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Oxidative Cleavage of DNA Mediated by Hybrid Metalloporphyrin–Ellipticine Molecules and Functionalized Metalloporphyrin Precursors†

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Received February 14, 1990; Revised Manuscript Received May 11, 1990

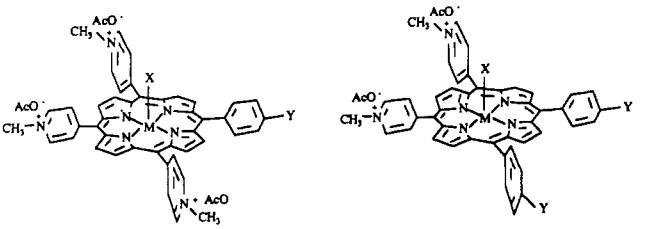
ABSTRACT: The nuclease activity of functionalized metalloporphyrins **1–8** and hybrid metalloporphyrin–ellipticine molecules **10–16** in the presence of potassium monopersulfate ($KHSO_5$) or magnesium monoperoxyphthalate (MMPP), water-soluble oxygen atom donors at physiological pH, toward double-stranded ϕX174 DNA is reported. The DNA cleavage efficiency as a function of the nature of functionalized metalloporphyrins, the length of the linkage between the two parts of the hybrid molecule, viz., metalloporphyrin and 9-methoxyellipticine, the nature of the central metal atom (Mn, Fe, or Zn), the ionic strength, and the nature of the oxygen donor has been studied. Single-strand breaks (SSBs) are observed on double-stranded DNA with a short incubation time of 2 min in the presence of manganese derivatives of both metalloporphyrins and hybrid molecules. Owing to their cytotoxic and nuclease activity, these new water-soluble hybrid molecules may be considered as efficient bleomycin models based on cationic metalloporphyrins.

The therapeutic activity of bleomycin, an efficient antitumoral antibiotic agent, is generally attributed to its DNA binding properties (Chien et al., 1977; Henichart et al., 1985) and to its ability to cleave DNA (Takeshita et al., 1978; d'Andrea & Haseltine, 1978). However, the DNA cleavage is observed in the presence of three cofactors: iron or copper salts, molecular oxygen, and an electron source (Sausville et al., 1978; Burger et al., 1981; Ehrenfeld et al., 1987). Such cleavage mainly occurs via single-strand breaks due to the abstraction of a hydrogen atom at the $C_{4'}$ position of the deoxyribose ring by a high-valent metal–oxo species strongly chelated by bleomycin (Povirk, 1983; Hecht, 1986; Stubbe & Kozarich, 1987; Pratviel et al., 1989a).

This mechanism involving several redox-active metals makes the modeling of bleomycin an interesting goal. All the modeling studies of bleomycin have been based on its structural duality: one part of the molecule is responsible for the DNA interaction (bithiazole, intercalating agent), whereas a second part is a strong chelator for metal ions [peptidic chain, EDTA[1] (Moser & Dervan, 1987; Youngquist & Dervan, 1987; Dervan, 1986), or a metalloporphyrin (Lown & Joshua, 1982; Lown et al., 1986; Hashimoto et al., 1983, 1986)].

Because our group is involved in oxidation reactions catalyzed by metalloporphyrins (Meunier, 1986), DNA cleavage by high-valent metal–oxo species (Fouquet et al., 1987; Bernadou et al., 1989; Pratviel et al., 1989a,b), and the mechanism of action of cytotoxic ellipticine derivatives (Meunier et al., 1988), we decided to synthesize hybrid metalloporphyrin–ellipticine molecules (Tadj & Meunier, 1988) on the basis of the association of a chelating agent, a metalloporphyrin, to an intercalating agent, an ellipticine. However, because of the presence of one hydrophobic metalloporphyrin moiety, these

Chart I: Structures of Cationic Functionalized Metalloporphyrins **1–8**



1: Y = NO_2; M = Mn
2: Y = NH_2; M = Mn
3: Y = NMe_3; M = Mn, Fe, Zn
4: Y = OH; M = Mn
5: Y = $NHCO(CH_2)_3N^+Me_3$; M = Mn
6: Y = $O(CH_2)_3N^+Me_3$; M = Mn
7: Y = OH; M = Mn
8: Y = $O(CH_2)_3N^+Me_3$; M = Mn

† This research was supported by the CNRS (DVAR) and by Pierre Fabre Médicaments (Castres). A fellowship from l'Association pour la Recherche sur le Cancer (ARC, Villejuif) to L. D. is gratefully acknowledged.

[1] Abbreviations: EDTA, ethylenediaminetetraacetic acid; MMPP, magnesium monoperoxyphthalate; 5-MF, 5-methylene-2-furanone.

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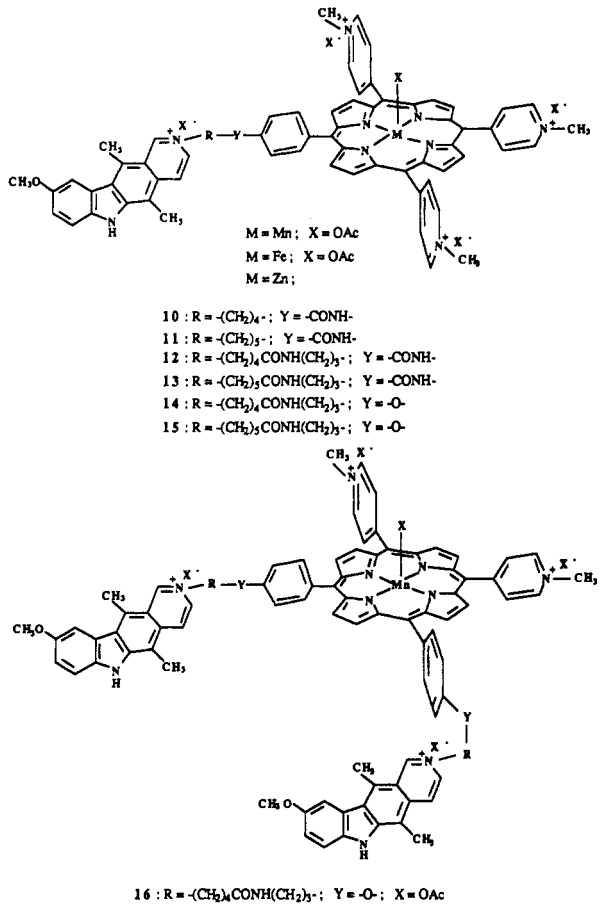
Chart II: Structures of Hybrid Metalloporphyrin–Ellipticine Molecules **10–16**



