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Mode of binding of camptothecins to double helix oligonucleotides † ‡

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We report an NMR study on the interaction of topotecan (Tpt) and other camptothecins (Cpts) with several double helix and single strand oligonucleotides. The results obtained by ^{31}P NMR spectroscopy, nuclear Overhauser experiments (NOE) and molecular dynamics (MD) simulations show that Cpt drugs do not intercalate into the double helix, as suggested by many authors. Phosphorus NMR spectra indicated that no deformation occurs at any level of the phosphodiester backbone, while 2D NOESY experiments allowed the detection of several contacts between the aromatic protons of Cpts and those of the double helix. Models of the drug/oligonucleotide complexes, built on the basis of NOE data, show that the drug is located at the end of the double helix, by stacking the A and B rings with the guanine or cytidine of the terminal CG base pairs, with a preference for the 3′-terminal end sites. Cpts interact with double strand, as well as with single strand oligomers, as can be seen from the NMR shift variation observed on the drug protons; but this shielding effect cannot be an evidence of intercalation, as it is largely due to external non-specific interactions of the positively charged drug with the negatively charged ionic surface of the oligonucleotide. The molecular weight of one of the complexes was obtained from the correlation time value. The conformational behaviour of the DNA fragment d(CGTACG)$_2$ was studied by MD simulations on a ns time scale in the presence of water molecules and Na^+ ions. Different models were examined and the deformations induced on the phosphodiester backbone by molecules that are known to intercalate, were monitored by MD simulations.

Introduction

DNA Topoisomerases (Topo) are nuclear enzymes that play an essential role in several DNA-functions, such as DNA replication and transcription.[1–4] They modify the topological state of DNA through the formation of transient breaks in the phosphodiester backbone of the DNA molecule. Two types of enzymes (Topo-I and Topo-II) exist in both prokaryotic and eukaryotic cells and are classified according to their mechanism of DNA breakage. Topo-I forms a single-strand break in DNA, remaining covalently linked to the 3′-phosphate through a tyrosyl residue. This intermediate is commonly referred to as a "cleavable complex". Under physiological conditions, Topo-I catalyses the religation of the broken strand. Camptothecins are the prototypical agents belonging to a class of antitumor drugs known as Topo-I poisons, because they have Topo I as a unique target.[5,6] The drug specifically inhibits the religation step, thus preventing release of DNA.[7,8] Models of interaction of camptothecins with Topo-I and DNA have been suggested on the basis of the X-ray analysis of the complex DNA/Topo-I,[9–11] and very recently Stewart and coworkers[12] reported the crystal structure of a topotecan/DNA/Topo-I ternary complex and gave an explanation of how Cpts specifically block DNA religation.

Actually, the mode of binding of Cpts to DNA in absence of the enzyme is still unclear and the published results on the DNA/Cpt interactions are confusing and contradictory. Cpt was first described as a prototypical Topo-I poison that exhibits little or no binding to either DNA or Topo-I alone.[13] More recently two clinically important Cpt derivatives, topotecan (Tpt) **1** and irinotecan, were shown to be capable of binding with DNA in the absence of Topo-I,[14–18] but no data on the molecular structure of Cpt/DNA complexes have been published. The results from linear dichroism spectroscopy appear difficult to interpret; binding in the major groove was suggested by some authors,[14] others claimed to have recognized two types of complexes with calf-thymus DNA,[15] and concluded for a binding in the minor groove.[16] Two NMR studies on Tpt/DNA binding have been reported,[17,18] but the results, based only on chemical shift values, are contradictory. Yao *et al.*[17] claimed a sequence specificity for duplex DNA containing dT, whereas Yang *et al.*[18] reported a specific intercalation of topotecan into dGdC rather than dAdT sequences. An intercalation type of binding has also been suggested by Pilch *et al.*[19]

Recently in our laboratories, several camptothecin derivatives, with potent cytotoxic activity, have been synthesised,[20–22] which have lipophilic substituents in position 7, some of them however containing polar groups, that afford a substantial solubility in water. We selected four of these compounds for a spectroscopic study, **2**–**5**. We then synthesised (this paper) two other camptothecins, which present the positive charge located in different sites of the molecule, *i.e.* at the end of a short or long side-chain, 20-*O*-alanylcamptothecin **6**[23] and 20-*O*-lysinyl-alanylcamptothecin **7**, respectively. We studied the interactions of oligonucleotides with camptothecins **2**–**7** and with topotecan **1**, by using ^{1}H and ^{31}P NMR spectroscopy, and specifically nuclear Overhauser experiments.[24] Oligonucleotides of different sequences were examined, *i.e.* the self-complementary duplexes d(CGTACG)$_2$ (TA), d(GCTAGC)$_2$, d(CGTATACG)$_2$ (TATA), d(CGACGTCG)$_2$ (ACGT), d(GCGATCGC)$_2$ (GATC),

† Electronic supplementary information (ESI) available: Chemical shift values, inter-proton distances obtained from MD simulations of CAP model for the complex d(CGTATACG)$_2$/Cpt **6** and molecular dynamics figures. See http://www.rsc.org/suppdata/ob/b3/b312780j/

‡ Dedicated to Professors Luciano Caglioti and Domenico Misiti on occasion of their 70th birthdays.

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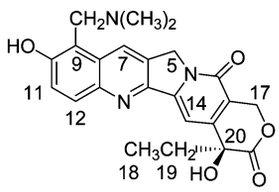
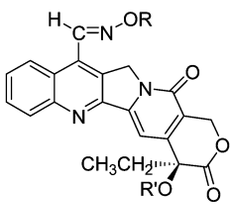
d(AAGAATTCTT)$_2$ (AATT), d(GCATCGCGATGC)$_2$ (CGCG), as well as the single strand oligomers d(CACGGCTGCA) (ss-GGCT), d(GCTTCCAAGTCG) (ss-CCAA) and d(ACATCAAAAAGGT) (ss-A5). They are considered as models for AT-rich and CG-rich sequences, with the CG base pairs at the end or in the middle of the strand.

