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Mode of Binding of the Cytotoxic Alkaloid Berberine with the Double Helix Oligonucleotide D(AAGAATTCTT)$_2$[†]

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Dedicated to Prof. Paola Vita Finzi on the occasion of her 70th birthday.

Abstract—Berberine, an isoquinoline plant alkaloid, belongs to the structural class of protoberberines. Recently, the ability of these compounds to act as Topoisomerase I or II poisons, was related to the antitumor activity. The binding of protoberberins to DNA has been studied and the partial intercalation into the double helix has been considered responsible for their activity. We have studied the interaction of berberine with the double helix oligonucleotides d(AAGAATTCTT)$_2$, d(GCGATCGC)$_2$, d(CGTATACG)$_2$, d(CGTACG)$_2$, 5′-d(ACCTTTTTGATGT)-3′/5(ACATCAAAAAGGT)-3′ and with the single strand 5′-d(ACATCAAAAAGGT)-3′, by ^{1}H, ^{31}P NMR and UV spectroscopy. Phosphorus resonance experiments were performed to detect small conformational changes of the phosphoribose backbone, in the case that an intercalation process occurs. Our data reveal that berberine does not intercalate into the duplexes studied, and binds preferentially to AT rich sequences. The structure of the complex with d(AAGAATTCTT)$_2$ was determined by using proton 2D NOESY spectra, which allowed to obtain several NOE contacts between the drug and the nucleotide. Structural models were built up by Molecular Mechanics (MM) and Molecular Dynamics (MD) calculations, by using the inter-proton distances derived from the NOE values. Berberine results to be located in the minor groove, lying with the convex side on the helix groove and presenting the positively charged nitrogen atom close to the negative ionic surface of the oligomer. The large ^{1}H chemical shifts variation, observed for the drug when it is added to the above duplexes, as well as to the single strand oligomer, was interpreted with non-specific ionic interactions. The binding constants were measured by UV and NMR spectroscopy. They are strongly affected by the ionic strength and by the self-association process, which commonly occurs with this type of drugs. A dimerisation constant was measured and the value was included in the calculations of the binding constants. The results obtained show that the non-specific ionic interactions represent the major contribution to the values of the binding constants. These parameters, as well as the protons chemical shift variation of the ligand, are thus not diagnostic for the identification of a drug/DNA complex.

Introduction

DNA Topoisomerases (Topo) represent the molecular target for many anticancer drugs. Two type of enzymes (Topo-I and Topo-II) exist in both prokaryotic and eukaryotic cells and are classified by their mechanism of DNA breakage.[1,2] Fewer Topo-I inhibitors have been discovered than Topo-II inhibitors. The mechanism of action of Topo-II inhibition has been largely studied, but in recent years, after the successful development of the camptothecin derivatives, other classes of compounds have been identified as Topo-I inhibitors and their mechanism of action is the object of intense studies.[3,4] Specifically the role that the drug–DNA interactions play in Topo-I inhibition is still unclear.[3,5] A model of interaction of camptothecin with Topo-I and DNA has been suggested on the basis of the X-ray analysis of the complex DNA/Topo-I,[6] but has not been proven by experimental results.

Synthetic protoberberines are potent poisons of Topo-I enzymes,[4,5] even though some of them affect Topo-II in a similar manner as Topo-I.[4] Berberine (**1**) represents one of the most studied among the naturally-occurring protoberberine alkaloids. Recent results indicate that berberine-induced apoptosis of human leukemia HL-60 cells is associated with down-regulation of nucleophosmin/B23 and telomerase activity.[7] Other notes on the antitumor activity of berberine are reported in ref 8.

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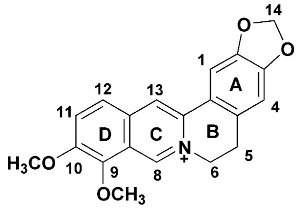
In contrast to camptothecins, protoberberines are known as DNA binders,[4,5] but the mode of binding is unclear. A partial intercalation of berberine into calf thymus DNA was first suggested[9] on the basis of ^{1}H NMR. However a fluorescence spectral studies[10] of berberine with calf thymus DNA suggested a groove binding rather than an intercalation process. Then, results obtained by the competition dialysis method[11] indicated that berberine can bind only to poly(dA)-[poly(dT)]$_2$ triplex DNA, and does not stabilise duplex DNA at all. Finally, a more recent study on four protoberberines[5] revealed that they unwind duplex DNA by approximately 11°, which should be consistent with an intercalative mode of interaction. A model was suggested,[5] which shows a portion of the ligand molecule intercalated into the double helix and another portion protruding into the minor groove. However, none of these studies presents a molecular structure of the complex drug/DNA derived from experimental geometrical parameters.

As a part of our research program on the structure determination of the complex between antitumour agents and oligonucleotides by NMR spectroscopy,[12,14–16] we report here the results of our investigation on berberine **1**. The self-complementary oligomers d(AAGAATTCTT)$_2$ (A_2T_2) and d(GCGATCGC)$_2$ (GATC) were used as models for AT- and CG-rich sequences respectively, in order to find a possible specificity of the interaction. ^{31}P and ^{1}H resonance experiments were performed to derive both the mode of binding and the details of the molecular structure. Specifically, ^{31}P resonance was used in order to detect even small changes in the geometry of the phosporibose chain due to the intercalation process;[12–15] ^{1}H Nuclear Overhauser Effect experiments (NOE) were used to obtain the inter-proton distance values between the ligand and the duplex.



