Cytotoxic Hybrid Molecules 'Metalloporphyrin–Ellipticine' having a High Affinity for DNA

Li Ding,[a] Guita Etemad-Moghadam,[a] Suzy Cros,[b] Christian Auclair,[c] and Bernard Meunier[a]*

[a] *Laboratoire de Chimie de Coordination du CNRS, 205 route de Narbonne, 31077 Toulouse Cedex, France*
[b] *Laboratoire de Pharmacologie et Toxicologie Fondamentales du CNRS, 205 route de Narbonne, 31077 Toulouse Cedex, France*
[c] *Institut Gustave Roussy, Laboratoire de Biochimie, 39 avenue C. Desmoulins, 94805 Villejuif Cedex, France*

Hybrid molecules 'cationic metalloporphyrin–intercalators' have been synthesized, which have a high affinity for double-stranded DNA and are able to cleave it at low concentrations; compound (**2**), an efficient bleomycin model based on a cationic metalloporphyrin, is cytotoxic towards murine leukaemia cells L1210 *in vitro*; iron and zinc analogues of (**2**) are less active, despite having the same affinity for nucleic acids.

Tris-pyridinium water-soluble manganese porphyrins exhibit a cytotoxicity towards murine leukaemia cells L1210 and a nuclease activity on DNA *in vitro*.[1] Attached to an intercalating agent, these tetrapyrrolic chelating molecules should provide good models for bleomycin, which is a glycopeptide antibiotic, antitumour agent, with two distinct structural entities: a bithiazole group for DNA binding and a metal chelating part.[2] The therapeutic activity of bleomycin is generally attributed to its DNA binding properties[3] and its ability to cleave DNA.[4] Such cleavages are mediated by several redox-active metals (*e.g.* iron, copper) which are strongly chelated by bleomycin and when reduced react with molecular oxygen.[2] This unique mechanism makes the modelling of bleomycin an interesting goal.

Here we report a biologically active model of bleomycin based on a metalloporphyrin linked to an intercalating agent (see Figure 1). Previous attempts to prepare bleomycin models by linking hemin to intercalators or ellipticine led to molecules which were non- or poorly-cytotoxic towards cancer cells.[5d] However, the porphyrin ligands used in these hybrid molecules were negatively charged or neutral and data described previously clearly indicate that the periphery of the porphyrin ring has to be positively charged to observe cytotoxic effects on whole cells.[1]

The porphyrin ligand was covalently linked to N^2-(valeric acid)-9-methoxyellipticinium acetate, (**1**).[1,5d] 9-Methoxyellipticine is an antitumour agent, but this pyridocarbazole derivative becomes a poor cytotoxic agent towards murine leukaemia L1210 cells [$ID_{50} > 23$ μM for (**1**)] after its quaternarization by a valeric acid group. However, (**1**) is still a good intercalating agent, with affinity constants of 2.5×10^6 and 4.9×10^6 $dm^3\ mol^{-1}$ for poly d(A–T) and poly d(G–C) respectively.[6]†

The resulting water-soluble hybrid molecule 'manganese-porphyrin–ellipticine' (**2**) is toxic towards L1210 cells. Its ID_{50}

† The binding constant K_{app} was determined as described by Baguley *et al.*,[6] under standard conditions at 25 °C, cacodylate buffer (0.01 M, pH 7.0) in NaCl solutions (10 mM) with ethidium bromide (1.26 μM) and polynucleotide (1 μM). Following the fluorescence of bound ethidium bromide in the presence of various concentrations of (**1**), the IC_{50} (the concentration of tested drug required to displace 50% of ethidium bromide from its binding sites) can be accurately estimated from the best fit to data prints obtained by a non-linear regression procedure. The association value K_{app} (**1**) of the ethidium bromide at the IC_{50} value, and K_{app}EB the association constant of ethidium bromide for the polynucleotide used were 9.5×10^6 and 9.9×10^6 $dm^3\ mol^{-1}$ for poly d(A–T) and poly d(G–C), respectively.