hybrid molecules have no high nuclease activity and are not cytotoxic on cells in vitro (Etemad-Moghadam et al., 1989). In order to improve the biological activities of these hybrid molecules, we recently developed the preparation of tris(pyridinium) water-soluble functionalized metalloporphyrins exhibiting a cytotoxicity toward murine leukemia cells L1210 in vitro (Chart I) (Meunier et al., 1988a; Ding et al., 1989). In addition, according to extensive studies of the interaction of porphyrins and metalloporphyrins with nucleic acids (Banville et al., 1986; Carvlin & Fiel, 1983; Pasternack et al., 1983a,b; Ward et al., 1986a), these cationic functionalized metalloporphyrins show a high affinity for DNA (K_{app} = 3.4 × 10^8 M^{-1} for poly[d(A-T)] and 1.0 × 10^8 M^{-1} for poly[d-(G-C)]) (Ding et al., 1989). So, we recently synthesized hybrid cationic metalloporphyrin–ellipticine by tethering the two parts of the molecule via various lengths of linker (Chart II) (Meunier et al., 1988a; Ding et al., 1990b). Contrarily to the former hybrid molecules based on hydrophobic porphyrins, the later, due to their cationic metalloporphyrin moiety, have a high affinity for DNA (K_{app} = 2.3 × 10^9 M^{-1} for poly[d(A-T)] and 2.8 × 10^8 M^{-1} for poly[d(G-C)]) and are cytotoxic toward L1210 cells in vitro. One can wonder whether this cytotoxicity is the result of their DNA cleavage ability.

Actually, DNA damages may be induced by porphyrin derivatives via (i) photoactivation (Moan, 1986; Kessel, 1984; Praseuth et al., 1986) or (ii) oxidative activation of iron- or manganese–porphyrin complexes by molecular oxygen in the presence of a reducing agent (Fiel et al., 1982; Aft & Mueller, 1983), by iodosylbenzene (Ward et al., 1986), by potassium monopersulfate (Fouquet et al., 1987; Dabrowiak et al., 1989; Bernadou et al., 1989), or by magnesium monoperoxyphthalate (Pratviel et al., 1989b). Moreover, metalloporphyrins linked to intercalating agents (Lown et al., 1986; Hashimoto et al., 1986) or oligonucleotides (Le Doan et al., 1987) are also able to cleave DNA.

In this paper, we report the nuclease activity of hybrid cationic metalloporphyrin–ellipticine molecules, **10–16**, and cationic functionalized metalloporphyrin precursors, **1–8**, in association with water-soluble, single oxygen atom donors [potassium monopersulfate ($KHSO_5$) and magnesium monoperoxyphthalate (MMPP)]. DNA breaks have been studied on duplex supercoiled ϕX174 DNA.

Experimental Procedures

Materials. The cationic functionalized metalloporphyrins **1–8** (Ding et al., 1990a), the manganese–tetrakis(4-*N*-methylpyridiniumyl)porphyrin **9** (Bernadou et al., 1989a), and the hybrid cationic metalloporphyrin–ellipticine molecules **10–16** (Ding et al., 1990b), were prepared according to the methods already described. Potassium monopersulfate or Oxone is the triple salt $2KHSO_5$, $KHSO_4$, K_2SO_4 and was obtained from Alfa Ventron. Magnesium monoperoxyphthalate or MMPP was purchased from Aldrich. The double-stranded ϕX174, supercoiled DNA (500 μg/mL) was obtained from Bethesda Research Laboratories. The Tris, Hepes, and disodium and monosodium phosphates for preparation of the various buffers were purchased from Sigma. The electrophoresis-grade agarose was from Bio-Rad Laboratories. Water used for all the solutions was distilled twice.

DNA Cleavage Experiments. (*a*) *ϕX174 DNA Digestion Conditions.* For all the experiments, commercial DNA was diluted in phosphate buffer (5 mM, pH 7.5). The reaction involved 5 μL of ϕX174 DNA (3.5 nM, 18.7 μM in base pairs), 5 μL of metalloporphyrin (**1–9**) or hybrid molecule (**10–16**) in a solution of 50 mM phosphate buffer (pH 7.5), 5 μL of 50 mM phosphate buffer (pH 7.5), and 5 μL of oxygen donor ($KHSO_5$ or MMPP) diluted in the same buffer. The preincubation time of DNA and metalloporphyrin was performed for about 15 min, and the digestion time was 2 min at 20 °C.

The influence of the ionic strength on the cleavage activity was investigated by increasing the concentration of NaCl in the medium. The reactions were carried out as described above but 5 μL of a NaCl solution of various concentrations in 50 mM phosphate buffer (pH 7.5; final concentration 25–500 mM) was used instead of the same buffer in standard conditions.

(*b*) *Electrophoresis.* Metalloporphyrin-mediated or hybrid molecule mediated DNA cleavage was monitored by agarose gel electrophoresis. Reactions were quenched by 5 μL of a "stopping reagent", and samples were kept on ice. The stopping reagent consisted of 250 mM Hepes buffer (pH 7.4) containing 75% glycerol and 0.05% bromophenol blue. It has been checked that the use of 50 mM Hepes (pH 7.4; final concentration in the quenched reaction samples) inhibits completely the cleavage reaction by rapid degradation of the oxygen donor (Bernadou et al., 1989). Reaction mixtures were then run in a 0.8% agarose slab horizontal gel containing ethidium bromide (1 μg/mL) at constant current (25 mA for 15 h) in 89 mM Tris–borate buffer and 2.5 mM EDTA (pH 8.3). Bands were located by UV light (254 nm) and photographed. Quantification of the amount of DNA (forms I, II, and III) was measured by densitometry (Hoefer GS-300).