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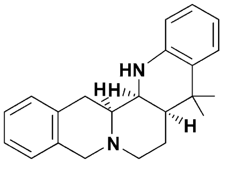

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Results and Discussion

Chemical shift variation of ^{1}H and ^{31}P NMR resonances

The addition of berberine to a solution of the double helix fragment d(AAGAATTCTT)$_2$ (DNA) induces the appearance of new peaks in the ^{1}H spectrum, due to the free and bound berberine protons in fast exchange (Fig. 1). Even at low values of drug/DNA ratio, $R = [\text{drug}]/[\text{DNA}] = 0.5$, the resonances of berberine are broad and move up-field with respect to the free drug, while those of the oligomer are unchanged. With an excess of drug ($R = 2$), also the resonances of the oligomer become broader, but the chemical shifts remain almost unchanged ($\Delta\delta = 0.05$–0.12 ppm). The binding process is fast with respect to the NMR time scale, as no separate signals were observed for the free and the bound species, even at low temperature (5 °C). The resonances of berberine were assigned by standard methods; the chemical shift values and the shift variation of berberine protons, obtained by addition of the drug to several oligonucleotides, are reported in Table 1. Similar Δδ values were found with

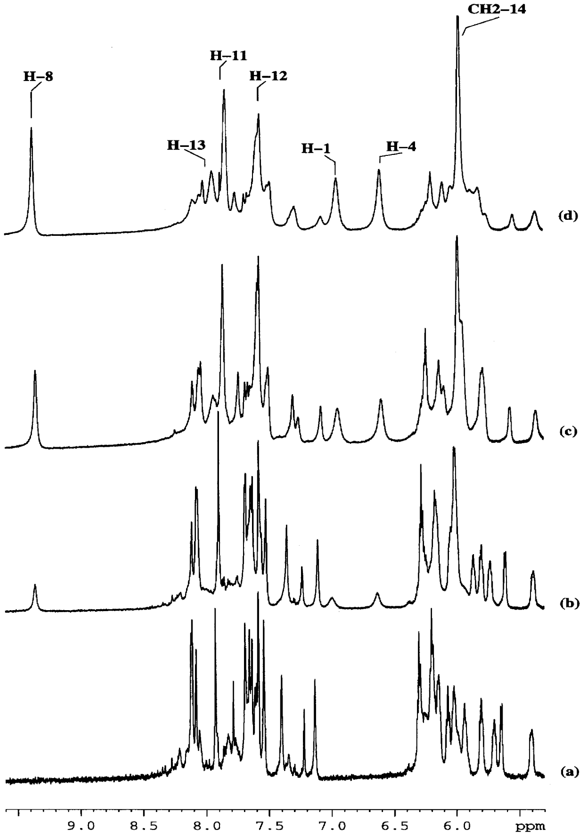

Figure 1. Low field region of 1D ^{1}H NMR spectra acquired at 20 °C, in D_2O, pH 5.8, 0.02 M NaCl, of the oligonucleotide A_2T_2 in presence of berberine at different ratios $R = [\text{drug}]/[\text{DNA}]$: (a) 0; (b) 0.5; (c) 1.0; (d) 2.0.

Table 1. ^{1}H chemical shift assignments for berberine (δ) and shift variation (Δδ) in the presence of several oligonucleotides[a]

		Δδ ($\delta_{\text{bound}} - \delta_{\text{free}}$)[b]					
Drug	Free[b]	A_2T_2	GATC	TATA	TA	T_5GA	A_5[c]
H-1	7.59	−0.54	−0.62	−0.49	−0.50	−0.69	−0.20
H-4	7.00	−0.38	−0.52	−0.38	−0.38	−0.57	−0.27
5-CH$_2$	3.25	−0.24	−0.26	−0.24	−0.24	−0.40	−0.39
6-CH$_2$	4.70	−0.17	−0.22	[d]	−0.19	[d]	−0.28
H-8	9.67	−0.29	−0.35	−0.35	−0.34	−0.50	−0.44
9-OMe	3.95	−0.13	−0.13	−0.18	−0.13	−0.30	−0.20
10-OMe	4.00	−0.15	−0.15	−0.20	−0.19	−0.30	−0.20
H-11	8.08	−0.20	−0.18	−0.23	−0.22	[d]	[d]
H-12	8.00	−0.38	−0.41	−0.40	−0.38	−0.51	−0.65
H-13	8.60	−0.55	−0.62	−0.49	−0.49	−0.67	−0.69
14-CH$_2$	6.11	−0.13	−0.14	[d]	−0.12	[d]	[d]

[a] Measured at 20 °C in D_2O, pH 5.6–5.8, 0.02 M NaCl, $R = [\text{drug}]/[\text{DNA}] = 1$.
[b] δ_{free} were measured at the concentration 5×10^{-5} M.
[c] Measured in absence of NaCl.
[d] Not detected.

AT-rich and CG-rich sequences, and also with shorter oligomers, as d(CGTACG)$_2$ (TA) and d(CGTATACG)$_2$ (TATA). A generalised up-field shift was observed for all protons of berberine, ranging from 0.30 to 0.70 ppm for the aromatic protons and from 0.12 to 0.30 ppm for the aliphatic moiety. Surprisingly, also with the single strand quite similar values were found; only H-1 and H-4 appear less shielded. The single strand 5′-d(ACATCA AAAAGGT)-3′ (ss-A$_5$) is one of the two strands of the non complementary duplex 5′d(ACCTTTTT GATGT)-3′/5′-(ACATCAAAAAGGT)-3′: this segment was selected in order to better compare the interactions with a single strand and with a duplex.

The shift variation effect must be considered as the sum of specific and non specific interactions. The external non specific ionic interactions of the positively charged berberine molecule with the negatively charged ionic surface of the oligonucleotide appear important, as shown by the strong shielding (0.2–0.7 ppm) observed with the single-strand fragment, for which intercalation or groove binding processes are excluded. It is then significant that different sequences gave the same values and that non-charged isoquinoline derivatives[17] as **2** did not show any shift variation. Furthermore, the binding constants decrease with the ionic strength (see below). Since the non-specific ionic interactions appear largely responsible for the proton chemical shift variation of the ligand, we shall not use these data to draw any conclusion about the type of interaction.

In contrast, we performed ^{31}P NMR experiments because the shift variation of phosphate resonances can provide a unique evidence of an intercalation process. The main factor which determines ^{31}P chemical shift variations in nucleotides is the conformation of the phosphodiester groups at the level of the P–O(5′) and P–O(3′) bonds, i.e., the values of the angles $\alpha = \mathrm{O(3')–P–O(5')–C(5')}$ and $\xi = \mathrm{C(3')–O(3')–P–O(5')}$. For a nucleotide in a B-DNA type conformation, the phosphate groups are normally found in the *gauche–gauche* conformation with angles of −60 and −90°, respectively. Even small changes in these angles are reflected in the values of the phosphorus chemical shifts. The intercalating molecule induces a deformation of the phosphodiester chain, such that the phosphates at the level of the interaction sites usually assume a *gauche–trans* conformation with angles of ca. −60 and 180°; this is associated with a low-field shift of ca. 1.5 ppm.[13] We have found a deshielding of 1.3 ppm on C$_5$pG$_6$, when daunomycin is intercalated into d(CGTACG)$_2$;[14] with other anthracyclines of the same family we have found[12,15] values ranging from 0.9 to 1.5 ppm.