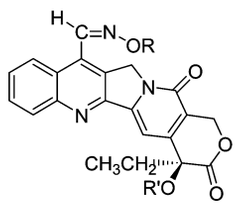


topotecan **1**



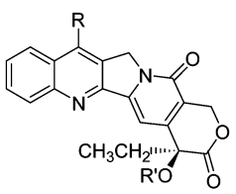
isomer *E*

2, 3, 4



isomer *Z*

2, 3, 4



5, 6, 7

Compd.	R	R'
2	$CH_2CH_2NH_2$	H
3	$CH_2CH_2NMe_2$	H
4		H
5	$CH_2CONH(CH_2)_3NH_2$ COO^-Na^+	H
6	H	$COCHCH_3$ NH_2
7	H	$COCHNHCOCH(CH_2)_4NH_2$ CH_3 NH_2

Results and discussion

For each camptothecin derivative, the equilibrium between the lactone and its corresponding ring-opened carboxylate form is pH dependent; at physiological pH the presence of the carboxylate form is significant, whereas it is practically absent at pH 4.5–5. As it is commonly accepted that the lactone is the active form of Cpts,[10,25] and since the presence of a carboxylate group should decrease the interaction with the ionic surface of the oligonucleotides, we performed our experiments at the lowest pH as possible, compatible with the stability of the oligonucleotide glycosidic bonds. *i.e.* in the range 5.5–6.5 of pH values. The presence of a small amount (*ca.* 10%) of the open form did not hamper the spectral analysis. On the contrary, for the oximes **2**–**4** the presence of *E*/*Z* stereoisomers in slow exchange with respect to the NMR time scale, induces broadening in the spectrum and increases the number of the signals; thus not all protons were assigned. This is an additional reason why we synthesised Cpts **6** and **7**. The configuration of the oximes was assigned by the shift values[26] and by NOE experiments. The *E* stereoisomer predominates: 83–88% for the lactone and 68–77% for the carboxylate form. The equilibrium is not affected by pH and remains constant with time.

Shift variations of ^{1}H and ^{31}P NMR resonances

Titration experiments performed with camptothecins (drug) on the solution of an oligonucleotide (DNA) show that the proton resonances of the drug become broad and move up-field with respect to the free drug, just after the addition of a small quantity, *i.e.* with R = [drug]/[DNA] = 0.25. The resonances of the oligomer, except for the imino NH protons of the terminal base pairs, are almost unchanged: a very small up-field shift ($\Delta\delta$ 0.0–0.18 ppm) was observed for all compounds, as reported for topotecan.[18] The chemical shift values are reported in the electronic supplementary material. Increasing the R = [drug]/[DNA] value from 0.2 to 2, the shielding remains constant, but the resonances of both the drug and the oligomer become broader (Fig. 1). No separate signals were observed for the free and bound species, because the binding process is fast with respect to the NMR time scale, even at low temperature (2 °C).

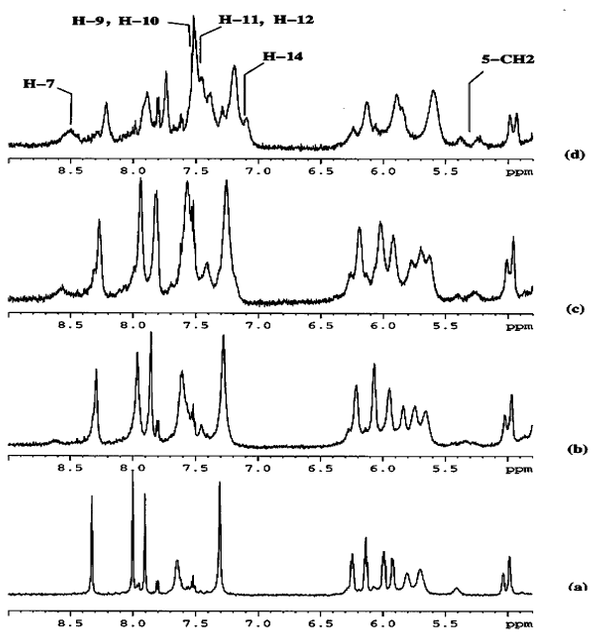


Fig. 1 Low field region of ^{1}H NMR spectra acquired at 20 °C, pH 7.0, 0.1 M NaCl, of d(CGTACG)$_2$ in presence of Cpt **2** at different ratios R = [drug]/[DNA]: (*a*) 0; (*b*) 0.5; (*c*) 1.0; (*d*) 2.0. 7-H stands for 7-CH=N.

We first examined the interaction with short oligonucleotides, such as d(CGTACG)$_2$ and d(CGTATACG)$_2$, since they are stable as double helices even at room temperature and all the phosphate resonances, already assigned,[27,28] are well separated. Since the hexamer d(CGTACG)$_2$ was used in a previous study[18] on **1**, we were able to obtain a significant comparison. The shift variations of the drug resonances are reported in Tables 1 and 2. The interaction of **1** with longer oligomers, induces shielding similar to those observed with d(CGTACG)$_2$, but it is significant that the same shielding was found for the single strands d(CACGGCTGCA) and d(GCTTCCAAGTCG) (Table 1). The up-field shift variations are in the range 0.2–0.5 ppm, and the same effects were observed for both lactone and carboxylate signals, in contrast with what has been reported.[17] We took some spectra at pH 7.2, as in these conditions the ratio between lactone and carboxylate is 65 : 35 and their resonances can easily be followed during the titration experiments.

Cpt **6** displays enhanced effects (Table 2). High $\Delta\delta$ values (0.6–0.8 ppm) were found for d(GCGATCGC)$_2$ and d(CGTATACG)$_2$, while the shielding is lower for AT-rich oligomers, duplex and single strand ($\Delta\delta$ 0.3–0.5 ppm); similar up-field shifts were found for **2**–**4** and **7**, whereas the titration experi-

Table 1 ^{1}H chemical shift variation ($\Delta\delta$) for topotecan (**1**) in the presence of several oligonucleotides[a]

	$\Delta\delta(\delta_{bound}-\delta_{free})$[b]						$\Delta\delta(\delta_{bound}-\delta_{free})$[c]	
	ACGT	GATC	CGCG[d]	ss-GGCT	ss-CCAA	A ATT	TA[d]	
							[e]	[f]
7-H	−0.27	−0.29	−0.29	−0.23	−0.30	−0.21	−0.13	−0.19
11-H	−0.38	−0.48	−0.27	−0.22	−0.20	−0.28	−0.29	−0.33
12-H	−0.41	[g]	−0.44	−0.35	−0.45	[g]	−0.42	−0.48
14-H	−0.24	−0.27	−0.37	−0.31	−0.33	[g]	−0.22	−0.17
18-Me	−0.04	−0.02	−0.04	−0.04	−0.06	−0.02	[g]	[g]

[a] Measured in ppm at 20 °C, solvent D_2O, R = [drug]/[DNA] = 1; δ_{free} were measured at a concentration of 5×10^{-5} M. The chemical shift values are reported in the electronic supplementary material. [b] pH 5.5–5.7, 0.02 M NaCl. [c] pH 7.3, 0.1 M NaCl. [d] The shielding on the imino NH protons of the terminal CG base pairs is $\Delta\delta = -0.45$ ppm for CGCG and −0.41ppm for TA oligomer, measured in H_2O at 10 °C. [e] Lactone signals. [f] Carboxylate signals. [g] Not detected.

