) of the crosslinked duplex is (are) degraded by the enzyme to give the ultimate product of digestion. The fact that the enzymatic reaction stops in the presence of the photoadduct might be explained by blockage of progression of the enzyme or by hindrance of its site for binding to DNA. A more precise analysis of the structure of the ultimate degradation product is in progress.

Conclusion and perspectives

The results presented herein confirm the formation of ODN photoadducts between a probe Ru sequence and a target sequence. High percentages of adduct are obtained even when there is only a single guanine residue in the target strand. Gel electrophoresis experiments clearly show that the I.P. of the involved guanine bases, their distance from the site of tethering and geometric factors originating from the linker, influence the formation of the ODN adduct. The electron transfer (the first step of the reaction in the formation of the adduct) detected by quenching of the luminescence of the complex is, to a first approximation, directly dependent on the I.P. of the guanine residue in the complementary strand. In contrast, the recombination of the radicals formed by the electron transfer seems to depend tremendously on geometrical constraints. This recombination reaction is more favourable when the guanine residues are on the 3′ side of the complementary strand as compared to the anchoring position of the complex. This geometrical favouring of the 3′ direction over the 5′ direction is in agreement with computer modelling results.

We might also extrapolate from the results described herein that the ODN adduct produced under illumination should be rather stable in living cells since the *N*-glycosidic bond of the damaged guanine residue is resistant to piperidine treatment. Moreover, the fact that the ODN adduct is able to block an exonuclease enzyme is of course very interesting for inhibition of a targeted gene.

Experimental Section

Automated DNA synthesis was carried out on an Expedite DNA synthesiser (Perkin – Elmer) by using standard β-cyanoethyl nucleoside phosphoramidite chemistry on a 1-μM scale. Bacteriophage T4 polynucleotide kinase was purchased from Pharmacia (9500 U mL^{-1}) and [γ^{32}P]-ATP (ATP = adenosine triphosphate; specific activity 3000 Ci mmol^{-1}) from Isotopchim. HPLC purification as well as HPLC analysis of oligonucleotides and conjugates were performed on a Waters system equipped with two M510 pumps, a M490E detector and a M680 system controller, with a μ-bondapak C-18 column (Macherey-Nagel Nucleosil: 10 × 250 mm, 7 μm). The following solvent system was used: solvent A, 20 mM ammonium acetate/CH_3CN, 95:5 (v:v); solvent B, CH_3CN; flow rate, 4 mL min^{-1}; a linear gradient from 0 to 30% B over 20 min was applied. Electrospray mass spectrometry (ES MS) analyses were carried out on a VG Platform (Micromass) in the negative mode.

Synthesis of functionalised ruthenium complex: The synthesis of the $[Ru(tap)_2dip]^{2+}$ complex was carried out according to a procedure published previously.[17a]

Synthesis of oligonucleotide – $[Ru(tap)_2dip]^{2+}$ conjugates: To achieve covalent coupling of the $[Ru(tap)_2dip]^{2+}$ complex to the oligonucleotide, the activated ester (succinimide ester) of the ruthenium complex and an amino-modified oligonucleotide were used according to the protocol described previously.[17a] The amino-modified oligonucleotides were prepared by using commercially available aminohexyl phosphoramidite (MMT-aminolinker) for introduction of the modification at the 5′-end, or the phosphoramidite of 5-aminopropyl-2′-deoxyuridine for introduction in the middle of the sequence. The different modified oligonucleotides were obtained in 50% isolated yield and characterised by ES MS: *m/z*: **Ru0**: calcd: 6077.5, found: 6076.2; **Ru0′**: calcd: 6213.5, found: 6210.7; **Ru2**: calcd: 6038.5, found: 6037.1; **Ru3**: calcd: 6038.5, found: 6036.5; **Ru6**: calcd:

6138.4, found: 6137.8; **Ru7**: calcd: 6168.4, found: 6168.4; **Ru8**: calcd: 6053.5, found: 6051.0; **Ru9**: calcd: 6053.5, found: 6051.8; **Ru10**: calcd: 6053.5, found: 6053.1; **Ru11**: calcd: 6053.5, found: 6053.2.

Spectroscopic studies: The duplex solutions (600 μL) used for quenching measurements were prepared at a concentration of 10 μM by dissolving the appropriate volume of conjugate and a volume of the complementary strand corresponding to an excess of 5 – 10% (as compared to the probe strand) in aqueous buffer (50 mM NaCl, 10 mM tris(hydroxymethyl)aminomethane (Tris), pH 7). The hybridisation of the two strands was realised by incubation at 90 °C for 5 minutes and then at room temperature for 1 hour. All the measurements were carried out in 600-μL quartz cells (UV Select, 1.0 × 0.2 cm) and data from at least three trials were used for all analyses. Emission spectra were recorded at room temperature on a Shimadzu RF-5001PC spectrofluorimeter equipped with a Hamamatsu R928 red-sensitive photomultiplier tube. The excitation wavelengths used were 379 and 422 nm; the spectra were recorded from 500 to 800 nm and corrected for the photomultiplier response. The emission maximum is centered at 646 ± 2 nm.