The addition of berberine to each of the oligonucleotides examined did not induce significant chemical shift variation of the phosphate signals in the ^{31}P NMR spectra ($\Delta\delta < 0.2$ ppm). We report in Table 2 these data and in Figure 2 the ^{31}P NMR spectrum of the oligonucleotide in absence and in presence of berberine. This result is a first evidence for excluding the intercalation of berberine into the double helix. The reported[5] unwinding angle of 11°, obtained by viscometric studies of protoberberines is probably too approximated and anyway it is significantly lower in magnitude than the unwinding angles found for classical intercalating agents, as also observed by the Authors. Furthermore the viscometric experiments have been performed on salmon testes DNA and might be difficult to compare the results with those obtained with short oligonucleotides.

On the other hand, fluorescence studies[10] of berberine with calf thymus DNA, have suggested that it binds to the double helix DNA with high affinity and the spectra changes are in contrast to an intercalation process.

Structure derivation of the complex of berberine with d(AAGAATTCTT)$_2$

Proton 2D NOESY spectra, performed by applying advanced methodologies for the quantitative analysis of NOE effects,[18] allowed to obtain specific interactions between protons of the ligand and protons of the double helix. NOE data were thus determinant to recognise the interaction site and to define the mode of binding of berberine. First of all, the resonances of the oligomer were assigned, for both the free and bound species, following standard methods (Table 3). The sequential assignment of the nucleotide units was performed by using the inter-residue NOE interactions between the aromatic protons of the bases and the ribose H-1′ and H-2′,2″ of the 5′-neighbour unit which are typical of B-DNA type double helix.[18] The results of the NOE experiments are reported in Table 4. A structural model of the free oligonucleotide was then built by using standard distances and angles for a B-DNA conformation. A minimisation run of this model generates a structure where the distances between the bases and the H-2″ ribose protons are in agreement with those obtained from the NOE data in Table 4. It results that the conformation of the oligomer is not like a classic B-DNA, but is slightly bent, the average twist angle being 37°.

The berberine protons at C-5, C-6, C-8 and those of the methoxy groups, all located on one side of the berberine molecule, show NOE contacts with the H-2 protons of the adenines in the minor groove, whereas H-11 and H-12 on the other side show contacts with the ribose H-1′ of cytidine. The protons on ring A, that is H-1, H-4 and 14-CH$_2$ show NOE interactions with the ribose protons H-4′ and H-5′,5″, which are all overlapped at 3.7–4.4 ppm and consequently have not been assigned. No NOE cross-peak was observed with the imino NH protons. On the basis of the results reported in Table 5, a manual docking of the drug into the minor groove of the helix was performed. Berberine appears with the convex side well adapted in the groove, thus offering the other side toward the sugar ring of cytidine C$_8$, which is actually confirmed by the NOEs involving H-11 and H-12.

The model of the complex was subjected to energy minimisation and restrained molecular dynamics calculations. A minimum number of restraints for the oligomer moiety was applied, that is the hydrogen bonds

Table 2. ^{31}P chemical shift assignments (δ) of the phosphates in the free oligonucleotides and in the complexes with berberine[a]

A_2T_2	Free	Bound	Δδ[b]	TA	Free	Bound	Δδ[b]	TATA	Free	Bound	Δδ[b]
A_1pA_2	−1.31	−1.19	+0.12	C_1pG_2	−1.00	−0.98	+0.02	C_1pG_2	−1.01	−1.00	+0.01
A_2pG_3	−1.00	−0.98	+0.02	G_2pT_3	−1.25	−1.21	+0.04	G_2pT_3	−1.38	−1.43	−0.05
G_3pA_4	−1.14	−1.12	+0.02	T_3pA_4	−1.06	−1.12	−0.06	T_3pA_4	−1.08	−1.07	+0.01
A_4pA_5	−1.40	−1.23	+0.17	A_4pC_5	−1.14	−1.12	+0.02	A_4pT_5	−1.38	−1.43	−0.05
A_5pT_6	−1.37	−1.40	+0.03	C_5pG_6	−0.92	−0.91	+0.01	T_5pA_6	−1.19	−1.25	−0.06
T_6pT_7	(−1.18)	(−1.10)	+0.08					A_6pC_7	−1.19	−1.25	−0.06
T_7pC_8	(−1.11)	(−1.07)	+0.04					C_7pG_8	−0.90	−0.91	−0.01
C_8pT_9	(−1.14)	(−1.10)	+0.04								
T_9pT_{10}	(−1.04)	(−1.07)	+0.03								

[a] Spectra acquired at 20 °C in D_2O, pH 5.6–5.8, 0.02 M NaCl. Similar values in parentheses may be interchanged.
[b] $\Delta\delta = \delta_{bound} - \delta_{free}$.

between the base pairs and the inter-proton distances between aromatic and ribose H-1′ protons derived from the NOE data (Table 4), while the restraints for the drug moiety are the distances derived from the inter-molecular NOE contacts reported in Table 5. In this way the double helix was allowed to move freely. A final structure, obtained by energy minimisation of the last 22 structures of the 47 ones obtained, is reported in Figure 3. The berberine molecule is thus located in the minor groove of the double helix of the oligonucleotide, at the level of A_4–T_7 and A_5–T_6 base pairs. It lies with the convex side on the helix groove, thus presenting the positive nitrogen atom close to the negative ionic surface. This proves how important are the ionic interactions between the drug and the ionic surface of the oligonucleotide for the formation of the complex. The ring A and the methylenedioxy group are external to the helix (Fig. 3b), while the aromatic protons H-11 and H-12 are close to the ribose of cytidine C_8 (Fig. 3a).

