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Influence of the nature of the porphyrin ligand on the nuclease activity of metalloporphyrin–oligonucleotide conjugates designed with cationic, hydrophobic or anionic metalloporphyrins

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ABSTRACT

The synthesis of metalloporphyrin–oligonucleotide conjugates with different metalloporphyrin moieties are described as well as the comparison of their *in vitro* nuclease efficiency toward a single-stranded DNA target. Between cationic, anionic and hydrophobic manganese porphyrins covalently linked to the oligonucleotide, the best nuclease activity was obtained with the cationic ones, suggesting that the affinity of the cleaver to the DNA target is a key factor.

INTRODUCTION

Several metalloporphyrin–oligonucleotide conjugates have been synthesized in the laboratory for various potential applications. The general aim was to associate the redox properties of the metalloporphyrin core (1) with the capacity of an oligonucleotide to hybridize specifically to its complementary sequence in order to target the reactivity of the metalloporphyrin moiety. One potential application is the design of reactive antisense oligonucleotides (2) able to mediate the irreversible degradation of biological targets (single-stranded DNA or RNA). The manganese metalloporphyrin can be activated in a bleomycin-like fashion with O_2 and a reductant or in the presence of $KHSO_5$ (3–6,14) to a high valent metal-oxo species (5,6) that is able to mediate oxidative damage on the targeted nucleic acids. In the present work, the nature of the metalloporphyrin covalently bound to the oligonucleotide vector was varied and this series of 'oligonucleotide-cleaver' afforded an opportunity to perform a strict comparison between the cationic, anionic and hydrophobic complexes in their ability to mediate oxidative damage on a single-stranded DNA target. One can find, in the literature, examples of reminiscent oxidative DNA cleavage by hydrophobic (7–9), anionic (10,11) and cationic (12–15) metalloporphyrins coupled to oligonucleotides, but no direct comparison of their nuclease efficiency was possible. This work was undertaken with conjugates designed with the same oligonucleotide vector, the same metal and the same linker-arm as well as under strictly identical experimental conditions. Thus, the highest efficiency of cationic metalloporphyrins as DNA cleavers in 'oligonucleotide-cleaver' was established unambiguously using two different modes of activation (reductant/O_2 or $KHSO_5$).

MATERIALS AND METHODS

Instrumentation

Oligonucleotides were synthesized on a Cyclone Plus DNA synthesizer from Milligen Biosearch. ^{1}H-FT-NMR spectra were recorded on Brucker AC 200 and AM 250 spectrometers. Mass spectrometry data were performed on Perkin Elmer SCIEX Api 100 or on Nermag R-10-10 instruments for electrospray (MS-ES) and desorption chemical ionisation (NH_3) (MS-DCI) measurements, respectively. HPLC analysis and purification steps of conjugates **1**, **2** and **3** were performed in the following conditions: eluents, A = 0.1 M TEAA (pH 6.5), B = CH_3CN; linear gradient, 10–90% B over 30 min; flow rate, 1 ml/min, on reverse phases C18 or C8 column: Nucleosil C18, 10 µ, 250 × 4.6 mm from Interchrom or Lichrosorb C8, 10 µ from Interchrom. HPLC profiles were followed at 260 and 468 nm using a diode array detector DAD 440 from Kontron.

Oligonucleotides: synthesis, purification and labelling

The two complementary oligodeoxyribonucleotides: 5′-NH_2-26mer anti-*rev* and 35mer *rev* (Fig. 1 for sequences) were synthesized by standard solid-phase β-cyanoethylphosphoramidite. The 26mer anti-*rev* was functionalized at the 5′-end with hexamethylenediamine as previously described (16). Purifications of 5′-NH_2-26mer anti-*rev* and 35mer *rev* were performed by HPLC and by electrophoresis, respectively (14). Concentrations of single-stranded oligonucleotides were determined by UV titration at 260 nm (17). The 5′-end of the 35mer *rev* target was labelled by ^{32}P using standard procedure with T4 polynucleotide kinase and [γ-^{32}P]ATP purchased from BioLabs and DuPont, respectively.