Emission lifetimes were measured by single-photon counting with an Edinburgh Instruments FL900 spectrometer equipped with a hypobaric nitrogen discharge lamp and a Hamamatsu R928 photomultiplier tube. The cell was kept at 25 °C with a Haake NB22 temperature controller.

Photocrosslinking experiments: Each complementary strand was 5′-labelled by treatment with [γ^{32}P]-ATP and T4 polynucleotide kinase at 37 °C for 30 minutes, and then hybridised with a slight excess of the corresponding Ru-containing oligonucleotide by incubation at 90 °C for 5 minutes and at room temperature for 1 hour.

Assay for photoreactivity: A solution containing ^{32}P-labelled duplex (2 pmol), Tris – HCl buffer (10 mM, pH 7) and NaCl (50 mM) in a total volume of 33 μL was illuminated at 4 °C for one hour with a mercury/xenon lamp (Oriel 200 W) by using a filter (2 M solution in KNO_3). A sample of this solution (2 μL) was dissolved in the stop solution (50% formamide, 25 mM EDTA, 0.1% xylene cyanol and 0.1% bromophenol blue). The reaction products were analysed by electrophoresis through a 20% denaturing (7 M urea) polyacrylamide gel (19:1 ratio of acrylamide to bisacrylamide) with TBE (90 mM Tris – borate, pH 8, 2 mM EDTA). DNA fragments were visualised by autoradiography with Kodak X-OMAT AR film and were counted with a PhosphorImager instrument.

Assay for stability: Piperidine treatment was carried out by adding piperidine (100 μL, 1 M) to the duplexes and heating the solution to 90 °C for one hour. The samples were then lyophilised and washed/lyophilised twice with water (100 μL). The dried residue was solubilised in the same aqueous solution as described above, and disposited on the gel.

G-specific treatment was performed by using dimethylsulfate (100 mM) in the presence of cacodylate buffer (100 mM, pH 7) in water for 30 minutes.

Enzymatic treatment of the illuminated duplex was performed with type III exonuclease from Pharmacia (200 units μL^{-1}). The enzyme (360 units) was added to the enzyme buffer (10 μL) in the presence of the isolated photoadduct and heated at 37 °C for one hour.

Computer modelling: A conformational search was performed on the metal complex attached to the oligonucleotides **Ru8** and **Ru10**. The bonds along the linker plus that between the phenyl ring and the C4 atom of the phenanthroline were rotated from 0 to 360° by fixed increments either of 120 or 180° depending on the number of torsion minima, so that all the torsion minima along the chain were included. The complex and the oligonucleotide were kept rigid during the conformational search. The JUMNA program[21] was used to construct a B-DNA-like three-dimensional model. The structure of the complex and the linker were calculated by using density functional theory (DFT) at the mPW1PW functional level[22] in combination with the 3 – 21G(d) basis set. All DFT calculations were performed with the Gaussian 98 program.[23] InsightII (Molecular Simulations Inc.) software was used to build models of the complex attached to the oligonucleotides through a thymine residue. A systematic search performed with the CHARMM program[24] gives rise to 52488 conformations for each sequence, a figure that makes the analysis of the whole ensemble of conformations unworkable. We eliminated conformations by imposing one energetic criterion and conditions of distance between the tap ligand and the guanine residue (see Results and Discussion). The first criterion was imposed since combinations of torsion values may be incompatible and lead to high energy structures because of atom clashes either within the linker or between the complex and the oligonucleotide. The second criterion was required to select conformations that could potentially lead to photoadduct formation. In the energetic selection, conformations whose nonbonded potential energy is higher than 10^7 kcal mol^{-1} were discarded. The choice of such a high value is guided by the fact that no energy minimisation can be performed because of the lack of reliable energy parameters for bonded terms. Thus, this selection allows elimination of conformations that could not be energy refined to a reasonable value without large changes of their conformations. Nonbonded parameters were taken from the CHARMM27 program,[25] with the exception of the charges for the linker and the complex, which were derived from the DFT calculations by using the ChelpG software.[26]

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[1] a) E. Uhlmann, A. Peyman, *Chem. Rev.* **1990**, *90*, 543 – 584; b) A. De Mesmaeker, R. Haner, P. Martin, H. E. Moser, *Acc. Chem. Res.* **1995**, *28*, 366 – 374; c) C. Hélène, J.-J. Toulmé, *Biochim. Biophys. Acta* **1990**, *1049*, 99 – 125.