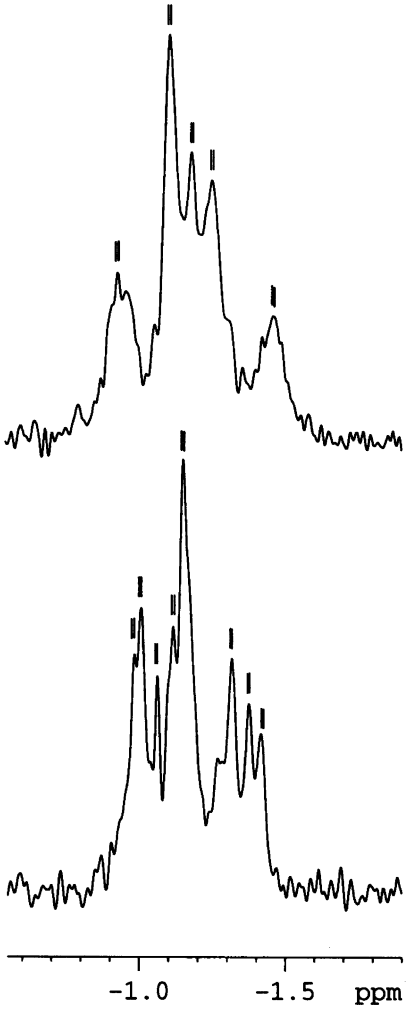

Figure 2. ^1H-decoupled ^{31}P NMR spectra acquired at 20 °C, in D_2O, pH 5.8, 0.02 M NaCl, of the oligonucleotide A_2T_2 in absence (a) and in presence (b) of berberine R = [drug]/[DNA] = 1.0.

Figure 3 might suggest that the complex presents in solution one single structure, which is not true. Small motions are allowed along the phosphodiester chains, thus many slightly different conformations exist in solution and the reported structure must be considered as a weighted average of all these conformations. In order to have an insight into this conformational equilibrium, we performed a more detailed analysis of the MD calculations at 600 K: every 2 ps a conformation was sampled and the torsion angles α, ξ, relative to each phosphate, δ and χ, relative to the ribose moieties, were measured and plotted as a graph (not reported here). δ is C(5′)–C(4′)–C(3′)–O(3′), χ is O(4′)–C(1′)–N(1)–C(2) for pyrimidine units and O(4′)–C(1′)–N(9)–C(4) for

Table 3. ^1H chemical shift assignments (δ) for $d(AAGAATTCTT)_2$ in the complex with berberine[a]

Residue	H-8	H-2	H-6	H-5	H-1′	H-2′	H-2″	H-3′
A_1	7.84	(7.69)			5.82	2.80	2.53	4.98
A_2	8.02	(7.67)			5.82	262	2.43	5.01
G_3	7.57				5.36	2.61	2.46	4.91
A_4	8.05	7.29			5.97	2.87	2.63	4.97
A_5	8.10	7.76			6.10	2.80	2.53	4.98
T_6			7.08		5.82	2.37	1.94	4.95
T_7			7.29		6.04	2.41	2.32	4.87
C_8			7.51	5.54	5.97	2.28	2.10	4.96
T_9			7.49		6.10	2.44	2.28	4.89
T_{10}			7.57		6.19	2.70	2.54	4.89
Base pair	Imino protons[b]							
G_3:C_8	12.20							
A_4:T_7	13.55							
A_5:T_6	13.45							

Similar values in parentheses may be interchanged.
[a] Measured at 20 °C in D_2O, pH 5.8, 0.02 M NaCl.
[b] Measured at 15 °C in H_2O/D_2O, pH 5.7, 0.02 M NaCl. NH protons of A_1:T_{10} and A_2:T_9 base pairs were not detected because of the 'fraying' process.[24]

Table 4. Inter-proton distances (Å) for d(AAGAATTCTT)$_2$ oligonucleotide, free and bound to berberine

	Free		Bound	
	Exp.[a]	Calcd[b]	I[c]	II[c]
H-8A$_2$···H-1′A$_1$	3.41	3.10	3.57	3.47
H-8A$_2$···H-2′A$_1$	2.52	2.95	3.07	5.11
H-8G$_3$···H-1′A$_2$	3.22	3.03	3.61	3.66
H-8G$_3$···H-2′A$_2$	2.70	2.42	2.23	2.20
H-8A$_4$···H-1′G$_3$	3.12	3.12	3.57	3.56
H-8A$_4$···H-2′G$_3$	2.12	2.26	2.95	2.62
H-8A$_5$···H-1′A$_4$	2.82	3.12	3.66	3.67
H-8A$_5$···H-2′A$_4$	2.23	2.24	2.57	2.16
H-6T$_6$···H-1′A$_5$	3.11	3.13	3.61	3.53
H-6T$_6$···H-2′A$_5$	2.70	2.39	2.18	2.92
H-6T$_7$···H-1′T$_6$	3.20	3.17	3.56	3.39
H-6T$_7$···H-2′T$_6$	2.34	2.27	2.38	3.60
H-6C$_8$···H-1′T$_7$	2.81	3.11	3.74	2.56
H-6C$_8$···H-2′T$_7$	2.33	2.30	2.42	3.10
H-6T$_9$···H-1′C$_8$	3.27	3.18	3.65	3.59
H-6T$_9$···H-2′C$_8$	2.21	2.35	2.24	2.27
H-6T$_{10}$···H-1′T$_9$	2.67	3.16	3.63	3.58
H-6T$_{10}$···H-2′T$_9$	2.02	2.26	3.03	3.23

The distances in the two strands I and II are different because the C_2 symmetry is destroyed by the binding to berberine.

[a] Obtained from NOE experiments.

[b] Obtained from the model of A$_2$T$_2$.

[c] Obtained from the model of the complex in Figure 3.

purine units. In this way it was possible to recognise the most populated conformations, which are in the *gauche–gauche* range for all phosphate*s* ($\alpha = 60$–$80°$, $\zeta = 90$–$100°$). The δ angles display values between 124 and 150°, coupled with glycosidic angles χ of 230–260° in agreement with the anticorrelation principle;[19] this corresponds to the *anti* orientation of the bases and to the ribose conformations within C-2′-*endo* and C-1′-*exo*.