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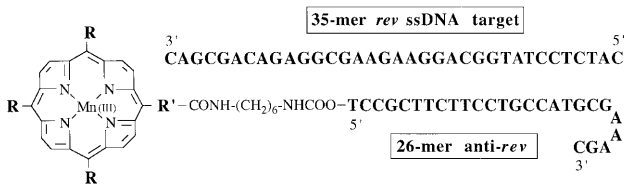


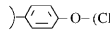
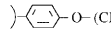
	R	R'	Metalloporphyrin nature
1			trianionic
2			hydrophobic
3			hydrophobic
4			tricationic

Figure 1. Structure of oligonucleotide–metalloporphyrin conjugates **1**–**4**. Variation of nature of metalloporphyrin part of 5′-metalloporphyrin-26mer anti-*rev* conjugates directed against a single-stranded DNA model 35mer *rev* target. For clarity, axial ligands of metalloporphyrins and cationic or anionic counter ions of *meso* substituents of the different metalloporphyrins have not been mentioned.

Preparation of metalloporphyrin precursors

Conjugate **1** (Fig. 1 for conjugate structures) was functionalized with an anionic metalloporphyrin, *meso*-tetrakis(4-carboxyphenyl)porphyrinato-manganese(III), as cleaver entity. This metalloporphyrin was prepared by metallation with $Mn(OAc)_2$ in DMF of the corresponding commercially available ligand. The new hydrophobic metalloporphyrins used to prepare conjugates **2** and **3** were obtained by total synthesis starting from pyrrole and the modified aldehyde derivatives as described below. The tricationic metalloporphyrin moiety of conjugate **4**, namely 5-[(4-(5-carboxy-1-butoxy)phenyl]-10,15,20-tris(4-*N*-methylpyridiniumyl) porphyrinato-manganese(III) was prepared according to ref. 18.

Preparation of 5-[4-(carboxy-1-butyloxy)phenyl]-10,15,20-tris(phenyl)-porphyrinato-manganese(III) 3d porphyrin precursor of conjugate 3

*5-(4-hydroxyphenyl)-10,15,20-tris(phenyl)porphyrin **3a***. A mixture of 4-hydroxybenzaldehyde (1.22 g, 10 mmol) in propionic acid (100 ml) was heated at 140°C with stirring. To this solution were successively and slowly added benzaldehyde (1.1 ml, 10 mmol) and pyrrole (1.4 ml, 20 mmol). This mixture was allowed to react for 1.5 h. After cooling the solution was neutralized with a solution of sodium hydroxide. After one night at 5°C, the pellet was filtered and washed with water. The crude material was dissolved in CH_2Cl_2-EtOH (9/1) and precipitated with 15 vol hexane. The supernatant was evaporated and the separation of the desired isomer was performed by column chromatography with silica gel with CH_2Cl_2-EtOH (99/1) as eluent and then with alumina with CH_2Cl_2-MeOH (97/3). The product was precipitated by addition of hexane to a CH_2Cl_2-EtOH (98/2) solution (40 mg, yield 2%). MS-DCI (NH_3) m/z: 631 (M^++1). Anal. Calcd for $C_{44}H_{30}N_4O$: C, 83.79; H, 4.79; N, 8.88. Found: C, 82.32; H, 4.69; N, 8.51. UV-vis (1.2 µM in CH_2Cl_2) λ, nm (ε, $M^{-1} \times cm^{-1}$): 594 (4×10^3), 550 (6×10^3), 514 (1.4×10^4), 418 (4×10^5, Soret band). ^{1}H NMR (250 MHz, DMSO-d_6) δ, p.p.m.: 10.10 (s, 1H, OH), 9.03 (d, 2H, J = 4.8 Hz, β-pyrrole), 8.93 (s, 6H, β-pyrrole), 8.34 (m, 6H, 2,6-phenyl), 8.13 (d, 2H, J = 8.4 Hz, 2,6-hydroxyphenyl), 7.95 (9H, 3,4,5-phenyl), 7.33 (d, 2H, J = 8.4 Hz, 3,5-hydroxyphenyl), –2.79 (s, 2H, NH).

