DNA Cleavage by a 'Metalloporphyrin-spermine-oligonucleotide' Molecule

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A 'manganese porphyrin-spermine-oligonucleotide' molecule, forms a stable triple helix structure (T_m = 42 °C), and cleaves a double-stranded DNA sequence of HIV-1.

Modified synthetic oligodeoxynucleotides (ODN) can be considered as new tools in the selective inhibition of gene expression and as potential drugs in anticancer or antiviral chemotherapy.[1] The target is a mRNA sequence in the antisense approach or a double-stranded DNA sequence in the antigene strategy.[2] Despite the low melting temperature of a triple helix (T_m values ranging from 20–30 °C for ODNs of 16–25 bases compatible with specific sequence recognition, see ref. 3 for few examples), HIV-1 transcription was inhibited by using a triple helix-forming oligonucleotide (TFO) that binds to the transcription initiation site.[4]

Here we report the preparation of a 'manganese porphyrin-spermine-oligonucleotide' molecule (Fig. 1) and its ability to cleave a double-stranded DNA target present on the 5′ side of U3 and also in the *pol* gene of HIV-1 genome. As cleaver moiety we used a tris(methylpyridiniumyl)porphyrinato–manganese(III) motif, Mn(TrisMPyP), because of its ability to perform oxidative DNA breaks when activated by potassium mono-persulfate, $KHSO_5$ (for recent articles on the oxidative DNA cleavage involving transition metal complexes, see ref. 5). The manganese porphyrin entity was linked to the 5′ end of the third strand *via* a polyamine, spermine, in order to improve the thermal stability of the triple helix[6] and to reduce the overall negative charge of the modified ODN (polyamines are positively charged *in vivo*, by addition of the four positive charges of the attached Mn(TrisMPyP) motif. Polyamines are known to bind to DNA,[7] to stabilise triplex formation[8] and to facilitate the penetration of cytotoxics into tumour cells.[9]

The ODNs used are given in Scheme 1:

5′-X-TTTTCTTTTGGGGGGT (16-mer, **TFO**)

5′-CCACTTTTTAAAAGAAAAGGGGGGACTGG (29-mer, **ODN 1**)

3′-GGTGAAAAATTTTCTTTTCCCCCCTGACC (29-mer, **ODN 2**)

20 15 1

Scheme 1 C is a 5-methylcytosine and X = OH or Mn(TrisMPyP-spermine)

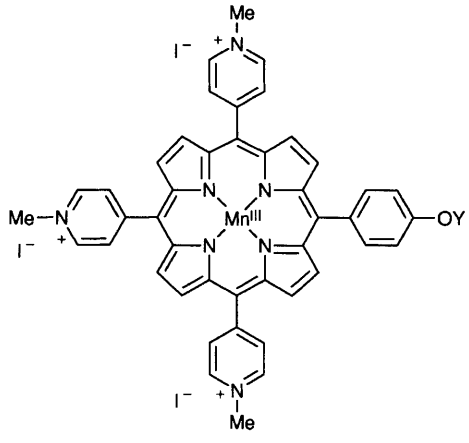
The triplex-forming oligonucleotide **TFO** containing a 5-methylcytosine is oriented parallel to the polypurine tract of **ODN 1** by Hoogsteen pairing.[3b] The Watson–Crick 29-mer duplex **ODN 1·ODN 2** contains the polypurine sequence present on the 5′ side of U3 of HIV-1 genome (positions 8626-8641 according to ref. 10). The same 16-purine sequence is also present in the *pol* gene (4331-4346).

The oligonucleotides were synthesised by standard solid-phase β-cyanoethylphosphoramidite chemistry on a Cyclone Plus from Milligen Biosearch. Concentrations of single-stranded oligonucleotides were determined at 260 nm.[11] The 16-mer **TFO** was functionalized at the 5′ position with spermine as previously described for diamines.[12] The functionalized 16-mer was purified on a 20% denaturing polyacrylamide gel. It should be noted that purification of G-rich oligonucleotide is somewhat complicated by the formation of G-quartets.[13]

The metallated cationic porphyrin precursor Mn(TrisMPyP-CO_2H) was prepared according to ref. 14 and activated by dicyclohexylcarbodiimide and 2-mercaptothiazoline. The yield of conjugate was 60% after purification (based on the spermine-linked oligonucleotide).