A correction factor, 1.47 ± 0.30, was used for the decreased stainability of form I DNA vs forms II and III, since supercoiled DNA (form I) has a lower ability to bind ethidium bromide (Bernadou et al., 1989). All form I percentage values

Table I: Nuclease Activity of Manganese-, Iron-, and Zinc-Porphyrin **3**-M in the Presence of $KHSO_5$[a]

compd	form (%) I	II	III
DNA (control)	90	10	0
3-Mn	17	83	0
3-Fe	78	22	0
3-Zn	88	12	0

[a] ϕX174 DNA (18.7 μM in bp) was incubated with 250 nM metalloporphyrin **3** (1 metalloporphyrin/76 bp) at ambient temperature for 2 min in the presence of 5 μM $KHSO_5$.

indicated in the tables are those after correction by this factor of 1.47.

Results and Discussion

Nuclease Activity of Cationic Functionalized Metalloporphyrins **1–8**

Influence of the Incubation Time and Concentration of $KHSO_5$. The influence of the concentration of $KHSO_5$ in the range 1–10 μM on the cleavage activity of manganese-functionalized porphyrins (250 nM), **1–8**, was studied. We observed the decay of supercoiled DNA (form I) as a function of the increasing concentration of $KHSO_5$. No significant breaks were quantified when the DNA was incubated with $KHSO_5$ at 5 μM for 2 min or at 10 μM for 1 min. However, for molecules **1–8**, we noted a higher nuclease activity under the former conditions. Consequently, we chose the following conditions: DNA concentration equal to 18.7 μM (bp) in the presence of 5 μM $KHSO_5$ for a 2-min incubation time.

Influence of the Preincubation Time. When we modified the preincubation period (10, 15, and 20 min) of metalloporphyrin with DNA, no significant difference in the cleavage efficiency could be evidenced. So, we used a preincubation time of 15 min.

Influence of the Nature of the Central Metal Atom. The cleavage activity of metalloporphyrins **1–8** varies as a function of the nature of the central metal atom, as already described for Mn-tetrakis(4-*N*-methylpyridiniumyl)porphyrin (**9**) (Bernadou et al., 1989). Under the same conditions, the manganese complex, **3**, is much more active than its iron analogue, whereas the zinc derivative is completely inactive (Table I). It must be noted that the cytotoxic activity of functionalized metalloporphyrins follows the same order (Ding et al., 1990a). These data strongly suggest that the biological activity of these molecules might be related to redox processes or oxygen atom transfer through the formation of reactive metal–oxo species, as previously suggested in studies on cytochrome P-450 models (McMurry & Groves, 1986; Meunier, 1986).

Influence of the Concentration of Metalloporphyrin Complexes. When the phage ϕX174 DNA was exposed to increased doses of manganese–porphyrin complexes in the presence of 5 μM $KHSO_5$ and 100 mM NaCl, the decay of supercoiled DNA (form I) and the concomitant appearance of relaxed circular DNA (form II) were observed. First of all, the data indicate that the manganese–porphyrin/monopersulfate system cleaves the double-stranded supercoiled ϕX174 DNA by means of single-strand breaks (SSBs), since the 50% decay of form I corresponds to the concomitant appearance of 50% of form II (data not shown). The band corresponding to linear form III can be seen only when the number of SSBs becomes sufficient. So, the double-strand breaks (DSBs) responsible for these linear forms of DNA have to be considered as resulting from the coincidence of two random SSBs. The data related to the influence of the manganese–porphyrin concentration on the cleavage of DNA are reported in Table II. For each concentration of the complex, the various percentages of forms I, II, and III and the corresponding number of SSBs per DNA molecule (S) are indicated. No breaks were observed when the DNA was incubated with $KHSO_5$ only or with the metalloporphyrin molecule alone at the various concentrations. When form I of ϕX174 DNA (18.7 μM bp) was incubated with the indicated concentration of Mn–porphyrin molecules at 20 °C for 2 min in the presence of 5 μM $KHSO_5$ and 100 mM NaCl (Figure 1), we observed a linear relationship between the manganese complex concentration and the number of SSBs per DNA molecule.

Table II: Cleavage of ϕX174 DNA in the Presence of Various Concentrations of Manganese-Functionalized Porphyrin Complexes **1**, **3**, **4**, and **5**, $KHSO_5$ Being the Oxidant

reagent	concn (nM)	form (%)[a] I	II	III	S[b]
DNA (control)	0	90	10	0	0
1	150	22	78	0	3.6
	250	23	77	0	3.5
	500	6	86	8	8.3
	1000	0	64	10	12.5
3	150	37	63	0	2.0
	250	30	70	0	2.6
	500	8	85	7	7.2
	1000	0	66	6	12.0
4	150	58	42	0	0.8
	250	64	36	0	0.6
	500	47	53	0	1.3
	1000	22	73	5	3.6
5	150				
	250	84	16	0	0.1
	500	65	35	0	0.6
	1000	44	56	0	1.5

[a] Form I ϕX174 DNA (18.7 μM in bp) was incubated with the indicated concentrations of metalloporphyrins **1**, **3**, **4**, and **5** at ambient temperature for 2 min in the presence of 5 μM $KHSO_5$ and 100 mM NaCl. [b] S = mean number of single-strand breaks per DNA molecule calculated from eq 1 or 2 with $h = 29$.

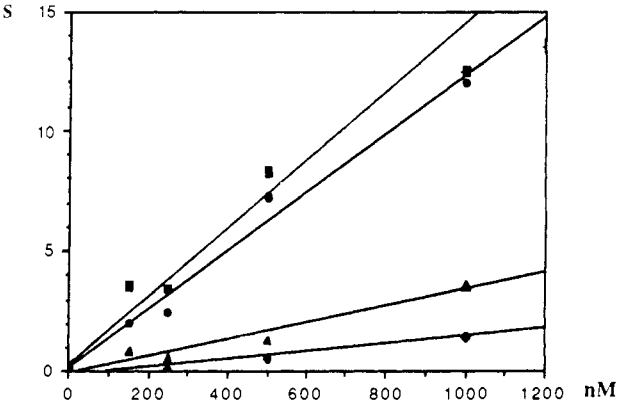


FIGURE 1: Influence of Mn–porphyrins **1** (■), **3** (●), **4** (▲), and **5** (◆) on the cleavage of ϕX174 DNA in the presence of potassium monopersulfate. ϕX174 DNA (18.7 μM in bp) was incubated for 2 min at ambient temperature with the indicated concentrations of metalloporphyrins and 5 μM $KHSO_5$ in phosphate buffer, pH 7.5.