Table 2 ^{1}H chemical shift assignment (δ) for camptothecins **2** and **6** with shift variation ($\Delta\delta$) in the presence of several oligonucleotides[a]

	free[b](δ)		$\Delta\delta(\delta_{bound} - \delta_{free})$					
	2[c]	**6**	**2** rTA	**2**[c] TA[d]	**6** GATC	**6** TATA[d]	**6** AATT	**6** ss-A5
7-H[e]	9.21	8.70	−0.55	−0.61[f]	−0.81	−0.58	−0.47	−0.51
9-H	8.24	8.18	−0.77	−0.82[g]	−0.79	−0.76	−0.40	[h]
10-H	7.91	7.77	[h]	−0.49	−0.72	−0.62	−0.28	−0.35
11-H	8.10	7.93	[h]	−0.70	−0.69	−0.78	−0.31	−0.29
12-H	8.10	8.12	[h]	−0.70	−0.67	−0.72	−0.34	[h]
14-H	7.66	7.44	−0.52	−0.56	−0.57	−0.57	−0.25	−0.26
5-CH_2	5.53	[h]	−0.10	−0.13	[h]	[h]	[h]	[h]
18-Me	1.00	1.03	+0.01	+0.01	−0.19	−0.21	−0.03	+0.01
19-CH_2	2.03	2.34	+0.05	+0.07	−0.26	−0.26	−0.06	[h]

[a] Measured in ppm, at 20 °C, solvent D_2O, pH 5.5–5.8, 0.01M NaCl, unless specified, R = [drug]/[DNA] = 1. The values correspond to the most abundant species in solution, *i.e.* lactone-oxime isomer *E* (80%) for **2**, lactone (>90%) for **6**. [b] δ_{free} were measured at the concentration of 5×10^{-5} M for **2** and 1×10^{-5} M for **6**. [c] pH 7.0, 0.1 M NaCl. [d] The shielding on the imino NH protons of the terminal CG base pairs is $\Delta\delta = -0.46$ ppm for TA and −0.35 ppm for TATA oligomer, measured in H_2O at 10 °C. [e] 7-CH=N for **2**. [f] For the carboxylate species, δ_{free} is 9.12 and $\Delta\delta$ is −0.49 ppm. [g] For the carboxylate species, δ_{free} is 9.05 and $\Delta\delta$ is −0.56 ppm. [h] Not detected.

ments performed with **5** were unsuccessful. We report in Table 2 the results for Cpt **2**, which displays the same effects for both lactone and carboxylate forms. Cpt **5** does not interact with the oligonucleotides examined, as shown by the absence of shift variation and line broadening and also by the absence of NOE interactions. This is explained by the presence of a negative charge on the molecule, which cannot be attracted by the ionic surface of the nucleotide.

The ^{1}H shielding effects are more or less spread over the whole drug molecule, but the values are significant only for the aromatic moiety and are independent of the distance from the positive charge. Unfortunately the shifts of the aromatic protons are affected by errors due to the self-aggregation process, which is relevant for all these molecules. For instance, the higher shielding observed for **6** *vs.* **1** can be explained with the self-aggregation, which is larger for **6** than for **1** (see later).

The $\Delta\delta$ values reported in Tables 1 and 2 were calculated by using the chemical shifts of the free drug obtained from the most diluted solution (10^{-5} M), in order to approach the values of the monomer; nevertheless the *Δδ cannot be used to obtain structural information for the following reasons*. (*i*) The shift variation effects on the drug molecule must be interpreted as the sum of specific and non-specific interactions. (*ii*) The external non-specific ionic interactions of a positively charged drug with the negatively charged ionic surface of the oligonucleotide are the main factor responsible for such effects, as recently found for berberine.[28] Also for Cpts, this is proven by the similar shielding observed for the interaction either with a single strand or with a duplex, and by the results obtained with the uncharged derivative **5**, which does not display any effect. It is significant that the stoichiometry of the process could not be deduced from the shielding on the drug protons, as the effect was already completed at R = 0.25. (*iii*) Shift variations of proton signals can also occur when a ligand intercalates between the base-pairs or binds to the minor groove. For instance, the intercalation of daunomycins between the CG base-pairs of $d(CGTACG)_2$, or $d(CGTATACG)_2$,[30] induces a significant up-field shift of the oligonucleotide resonances at the level of the intercalation sites, *i.e.* imino NH of G_2:C_5 base pairs $\Delta\delta$ 0.6–0.7 ppm, 5-H (C_1) and 5-H (C_5) $\Delta\delta$ 0.5 ppm. By contrast, the shift variation of the drug resonances was difficult to interpret, because daunomycins are also strongly affected by the self-aggregation process. The ^{1}H chemical shift variation in these cases is the sum of the effects of all these processes, which may have opposite sign, but we can only observe the average of all these $\Delta\delta$ values, and consequently it is risky to use the shielding effects observed on the drug to draw conclusions about the type of interactions.

On the other hand, it is the shift variation of the ^{31}P resonances that can provide 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 α = O(3′)–P–O(5′)–C(5′) and ξ = C(3′)–O(3′)–P–O(5′). For a nucleotide in a classical B-DNA type conformation, the phosphate groups are normally found in the *gauche–gauche* conformation with angles of −60° and −90° respectively. Changes in these angles are reflected in the values of the phosphorus chemical shifts. The intercalating molecule induces a deformation of the phosphodiester chain, which usually assumes a *gauche–trans* conformation with angles of −60° and 180°; this is associated with a low-field shift of 1.0–1.5 ppm.[29] The addition of Cpts to each of the oligonucleotides examined did not induce significant chemical shift variations of the phosphate signals in the ^{31}P NMR spectra ($\Delta\delta$ < 0.2 ppm).