*5-[4-[5-(ethoxycarbonyl)-1-butyloxy)phenyl]-10,15,20-tris(phenyl)porphyrin **3b***. To a solution of **3a** (40 mg, 63 µmol) in dry DMF (5 ml) under nitrogen was added powdered sodium hydroxide (60 mg, 1.5 mmol) and the mixture was stirred for 1 h. Ethyl 5-bromovalerate was then added (29 µl, 183 µmol) and the mixture was stirred for 3 h at room temperature. Formation of the product was monitored by TLC on silica with CH_2Cl_2-EtOH (99/1) as eluent. Water and dichloromethane were added to the solution to extract the desired product. The organic layer was washed several times with water, dried over sodium sulphate and evaporated to dryness. The product was purified on a silica gel column with a CH_2Cl_2-EtOH (99/1) as eluent (20 mg, yield 42%). MS-DCI (NH_3) m/z: 759 (M^++1). Anal. Calcd for $C_{51}H_{42}N_4O_3$: C, 80.45; H, 5.58; N, 7.38. Found: C, 79.65; H, 5.58; N, 7.06. ^{1}H NMR (200 MHz, CD_2Cl_2) δ, p.p.m.: 8.90 (8H, β-pyrrole), 8.25 (6H, 2,6-phenyl), 8.13 (d, 2H, J = 8.6 Hz, 2,6-hydroxyphenyl), 7.79 (9H, 3,4,5-phenyl), 7.30 (d, 2H, J = 8.6 Hz, 3,5-hydroxyphenyl), 4.28 (t, 2H, J = 6 Hz, OCH_2), 4.19 (q, 2H, J = 7 Hz, $COOCH_2$), 2.51 (t, 2H, J = 7 Hz, CH_2COO), 2.00 (m, 4H, 2 CH_2), 1.32 (t, 3H, J = 7 Hz, CH_3), –2.82 (s, 2H, NH).

*5-[4-(carboxy-1-butyloxy)phenyl]-10,15,20-tris(phenyl)porphyrin **3c***. To a solution of **3b** (20 mg, 26 µmol) in DMF (5 ml) was added powdered sodium hydroxide (27 mg, 675 µmol) and 100 µl water and the mixture was stirred for 4 h at room temperature. Formation of the product was monitored by TLC on silica with CH_2Cl_2-EtOH (98/2) as eluent. The mixture was neutralized with a solution of citric acid (pH 2). This water solution was extracted by dichloromethane to extract the desired product. The organic layer was washed several times with water, dried over sodium sulphate and evaporated to dryness. The product was purified on a silica gel column with a CH_2Cl_2-EtOH (98/2) as eluent (4 mg, yield 21%). MS-DCI (NH_3) m/z: 731 (M^++1). ^{1}H NMR (200 MHz, CD_2Cl_2) δ, p.p.m.: 8.90 (8H, β-pyrrole), 8.23 (6H, 2,6-phenyl), 8.13 (d, 2H, J = 8.6 Hz, 2,6-hydroxyphenyl), 7.77 (9H, 3,4,5-phenyl), 7.28 (d, 2H, J = 8.7 Hz, 3,5-hydroxyphenyl), 4.27 (t, 2H, J = 6.7 Hz, OCH_2), 2.60 (t, 2H, J = 6.7 Hz, CH_2COO), 2.06 (m, 4H, 2 CH_2), –2.82 (s, 2H, NH).

*5-[4-(carboxy-1-butyloxy)phenyl]-10,15,20-tris(phenyl)porphyrinato-manganese(III) **3d***. Porphyrin **3c** (4 mg, 5.5 µmol) was metallated with manganese(II) acetate tetrahydrate (7 mg, 28.5 µmol) in DMF (2 ml) in the presence of triethylamine (4 µl, 28.5 µmol) at 140°C during 1 h. Dichloromethane was added to the solution and the organic layer was washed several times with water, dried over sodium sulphate and evaporated to dryness (4 mg, yield 95%). MS-DCI (NH_3) m/z: 784 (M^++1). UV-vis (10 µM in CH_2Cl_2) λ, nm (ε, $M^{-1} \times cm^{-1}$): 472 (90×10^3, Soret band).