Quantification of DNA Cleavage. The mean number of single-strand breaks S is calculated from either eq 1 (Bernadou et al., 1989) or eq 2 (Hertzberg & Dervan, 1984; Freifelder & Trumbo, 1969) depending on the presence or absence of form III. In the absence of form III release, and with [I] + [II] = 1, S can be written as

$$S = 4 \ln I_0/I + 3(I - I_0) \quad (1)$$

[for a detailed discussion on this equation, which takes into

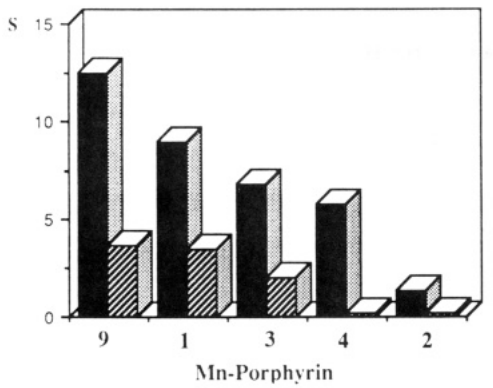


FIGURE 2: Influence of the nature of the oxygen donor [$KHSO_5$ (hatched) and MMPP (solid)] (5 μM) on the cleavage ability of various Mn-functionalized porphyrins, **1–4** (150 nM), incubated with ϕX174 DNA (18.7 μM in bp) for 2 min at ambient temperature.

count the accessibility difference of cleavage sites of form I and II, see Bernadou et al. (1989)].

But, when the cleavage reaction leads to the formation of linear DNA molecules (form III), the number of single-strand breaks S can be calculated (Hertzberg & Dervan, 1984; Freifelder & Trumbo, 1969) from

$$[I] + [II] = \{1 - [S(2h + 1)/2L]\}^{S/2} \qquad (2)$$

where h is the distance between nicks on opposite strands needed to produce linear form III ($h = 29$) and L is the total number of DNA base pairs in ϕX174 (5386 bp).

Influence of the Nature of the Functionalized Aryl Group in the Meso Position of Metalloporphyrin. The DNA cleavage activity of metalloporphyrins **1–8** varies as a function of the nature of the aryl groups at the meso position of the macrocycle. Figure 2 illustrates the variation of SSB per DNA as a function of the structure of metalloporphyrins. The manganese–tris(4-*N*-methylpyridiniumyl)(4-X-phenyl)-porphyrins functionalized by a 4-nitrophenyl (**1**, X = NO_2), 4-(trimethylamino)phenyl (**3**, X = N^+Me_3), or 4-hydroxyphenyl (**4**, X = OH) group have a high cleavage activity ($6.3 < S < 10.5$), comparable to the nuclease activity of the manganese–tetrakis(4-*N*-methylpyridiniumyl)porphyrin derivative, **9**. Surprisingly, we observed a loss of activity for the manganese–tris(pyridiniumyl)(4-aminophenyl)porphyrin, **2**, which also contains three *N*-methylpyridinium groups at the meso position of the macrocycle and three positive charges on the periphery of the porphyrin entity.

For the manganese tris(pyridiniumyl)porphyrins functionalized by one or two ammonium groups via a methylene chain covalently attached to a 4-amino- or 4-hydroxyphenyl group at the meso position of the macrocycle, **5**, **6**, **8**, or for the manganese derivative of the *cis*-bis(4-*N*-methylpyridiniumyl)bis(4-hydroxyphenyl)porphyrin, **7**, we noted a highly reduced nuclease activity. This phenomenon might be attributed to variations of the number and the space distribution of positive charges on the porphyrin periphery.

The comparison of the nuclease activity of porphyrins **9**, **1**, **3**, **4**, and **7** shows that the porphyrin with four pyridinium groups and four positive charges, **9**, is more active than their three pyridinium homologues. The bis(pyridinium) complexes, **7** and **8**, with two positive charges were almost inactive. However, for porphyrins **5**, **6**, and **8** with four positive charges on the porphyrin entity, the loss of cleavage efficiency was presumably due to the presence of functionalized linkers which prevent a good interaction between highly active metal–oxo species and the target on DNA. Finally, it is noteworthy that reducing the number of pyridinium moieties from four to two leads to a decrease of cleavage ability of the cationic metalloporphyrins.

Table III: Influence of the Nature of the Oxygen Donor ($KHSO_5$, MMPP) on the Cleavage Ability of Mn–Porphyrins **1–4**[a]

	S[b]	
reagents	$KHSO_5$	MMPP
1	3.4 (3.6)[c]	9.0 (6.5)[c]
2	0.2 (<0.1)	1.3 (0.4)
3	2.0 (2.0)	6.8 (3.5)
4	0.2 (0.8)	5.8 (2.0)
9	3.6 (12)	12.5 (35)

[a] Form I ϕX174 DNA (18.7 μM) was incubated with Mn–porphyrins **1–4** and with **9** (150 nM) and 5 μM $KHSO_5$ or MMPP at ambient temperature for 2 min. [b] S was calculated from eq 1 or 2 with $h = 29$. [c] Data in parentheses have been obtained in the presence of 100 mM NaCl.

Without additional information on the exact nature of the interactions of these manganese–porphyrin complexes with DNA, it is not possible to go further in this discussion. However, the minor groove might be an important interaction site, since preliminary data indicate that 5-methylene-2-furanone (5-MF) is detected as a sugar degradation product in metalloporphyrin-mediated DNA cleavage (Bernadou et al., 1989b). This product is also observed by Sigman in bis-(phenanthroline)copper-mediated DNA cleavage (Goyne et al., 1987) and results from a hydroxylation reaction at the anomeric C–H bond.