Table 5. Inter-molecular NOE interactions and inter-proton distances between berberine and oligonucleotide protons in the complex with d(AAGAATTCTT)$_2$

Berberine protons[a]	Nucleotide protons[a]	Distances(Å)[b]	
		I	II[c]
H-8	H-2 A$_5$ (*m*)[d]	2.41	5.50
H-8	H-1′ T$_7$ (*w*)	7.16	4.22
H-11	H-1′ C$_8$ (*w*)	16.04	2.92
H-12	H-1′ C$_8$ (*m*)	14.35	4.26
6-CH$_2$[e]	H-2 A$_5$ (*m*)	3.70	2.95
5-CH$_2$[e]	H-2 A$_5$ (*m*)	2.60	4.34
9-OCH$_3$	H-2 A$_5$ (*w*)	4.16	7.79
10-OCH$_3$	H-2 A$_4$ (*w*)	3.72	14.78
H-1	H-4′ T$_7$	3.46	2.80
	H-5′ T$_7$[f]	4.10	4.07
H-4	H-4′ T$_6$,	7.79	3.11
	H-4′ C$_8$,[f]	3.77	10.32
	H-5″ C$_8$	2.25	10.52
14-CH$_2$	H-4′ T$_7$,	4.13	5.45
	H-5′ T$_7$[f]	6.19	4.07

[a] Acquired at 20 °C, in D$_2$O, pH 5.8, 0.02 M NaCl.

[b] Obtained from the final structure of the complex after MD calculations. The distance between H- 5 and H-6 proton of cytidine (2.45 Å) was taken as reference.

[c] Strand I and strand II, as in Figure 3.

[d] Intensity of the NOE peaks, as quantified by Felix program and transformed in inter-proton distances: 1.0–2.5 Å (*s*), 2.5–3.5 Å (*m*), 3.5–5.0 Å (*w*).

[e] Average value for the two methylene protons.

[f] NOE cross-peaks with H-4′,5′,5″ were detected but all protons are overlapped, consequently they were not assigned.

The oligonucleotide moiety thus maintains in the complex the conformation of a B-DNA, just slightly bent as in the free state. This is also shown by the inter-proton distance values reported in Table 4 and by the average twist angle of the double helix in the complex (40°), which corresponds to an unwinding angle of only 3°. Finally we observed an increase of the minor groove width at the level of A$_4$ and A$_5$ units from 7.7–7.9 to 10–11 Å, respectively. The minor groove width is defined by the distance between C-1′ of A$_4$ and C-1′ of C$_8$ on the complementary strand. This finding is expected in the case of a minor-groove binder.

Computer model studies, performed on poly[d(A-T)]$_2$ by Pilch et al.,[5] have suggested that the interaction between protoberberines and DNA can be described by a partial intercalation of the ligand molecule (isoquinoline portion) into the base pairs, while rings A and B protrude into the minor groove of the host duplex. In order to verify if our experimental data could be consistent also with this mode of binding, we inserted the berberine molecule in different orientations, with the isoquinoline moiety intercalated between the base pairs at the level of adenines A$_4$, A$_5$ and with the rings A and B protruding in the groove of the helix. In all these cases, significant contacts occur between the aromatic protons of rings C and D, that is H-11,12,13 with H-2 of adenine A$_4$, and consequently strong NOE peaks should appear in the NOESY spectra. Since these NOE interactions are absent in our spectra, but in contrast the same protons showed NOE contacts with another base (C$_8$), we again can exclude this intercalation mode of binding for berberine. In addition, it must be observed that all the NOEs between the aromatic protons of the bases and the ribose protons of the 5′-neighbour unit, typical of a B-DNA type conformation, were also found in the complex, as reported in Table 4. In the presence of an intercalating agent the sequence of these characteristic NOE interactions breaks off at the level of the bases involved in the intercalation, just as observed for anthracyclines.[12]

That berberine behaves as a minor groove binder, is also indirectly shown by the experiments with the CG-rich oligomer d(GCGATCGC)$_2$: although the ionic interactions are very similar to those of the AT-rich oligomer above described, the NOESY spectra showed very few contacts (with C$_2$, A$_4$ and T$_5$), which did not allow to build a model of the complex. Experiments with the oligomer d(CGTACG)$_2$ were also unsuccessful. The CG-rich sequences actually present minor grooves of smaller dimension with respect to those of AT-rich sequences, thus making more difficult the accommodation of the ligand. Furthermore the AT-rich minor groove has a relatively negative electrostatic potential,[20] that significantly enhances its ability to bind positively charged molecules. In the case of d(GCGATCGC)$_2$, the NH$_2$ group of guanine G$_3$ creates a steric hindrance for a stable binding to the AT site.

