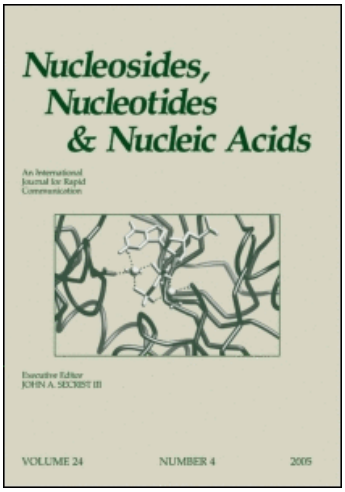

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DESIGN AND SIMPLE ROUTES OF SYNTHESIS OF OLIGONUCLEOTIDE CONJUGATES FOR STUDIES OF DNA TRIPLE HELIX FORMATION

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ABSTRACT

A series of oligonucleotides conjugated to intercalators, as well as fluorescent and lipophilic substances, minor groove binders and photoactive molecules were synthesized for studies of their ability to form a stable triple helix. Purine-rich short double stranded DNA fragments from HIV-1 genome and pyrimidine 16-mer oligodeoxyribonucleotide were used as models. A conjugate of a dipyrido[3,2-a:2′,3′-c]phenazine-ruthenium (II) complex and a triple helix-forming oligonucleotide was constructed. Upon sequence-specific duplex and triplex formation of the conjugate, the ruthenium complex becomes highly fluorescent. The attached ruthenium complex induces a stabilization of the DNA triple helix and a significant increase of the time of residence of the third strand on the duplex.

Oligonucleotides, which can form complexes with DNA by triple helix formation (anti-gene strategy), represent a promising class of potential therapeutic agents acting directly on gene expression (1–3). However, the reversibility of the formation of the complex, and its low temperature and pH stability could be cited as one of the most serious problems of this strategy.

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The conjugation of oligonucleotides to duplex or triplex-specific ligands stabilizing these complexes is one of the possible ways to solve this problem. We have developed a series of methods for the functionalization of oligonucleotides at their termini by amino, sulfhydryl, thiophosphate or carboxyl groups. The method involves different chemical reactions and linkers in both organic and aqueous media using terminal phosphate activation (4). Experimental conditions for subsequent coupling to oligonucleotides of ligands containing aliphatic and aromatic amines, aromatic alcohols, carboxylic acids, sulfhydryl groups, alkylating functional groups, aldehydes and other reactive nucleophiles and electrophiles were established. The elaborated methods were applied for covalent coupling of intercalators, as well as fluorescent and lipophilic substances, minor groove binders and photoactive molecules. The protocols will be published elsewhere (5).

Among various ligands, an intercalating ligand that has a strong affinity to double-stranded DNA, dipyrido[3,2-a:2′,3′-c]phenazine-ruthenium (II) complex, attracted our attention (6–8). The free complex is not fluorescent in aqueous solution but exhibits strong fluorescence once bound to double helical DNA (9,10). We attached an alkylating ruthenium *bis*(1,10-phenanthroline)(2-isocyano-2-[bromoacetyl-(aminopropyl)-aminocarbonyl]-2,3-dihydro-1H-cyclopenta[b]-benzodipyrido [b:3,2-h:2′,3′-j]-phenazinium complex([Ru(phen)$_2$Dppz^{++}]) to terminally modified 16-mer oligonucleotide that forms triple helix with the oligopurine-oligopyrimidine fragment from genes *pol* and *nef* of HIV proviral genome (11). The schema for the synthesis is shown in Figure 1 and sequences of target 29-mer HIV proviral fragment and 16-mer triple helix-forming oligonucleotide are shown in Figure 2. A sulfhydryl group was generated at the 5′-terminus of the oligonucleotide according to the method described earlier (12) using cystamine. The coupling reaction is followed by reduction of the disulfide group by dithiothreitol. Then the SH group was alkylated by the [Ru(phen)$_2$Dppz^{++}] bromoderivative and the resulting conjugate was purified by HPLC.