Influence of the Nature of the Oxygen Donor ($KHSO_5$ or MMPP). The cleaving activity of metalloporphyrins **1–4** (150 nM; 1 metalloporphyrin/127 bp) in 40 mM phosphate buffer associated with $KHSO_5$ (5 μM) or MMPP (5 μM) over an incubation time of 2 min with ϕX174 DNA has been compared (Table III; Figure 2). As described elsewhere (Pratviel et al., 1989b), control experiments have shown that MMPP alone does not react with DNA over an incubation time of 10 min.

So, for the same oxygen donor concentration, the MMPP/metalloporphyrin system is more efficient than the $KHSO_5$/metalloporphyrin one. However, the difference in efficiency between the two oxygen donors depends on the structure of the functionalized porphyrins. Thus, for compounds **1** and **3**, as already described for Mn–TMPyP (Pratviel et al., 1989b), the MMPP/metalloporphyrin system is about 3 times more efficient than the $KHSO_5$/metalloporphyrin one, while for **2** this difference is 6 times and for **4** the MMPP system is 25 times more active than the $KHSO_5$ one. This phenomenon could be attributed to electrostatic and/or stacking interactions of the oxygen donor with metalloporphyrins and DNA, which then leads to a difference in the cleavage efficiency of metalloporphyrins with different oxygen donors.

Variation of the Concentration of Salts. The influence of the ionic strength on the cleavage activity of manganese-functionalized porphyrins **1–4** in the presence of $KHSO_5$ and MMPP has been studied (Figure 3).

Under the same conditions, when ϕX174 DNA is incubated with a 150 nM manganese–porphyrin, **1–4** (1 Mn–porphyrin/127 bp), in phosphate buffer (40 mM final concentration) with or without 100 mM NaCl, in the presence of $KHSO_5$ (5 μM) or MMPP (5 μM), (i) we observed a decrease of the cleavage efficiency with MMPP/100 mM NaCl compared to MMPP while with Mn–TMPyP, under the same conditions, we noted an opposite effect (Figure 4), and (ii) with $KHSO_5$ as oxygen donor, no significative modifications are noticeable either in the presence or in the absence

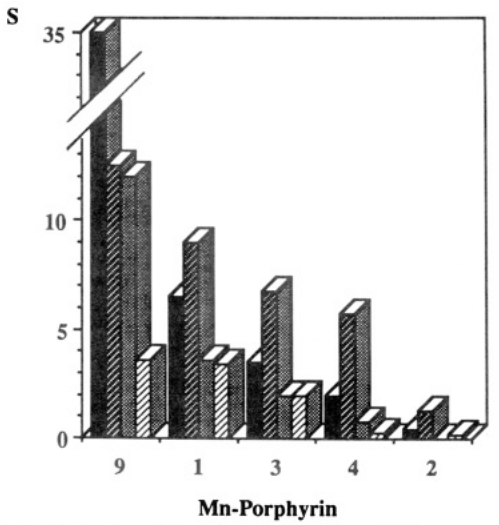


FIGURE 3: Single-strand breaks as a function of the concentration of salts in the cleavage of ϕX174 DNA by the Mn-functionalized porphyrin (**1–4**)/MMPP system. Form I ϕX174 DNA (18.7 μM in bp) was incubated at ambient temperature for 2 min with 150 nM metalloporphyrin and 5 μM MMPP in the presence (solid) or absence (dark hatched) of 100 mM NaCl or 5 μM $KHSO_5$ in the presence (stippled) or absence (light hatched) of 100 mM NaCl in phosphate buffer.

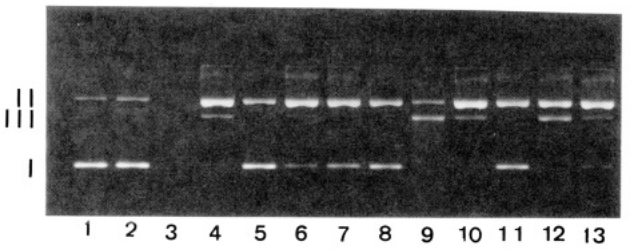


FIGURE 4: Agarose gel electrophoretic pattern of ϕX174 DNA stained with ethidium bromide. Effect of NaCl concentration on the cleavage efficiency of the metalloporphyrin (**1–5** and **9**)/MMPP system. ϕX174 DNA (18.7 μM in bp) was incubated with 250 nM metalloporphyrin and 5 μM MMPP in 40 mM phosphate buffer, pH 7.5, with or without 100 mM NaCl, for 2 min at room temperature. Lane assignment: (1) DNA control, 100 mM NaCl. (2) MMPP control, 100 mM NaCl. Cleavage reaction in the presence of NaCl: (3) **9**; (4) **1**; (5) **2**; (6) **3**; (7) **4**; (8) **5**. Cleavage reaction without NaCl: (9) **9**; (10) **1**; (11) **2**; (12) **3**; (13) **4**.

of 100 mM NaCl, in contrast to the case of Mn–TMPyP which presents maximal cleavage efficiency with $KHSO_5$ at 100 mM NaCl.

So, as already described, MMPP is more efficient than $KHSO_5$ as an oxygen donor. However, beyond 150–500 mM NaCl, a decrease of the nuclease activity is observed for both oxidants and for metalloporphyrin derivatives **1–4**.

Nuclease Activity of Hybrid Cationic Metalloporphyrin–Ellipticine Molecules **10–16**

The standard DNA digestion conditions were the same as for metalloporphyrins **1–9**. Thus, reactions occurred with 3.5 nM ϕX174 DNA (18.7 μM in bp) in 40 mM phosphate buffer, pH 7.5 (final concentration), in the presence of hybrid molecule (**10–16**) and of an oxygen donor such as $KHSO_5$ or MMPP (5 μM) during an incubation time of 2 min. The preincubation time was 15 min.