Table 3 ^{31}P chemical shift variation ($\Delta\delta$) for the phosphates of the oligonucleotides bound to topotecan **1** and to camptothecins **2** and **6**[a]

TA	**1**[b] $\Delta\delta$	**2**[c] $\Delta\delta$	TATA	**6**[d] $\Delta\delta$
C_1pG_2	0.00	+0.07	C_1pG_2	+0.02
G_2pT_3	−0.06	+0.01	G_2pT_3	+0.00
T_3pA_4	−0.02	−0.03	T_3pA_4	0.00
A_4pC_5	−0.08	−0.03	A_4pT_5	0.00
C_5pG_6	−0.10	+0.07	T_5pA_6	−0.03
			A_6pC_7	+0.02
			C_7pG_8	−0.02

[a] Spectra measured in ppm at 20 °C, solvent D_2O. $\Delta\delta = \delta_{bound} - \delta_{free}$. The chemical shift values are reported in the electronic supplementary material. [b] pH 6.3, 0.1 M NaCl. [c] pH 7.2, 0.1 M NaCl. [d] pH 5.8, 0.01 M NaCl.

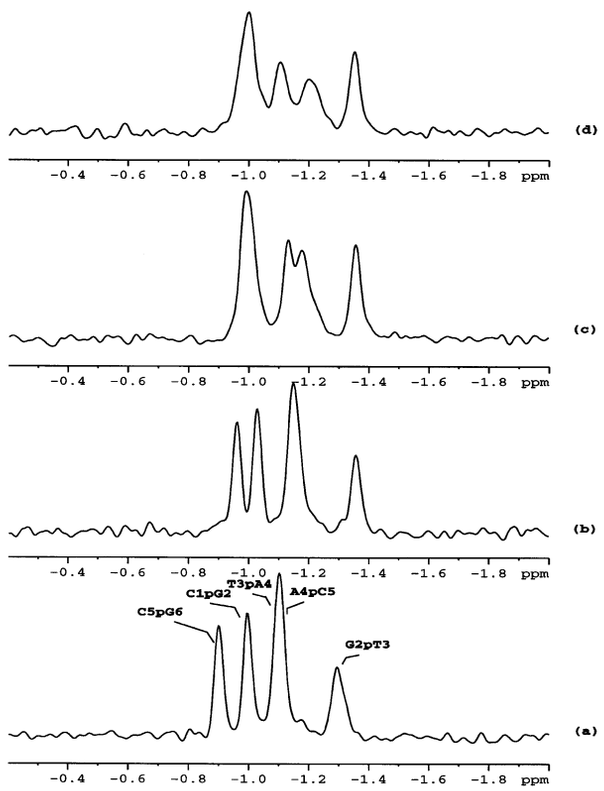


Fig. 2 ^{1}H-decoupled ^{31}P NMR spectra acquired at 20 °C, pH 6.3, 0.1 M NaCl of $d(CGTACG)_2$ in presence of topotecan **1** at different ratios R = [drug]/[DNA]: (a) 0; (b) 0.5; (c) 1.0; (d) 2.0.

We report these results in Table 3 and in Fig. 2 the ^{31}P NMR spectrum of the oligonucleotide $d(CGTACG)_2$ in absence and in presence of **1**.

Therefore the intercalation of Cpts into the double helix appears extremely improbable, as the shift variations of the phosphate resonances of the phosphodiester backbone and those of the inner imino NH protons are negligible. The ^{1}H up-field shifts observed for the drug molecule are not a consequence of the drug intercalation into the base pairs of the double helix, as claimed for topotecan,[18] but they are mainly due to external ionic interactions.

The up-field shift ($\Delta\delta$ = 0.35–0.45) observed for the imino NH protons of the terminal CG base-pairs presents some interest. This finding, together with the shielding effects on the drug protons, especially in the case of Cpts **2** and **6**, suggests a *preference for the CG-rich sequences*. In order to confirm this preference, we measured the binding constants of the interaction process with different oligonucleotides, by using UV spectroscopy.

Table 4 Binding constant values (K) for the interaction with oligonucleotides of 20-alanylcamtotecin **6** and topotecan **1**[a]

Oligonucleotide	K (M^{-1}) **6**	K (M^{-1}) **1**
$d(CGTACG)_2$[b]	$(2 \pm 0.1) \times 10^4$	$(2 \pm 0.2) \times 10^4$[c]
$d(CGTATACG)_2$	$(2 \pm 0.5) \times 10^4$	$(1 \pm 0.3) \times 10^4$
$d(GCGATCGC)_2$	$(1 \pm 0.1) \times 10^4$	$(1 \pm 0.1) \times 10^4$
$d(AAGAATTCTT)_2$	$(3 \pm 0.2) \times 10^3$	$(9 \pm 0.5) \times 10^2$
d(ACATCAAAAAGGT)	$(9 \pm 0.3) \times 10^2$	$(5 \pm 0.3) \times 10^2$
d(CACGGCTGCA)	$(4 \pm 0.2) \times 10^3$	$(3 \pm 0.3) \times 10^3$

[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 non- linear equations and using MATLAB software (v. 5.1). The dimerization constant K_D was included in the calculations. For **6**, $K_D = (2 \pm 0.2) \times 10^4$ M^{-1}; for **1**, $K_D = (2 \pm 0.5) \times 10^3$ M^{-1} in 0.02 M NaCl and $(1 \pm 0.1) \times 10^3$ M^{-1} in 0.1 M NaCl. [b] The binding constant for the interaction of Cpt **2** with $d(CGTACG)_2$ is $K = (5 \pm 0.1) \times 10^4$ M^{-1}, pH 7.0, 0.1 M NaCl, $K_D = (1 \pm 0.2) \times 10^3$ M^{-1}. [c] 0.1 M NaCl.

Binding constants and self-association constants

As the self-association process may strongly affect the measure of the binding constants,[28] we performed dilution experiments by NMR and UV spectroscopy. The up-field variation of the proton chemical shifts, observed in the NMR spectra, indicates that camptothecins are still aggregated even at the lowest possible concentration for NMR measurements (10^{-5} M). Thus we used UV spectroscopy (concentration range 10^{-5}–10^{-7} M) in order to estimate the aggregation phenomenon and also for the titration experiments with the oligonucleotides. A dimerisation process has been considered[32,33] as a sufficient approximation of the aggregation process, provided that the concentration is below the isosbestic point; at higher concentration, most planar aromatic compounds continue to associate into higher aggregates. The isosbestic point was found at 463 and 409 nm for **1** and for **6**, respectively. We thus obtained the dimerisation constant values, which are in line with that reported[34] for topotecan. The self-association in the case of Cpt **6** appears one order of magnitude stronger than that observed for **1**, which is explained by the presence of the unsubstituted quinoline moiety. Then, the dimerisation constants were used for the calculations of the binding constants (Table 4). Both **1** and **6** show the same values for the interaction with the CG-rich model $d(GCGATCGC)_2$, as well as with the sequences $d(CGTACG)_2$ and $d(CGTATACG)_2$. The binding constants for the AT-rich sequences are significantly lower, for both compounds. Cpt **2** displays the same behaviour. These data show a preference for the CG-rich sequences, confirming what was suggested by the ^{1}H chemical shift results. However, these parameters, as well as the proton chemical shift variation of the ligand, are not diagnostic to define the mode of binding to DNA.