As already observed for the free complex (13,14), the fluorescence at 600–630 nm increases significantly when double-stranded DNA is added due to intercalation of the complex into DNA. But, in contrast with the free complex, for a conjugate such increase is sequence-specific. It is observed only when a complementary DNA single strand or a double-stranded fragment that can form a triple helix is added (Fig. 3). It may be concluded that intercalation of coupled [Ru(phen)$_2$Dppz^{++}] occurs only when it is delivered to its target by a complementary or a triple helix-forming oligonucleotide. The increase of fluorescence is also time-dependent (data not shown). It occurs in several seconds after addition in case of duplex formation, while in case of triplex formation it is slow and reaches a plateau only after 5–6 hours of incubation. This fact reflects different velocity of the duplex and triplex formation (15) and permits studies of the kinetics of complex formation by fluorescent methods.

Formation of a triple helix by [Ru(phen)$_2$Dppz^{++}]-oligonucleotide conjugate was demonstrated by electrophoresis on a non-denaturing polyacrylamide gel with

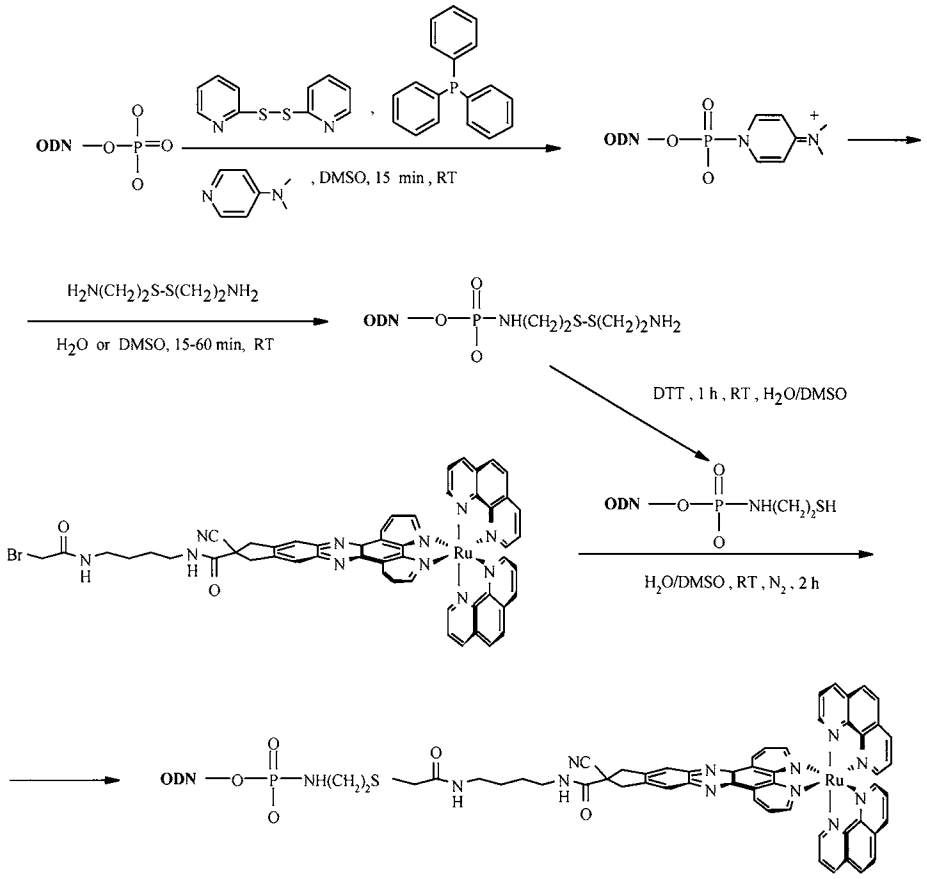

Figure 1. Schema of the synthesis of oligonucleotide - ruthenium complex conjugate.

^{32}P-labeled strand HIV-D2. Under these conditions (described on Fig. 4) the triple helix migrates as a retarded band compared to the single strand and duplex. As is seen from Figure 4, both non-modified oligonucleotide HIV-T and its conjugate with [Ru(phen)$_2$Dppz^{++}] form stable triplexes. Their stability was estimated by the method of thermal denaturation. As is seen from Figure 5, the temperature of transition triplex-duplex for [Ru(phen)$_2$Dppz^{++}]-conjugate is 46°C whereas the control triplex with non-modified oligonucleotide dissociates at 34°C. Thus, the

HIV-T : 5′-TTT-T*C*T-TTT-*CCC*-*CCC*-T-3′

HIV-D1: 5′-CCA-CTT-TTT-AAA-AGA-AAA-GGG-GGG-ACT-GG-3′

HIV-D2: 3′-GGT-GAA-AAA-TTT-TCT-TTT-CCC-CCC-TGA-CC-5′

Figure 2. Nucleotide sequence of 16-mer oligopyrimidine fragment HIV-T and target 29-mer double stranded fragment from HIV proviral genome. 5-methylated cytosines are indicated in italics.