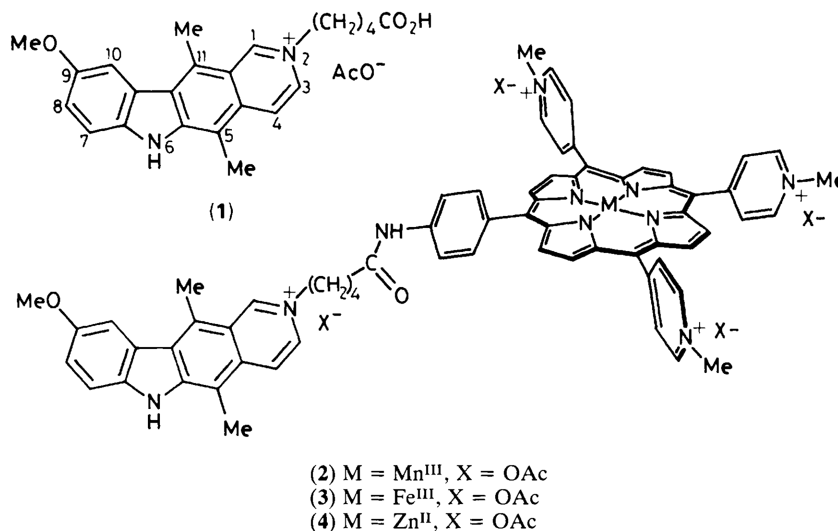


Figure 1. Structures of hybrid molecules of 'metalloporphyrin–ellipticine,' (**2**)—(**4**).

Table 1. Cytotoxicity towards murine leukaemia L1210 cells (ID_{50}), affinity constants for the nucleic acids, and nuclease activity of hybrid molecules (**2**) to (**4**).

	ID_{50}[a] (L 1210) /μM	K_{app}[b]/$dm^3\ mol^{-1}$ poly d(A–T)	poly d(G–C)	Runs	Cleaved supercoiled ΦX174 DNA[c]/% I	II	III
(**1**)	>23	2.5×10^6	4.9×10^6		—	—	—
(**2**)	0.80 ± 0.17	2.3×10^9	2.0×10^8	1[d]	64	36	—
				2[e]	16	78	6
				3[f]	58	42	—
(**3**)	1.61	—	—	4[d]	87	13	—
				5[e]	80	20	—
				6[f]	85	15	—
(**4**)	>7.5	2.0×10^9	1.9×10^8	7[d]	85	15	—

[a] ID_{50}: dose which reduces by 50%, after 48 h, the L1210 cell growth as compared to controls (the standard deviation based on four independent experiments is reported in the case of (**2**); the other ID_{50} values are the mean values of two or three determinations). [b] K_{app}: affinity constant measured by competition of ethidium bromide in NaCl solution (10 mM) with polynucleotides. [c] DNA breaks obtained after activation of the hybrid metalloporphyrins (**2**) and (**3**) by $KHSO_5$. I, II, and III are the various forms of ΦX174 DNA: covalently closed circular (form 1), nicked circular (form II), and linear (form III). [DNA] = 3.5 nM. Pre-incubation time 15 min, incubation time 1 min. For all experiments, the reaction took place in phosphate buffer (40 mM, pH 7.4) (final concentration). [d] [$KHSO_5$] = 10 μM, [hybrid molecule] = 1 μM. [e] [$KHSO_5$] = 10 μM, [hybrid molecule] = 4 μM. [f] [$KHSO_5$] = 5 μM, [hybrid molecule] = 1 μM; NaCl (100 mM) also present.

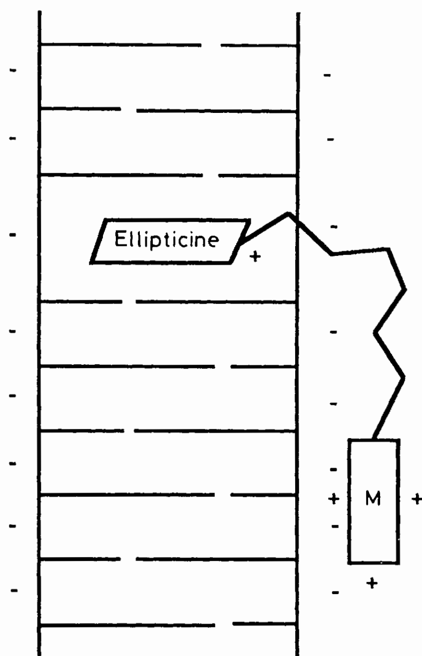
value is 0.80 μM (see Table 1), close to that of the free tris-pyridinium manganese porphyrin alone (ID_{50} = 0.62 μM). It is not possible yet to say if (**2**) is a pro-drug of the metalloporphyrin entity or if the observed cytotoxicity is related to the entire hybrid molecule. However, as seen for tris-pyridinium metalloporphyrin derivatives, the cytotoxicity of hybrid molecules is dependent on the nature of the central atom. The iron derivative (**3**) is less toxic than (**2**) (ID_{50} = 1.6 μM) and the zinc derivative (**4**) has a marginal activity (ID_{50} > 7.5 μM), mainly due to photoactivation.‡

K_{app} values, the affinity constants of manganese- and zinc-hybrid molecules (Table 1) towards polynucleotides, have been determined with poly d(A–T) and poly d(G–C) using the ability of these hybrid molecules to compete with the binding of ethidium bromide to the polynucleotides.[6]† Within the experimental error, the affinity constants are not modulated by the central metal, but a slight preference is observed for poly d(A–T) compared to poly d(G–C). The K_{app} of (**2**) has been determined as a function of ionic strength.[8] Record's plots (log K_{app} *vs.* log [Na^+]) for these data are linear with slopes of 1.94±0.18 and 1.82±0.12 for poly d(A–T) and poly d(G–C), respectively, consistent with the involvement of two positive charges in the binding process.