NOE experiments

The ^{1}H NOE experiments, allowing the detection of specific interactions between protons of the ligand and protons of the duplex, were performed in order to recognize possible preferred interaction sites. The resonances of the oligomers were assigned, for both free and bound species, following well established procedures[24] for the analysis of double stranded oligonucleotides. The sequential assignment of the nucleotide units was performed by detecting the NOE cross-peaks between the aromatic protons of the bases and the 1′,2′,2″-H ribose protons of the 5′-neighbour unit, thus allowing the B-DNA conformation of the double helix to be recognized. The formation of the complex was followed by titration experiments with the drug on the nucleotide solution and the NOESY spectra were acquired with R = [drug]/[DNA] = 1, 1.5 and 2. The results are reported in Table 5.

Table 5 Inter-molecular NOE interactions between protons of the drugs **1**, **2** and **6** and protons of the nucleotides[a]

d(CGTACG)$_2$	/1	/6	/2[b]	d(GCTAGC)$_2$	/1
8 -H (G$_6$) ··· 11-H	m	m		1′-H (C$_6$) ··· 11-H	w
2′-H (C$_5$) ··· 11-H	w	w	w	5 -H (G$_8$) ··· 11-H	w
2′-H (G$_6$) ··· 11-H	m	w	w	2′-H (G$_5$) ··· 12-H	w
8 -H (G$_6$) ··· 12-H	w	w			
2′-H (G$_6$) ··· 12-H	w				
2″-H (G$_6$) ··· 12-H	w				

d(GCGATCGC)$_2$	/1	d(GCGATCGC)$_2$	/6
6 -H (C$_8$) ··· 11-H	m	8 -H (G$_7$) ··· 11-H	w
1′-H (C$_8$) ··· 11-H	w	2′-H (C$_8$) ··· 12-H	w
1′-H (C$_8$) ··· 12-H	m		
2′-H (G$_7$) ··· 12-H	m		

d(CGACGTCG)$_2$	/1	d(CGTATACG)$_2$	/6[c]
2′-H (C$_7$) ··· 11-H	w	8 -H (G$_8$) ··· 10,11-H[d]	w
8-H (G$_8$) ··· 11-H	w	2′-H (G$_8$) ··· 10,11-H	w
8-H (G$_8$) ··· 12-H	w	8-H (G$_8$) ··· 12-H	w

[a] Acquired at 20 °C, in D_2O, pH 5.5–5.8, 0.01 M NaCl, unless specified. 2′-H and 2″-H stand for low field and up field proton respectively. The intensities of the signals were estimated as follows: w = 4–5 Å, m = 3–4 Å, s = 2–3 Å, by using as reference the cross-peak of 5-H/6-H of cytidines (2.5 Å). Zero NOEs were observed, in H_2O at 10–15 °C, for the imino NH and 2-H protons of the bases. [b] pH 7.0. [c] Acquired at 15 °C. [d] The signals of the two protons are overlapped.

The interaction of **1** with d(CGTACG)$_2$ leads to NOE contacts involving the 11,12-H aromatic protons of the drug, the adenine and guanine 8-H base protons and the 2′,2″-H sugar protons of G$_6$, while 11-H of the drug also interacts with 2′-H of C$_5$. Zero NOEs were instead found for the imino NH, for adenine 2-H and for protons of TA residues. The results obtained with longer sequences are more significant: with the AT-rich oligomer d(AAGAATTCTT)$_2$ no NOE peaks were observed, whereas the interaction with CG-rich models such as d(GCGATCGC)$_2$ and d(CGACGTCG)$_2$ leads to NOE contacts involving only the terminal CG residues. The interaction with d(CGACGTCG)$_2$, which presents the CG base-pairs also in the middle of the double helix, confirms the preference for the terminal ends, and it is interesting to observe that the interaction with d(GCGATCGC)$_2$ involves the protons of C$_8$ and G$_7$, but not those of G$_1$ and C$_2$, *showing a preference for the 3′-terminal ends*. In order to confirm this finding, we examined the oligomer d(GCTAGC)$_2$ in comparison with d(CGTACG)$_2$: also in this case the 3′-terminal ends are preferred, as follows from the NOE contacts between 11,12- H of **1** and the C$_6$ and G$_5$ base protons.

Camptothecins **6** and **7**, without substituents on rings A and B, were synthesized in order to improve the claimed intercalation property of these drugs, while the positively charged long chain of **7** was expected to interact with the ionic surface near the minor groove. Actually, Cpt **7**, after the addition of the oligonucleotide, shows too broad signals to allow NOE experiments, while Cpts **2**, **4** and **6** present NOE contacts with d(CGTACG)$_2$ similar to those observed for **1**, *i.e.* with the terminal base pairs, G$_6$ and C$_5$ (Table 5). The NOE contacts found with d(GCGATCGC)$_2$ involve the protons of C$_8$ and G$_7$, showing, as in the case of Tpt, a preference for the 3′-terminal ends. The interaction of **6** with d(CGTATACG)$_2$ leads to a sufficient number of data to derive a structural model of the complex. A correlation time of 2.5 ns (with a cross-relaxation rate of 0.4 s^{-1}) was obtained for the complex **1**/d(CGTACG)$_2$ by using the NOE interaction between protons of known distance, 5-H and 6-H of cytidines (2.45 Å). In order to estimate the dimension of the complex, we calculated with the correlation time value, through the Debye–Stokes–Einstein equation,[35] an average molecular volume of 110×10^{-23} cm^3, which corresponds to the molecular weight of d(CGTACG)$_2$ plus 1.5 molecules of topotecan. When a spine of 50 water molecules is included into the double helix, the molecular weight corresponds to a complex with 1.2 Tpt molecules. Thus, taking into account the approximation of the method, this molecular volume is consistent with the presence in solution of monomeric species of the complex, which reasonably has a ratio $R = 1$.

Structure derivation and MD simulations

The structural models of the free oligonucleotides and of the complexes with **1** and **6** were built by using standard distances and angles for a B-DNA type conformation. We started by studying the structure of the duplex d(CGTACG)$_2$ inserted in a box of water molecules and in the presence of Na^+ ions to neutralize the charges. We performed 2 ns of MD calculations at 300 K with GROMOS96 force field. Hydrogen bonding restraints were applied to all the base pairs. The results show (Fig. 3) that the hexamer remains in a B-DNA conformation, slightly bent at the level of T$_3$:A$_4$ base pairs, in agreement with the NOE data (not reported here).