Preparation of 5-[4-carboxyphenyl]-10,15,20-tris(phenyl) porphyrinato-manganese(III) 2b, porphyrin precursor of conjugate 2

*5-[4-carboxyphenyl]-10,15,20-tris(phenyl)porphyrin **2a***. A mixture of 4-carboxybenzaldehyde (5 g, 33 mmol), propionic acid (100 ml) and acetic anhydride (5 ml) was heated at 140°C with stirring. As previously described for the synthesis of compound **3a**, benzaldehyde (6 ml, 56 mmol) and pyrrole (5.3 ml, 76 mmol)

were added. The reaction was allowed to take place for 1.5 h. The subsequent treatment of the reaction mixture was as described above for porphyrin **3a**. The crude product was dried and purified by column chromatography on silica gel with CH_2Cl_2-EtOH (97/3) as eluent. To complete the purification, the porphyrin was precipitated by addition of acetonitrile to a CH_2Cl_2-EtOH (9/1) solution of **2a** (251 mg, yield 2%). MS-DCI (NH_3) m/z 659 (M^++1). Anal. Calcd for $C_{45}H_{30}N_4O_2$: C, 82.05; H, 4.59; N, 8.50. Found: C, 80.79; H, 4.77; N, 8.17. UV-vis [2 µM in CH_2Cl_2-MeOH (9/1)] λ, nm (ε, $M^{-1} \times cm^{-1}$): 514 (23×10^3), 418 (47×10^4, Soret band). ^{1}H NMR (200 MHz, DMSO-d_6) δ, p.p.m.: 8.96 (s, 8H, β-pyrrole), 8.50 (d, 2H, J = 8.2 Hz, ArCOOH), 8.44 (d, 2H, J = 8.2 Hz, ArCOOH), 8.34 (m, 6H, phenyl), 7.96 (m, 9H, phenyl), –2.82 (s, 2H, NH pyrrole).

5-[4-carboxyphenyl]-10,15,20-tris(phenyl)porphyrinato-manganese(III) ***2b***. Porphyrin **2a** (100 mg, 0.15 mmol) was metallated with manganese(II) acetate tetrahydrate (186 mg, 0.76 mmol) in the presence of 2,4,6-collidine (390 µl, 2.96 mmol) in DMF (25 ml). The mixture was refluxed for 1 h. Metallation was monitored by UV-vis. The reaction medium was cooled to room temperature and precipitated by water addition (64 mg, yield 55%). UV-vis (6.5 µM in CH_2Cl_2) λ, nm (ε, $M^{-1} \times cm^{-1}$): 612 (9.7×10^3), 576 (1×10^4), 472 (1×10^5, Soret band).

The hydrophobic metalloporphyrins are pentacoordinated with an acetate group as axial ligand. The water-soluble metalloporphyrins are hexacoordinated with two water molecules as axial ligands and consequently bear an additional positive charge, these axial ligands and the positive charges are not represented on Figure 1 for clarity. The anionic counter ions of the positively charged *meso* substituents of tricationic metalloporphyrin (iodide in the case of compound **4**) are compatible with the activation mode used to prepare the activated ester of these metalloporphyrins, so they were not exchanged for alternative ones. As indicated above, these anionic counter-ions and cationic ones (Na^+) of the anionic metalloporphyrin precursor of conjugate **1** were omitted for clarity from Figure 1.

Preparation of oligonucleotide–metalloporphyrin conjugates

The synthesis of conjugates **1–4** consisted in the covalent attachment, through the formation of a peptide linkage, of the 5′-NH_2-end of the 26mer anti-*rev* oligonucleotide and the carboxylic terminal function of the different metalloporphyrin precursors described above.

Synthesis of conjugate ***1***. The activation of the metalloporphyrin precursor was carried out in dry DMF (dried over calcium sulphate). To 45 µl of a 5 mM DMF solution of *meso*-tetrakis (4-carboxyphenyl)porphyrinato-manganese(III) (0.23 µmol, 1 equiv.) were added 9 µl 200 mM 4-methylmorpholine (NMM) solution in DMF (0.9 µmol, 4 equiv.) and 16 µl of both 25 mM benzotriazol-1-yloxy-tris(dimethylamino)phosphonium (BOP) and hydroxybenzotriazole (HOBt) solutions (0.23 µmol, 1 equiv.). After 1 h at 50°C, activation was analyzed by TLC on silica gel (eluent: $CHCl_3$/CH_3OH, 1/1, v/v): 50% *meso*-tetrakis (4-carboxyphenyl)porphyrinato-manganese(III) (R_f = 0.33) was converted to a monoactivated main product (R_f = 0.6) and to some minor polyactivated metalloporphyrins migrating more rapidly. This solution was then added to the 5′-NH_2-26mer *rev* oligonucleotide (22 nmol, 5 OD_{260}) diluted in 90 µl 2 M borate buffer (pH 8). After 1 h at 37°C, 5 vol water were added and incubation was extended for an extra 1 h at 37°C in order to hydrolyze the majority of activated esters and activating reagents and to facilitate the purification steps. The reaction products were then precipitated with 0.3 M (final concentration) sodium acetate buffer (pH 5.2) and 6 vol ethanol to remove the excess of free porphyrin, washed with 90% ethanol and redissolved in 100 µl water. Conjugate **1** was purified by HPLC on a reverse phase C18 column, lyophilized, dissolved in 200 µl 1.5 M sodium acetate buffer (pH 5.2), precipitated with 1.3 ml ethanol 1 h at –20°C, washed with 2 × 1 ml cold ethanol and lyophilized. Conversion of the starting oligonucleotide (R_t = 10 min) to conjugate **1** (R_t = 13.2 min) was 70% and the yield was 40% after total purification. MS-ES: observed mass, 8826.1 ± 1.4; calculated mass, 8825.0.