Melting curves were obtained by measuring the absorbance of the target duplex and conjugate **1** from 10 to 90 °C in 25 mmol dm^{-3} Tris·HCl (pH 7.1), 100 mmol dm^{-3} NaCl, 10 mmol dm^{-3} $MgCl_2$ and 2 mmol dm^{-3} free spermine medium. The melting point of the triplex (42 ± 1 °C) was determined from the curve corresponding to the difference between the triplex absorbance (A_t) and the duplex absorbance (A_d) plotted *versus* temperature (Fig. 2). The presence of spermine as linker with two protonable sites within the tether contribute to the stabilisation of the triplex. The melting point of this triplex is now above 37 °C. The melting point is only 30 °C when the spermine linker is replaced by an aliphatic diamine like hexamethylenediamine.

The 29-mer target duplex **ODN 1·ODN 2** was labelled at the 5′-end of the pyrimidine rich strand **ODN 2** by reaction with γ^{32}P-ATP and T_4 polynucleotide kinase. In cleavage experiments triplex formation was obtained by incubation of the duplex **ODN 1·ODN 2** (10 nmol dm^{-3}) and conjugate **1** (100 nmol dm^{-3} to 10 μmol dm^{-3}) in a 8 μl volume containing 25 mmol dm^{-3} Tris·HCl (pH 7.1), 100 mmol dm^{-3} NaCl, 10 mmol dm^{-3} $MgCl_2$ and 2 mmol dm^{-3} of free spermine. The hybridization mixture was heated at 90 °C for 5 min and slowly



Y = $(CH_2)_4CONH(CH_2)_3NH(CH_2)_4NH(CH_2)_3NHCO_2$–$T_4CT_4G_6T$–$^{3'}OH$

Fig. 1 Structure of Mn(TrisMPyP-spermine-5′-TFO) conjugate **1**

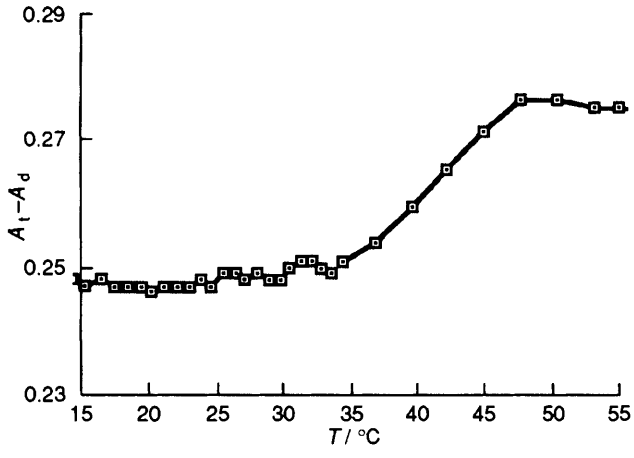


Fig. 2 Melting curve of the triple-helix described in Scheme 1 with X = Mn(TrisMPyP-spermine)

cooled to room temperature within 4 h and stored at 4 °C for 15 h to complete the hybridization process.