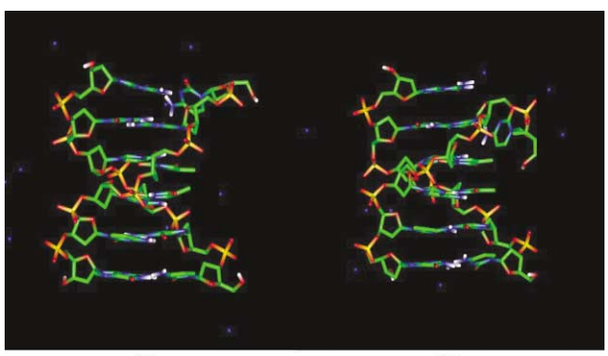


Fig. 3 Structure of d(CGTACG)$_2$ generated by GROMOS96; (a) initial MD step; (b) after 2 ns MD

The same system was subjected to 10 ps of simulations with CVFF force field, in the presence of a sphere of water molecules and Na^+ as counterions, by using the DISCOVER module of INSIGHT II. The results are in line with those obtained with GROMOS96, despite the shorter simulation time. Monitoring the α and ζ angles relative to each phosphate every 1 ps, we observed the *gauche*$^-$, *gauche*$^-$ conformations typical of a B-DNA, with α values ranging from 60° to 90° and with ζ values around 90°. A slight amount (~25%) of ζ T$_3$ angles were found with values of 150–180°.

Comforted by these results, which show transitions from canonical angles occurring on the ps time scale, and since the simulations on a ns time scale are computationally too expensive, we performed the MD study on the complexes drug/nucleotide, by using the simulations with CVFF force field on the ps time scale, in presence of water and sodium cations.

In order to rationalize the different mode of binding, structural models of the complexes **1**/d(CGTACG)$_2$ and **6**/d(CGTACG)$_2$ were built on the basis of our NOE results and considering also the binding mode suggested by Pommier[10] and Pilch[11] for the system Cpt/Topo-I/DNA and the X-ray structure of the ternary complex Tpt/Topo-I/DNA.[12] At first, the Tpt molecule was located at the terminal end of the oligonucleotide outside of the double helix, by stacking the A and B rings with the terminal G$_6$ base, starting from a minimized structure of the oligomer (CAP- model). In an alternative system, Tpt was intercalated between the base pairs at the level of C$_5$pG$_6$ (Sandwich model, SM), positioning the planar A and B rings either parallel to the base pairs, as in the X-ray structure of the ternary complex[12] (SM1 model) or perpendicu-

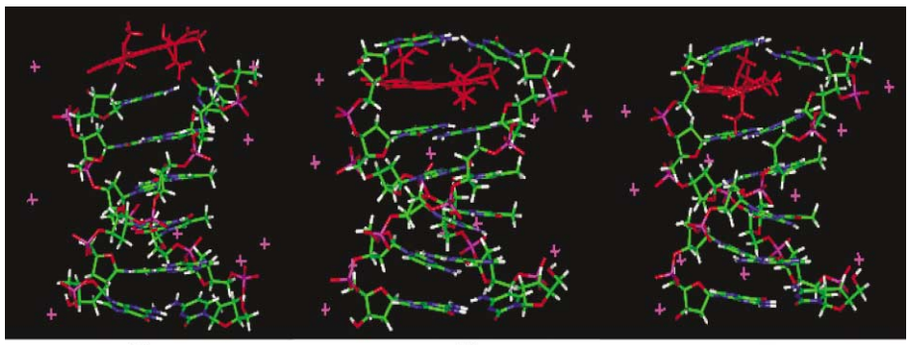


Fig. 4 Energy minimized molecular models of the complexes between topotecan **1** and d(CGTACG)$_2$: (a) CAP model, built on the basis of the experimental NOE data; (b) SM1 model and (c) SM2 model, built (b) on the basis of the X-ray structure of the ternary complex Tpc/Topo-I/DNA,[12] and (c) considering the binding mode suggested in Refs. 10 and 11. Figs. (b) and (c) show the strong deformations of the phosphodiester backbone of the double helix, as a consequence of the intercalation of the drug.

lar to the base pairs of the double helix[10,11] (SM2 model). In all cases, we performed MD calculations (up to 87 ps) and analyzed the explored dihedral angles α and ζ of the backbone. The convergence of the energy was ensured and the stability of the system was checked: the plot of the total energy of the complexes drug/nucleotide *vs.* time of the MD simulation showed that after only 2 ps the systems reached the equilibrium.

NOE restraints between Tpt and the oligonucleotide, together with the hydrogen bonds of the base pairs, were applied. In the case of CAP model, after 20 ps all the phosphate groups remained in the same conformation as the free oligomer. On the contrary, when Tpt was intercalated parallel between the base pairs as in the SM1 model, we observed just after 10 ps a complete conformational change from *gauche*$^-$ to *trans* (170°–180°) of ζ T_3 angle on one strand, and a partial change from *gauche*$^-$ to *trans* of ζ G_2 on the opposite strand. Similar deformations occur in the complex with the drug inserted perpendicular to the base pairs (SM2 model), *i.e.* a complete conformational change of ζ T_3 angle (180°) for both strands (Fig. 4). If the conformational changes observed in models SM1 and SM2 would occur in solution, it should be reflected in a chemical shift variation of the ^{31}P resonances of phosphate groups. By contrast, the presence of the drug at the end of the oligomer, as in the CAP-model, does not affect the torsional angles of the phosphoribose backbone and consequently no chemical shift variation is expected in the ^{31}P NMR spectra, which was just observed experimentally.

The comparison of the intermolecular distances between Tpt and d(CGTACG)$_2$ in the different complexes (Table 6) show *a best fit with our experimental data in the case of CAP-like model, where the drug is located at the end of the helix*, *i.e.* all the distances corresponding to the observed NOEs are within 4.4 Å, and those corresponding to significant zero NOE are > 5 Å. By contrast, in the SM1 model, the distances 2′-H(G_6) ··· 12-H(Tpt), 1′-H(G_6) ··· 12-H(Tpt) and 2″-H(C_5) ··· 11-H(Tpt) do not fit with the experimental data. The same occurs for the SM2 model, where the distances between the sugar protons of G_6 and C_5 and the drug protons 11,12-H are much longer than the experimental ones. In addition, two distances between 14-H and the sugar protons of G_6 and C_5 are too short and thus disagree with the observed zero NOEs.