Synthesis of conjugates ***2*** *and* ***3***. To 100 µl of 1 mM solutions of the metalloporphyrin precursors 5-(4-carboxyphenyl)-10,15,20-tris(phenyl)porphyrinato-manganese(III) **2b** and 5-[4-(carboxy-1-butyloxy)phenyl]-10,15,20-tris(phenyl)porphyrinato-manganese (III) **3d** (0.11 µmol, 1 equiv.) in dry DMF (DMF dried over barium oxide, distilled and kept over 4 Å molecular sieves) the following were added: 0.25 µl NMM (2.2 µmol, 20 equiv.), 20 µl of both 38.5 mM solutions of BOP and HOBt (0.77 µmol, 17 equiv. of both reagents) in dry DMF. After 15 min at 60°C, solutions of benzotriazole activated esters were added to the 5′-NH_2-26mer *rev* oligonucleotide (22 nmol, 5 OD_{260}) dissolved in 60 µl 20 mM MOPS buffer (pH 7.5). The resulting solvent medium for these coupling reactions was buffer/DMF mixture, 30/70. Coupling reactions were allowed to take place over 18 h at 60°C. Then 200 µl dichloromethane were added on the coupling reaction media and the coupling products were extracted with water (2 × 200 µl). HPLC was used to monitor the progress of the coupling reaction and to purify conjugates **2** and **3**. These HPLC analyses and purification steps were performed on a reverse-phase C8 column. Conversion of the starting oligonucleotide (R_t = 12.5 min) to conjugates **2** and **3** (R_t = 20.8 min) were 80 and 40%, respectively. After purification of the crude samples by HPLC, the purified compounds were dissolved in a 0.3 M sodium acetate buffer (pH 5.2) and precipitated by the addition of 3 vol cold ethanol in order to remove triethylammonium counter-ions of phosphate groups of the oligonucleotide moiety of conjugates. After a night at –20°C, supernatants were removed and pellets were washed with cold 90% ethanol. MS-ES: observed mass, 8693.8 ± 0.7 and 8764.9 ± 1.0; calculated mass, 8693.0 and 8765.1 for conjugates **2** and **3**, respectively.

Synthesis of conjugate ***4***. Conjugate **4** was synthesized, analyzed and purified as previously described (14).

Spectrophotometric data relative to oligonucleotides and conjugates

The spectrophotometric data of oligonucleotides and conjugates were as follows. Oligonucleotidic precursor 5′-NH_2-26mer anti-*rev*, calculated ε_{260} in water: 230×10^3 $M^{-1} \times cm^{-1}$. Conjugates **1–4** showed the two typical absorbances, one corresponding to the Soret band of the metalloporphyrin moiety at λ = 468 nm and the other one at λ = 260 nm for the oligonucleotide part. These conjugates were characterized by a visible/UV ratio obtained by the diode-array spectra of the purified conjugate HPLC peak: A_{468}/A_{260} = 0.38, 0.44, 0.39 and 0.47 for compounds **1–4**, respectively. The concentrations of the

conjugates were determined spectrophotometrically at 260 nm as for single-stranded oligonucleotides.

Cleavage experiments

The comparative *in vitro* study of the nuclease activity of the conjugates **1**–**4** were performed on the 5′-[^{32}P]-35mer *rev* target as already described (14).