Viscosimetric studies[9] on calf thymus DNA with (**2**) indicate that the slope value of the plot of log η/η_0 *vs.* log $(1+2r)$§ is 2.2, as expected for a mono-intercalating agent. The ellipticine moiety of (**2**) is presumably intercalated between DNA

‡ For the zinc derivative, the main nuclease activity may be due to the photoactivation of (**3**),[7] rather than to monopersulphate derived species.

§ η and η_0 are the intrinsic viscosity values of sonicated calf thymus DNA in the presence or in the absence of tested drug and r is the number of molecules bound per nucleotide (see ref. 9 for more experimental details on viscosimetric data).



Scheme 1. Interactions of the hybrid 'metalloporphyrin–ellipticine' (**2**) with double-stranded DNA.

base pairs with the positively charged manganese porphyrin entity strongly interacting with hydrophobic and electrostatic contributions, on the outside of the nucleic acid (minor groove?), Scheme 1.

The nuclease activities of (**2**) and (**3**) have been reported in Table 1 (runs 1 to 6).¶ For a concentration of hybrid molecules of 4 μM in the presence of 10 μM $KHSO_5$, and an incubation time of 1 min, 78% of DNA (3.5 nM ΦX174 DNA) is cleaved by (**2**) to the relaxed circular form and 6% to the linear form (run 2). Under the same conditions, the iron analogue (**3**) is less efficient: 80% of form I are still present, with 20% of form II (run 5). The zinc analogue (**4**) is completely inactive (run 7). The increase of the reaction mixture ionic strength, by NaCl (100 mM), increases slightly the cleavage efficiency.

As described above for simple metalloporphyrins, the manganese derivative is a more effective cytotoxin and DNA cleaver than the iron compound, despite having the same affinity constant. These data suggest that the biological activities of these hybrid molecules are related to redox processes or to oxygen atom transfer by the formation of reactive metal-oxo species, as suggested previously for bleomycin.[2]

The data obtained indicate that the two main properties (cytotoxicity and DNA cleavage) of the tris-pyridinium manganese porphyrin moiety are maintained when this pharmacophore is linked to a vector. Work on the linkage of these biologically active water-soluble metalloporphyrins to more sophisticated vectors is in progress. Compared to other chelating agents which have been used as artificial nucleases, one advantage of metalloporphyrins is the absence of metal exchange *in vivo,* which has been confirmed recently for anionic manganese porphyrin derivatives.[10] In conclusion, the tris-pyridinium manganese porphyrin moiety is a cytotoxic DNA cleaver which can be linked easily to various molecules for targetting to specific nucleic acid sequences, and might have a real future in pharmacology for the design of new DNA damaging drugs.

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¶ DNA cleavage studies were performed on supercoiled ΦX 174 DNA (purity: 90%) under the following conditions. *ΦX174 DNA digestion conditions.* For all the experiments, DNA was diluted in phosphate buffer (5 mM; pH 7.4). The reaction involved 5 μl of ΦX174 DNA (3.5 nM), 5 μl of metalloporphyrin solution in phosphate buffer (50 mM, pH 7.4) (1 and 4 μM, final concentrations), 5 μl of phosphate (50 mM, pH 7.4) (runs 1, 2, 4, 5, 7, Table 1) or 5 μl of NaCl solution in the same buffer (100 mM) (runs 3 and 6), and 5 μl of $KHSO_5$ diluted in the same buffer (5 and 10 μM, final concentration). Digestion time was 1 min at 20 °C. The preincubation time of DNA with metalloporphyrin was 15 min. *Electrophoresis.* 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 7.2) containing 75% glycerol and 0.05% Bromophenol Blue. We have checked that 50 mM HEPES buffer (pH 7.2) (final concentration in the quenched reaction samples) degrades more than 90% of $KHSO_5$ in 1 min. Control experiments show no DNA strand scission by the degradation products. Reaction mixtures were then run in a 0.8% agarose slab horizontal gel containing ethidium bromide (1 μg ml^{-1}), at constant current (25 mA for 16 h), in Tris-borate [Tris = tris(hydroxymethylaminomethane) buffer (89 mM, pH 8.3). Bands were located using u.v. light and photographed. The DNA (forms I, II, and III) was quantified by densitometry (Hœfer GS-300). The fluorescence of form I was corrected (correction factor 1.47, standard error 0.3).

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