The simulations performed with Cpt **6** show for the CAP-model the best fit with the experimental data, in line with the results obtained with Tpt. The intermolecular distance values are reported in the electronic supplementary material. The interactions with the 8-mers d(CGTATACG)$_2$ and d(GCGATCGC)$_2$ gave similar results, *i.e.* the drug is accommodated at the end of the duplex, over the terminal guanine or cytidine respectively. Fig. 5 reports the structural model of the complex between Cpt **6** and d(CGTATACG)$_2$.

Table 6 Inter-proton distances (Å) obtained from MD simulations of different models for the complexes oligonucleotide/drug[a]

	d(CGTACG)$_2$/Tpt**1**		/Cpt **6**	
Distance (Å)	CAP[b]	SM1[c]	SM2[d]	CAP[b]
8-H (G_6) ··· 11-H	3.49	3.73	3.67	3.45
2′-H (C_5) ··· 11-H	4.40	2.93	**6.58**	4.80
2′-H (G_6) ··· 11-H	3.93	3.29	**7.27**	3.78
8-H (G_6) ··· 12-H	4.25	3.51	2.69	4.15
2′-H (G_6) ··· 12-H	2.34	**5.00**	**6.20**	2.30
1′-H (G_6) ··· 11-H	5.69	5.58	7.22	5.75
2″-H (C_5) ··· 11-H	5.36	**2.82**	5.88	5.30
1′-H (G_6) ··· 12-H	5.00	**4.17**	5.57	7.62
1′-H (G_6) ··· 14-H	6.60	5.19	**4.29**	6.25
2′-H (C_5) ··· 14-H	10.5	7.46	**3.86**	9.61

[a] The numbers in bold represent values in disagreement with the experimental data. [b] CAP model, with the drug located at the 3′-terminal ends. [c] Sandwich model, with the drug intercalated between C_5G_6, parallel[12] to the base pairs. [d] Sandwich model, with the drug intercalated between C_5G_6, perpendicular[10,11] to the base pairs.

The structure of the hexamer d(GCTAGC)$_2$ is like that found for d(CGTACG)$_2$. When the drug, **1** or **6**, is positioned at the terminal end of the duplex, following the NOE contacts, we can observe that the drug remains located over cytidine C_6. Similarly, in the complex with d(GCGATCGC)$_2$ the drugs do not stack over guanine G_1 but over cytidine C_8, *confirming the preference for the 3′-terminal ends.* An inspection of the models in Figs. 5 and 4a shows that the sugar of the 5′-terminal creates some steric hindrance to the stacking of the drug molecule over its own base, for instance C_1 in the complex with d(CGTATACG)$_2$, whereas the approach to the complementary base G_8 is easier. Distances compatible with possible hydrogen bonds were not revealed, and also ionic interactions involving the partial positive charge on the amino groups do not appear responsible for this preference.

Finally, we studied in more detail by MD the deformations induced on the backbone of the double helix by an intercalating ligand, comparing the results with SM1 and SM2 models of Tpt complex. We used a classical intercalating molecule, morpholinodoxorubicin,[30] and the oligonucleotide d(CGTACG)$_2$. This system has been extensively studied in our laboratory; we have found a deshielding of 1.3 ppm on C_5pG_6, when daunomycin is intercalated into d(CGTACG)$_2$;[27] with other anthracyclines of the same family we have found[30,31] values ranging from 0.9 to 1.5 ppm. The insertion of a flat molecule between the base pairs of a duplex mimics the increase of one base pair of the double helix, and this must induce a conformational change at the phosphate angles. The MD simulations of the complex with morpholinodoxorubicin show, as expected from

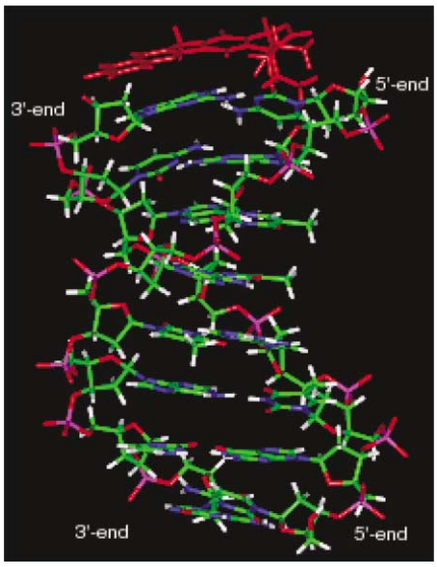


Fig. 5 Energy minimized structural model of the complex between Cpt **6** and d(CGTATACG)$_2$ built on the basis of the experimental NOE data. The model shows that the 3′-terminal end is more accessible to the stack of the drug.

previous studies,[30] conformational transitions at the level of ζ C_5 and α G_6 phosphate angles on one strand and at the level of ζ C_1 and α G_2 on the opposite strand, which correspond to the interaction sites. If topotecan is intercalated between the CG base pairs, as suggested by some authors[16,17] and represented in the models SM1 and SM2, the strain caused on the backbone appears reflected mostly on T_3pA_4, while minor deformations appear at the level of C_1pG_2 for SM1 model, at C_5pG_6 and G_2pT_3 for SM2 model. Morpholinodoxorubicin presents, but topotecan does not, a flexible portion of the molecule, *i.e.* daunosamine and morpholine rings, which lie at the level of the minor groove, as shown by the NOE interactions with T_3 and A_4 protons.[30] The interactions of daunosamine and morpholine moieties with T_3 and A_4 phosphate groups are precisely those that hold the drug in place, forbidding any possible conformational change at this level, with the result of relaxing all the conformational strain only at the level of the intercalation site. With Tpt, the stabilization of the duplex by interactions at the minor groove cannot occur and consequently the deformation of the helix takes place predominantly at the next phosphate relative to the intercalation site, and this should be reflected in the ^{31}P NMR spectrum.