[2] a) D. Praseuth, A. L. Guieysse, C. Hélène, *Biochim. Biophys. Acta* **1999**, *1489*, 181 – 206; b) T. LeDoan, L. Perrouault, D. Praseuth, N. Habhoub, J.-L. Décout, N. T. Thuong, J. Lhomme, C. Hélène, *Nucl. Acids Res.* **1987**, *15*, 7749 – 7760; c) H. E. Moser, P. B. Dervan, *Science* **1987**, *238*, 645 – 650.

[3] a) S. Akhtar, S. Agrawal, *Trends Pharmacol. Sci.* **1997**, *18*, 12 – 18; b) I. Tamm, B. Dörken, G. Hartmann, *Lancet* **2001**, *358*, 489 – 497.

[4] a) S. Akhtar, M. D. Hughes, A. Khan, M. Bibby, M. Hussain, Q. Nawaz, J. Double, P. Sayyed, *Adv. Drug Delivery Rev.* **2000**, *44*, 3 – 21; b) S. Ylä-Herttuala, J. F. Martin, *Lancet* **2000**, *355*, 213 – 222.

[5] a) S. L. Beaucage, R. P. Iyer, *Tetrahedron* **1993**, *49*, 1925 – 1963, and references therein; b) S. L. Beaucage, R. P. Iyer, *Tetrahedron* **1993**, *49*, 6123 – 6194, and references therein; c) J. Goodchild, *Bioconjugate Chem.* **1990**, *1*, 165 – 187, and references therein; d) C.-H. Tung, S. Stein, *Bioconjugate Chem.* **2000**, *11*, 605 – 618, and references therein; e) S. T. Crooke, *Antisense Drug Technology-Principles, Strategies and Applications*, M. Dekker, New-York, Basel, **2001**.

[6] a) M. Boidot-Forget, M. Chassignol, M. Takasugi, N. Thuong, C. Hélène, *Gene* **1988**, *72*, 361 – 371; b) H. Han, P. B. Dervan, *Proc. Natl. Acad. Sci. U.S.A.* **1993**, *90*, 3806 – 3810.