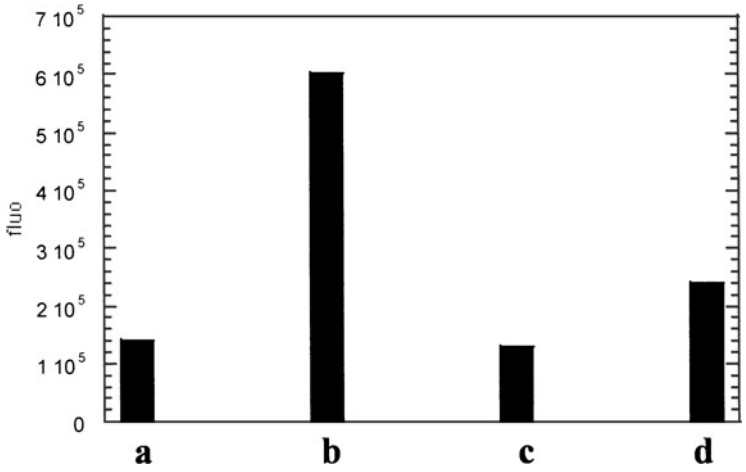

Figure 3. Fluorescence of 2.7×10^{-7} M Δ-HIV-T-Ru a) with no additive, b) after addition of 1 equiv. of double-stranded DNA HIV-D1/HIV-D2, c) after addition of 1 equiv. of a nonfitting double-stranded DNA and d) after addition of 1 equiv. (refering to the number of bases) of calf thymus DNA (all samples in 50 mM MES buffer, pH 6.0, 50 mM NaCl, 5 mM $MgCl_2$).

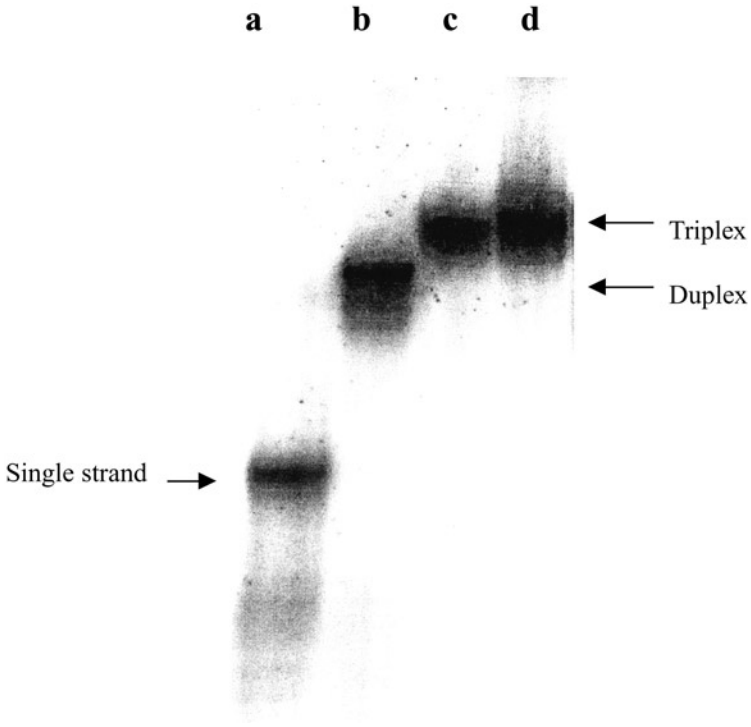

Figure 4. Gel electrophoresis at 10°C (non-denaturating polyacrylamide gel; 20% in 50 mM MES buffer, pH 6.0) of a) 50 nM oligonucleotide HIV-D1 (5′-^{32}P), b) 50 nM double stranded DNA HIV-D1/HIV-D2, c) the triplex formed from 50 nM double stranded DNA HIV-D1/HIV-D2 and 20 μm non-modified HIV-T and d) the triplex formed from 50 nM double stranded DNA HIV-D1/HIV-D2 and 20 μM conjugate Δ-HIV-T-Ru.