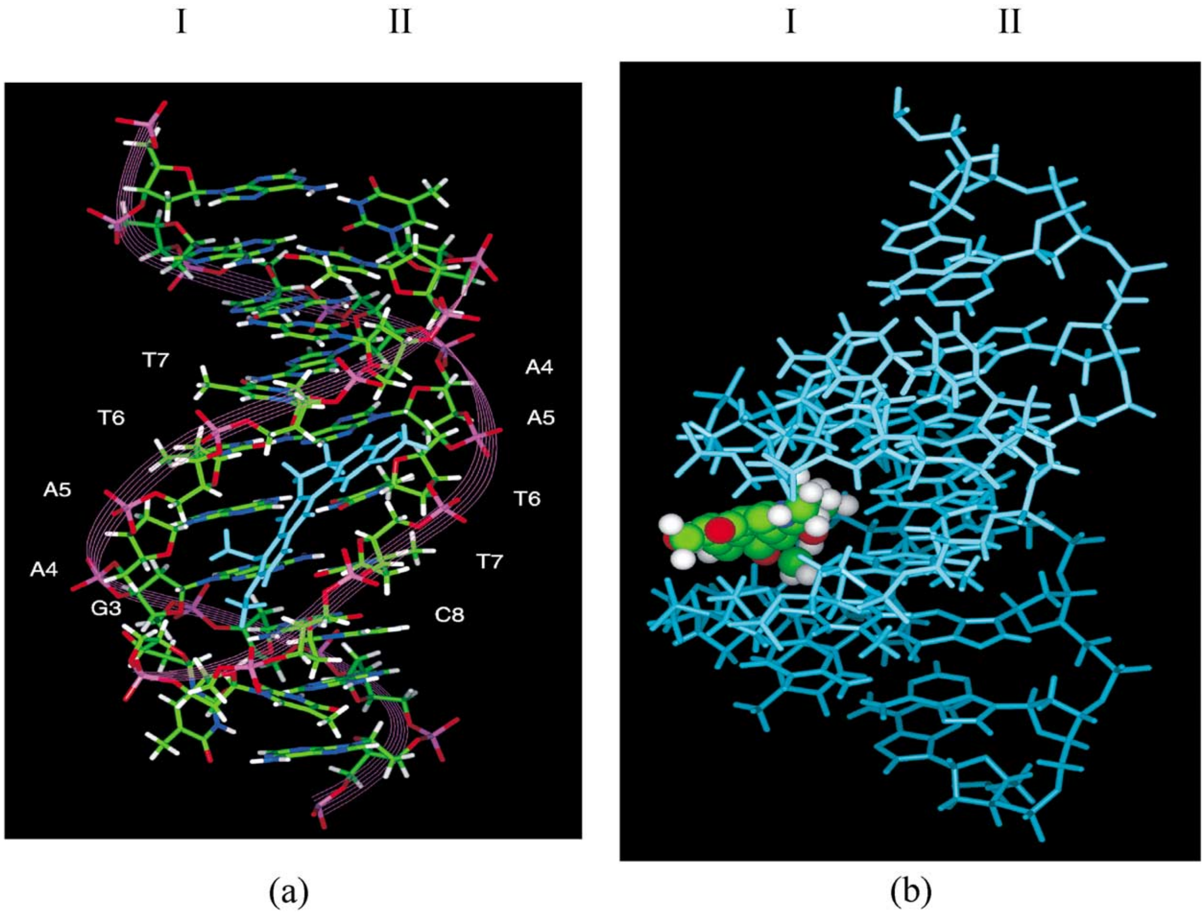

Figure 3. Energy minimized molecular model of the complex between berberine and the oligonucleotide A_2T_2. The C_2 symmetry of the double helix is destroyed in the complex by the presence of the ligand; thus the two strands are non-equivalent and are labelled I and II (strand on the left and on the right side, respectively): (a) frontal view; (b) view of the berberine located in the minor groove.

Binding constant and self-association constant

The binding of berberine to calf-thymus DNA has been studied by UV spectroscopy and fluorescence quenching experiments.[10] However, no binding constant value was reported, as too small hypochromism and no frequency shift were observed in the experimental conditions used. Thus we measured, by UV spectroscopy, the binding constants relative to the interaction of berberine with $d(AAGAATTCTT)_2$, $d(GCGATC\ GC)_2$ and with the single strand 5′-d(ACATCAA AAAGGT)-3′ (Table 6). Since other equilibria may affect the binding of these drugs to DNA, such as the melting of the double helix, the self-aggregation of the drug and the non specific ionic interactions as well, we have considered these processes.

The melting equilibrium of the double helix depends on the temperature and on the salt concentration. This can easily be monitored by NMR spectra, following the frequencies of the ribose (H-1′) and of the base protons. In the case of the duplexes studied, the melting process can be neglected in the experimental conditions used (25 °C and 0.02 M NaCl), because they were entirely (95%) as double helices. We also performed UV melting experiments, in order to see whether berberine stabilises or not the double helix of a short DNA fragment: the melting temperature of $d(AAGAATTCTT)_2$ (39 °C) increases of 3 °C in presence of berberine. The stabilisation is thus poor or null as found[16] for tallimustine, a minor groove binder of the distamycin-A family.

Table 6. Association constant values (K) for the interaction berberine/oligonucleotides[a]

Oligonucleotide	K^b (M^{-1})	K^c (M^{-1})
$d(AAGAATTCTT)_2$	$(2\pm0.2)\cdot10^4$	
	$(1\pm0.1)\cdot10^{4d}$	$(1\pm0.1)\cdot10^4$
	$(7\pm0.5)\cdot10^{3e}$	
$d(GCGATC\ GC)_2$	$(1\pm0.1)\cdot10^4$	$(9\pm0.1)\cdot10^3$
5′-d(ACATCAA AAAGGT)-3′	$(1\pm0.1)\cdot10^3$	$(1\pm0.1)\cdot10^3$

[a] pH 5.8, 0.02 M NaCl, 20 °C unless specified. The values were derived from the experimental data by solving a system of a non-linear equations and using MATLAB software (v. 5.1). The dimerization constant $K_D = 1.0 \times 10^5 M^{-1}$ was included in the calculations. The values obtained are in line with those reported for the interaction of coralyne with calf thymus DNA ($1.25 \times 10^4 M^{-1}$),[11] and of palmatine with salmon DNA ($7.9 \times 10^4 M^{-1}$)..[5]

[b] Obtained by UV.

[c] Obtained by ^1H NMR.

[d] 0.1 M NaCl.

[e] 1 M NaCl.

In order to estimate the importance of the self-aggregation process, dilution experiments were performed by UV spectroscopy in the concentration range 10^{-5}–10^{-7} M. The value of the dimerisation constant thus obtained, $K_D = (1 \pm 0.1) \cdot 10^5\,M^{-1}$ is in agreement with that reported[21] for coralyne, a planar isoquinoline alkaloid similar to berberine. Then, titration experiments with the above said oligomers were performed by means of both UV and NMR spectroscopy. When the dimerisation constant K_D is included in the calculations of the binding constants K (see Experimental), the value of K decreases of one order of magnitude; consequently the aggregation process may significantly affect the results. UV titration experiments with d(AAGAATTCTT)$_2$ oligomer were performed at different salt concentration, in order to evaluate the ion effects. The binding constant significantly decreases with the ionic strength. That the K values obtained with the CG-rich and the AT-rich oligomers are approximately the same also indicates that the non-specific ionic interactions represents the major contribution to the value of the binding constants. The experiment performed with the single strand 5′-d(ACATCAAAAAGGT)-3′ shows a binding constant of one order of magnitude lower, because the charge density on the single strand is lower than in the duplex.