RESULTS

Hybrid molecules **1–4** (see Fig. 1 for structures) were prepared in order to study the influence of the porphyrin ligand on the nuclease activity of such conjugates oligonucleotide–metalloporphyrin. To perform a direct and strict comparison of the activity of conjugates **1–4** as a function of the nature of their metalloporphyrin moiety, all these conjugates were designed with the same oligonucleotidic part, namely the 26mer anti-*rev*. This oligonucleotide contains a 18 nucleotide long sequence directed against a single-stranded DNA 35mer target (for the sequence of the 35mer *rev* target see Fig. 1) corresponding to the initiation site of the HIV-1 *rev* gene [5507–5541 according to ref. (19)]. At the 3′-end of this 18mer oligonucleotide was added a highly stable minihairpin in order to enhance the metabolic stability of the oligonucleotide vector in culture medium (13,20,21).

Preparation of conjugates

All conjugates **1–4** were prepared by linking via a peptidic bond the 5′-NH_2 end of the oligonucleotide to a carboxylic acid function of a functionalized metalloporphyrin. Conjugates **1**, **2** and **3** were prepared using BOP and HOBt as activating agents of the carboxylic residue of the metalloporphyrin moiety. This procedure was previously used in the case of various conjugates synthesized through the attachment of a metalloporphyrin to the 26mer anti-*rev* oligonucleotide (14), but in the case of anionic and hydrophobic metalloporphyrins (conjugates **1**, **2** and **3**) some modifications were necessary in order to obtain efficient coupling reactions.

To prepare compound **1**, the activation step was carried out with only one equivalent of both cofactors BOP and HOBt. This protocol was used in order to activate only one carboxylic acid function over the four ones of the tetraanionic porphyrinic ligand. Monitoring of the activation step by a TLC analytical method allowed us to stop it when a high ratio of benzotriazole-monoactivated ester was generated. We noticed that for conjugate **1**, the coupling reaction was only possible in a medium containing a high concentration of borate salts in order to avoid the repulsive electrostatic interactions between the polyanionic oligonucleotide and the anionic metalloporphyrin precursor.

Conjugates **2** and **3** were prepared with hydrophobic metalloporphyrins which differed only by the presence, in the case of compound **3**, of an aliphatic linker between the aromatic *meso* substituents of the metalloporphyrin and the carboxylate function attached to it. The same procedures for activating and coupling reactions were used to synthesize these two compounds. A longer reaction time (18 h) and a higher temperature (60°C) than that used generally (13,14) were found to be the optimal conditions for the coupling step. To remove the excess of free metalloporphyrins from the reaction medium, dichloromethane was added, followed by successive water extractions of this organic layer which proved to be successful.

Comparative study of nuclease activity of conjugates as a function of the nature of their metalloporphyrin part

The relative abilities of conjugates **1–4** to induce oxidative damage on a single-stranded DNA target were examined in both oxidant and reducing initiating conditions. The conjugate and the target used to perform these experiments were equimolar, i.e. 10 nM of each. Activation of the cleaver entity of the conjugates in oxidative conditions was performed by the addition of potassium monopersulfate ($KHSO_5$) at a final concentration of 1 mM, subsequently the reaction lasted 1 h at 4°C. Cleaving reactions in reducing conditions involved an activation of the metalloporphyrin with dithiotreitol (DTT) and O_2 as cofactors and incubations lasted 15 h at 37°C. The final concentration of DTT in the reaction media was 0.1 mM, the previously found optimal concentration of reducing agent to activate the cationic metalloporphyrins in such conditions (14). The resulting samples were analyzed by PAGE before and after piperidine treatment. Such treatment reveals (i) some oxidative damage detected as smears on gels and corresponding to nucleic acid bases lesions, and (ii) cross-links (14,15).