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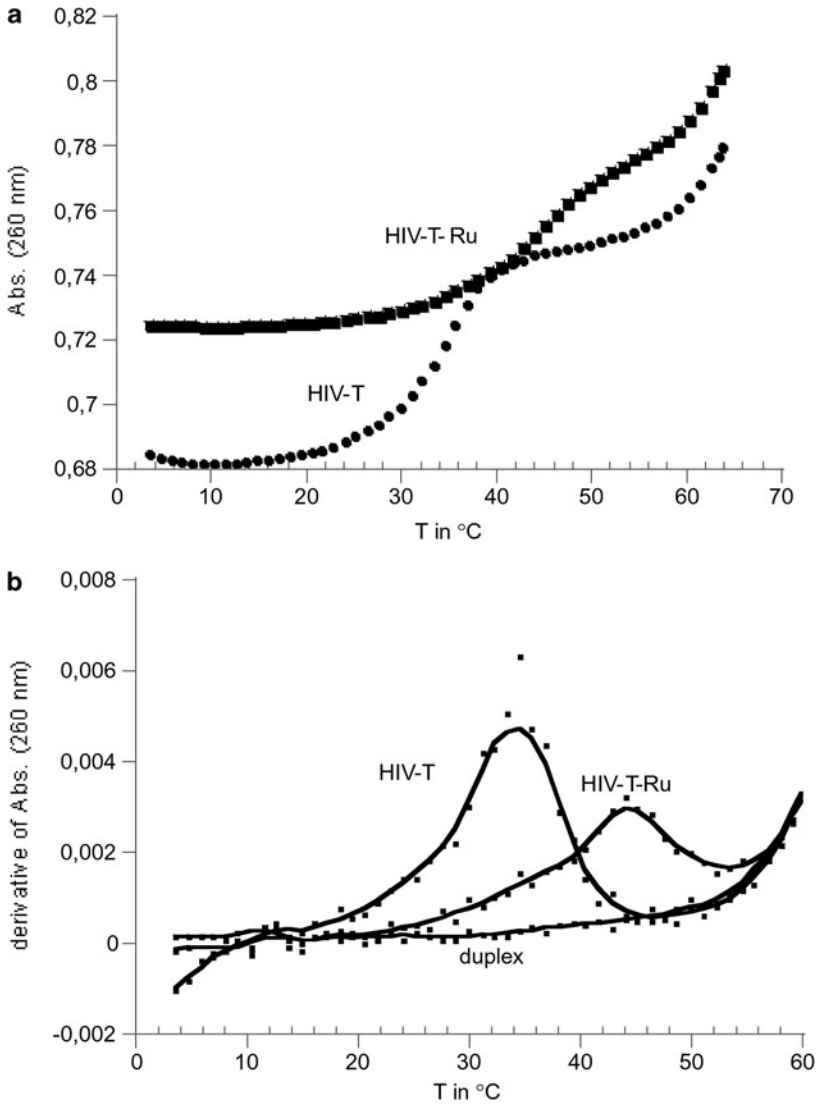

Figure 5. a) Thermal denaturation of the triple helices formed from 1.3 μM double stranded DNA HIV-D1/HIV-D2 and 1.7 μM non-modified HIV-T or 1.7 μM conjugate Δ-HIV-T-Ru in 10 mM cacodylate buffer pH 6.0, 0.2 M NaCl, 10 mM $MgCl_2$; b) the derivative of the above curves.

triplex stabilization by the intercalator results in a 12°C increase in the temperature. Plasmon resonance biosensor analysis of the system made on BIAcore™ 2000 instrument (16,17) allowed determination of the third strand residence time in the triplex. Thus, the half-life period of non-modified triplex was estimated as $t_{1/2} =$ 17 min., whereas for ruthenium-containing triplex it was about 90 min.

In conclusion, we have obtained the oligonucleotide-intercalating ruthenium complex conjugate using a simple and rapid chemical method of synthesis. This

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conjugate forms a stable fluorescent triple helix with a target double-stranded DNA that has a longer life time and dissociates at a higher temperature compared to the unmodified oligonucleotide. Fields of potential use of this conjugate include anti-gene applications, studies of duplex and triplex formation kinetics and cell penetration by fluorescent methods; photochemical modifications of DNA (18) and electron transfer studies (19).

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