[7] D. M. Perrin, A. Mazumder, F. Sadeghi, D. S. Sigman, *Biochemistry* **1994**, *33*, 3848 – 3854.
[8] S. A. Kane, S. M. Hecht, J. Sun, T. Garestier, C. Hélène, *Biochemistry* **1995**, *34*, 16715 – 16724.
[9] a) B. Mestre, M. Pitie, C. Loup, C. Claparols, G. Pratviel, B. Meunier, *Nucleic Acids Res.* **1997**, *25*, 1022 – 1027; b) L. De Napoli, S. De Luca, G. Di Fabio, A. Messere, D. Montesarchio, G. Morelli, G. Piccialli, D. Tesauro, *Eur. J. Org. Chem*. **2000**, 1013 – 1018.
[10] a) J. Telser, K. A. Cruickshank, K. S. Schanze, T. L. Netzel, *J. Am. Chem. Soc.* **1989**, *111*, 7221 – 7226; b) W. Bannwarth, W. Pfleiderer, F. Müller, *Helv. Chem. Acta* **1991**, *74*, 1991 – 1999; c) D. J. Hurley, Y. Tor, *J. Am. Chem. Soc.* **2002**, *124*, 3749 – 3762.
[11] a) D. B. Hall, R. E. Holmlin, J. K. Barton, *Nature* **1996**, *382*, 731 – 735; b) P. J. Dandliker, R. E. Holmlin, J. K. Barton, *Science* **1997**, *275*, 1465 – 1468; c) N. J. Turro, J. K. Barton, *J. Biol. Inorg. Chem.* **1998**, *3*, 201 – 209; d) G. N. Grimm, A. S. Boutorine, P. Lincoln, B. Nordén, C. Hélène, *ChemBioChem* **2002**, *3*, 324 – 331.
[12] a) E. Boone, G. B. Schuster, *Nucl. Acids Res.* **2002**, *30*, 830 – 837; b) M. E. Nunez, D. B. Hall, J. K. Barton, *Chem. Biol.* **1999**, *6*, 85 – 97; c) M. E. Nunez, J. K. Barton, *Curr. Opin. Chem. Biol.* **2000**, *4*, 199 – 206; d) B. Giese, *Acc. Chem. Res.* **2000**, *33*, 631 – 636; e) K. Nakatani, C. Dohno, I. Saito, *J. Am. Chem. Soc.* **1999**, *121*, 10854 – 10855.
[13] a) B. Keppler, *Metal Complexes in Cancer Chemotherapy*, Verlag Chemie, Weinheim, **1993**; b) W. R. Waud in *Cancer Chemotherapy Agents* (Ed.: W. O. Foye), American Chemical Society, Washington, **1995**.
[14] a) J. Reedijk, *Chem. Commun.* **1996**, 801 – 806; b) J. P. Whitehead, S. J. Lippard in *Metal Ions in Biological Systems*, M. Decker, New York, **1996**; c) P. M. Takahara, A. C. Rosenzweig, C. A. Frederick, S. J. Lippard, *Nature* **1995**, *377*, 649 – 652.
[15] a) J.-P. Lecomte, A. Kirsch-De Mesmaeker, M. M. Feeney, J. M. Kelly, *Inorg. Chem.* **1995**, *34*, 6481 – 6491; b) M. M. Feeney, J. M. Kelly, A. B. Tossi, A. Kirsch-De Mesmaeker, J.-P. Lecomte, *J. Photochem. Photobiol. B* **1994**, *23*, 69 – 78; c) J. M. Kelly, M. M. Feeney, L. Jacquet, A. Kirsch-De Mesmaeker, J.-P. Lecomte, *Pure Appl. Chem.* **1997**, *69*, 767 – 772.
[16] L. Jacquet, D. R. Davies, A. Kirsch-De Mesmaeker, J. M. Kelly, *J. Am. Chem. Soc.* **1997**, *119*, 11763 – 11768.
[17] a) I. Ortmans, S. Content, N. Boutonnet, A. Kirsch-De Mesmaeker, W. Bannwarth, J.-F. Constant, E. Defrancq, J. Lhomme, *Chem. Eur. J.* **1999**, *5*, 2712 – 2721; b) D. Garcia-Fresnadillo, N. Boutonnet, S. Schumm, C. Moucheron, A. Kirsch-De Mesmaeker, E. Defrancq, J.-F. Constant, J. Lhomme, *Biophys. J.* **2002**, *82*, 978 – 987.
[18] S. Schumm, M. Prévost, D. Garcia-Fresnadillo, O. Lentzen, C. Moucheron, A. Kirsch-De Mesmaeker, *J. Phys. Chem.* **2002**, 106, 2763 – 2768.
[19] A. Kirsch-De Mesmaeker, J.-P. Lecomte, J. M. Kelly, *Top. Curr. Chem.* **1996**, *177*, 25 – 76.
[20] A. A. Levin, *Biochim. Biophys. Acta* **1999**, *1489*, 69 – 84.
[21] R. Lavery, K. Zakrzewska, H. Sklenar, *Comput. Phys. Commun.* **1995**, *91*, 135 – 158.
[22] C. Adamo, V. Barone, *J. Chem. Phys.* **1998**, *108*, 664 – 675.
[23] Gaussian 98 (Revision A.7), M. J. Frisch, G. W. Trucks, H. B. Schlegel, G. E. Scuseria, M. A. Robb, J. R. Cheeseman, V. G. Zakrzewski, J. A. Montgomery, R. E. Stratmann, J. C. Burant, S. Dapprich, J. M. Millam, A. D. Daniels, K. N. Kudin, M. C. Strain, O. Farkas, J. Tomasi, V. Barone, M. Cossi, R. Cammi, B. Mennucci, C. Pomelli, C. Adamo, S. Clifford, J. Ochterski, G. A. Petersson, P. Y. Ayala, Q. Cui, K. Morokuma, D. K. Malick, A. D. Rabuck, K. Raghavachari, J. B. Foresman, J. Cioslowski, J. V. Ortiz, A. G. Baboul, B. B. Stefanov, G. Liu, A. Liashenko, P. Piskorz, I. Komaromi, R. Gomperts, R. L. Martin, D. J. Fox, T. Keith, M. A. Al-Laham, C. Y. Peng, A. Nanayakkara, C. Gonzalez, M. Challacombe, P. M. W. Gill, B. G. Johnson, W. Chen, M. W. Wong, J. L. Andres, M. Head-Gordon, E. S. Replogle, J. A. Pople, Gaussian, Inc., Pittsburgh, PA, **1998**.
[24] B. R. Brooks, R. E. Bruccoleri, B. D. Olafson, D. J. States, S. Swminathan, M. Karplus, *J. Comp. Chem.* **1983**, *4*, 187 – 217.
[25] N. Foloppe, A. D. J. McKerell, *J. Comp. Chem.* **2000**, *21*, 86 – 104.
[26] C. M. Breneman, K. B. Wiberg, *J. Comp. Chem.* **1990**, *11*, 361 – 364.

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