Cleavage patterns (Fig. 2) obtained for conjugate **4** having a tricationic metalloporphyrin have been already reported (13,14). When $KHSO_5$ initiation was carried out, an intense smear extending from the 35mer full length band to a fragment corresponding to the G_{22} band of the Maxam–Gilbert ladder was observed. After alkaline treatment, this smear was resolved as discrete bands corresponding to various fragments resulting exclusively from oxidative lesions on guanine residues. The activation of cationic metalloporphyrin conjugate in the presence of air and DTT provided, before alkaline treatment, only a broadening of the 35mer full length band. After alkaline treatment, a major band corresponding to the G_{27} fragment and four minor bands corresponding to G_{33}, G_{31}, G_{25} and G_{24} fragments appeared. Conjugates **1**, **2** and **3** designed with anionic and hydrophobic manganese porphyrins led to similar cleavage patterns. With these conjugates, a nuclease activity was only observed when $KHSO_5$ was used as activating agent. No cleaving activity was observed with DTT and O_2 as cofactors. In the presence of $KHSO_5$ the cleaving reaction showed, before alkaline treatment, particular PAGE profiles that were characterized by (i) a broadening of the band corresponding to the starting material and by (ii) the presence of two slow migrating bands localized above the 35mer full length fragment. When the reaction mixtures were submitted to piperidine treatment, these two slow migrating species and part of the full length material were transformed to five discrete bands of various intensity. As for conjugate **4**, guanine residues appeared as the exclusive targets of the anionic and hydrophobic cleavage systems. The major site of oxidative lesions induced by these conjugates was observed at G_{27}, minor sites were G_{33}, G_{31}, G_{25} and G_{24}. In fact the cleavage pattern obtained with compound **4** in the presence of a reductant and O_2 or in the presence of 1 mM $KHSO_5$ at a reaction time of 30 s (instead of 1 h in the present work, the cleavage pattern at 30 s was analyzed in ref. 14) was identical to that obtained with conjugates **1**, **2** and **3** when using $KHSO_5$. Concerning the cleaving efficiencies of each compounds, compound **4** led to 80 and 50% of oxidative degradation of the target with $KHSO_5$ and DTT/O_2 as cofactors, respectively. In the case of compounds **1**, **2** and **3** no significant degradation was induced on the single-stranded DNA when reducing agents and O_2 were used to initiate

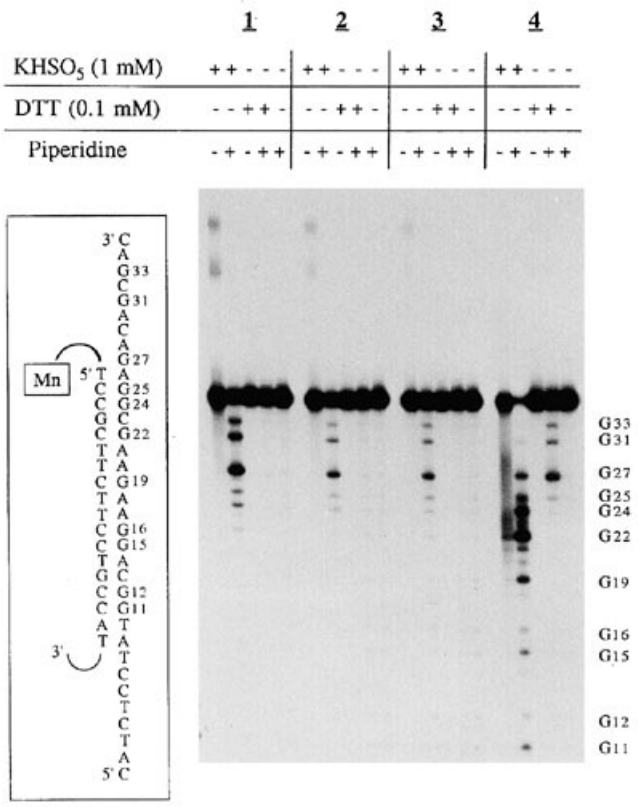


Figure 2. PAGE analysis of the cleavage of the 5′-labelled 35mer *rev* target (10 mM) by conjugates **1**–**4** (10 nM). For each compound, the two lanes were obtained with $KHSO_5$ at a final concentration of 1 mM and for a cleavage reaction time of 1 h at 4°C. The two following lanes were obtained with dithiotreitol at a final concentration of 0.1 mM and O_2 as initiating agents of the cleavage reaction which lasted 15 h at 37°C. The last lane represents the control reaction when the target/conjugate duplex was incubated without activating agent for 15 h at 37°C. For both $KHSO_5$ and DTT activation modes, and for control reactions, an alkaline treatment in the presence of piperidine 1 M for 1 h at 90°C was performed when indicated.

the cleavage reaction. When using $KHSO_5$ with conjugate **1**, based on an anionic cleaver entity, the degradation yield determined after alkaline treatment was ~60%. Compounds **2** and **3** designed with hydrophobic metalloporphyrins led to ~20% of oxidative degradation of the 35mer *rev* target when activated by $KHSO_5$. Quantification of the degradation of the starting material were measured by densitometry from underexposed autoradiograms.