For cleaving experiments, 2 μl of a 5 mmol dm^{-3} $KHSO_5$ solution were added at 4 °C to 8 μl of the hybridization mixture and the reaction was allowed to proceed for 1 h. The reaction was stopped by the addition of 1 μl of 1 mol dm^{-3} Hepes buffer (pH 8). Samples were then diluted with 1 μl of yeast tRNA (10 mg ml^{-1}) and 100 μl of 0.3 mol dm^{-3} sodium acetate (pH 5.2), precipitated with 300 μl of ethanol and finally rinsed with 70% aqueous ethanol and lyophilised. Fragments of DNA were analysed on a 20% denaturing polyacrylamide gel (Fig. 3).[15]

When the targeted manganese porphyrin was activated by potassium monopersulfate (ref. 16 for the formation and the reactivity of oxomanganese porphyrin complexes in water solutions), DNA breaks were observed as individual bands, but mainly at two sites centred at T_{15} (major site) and T_{20} (minor site). The cleavage products are eluting similarly to the Maxam–Gilbert sequencing ladder products (*i.e.* with a 3′-phosphate end), without smears as previously reported for a cationic manganese porphyrin linked to an oligonucleotide by an aliphatic diamine in antisense strategy.[15] Breaks occurred at low DNA cleaver concentration (1–10 μmol dm^{-3}, lanes 5–7). The main cleavage sites centred at T_{15} and T_{20} indicate that the metalloporphyrin moiety is interacting with the triple helix structure. However the bands observed beyond $T_{20}A_{21}$ suggest that the cleaver is able to cleave the DNA target outside of the triplex structure. We are currently working on the detailed mechanism of the double-stranded DNA cleavage by this metalloporphyrin-spermine-TFO in order to determine the groove where breaks are generated and the interactions of the metalloporphyrin and the spermine linker with the double-stranded DNA target.

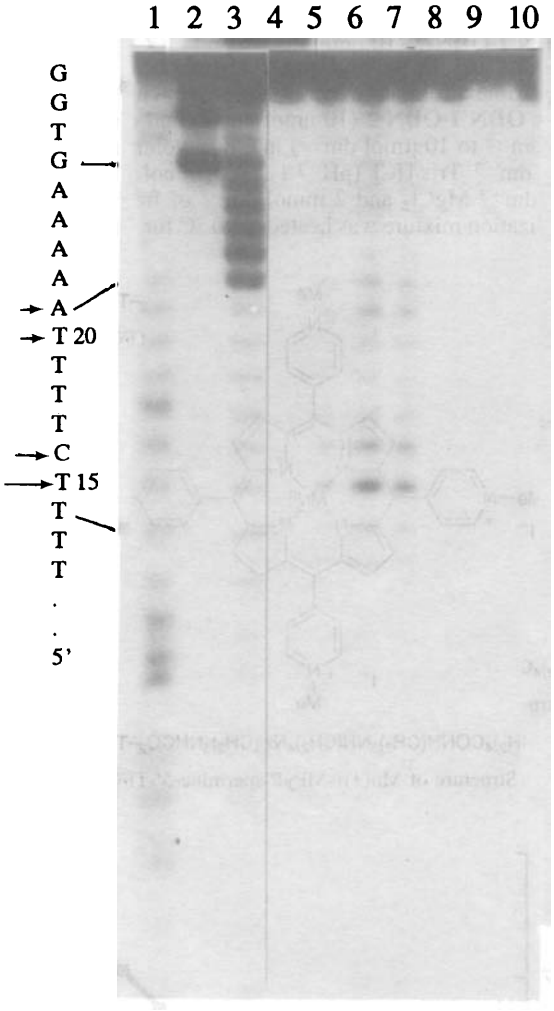


Fig. 3 Analysis by electrophoresis of the cleavage of the duplex target **ODN 1**·**ODN 2** by the triplex-forming conjugate **1** (**ODN 2** is labelled at its 5′-end). Lanes 1 to 3: Maxam–Gilbert, C + T, G, A + G. Lanes 4 to 7: 100 nmol dm^{-3}, 1, 5 and 10 μmol dm^{-3} of the conjugate **1** respectively, with 1 mmol dm^{-3} $KHSO_5$. Lane 8: target duplex only (control). Lane 9: target duplex and 5 μmol dm^{-3} of the conjugate **1** without $KHSO_5$ (control). Lane 10: target duplex with 1 mmol dm^{-3} $KHSO_5$ (control). Arrows are proportional to DNA breaks measured by densitometry.

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