Influence of the Nature of the Central Metal Atom. When the phage ϕX174 DNA was incubated with hybrid molecule **10** with manganese, iron, or zinc as central metal atom, in the presence of $KHSO_5$ for 2 min, we observed the same order of nuclease activity as for the metalloporphyrin precursors (Table IV). Thus, the manganese derivative was more active

Table IV: Nuclease Activity of the Manganese, Iron, and Zinc Hybrid Molecules **10**–M[a]

compd	form (%) I	II	III
DNA (control)	90	10	0
10–Mn	16	78	6
10–Fe	80	20	0
10–Zn	80	20	0

[a] ϕX174 DNA (18.7 μM) was incubated with 1 μM hybrid molecule **10** (1 hybrid molecule/19 bp) in the presence of $KHSO_5$ at 20 °C for 2 min.

Table V: Cleavage of ϕX174 DNA by Hybrid Molecules **10** and **13** as a Function of the Nature of the Oxygen Donor ($KHSO_5$ or MMPP)

	compd	S^c at hybrid molecule concn (nM) of 250	500	1000	2000	3000	CC_{50} (nM)
$KHSO_5$[a]	**10**	0.1	0.2	0.8	2.0	5.8	1500
	13	2.1	4.1	7.6[d]	11	15	170
MMPP[b]	**10**	2.0[d]	3.5[d]	6.0	9.0	11	170
	13	5.0	5.8[d]	8.5	11	14	120

[a] Form I ϕX174 DNA (18.7 μM in bp) was incubated with 5 μM $KHSO_5$ in the presence of the indicated concentrations of hybrid molecule **10** or **13** at ambient temperature for 2 min. [b] Under the same conditions as for footnote *a*, MMPP being used instead of $KHSO_5$. [c] S = mean number of single-strand scissions per DNA molecule; S was calculated from eq 1 or 2 with h = 29. [d] The indicated value is the average of four independent experiments (in these cases, the standard deviation is 5–10% of the indicated value).

than the iron complex, and the zinc analogue showed no activity (at a concentration of 4 μM for the iron and zinc complexes **10**, a difference in activity is observed in favor of the iron complex). Since the cytotoxic activity (ID_{50}) is also dependent on the nature of the central metal atom and follows the same order, despite the same affinity constant (Ding et al., 1989), we may consider that the biological activity of the hybrid molecules is essentially related to the manganese–tris(pyridinium)porphyrin moiety. The intercalating agent is there to increase the affinity of the molecule for DNA.

Influence of the Nature of the Oxygen Donor ($KHSO_5$ or MMPP). Data to determine the CC_{50} (nM) values of the hybrid molecules, **10** and **13**, are listed in Table V. The CC_{50} is defined as being the hybrid molecule concentration which cleaves 50% of form I after 2 min in the presence of an oxygen donor ($KHSO_5$ or MMPP) and is obtained by plotting the percentage of form I or II versus the hybrid molecule concentration.

Under the same conditions, hybrid molecule **13** (13 bonds, i.e., a molecule with a chain of 13 bonds between the ellipticine and the porphyrin moieties) is more active than **10** (7 bonds). However, the difference in nuclease activity between the two molecules is also highly dependent on the nature of the oxidant. Comparative studies indicate that **13** is almost 9 times more active than **10** in the presence of $KHSO_5$, while the activity ratio becomes only 1.5 in the presence of MMPP. These data suggest that the difference in nuclease activity decreases when the cleavage occurs in the presence of an efficient oxygen donor such as MMPP.

The comparison of the nuclease activity of manganese complex **10** in the presence of $KHSO_5$ (CC_{50} = 1500 nM) and in the presence of MMPP (CC_{50} = 170 nM) indicates that maximal cleavage activity is observed in the presence of MMPP with 40 mM phosphate buffer (in these conditions, MMPP is 9 times more active than $KHSO_5$) (Table V). For manganese complex **13**, this ratio is only 1.5. Since hybrid

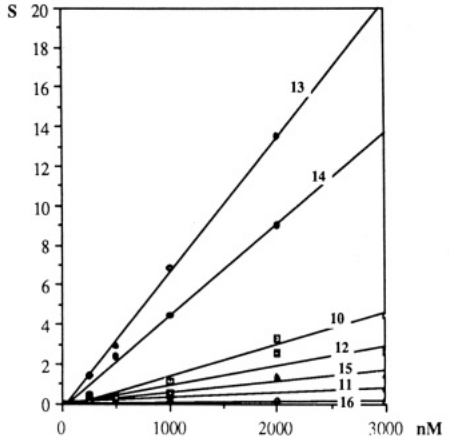


FIGURE 5: Influence of the concentrations of Mn–hybrid molecules **10–16** on the cleavage of ϕX174 DNA (18.7 μM in bp) in the presence of 5 μM $KHSO_5$ with 100 mM NaCl, for 2 min at ambient temperature.

Table VI: Comparative DNA Cleavage Efficiency of Hybrid Molecules **10–15** with Various Lengths of Linkage (7–13 Bonds)[a]

compd	$S^{b,e}$	$S^{c,e}$	$S^{d,e}$
10	0.15	0.42	3.55
11	0.06	0.17	0.42
12	0.23	0.3	1.75
13	1.28	2.75	5.75[f]
14	0.35	1.87	5.0[f]
15	0.28	0.37	1.38

[a] Form I ϕX174 DNA (18.7 μM) was incubated with hybrid molecules **10–15** (500 nM; 1 hybrid molecule/38 bp) and 5 μM of oxygen donor at ambient temperature for 2 min. [b] $KHSO_5$ as oxygen donor. [c] $KHSO_5$ as oxygen donor in the presence of 100 mM NaCl. [d] MMPP as oxygen donor. [e] S = mean number of single-strand scissions per DNA molecule; S was calculated from eq 1 with $h = 29$. [f] S was calculated from eq 2 with $h = 29$.

molecule **13** is more active than **10**, this phenomenon might be explained by the fact that the efficiency of MMPP reaches a plateau compared to that of $KHSO_5$ when the oxygen donor is associated to highly active metalloporphyrins.

Influence of the Concentration of Hybrid Molecules. The data listed in Table V also indicate that the hybrid molecule/$KHSO_5$ (or MMPP) system cleaves the double-stranded supercoiled ϕX174 DNA by means of single-strand breaks (SSBs), similarly to their metalloporphyrin precursors.