DISCUSSION

The aim of this work was to perform a strict comparison of the nuclease activity of different metalloporphyrins when covalently bound onto an oligonucleotide. Nuclease activity was studied on the same single-stranded DNA target and with two different ways of activation of the metalloporphyrin core of the conjugates. Thus quantitative and qualitative analyses of their nuclease properties can be discussed. If we first consider the quantitative aspects of this comparative study, i.e. the percentages of degradation of the 35mer *rev* target by these different conjugates, we can notice that the cationic metalloporphyrin conjugate **4** was the most efficient for both types of activation mode. In addition, they all present a significant higher reactivity with $KHSO_5$ as cofactor. In such conditions it is possible to degrade almost totally the target with only one equivalent of compound **4** with respect to the 35mer *rev* ss-DNA target. In the same conditions, the hybrid molecule **1** led to ~60% and compounds **2** or **3** to 20% of degradation of the target. With a reducing agent as activator (DTT/O_2 system), lower amounts of damage were observed: a single equivalent of conjugates **1**, **2** and **3** with respect to the target had no nuclease activity, whereas conjugate **4** led to ~50% of degradation of the target. These results highlight the benefit of the attachment of a cationic porphyrinic ligand over anionic or hydrophobic ones on an oligonucleotidic vector when an oxidative cleavage of a DNA target is concerned. This can be explained simply by the nature of the interaction of the metalloporphyrin with the DNA target: cationic metalloporphyrins exhibit a higher nuclease efficiency because the positive charges present at the periphery of the ligand give strong and favorable electrostatic interactions with the polyanionic DNA target and favour close contacts of the generated high-valent metal-oxo species with the different possible oxidation sites on DNA (C-H bonds or sugar units and/or electron-rich nucleobases like guanine residues). Oxidation of the DNA target thus competes favourably with the self-degradation of the activated metal-oxo species in the case of cationic metalloporphyrin conjugates compared to neutral or anionic metalloporphyrin conjugates having less DNA affinity. In addition the better efficiency, and also the different cleavage profile of compound **4** in the presence of $KHSO_5$, is due to its catalytic behavior as previously demonstrated (14). With a short incubation time (30 s) the cleavage profile was similar to that observed with conjugates **1–3**, but after 1 h of incubation the observed profile corresponds to an over-oxidation of the target, leading to a different gel pattern, that one presented in Figure 2.

Furthermore, the length of the linker joining the oligonucleotide to the hydrophobic metalloporphyrin did not appear as a parameter which can significantly disturb the nuclease activity of conjugates. Compounds **2** and **3** which differ only on this structural point exhibited the same efficiency when activated by $KHSO_5$.

Concerning the mechanism of DNA damage mediated by the metalloporphyrin–oligonucleotide conjugates, no strong differences were noted. For both activating agents and with the different metalloporphyrin entities, guanine oxidation was the main observed event. The first site of attack was always G_{27}, the G residue located in the near proximity of the reactive entity. Before piperidine treatment, damage appeared as a broadening of the band corresponding to the full length target associated to some minor cross-link products in the case of non-cationic metalloporphyrins or to a smear of degradated DNA fragments in the case of cationic metalloporphyrins. Piperidine treatment transformed these alkali-labile lesions to fragments of DNA that have lost one deoxyguanosine nucleotide unit. The exact chemical mechanism underlying these damages is not fully known at the present time and is far beyond the present study.

Conclusion

Because cationic metalloporphyrins are endowed with high affinity for nucleic acids due to their positive charges, their covalent coupling onto an oligonucleotide provided very efficient tailored artificial nucleases compared to the corresponding anionic or hydrophobic porphyrin derivatives. These oligo-

nucleotides conjugates with a cationic manganese porphyrin were the only ones able to cleave efficiently the DNA target with a 1 to 1 ratio at 10 nM.

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