Conclusions

The interaction of berberine with several oligonucleotides, studied by NMR spectroscopy, showed that berberine binds preferentially to AT-rich sequences. Phosphorus resonance spectra and NOE experiments allowed to exclude the intercalation of berberine into the double helix, claimed by some authors[5] on the basis of viscometric studies. On the contrary it binds to the minor groove of duplexes rich in AT. 2D NOE experiments allowed to detect several contacts between protons of berberine and protons of the self-complementary oligomer d(AAGAATTCTT)$_2$. With the inter-proton distances thus derived, a structural model of the complex was obtained. The berberine molecule is located in the minor groove of the double helix of the oligonucleotide, at the level of A_4–T_7 and A_5–T_6 base pairs. It lies with the convex side on the helix groove, thus presenting the positive nitrogen atom close to the negative ionic surface. The ring A and the methylenedioxy group are external to the helix, while the aromatic protons H-11 and H-12 are close to the ribose of cytidine C_8. The oligonucleotide moiety maintains in the complex the conformation of a slightly bent B-DNA. The sequence of the characteristic NOE interactions is not broken off by the presence of berberine ligand, as occurs with intercalant molecules. This is a further evidence for excluding an intercalation process.

The non-specific ionic interactions between the positively charged drug molecule and the negatively charged ionic surface of the nucleotide play an important role in the binding process, but they are not sufficient for the formation of the complex. This is proved by the experiments with the CG-rich oligomer d(GCGATCGC)$_2$: although the ionic interactions are very similar to those of the AT-rich oligomer d(AAGAATT CTT)$_2$, only a few and weak NOE signals were detected. The non-specific ionic interactions are responsible of the large chemical shift variation observed for the protons of the drug, when solutions of drug and nucleotide are mixed. This occurs with both AT-rich and CG-rich duplexes, as well as with a single strand oligonucleotide. The binding constants measured by NMR, and also by UV spectroscopy are strongly affected by the self-association phenomenon, which is significant for berberine (the dimerisation constant K_D is $(1 \pm 0.1 \times 10^5\,M^{-1})$. The binding constant values, obtained with the AT- rich and with the CG-rich oligomer are of the same order of magnitude, $K = 10^4\,M^{-1}$, and are comparable to the value found for the single oligomer 5′-d(ACATCAAAAAGGT)-3′($K = 10^3\,M^{-1}$), taking into account that the single strand has a lower charge density than the duplex. This means that the non-specific ionic interaction process represents the major contribution to the values of the binding constants. Consequently these parameters, as well as the proton chemical shift variation of the ligand are not diagnostic for the identification of a drug–DNA complex.

It has been suggested[5] by a computer model study that the interaction of the drug with the enzyme occurs in the minor groove of the host DNA duplex. If this is true (any experimental evidence is lacking), the ability of berberine to act as a minor groove binder might explain the inhibition activity on Topo-I enzyme. However, other related questions require an answer, for instance why molecules, which are not groove binders are Topo-I poisons. The mechanism of this process is quite complex and additional studies are needed in order to understand the nature of the drug–DNA–enzyme interactions.

Experimental

Oligodeoxynucleotides, synthesised by solid phase, and berberine chloride were purchased from Roche-Diagnostics and Fluka respectively.

NMR experiments

The NMR spectra were recorded on a Bruker AMX 600 spectrometer operating at a frequency of 600.13 MHz for ^{1}H nucleus and at 242.94 MHz for ^{31}P nucleus. The chemical shifts (δ) were measured in ppm and referenced for the protons to the residual water signal set at 4.78 ppm, and for ^{31}P resonances to external methylenediphosphonic acid (MDA). Estimated accuracy is within 0.03 ppm for protons and within 0.05 ppm for phosphorus. D_2O and H_2O–D_2O (90:10 v/v) were used as solvent. The oligonucleotides were dissolved in presence of a minimum amount of NaCl (0.02 M) in order to stabilize the double helix. pH was adjusted to 5.6–5.8 and the final concentration resulted in the range 1–3 mM. As berberine is not soluble enough in water, the NMR titration experiments were performed by adding increasing amounts of a 12 mM solution of berberine dissolved in DMSO-d_6 to the oligonucleotide solution.

NOESY spectra were acquired in the phase sensitive TPPI mode, with 2K × 512 complex FIDs, spectral width of 6666.667 Hz, recycling delay of 1.3 s, 72 scans, at temperature of +20 and 15 °C. Mixing times from 50 to 300 ms. TOCSY[22] spectra were recorded with the use of MLEV-17 spin-lock pulse (field strength 7576 Hz, 60 ms total duration). All spectra were transformed and weighted with a 90° shifted sine-bell squared function to 1K × 1K real data points. Water suppression was achieved by presaturation technique, placing the carrier frequency on the H_2O resonance. In the case of H_2O/D_2O solutions, the spectra were measured at 15 °C by using gradient-based pulse programs, capable of suppressing the water signal and minimise the magnetisation loss due to saturation transfer.

The assignment of proton resonances for berberine and for the oligonucleotides, free and bound, were performed by using NOESY and TOCSY experiments. The sequential assignment of nucleotide units in the free nucleotide and in the complex was performed by applying well established procedures for the analysis of double stranded oligomers in the B-DNA form.[18] TOCSY experiments provided the easy detection of thymine 5-Me/H-6 and cytidine H-5/H-6 spin systems, as well as all intra-ribose connectivities. The inter-residue NOEs between the aromatic protons of the bases (H-5 for thymine and H-8 for purines) and the ribose H-1′ and H-2′,2″ of the 5′-neighbour unit allowed to establish the sequential assignments. H-3′ protons were assigned via TOCSY with H-2′,2″. The aromatic H-2 protons of the adenines were assigned by means of the NOE interactions with the imino NH protons of the same AT base-pair. The assignments of NH protons for d(CGTATACG)$_2$[23] and for 5′d(ACCTTTTTGATGT)-3′/5′-(ACATCAAAAAGGT)-3′[16] have previously been reported. For d(AAGAATTCTT)$_2$, the imino NH of the sole guanine was easily recognised by the NOE with NH_2 protons of the complementary cytidine, which in turn were assigned by the NOE with H-5 of the same base. Other two imino protons were detected, those of the base pairs A_4:T_7 and A_5:T_6, attributed on the basis of the NH–NH inter-residue NOE interactions with G_3 and A_4, respectively. Those of the two terminal base pairs were missing, because the chain terminals are more flexible and the 'fraying' process usually occurs at this temperature in the terminal part of the double helix,[24] leading to a fast exchange of the NH protons with the solvent.