Data related to the influence of the concentration of the manganese hybrid molecules **10–16** on the cleavage of DNA are illustrated by Figure 5. No breaks are observed when a hybrid molecule is incubated alone for 2 min at the various concentrations described here. A linear relationship is observed between the manganese hybrid molecule concentration and the number of SSBs per DNA molecule (Figure 5). We also noted that the cleavage efficiency varies as a function of the linker length and of the ionic strength (with or without 100 mM NaCl) (vide infra).

Influence of the Length of the Linker. The DNA cleaving activity of manganese hybrid molecules increases with the length of the linker between the two parts (intercalator and chelating group) of the molecule, according to the following order (Figures 5 and 6, Table VI): **13** (13) > **14** (11) > **10** (10) > **12** (12) ≥ **15** (12) > **11** (8) (the number of single bonds in the linker is indicated in parentheses). This phenomenon might be correlated with a modification of the position of the hybrid molecules with respect to their target(s) since their interactions with DNA is attributed to the intercalation of the ellipticinium moiety between DNA base pairs and a strong interaction of the positively charged manganese–porphyrin

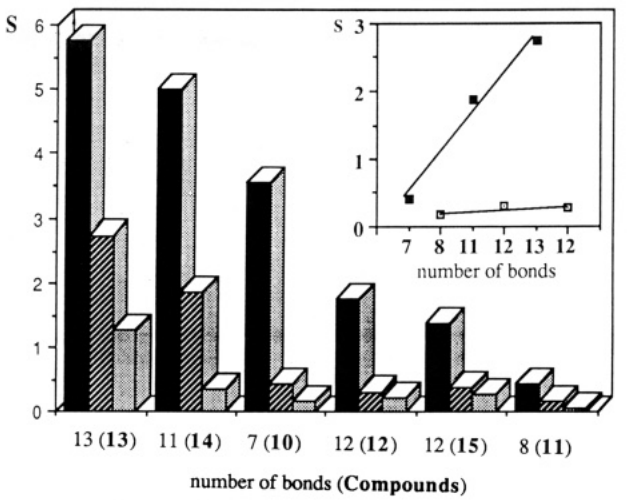


FIGURE 6: Influence of the length of the linker on the DNA cleavage of hybrid molecules **10–15**. ϕX174 DNA (18.7 μM in bp) was incubated with 500 nM hybrid molecules **10–15** (stippled) in the presence of 5 μM $KHSO_5$, (hatched) in the presence of 5 μM $KHSO_5$ with 100 mM NaCl, and (solid) in the presence of 5 μM MMPP, for 2 min at ambient temperature. Inset: single-strand breaks as a function of the number of bonds between the two parts of hybrid molecules **10–15** in the presence of $KHSO_5$ with 100 mM NaCl. (■) Odd and (⊡) even number of bonds.

entity outside the nucleic acid (Ding et al., 1989). Since 5-MF is also observed as the main sugar degradation product, the metalloporphyrin is probably inside of the minor groove of B DNA. Consequently, the longer linkers facilitate the approach of the high-valent manganese–oxo porphyrin species to the target on nucleic acids (viz., $C_{1'}$ or $C_{4'}$ of deoxyribose rings located inside the minor groove).

When we reported the single-strand breaks as a function of the length of the linker as shown in Figure 6, we observed that *hybrid molecules with an odd number of bonds between the two parts of the molecule*, i.e., **10**, **14**, and **13** with 7, 11, and 13 bonds, respectively, *are more active than molecules with an even number of bonds*, i.e., **11**, **12**, and **15**, with 8, and 12 bonds, respectively. These two classes of molecules actually appeared on two distinct curves with a slope ratio of 19 (Figure 6, inset). This strong influence of the linker length on the nuclease activity of these hybrid molecule indicates that the active species, responsible for the DNA cleavage, is not a diffusive entity. In addition, since two possible targets ($C_{1'}$–H bonds) on the same strand are separated by ≈4 Å, one can expect that, if n bonds is the correct number of linkages to adjust a metal–oxo species in front of a C–H bond of a sugar ring, the $n + 1$ molecules will have the metal center away from the target, and again, $n + 2$ will fit with C–H bonds of the next sugar ring.

Influence of the Nature of the Linker Group (Carboxamido or Ether Function). We have compared the nuclease activity of manganese hybrid molecules **12** and **15** having their tris-(pyridinium)porphyrin moiety covalently attached by the same length of linker (12 bonds) to the ellipticinium entity via a carboxamido or an ether group (Figure 7). We observed almost the same number of single-stranded breaks S for both molecules whatever the nature of the oxidant. Consequently, the eventual rigidity of the carboxamido linkage in comparison with the ether function has no noticeable effect on cleavage ability of the hybrid molecules. As to the effect of the chain, the linker length is the main factor controlling the nuclease activity of these hybrid molecules.

As also shown in Figure 6, MMPP appears as a much more efficient oxygen donor than $KHSO_5$. However, the cleavage

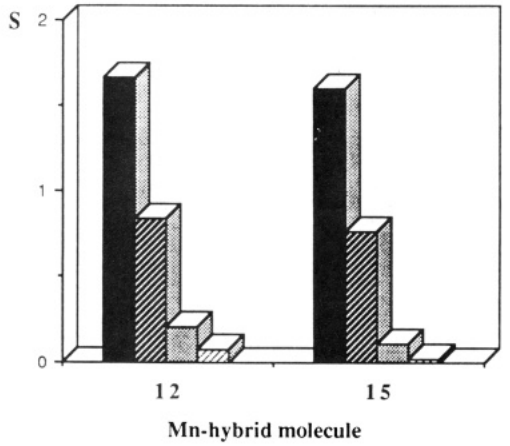


FIGURE 7: Comparative efficiency of Mn–hybrid molecules **12** and **15**, having the same length of linker (12 bonds) but distinguishable only by the nature of the linker group, carboxamido or ether function, on DNA cleavage in the presence of an oxygen donor. Form I ϕX174 DNA (18.7 μM in bp) was incubated at ambient temperature with 250 nM hybrid molecule **12** or **15** and 5 μM $KHSO_5$ (light hatched), 5 μM $KHSO_5$ with 100 mM NaCl (stippled), 5 μM MMPP (dark hatched), or 5 μM MMPP with 100 mM NaCl (solid) for 2 min. S = single-strand scissions per DNA molecule calculated from eq 1.