The assignment of phosphate groups in the free DNA and in the complex with berberine was performed by 2D ^{1}H–^{31}P HMBC NMR experiments. The heteronuclear spectra were acquired using the pulse program with gradients present in the XWINNMR software library. 80 × 1K FIDs were collected, a spectral width of 2600 and 243 Hz was used for ^{1}H and ^{31}P respectively, 332 scans, at temperature 20 °C. All spectra were transformed and weighted with a EM function (2 Hz) to 1K × 512 real data points. This experiment has been used to correlate the H-3′ ribose protons of DNA with the phosphorus nucleus of the 3′-neighbour unit, which are connected by three-bond coupling. The phosphates of d(CGTACG)$_2$ have already been assigned.[14]

The binding constants (K) for the nucleotide–drug interaction were obtained by both ^{1}H NMR and UV spectroscopy. The NMR titration experiments were performed as described above by following the shift variation of H-8 and H-1.

UV experiments

The UV spectra were recorded at 25 °C on a Perkin-Elmer Lambda 40 UV–vis spectrophotometer and at variable temperature for the melting experiments, on a JASCO W-550 spectrophotometer equipped with a thermostated cell. Melting of the DNA in absence and in presence of berberine was followed through UV absorbance. Dilution experiments on berberine were performed starting from a concentrated solution (10^{-5} M), which was diluted to 10^{-7} M, in presence of 0.02, 0.1 and 1 M NaCl. For the aggregation process a dimerisation model, eq 1, was considered to be a sufficient approximation,[21,25] thus the dimerisation constant, K_D, was calculated by using eqs 2–4:

$$2\ \text{drug} \overset{K_D}{\rightleftarrows} (\text{drug})_2 \tag{1}$$

$$K_D = x_D/\left(x_M^2[\text{drug}]_0\right) \tag{2}$$

$$x_M = (\lambda_{obs} - \lambda_D)/(\lambda_M - \lambda_D) \tag{3}$$

$$x_M + x_D = 1 \tag{4}$$

where $[\text{drug}]_0$ is the total concentration of berberine, λ_{obs} is the observed UV wave length, λ_M, λ_D, x_M and x_D are the wave lengths and the molar fractions of the monomeric and dimeric species respectively.

The binding constants (K) for the nucleotide–drug interaction were obtained by titration experiments, performed by adding increasing amounts of a 4.0×10^{-6} M solution in water of the drug to a 1.5×10^{-5} M solution of the oligonucleotide. The inverse experiment was also performed, that is the addition of a solution of the oligonucleotide to the solution of the drug, obtaining the same results. Then the binding constants K were calculated by considering the following equilibria, eqs 5 and 6:

$$2\ \text{drugs} \overset{K_D}{\rightleftarrows} (\text{drug})_2 \tag{5}$$

$$\text{duplex} + \text{drug} \overset{K}{\rightleftarrows} \text{duplex} - \text{drug} \tag{6}$$

The binding constant is thus defined by eq 7 :

$$K = [\text{duplex} - \text{drug}]/[\text{duplex}][\text{drug}] \qquad (7)$$

The concentrations of the molecular species are related as follows:

$$[\text{duplex}]_0 = [\text{duplex}] + [\text{duplex} - \text{drug}] \qquad (8)$$

$$[\text{drug}]_0 = [\text{drug}] + \left[(\text{drug})_2\right] + [\text{duplex} - \text{drug}] \qquad (9)$$

where $[\text{duplex}]_0$ and $[\text{drug}]_0$ are the total concentrations (i.e., free and bound) of the double helix and drug, respectively. The concentration of the oligonucleotides, $[\text{duplex}]_0$, was measured by UV absorbance ($\varepsilon = 154{,}000\,M^{-1}\,cm^{-1}$ for the 10-mer A_2T_2; $\varepsilon = 55{,}500\,M^{-1}\,cm^{-1}$ for the 8-mer AT).

Eqs 8 and 9 may be rewritten in terms of the concentrations of the free species:

$$[\text{duplex}]_0 = [\text{duplex}] + K[\text{duplex}][\text{drug}] \qquad (10)$$

$$[\text{drug}]_0 = [\text{drug}] + K_D[\text{drug}]^2 + K[\text{duplex}][\text{drug}] \qquad (11)$$

Eqs 10 and 11 represent a system of a non-linear equations, which was solved by using MATLAB software (v. 5.1).

Molecular modelling

Molecular models were built using a Silicon Graphics 4D35GT workstation running the Insight II and Discover software. Molecular mechanics (MM) and molecular dynamics (MD) were carried out using both CVFF and AMBER forcefield. The starting geometry of berberine and of the 10-mer A_2T_2 were generated using standard bond lengths and angles. The structure of the oligonucleotide in the canonical B-DNA form was energy minimised by using both forcefields; a scaling factor of 0.5 was used for 1–4 interactions in the case of AMBER. A distance-dependent relative permittivity $\varepsilon = 4.0\,r$ was used to simulate the solvent effect. No other restraints were applied except for the hydrogen bonds between the base pairs (1.7–1.9 Å). The volume of the NOE peaks, relative to the aromatic protons of the bases and the ribose protons in the free oligomer, were integrated and transformed into inter-proton distances by using FELIX software included in the Insight II and Discover programs. The berberine structure was energy minimized using a CVFF force-field and then manually docked into the minor groove of the double helix. The berberine-DNA complex was then constructed by interactive graphics, qualitatively following the NOE data. The model was energy minimized and a subsequent restrained molecular dynamic calculation was performed for 90 ps at 600 K temperature, sampling the trajectory every 2 ps. The following restraints were applied: the distances between berberine and the oligonucleotide protons, the distances between the aromatic protons of the bases and the ribose H-1′ protons of the five-neighbour units, all obtained from NOE data (Table 4) and the hydrogen bonds between the base pairs. Of the 47 structures thus generated, the first 25 ones were discarded, leaving the system to equilibrate; the last 22 structures were then selected, averaged and subsequently minimized with the restraints said before. From the final structure thus obtained, we derived the inter-proton distances, the helical twist angles, the α, ζ, δ and χ torsion angles. During restrained MM and MD, a force constant of 41.8 KJ mol^{-1} $Å^{-2}$ was applied for all distance restraints.

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