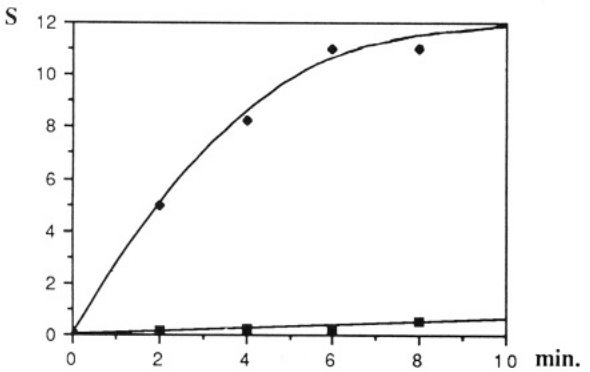


FIGURE 8: Comparative efficiency of Mn–porphyrin–ellipticine **14** (◆) and Mn–porphyrin–bis(ellipticine) **16** (■) (1 μM) on DNA cleavage in the presence of 5 μM MMPP at ambient temperature. The number of single-strand scissions per DNA molecule (S, calculated from eq 1 for **16** and from eq 2 for **14**) was determined as a function of the incubation time (2–8 min).

activity of the Mn–hybrid molecule/$KHSO_5$ system can be increased in the presence of 100 mM NaCl as already described.

Influence of the Number of Intercalator Moieties. The comparison of the nuclease activity of hybrid molecules **14** and **16** having one and two ellipticinium groups, respectively, in the presence of $KHSO_5$ or MMPP as oxygen donor shows a noticeable difference between the two structures (Figure 8). Indeed, **16** shows almost no nuclease activity, whatever the incubation conditions, while the cleavage activity of **14** increases in the presence of MMPP. This phenomenon is tentatively attributed to the loss of a degree of freedom for **16**, which do not allow the right fit of the metalloporphyrin moiety inside of the minor groove.

Variation of the Concentration of Salts. The affinity constant (K_{app}) of hybrid molecule **10** toward polynucleotides (poly[d(A-T)] and poly[d(G-C)]) has been determined as a function of ionic strength and indicates the involvement of two positive charges in the binding process (Ding et al., 1989). We also observed that K_{app} is not modulated by the central metal atom but presents a slight preference for poly[d(A-T)] compare to poly[d(G-C)] (Ding et al., 1989). Moreover, cationic manganese–tetrakis(4-*N*-methylpyridiniumyl)porphyrin (**9**) is known to bind, through electrostatic forces, to the outside of DNA with a significant preference for A-T sequences compared to G-C sequences (Pasternack et al., 1983b; Ward et al., 1986a).

Table VII: Influence of the Concentration of Salts on the Nuclease Activity of Mn Hybrid Molecules **10–16**[a]

hybrid molecule	S			
	$KHSO_5$ alone	$KHSO_5$/100 mM NaCl	MMPP alone	MMPP/100 mM NaCl
10	<0.1	0.2	1.2	1.2
11	0	<0.1	0.4	1.5
12	0.1	0.2	0.8	1.7
13	1.6	2.3	5.0	5.5
14	0.2	0.5	2.7	2.5
15	<0.1	0.1	0.8	1.6
16	0	0	<0.1	0.1

[a] Form I ϕX174 DNA (18.7 μM) was incubated with Mn hybrid molecules **10–16** (250 nM) and 5 μM of oxygen donor at ambient temperature for 2 min. S = mean number of single-strand scissions per DNA molecule. S was calculated from eq 1 or 2 with h = 29.

Consequently, as previously described for **9** (Bernadou et al., 1989), the nuclease activity of these hybrid molecules might be strongly dependent on the salt concentration. Data related to the influence of the ionic strength on their cleavage activity are reported in Table VII.

As seen above, the nuclease activity of hybrid molecules is affected by the length of the linker and the even or odd number of bonds between the two parts of the molecule. So, we studied the influence of ionic strength on the DNA cleavage for the two extreme cases, viz., **11** (8 bonds) and **13** (13 bonds), in the presence of $KHSO_5$ or MMPP in 40 mM phosphate buffer with various concentrations of NaCl (0–500 mM) for 2 min (data not shown).

In general, for hybrid molecules **11–16** in the presence of MMPP, the highest activity is observed with 100 mM NaCl. In addition, when $KHSO_5$ is used as the oxygen donor, maximal cleavage is also obtained in the presence of about 50–100 mM NaCl. However, the addition of NaCl (over 150 mM) decreases the cleavage activity and has an inhibitory effect in both cases.

Therefore, we assume that the influence of the ionic strength on the nuclease activity of hybrid molecules varies as a function of (i) the length of the linker between the intercalator moiety and the metalloporphyrin entity and (ii) the nature of the oxygen donor (MMPP or $KHSO_5$). No clear attribution to a specific factor can be inferred from the present study, but we may assume the existence of an interaction of the hybrid molecule with DNA and with the oxygen donor.

CONCLUSIONS

The hybrid cationic metalloporphyrin–ellipticine molecules and the functionalized metalloporphyrin precursors, associated with a single oxygen atom donor such as $KHSO_5$ or MMPP, may be considered as efficient DNA cleaving systems. The comparative efficiency of these systems on supercoiled ϕX174 DNA enlightens the influence of various structural factors. The fact that manganese porphyrins have the highest nuclease activity compared to their iron and zinc analogues, following the same order as cytotoxicity, can be attributed to a mechanism of DNA breaks involving metal–oxo species, as previously suggested for bleomycin. Consequently, these cytotoxic DNA-cleaver hybrid metalloporphyrin–ellipticine molecules can be considered as efficient bleomycin models based on cationic metalloporphyrins.

Moreover, this study shows that the active moiety of the hybrid molecule is the metalloporphyrin entity and that el-

lipticine only increases the affinity of the molecule for DNA. So, this new series of cytotoxic functionalized metalloporphyrins can be linked easily to various molecules for targeting to specific nucleic acid sequences and may have a real future in DNA footprinting experiments or in pharmacology for the design of new DNA damaging drugs.

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