Conclusions

The results, obtained by NMR spectroscopy and MD studies on the interactions of Cpt drugs with several oligonucleotides, show that *Cpt drugs do not intercalate into the double helix, as suggested by many authors.*[18,19] This conclusion is based on the experimental evidence from ^{31}P NMR spectra, which is confirmed by MD simulations. The negligible shift variation of the phosphate resonances indicates that *no deformation occurs at any level of the phosphodiester backbone.* Conformational changes at the phosphate angles are known to occur with intercalating molecules, and are reflected in significant (1–1.5 ppm) low field shift of the phosphorus frequency.[27,29–31] MD simulations, in presence of a sphere of water molecules and Na^+ counterions were performed with our molecules and with a classical intercalator, morpholinodoxorubicin, in order to have a structural picture of the deformations on the double helix. When topotecan is intercalated between the CG base pairs of d(CGTACG)$_2$, as in SM1 model, a complete conformational change from *gauche*$^-$ to *trans* was observed for ζ T_3 angle on one strand, and a partial change for ζ G_2 on the opposite strand. In the case of SM2 model, the conformational change was observed for ζ T_3 angle on both strands. If the conformational changes observed in models SM1 and SM2 occurred in solution, this should have been reflected in a chemical shift variation of the ^{31}P resonances of phosphate groups, which was not found experimentally.

These results are in agreement with the early finding[13] whereby Cpt does not intercalate in native duplex DNA. Cpts, however, interact with double strand, as well as with single strand oligomers, as can be seen from the NMR shift variation observed on the drug protons. But *this shielding effect cannot be evidence of intercalation, as suggested,*[18] because it is largely due to external non-specific ionic interactions of the positive charged drug with the negatively charged ionic surface of the oligonucleotide. Similarly, no conclusion can be drawn from the results obtained by linear dichroism.[14–16] The negative LD signal, with angles of 55° or 59° provides the relative orientation of the drug chromophore with respect to the long axis of the helix, but the experimental data are ambiguous as they offer a double interpretation.

2D NOESY experiments allowed the detection of several contacts between the aromatic protons of Cpt molecules and those of the double helix, specifically with protons of the CG base pairs at the terminal ends of the oligomers, whereas zero NOEs were found with the imino NH and with 2-H protons of adenine and guanine bases, located in the inner part of the duplex. Moreover no NOEs were observed with the AT rich oligomer d(AAGAATTCTT)$_2$. These results show that, in the experimental conditions examined, an *intercalation between the base pairs, as well as an external binding in the minor or in the major groove, can be excluded, but indicate a stacking with the terminal bases.* The up field shift ($\Delta\delta$ = 0.35–0.45 ppm) observed only for the imino NH protons of the terminal base pairs confirms these results. The molecular weight of the complex, obtained from the measured correlation time value, suggests the presence of monomeric species in solution, with the ratio $R = 1$.

It is interesting to observe that *guanine does not represent the preferred site for the stacking, but camptothecins display a preference for the CG base-pairs of the 3′-terminal ends*, as shown by the NOE contacts found in the complexes with the d(GCTAGC)$_2$ and d(GCGATCGC)$_2$, where Cpts stack over the 3′-terminal cytidine.

Models were built on the basis of the NOE contacts, with Cpts located on the top of the double helix, by stacking the A and B rings of the drug with the terminal base, guanine or cytidine (CAP-model). An angle of 60–70° between the plane of the aromatic A and B rings and the principal axis of the nucleotide can be derived by the model. The presence of the drug in this position does not induce any deformation along the double helix, and gives a best fit with the NOE data.

Does the fraying process at the terminal base-pairs[36] play a role in the formation of the complex with the oligonucleotide? Actually the open form, in equilibrium with the duplex, is expected to make the insertion of the drug easier. The fraying process in the oligonucleotides, experimentally shown by the fast exchange of the imino NH protons of the terminal base pairs, does not interfere with the conformation of the rest of the molecule.

The binding mode of camptothecins by stacking with the guanine or cytidine of the terminal CG base pairs, presented in this paper, is not conflicting with the X-ray structure of the ternary complex Tpt/DNA/Topo-I.[12] Actually, the intercalation binding pocket in the ternary complex can form only after the first transesterification (with tyrosine), which effectively opens the backbone of one strand of the duplex. Then a further space for the pocket is created in the other uncleaved strand by conformational changes of the phosphodiester angles, as occurs with intercalating ligands. Therefore, without the action of the enzyme, which provides the tyrosine for the esterification of one phosphate and thus causes the break of the backbone, the

intercalation of the drug must be too difficult, in spite of the preference for the stacking to the CG base pairs.

Experimental

Materials

Oligodeoxynucleotides, synthesised by solid phase, were purchased from Roche-Diagnostics. (*S,S*)-camptothecin-20-*t*-Boc-alaninate was synthesised by following Ref. 22, and then was deprotected by using TFA (20%) in CH_2Cl_2 (DCM). The reaction was stirred at room temperature for 2 h to provide Cpt **6**. Cpt **6** (1 mmol) was suspended in a solution of Boc-Lys(Boc)-OH (1 mmol) in DMF and DCM and cooled in an ice bath. Then, Bop reagent[37] (1 mmol) and diisopropyl-ethylamine (2.2 mmol) were added. The reaction was stirred at 0 °C for 30 min and then at room temperature for 20 h. DCM and DMF were removed *in vacuo* and the residue was taken up in DCM. The DCM phase was washed with saturated NaCl solution, with 2% $KHSO_4$, 5% $NaHCO_3$ and water. After chromatography with DCM/MeOH (98 : 2), the product was deprotected using TFA (20%) in DCM to give 20-*O*-lysinyl-alanyl-camptothecin **7**.

NMR experiments

The NMR spectra were recorded on a Bruker AMX 600 spectrometer group 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 the presence of a minimum amount of NaCl (from 0.01 M to 0.1 M) in order to stabilize the double helix. The pH was adjusted to the values reported in the Tables, and the final concentration resulted in the range 1–3 mM. The chemical shift values are reported in the electronic supplementary material

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 °C, +15 °C and +10 °C. Mixing times from 50 ms to 300 ms. TOCSY[38] spectra were acquired 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 the pre-saturation technique, placing the carrier frequency on the H_2O resonance. In the case of H_2O–D_2O (90 : 10 v/v) solutions, the spectra were measured at 10 °C by using gradient-based pulse programs, capable of suppressing the water signal and minimize the magnetisation loss due to saturation transfer.

The ^{1}H assignments for free and bound camptotecins were performed by using NOESY and TOCSY experiments. The assignments for the free oligonucleotides TA,[27] TATA,[39] AATT[28] and ss-A_5[28] have previously been reported. 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.[24] The assignments of the phosphate groups for the oligonucleotides TA[27] and TATA[28] have previously been reported.

UV experiments

The UV spectra were recorded at 25 °C on a Perkin-Elmer Lambda 40 UV-VIS spectrophotometer. Dilution experiments were performed starting from a concentrated solution (10^{-4} M) of the drug, which was diluted to 10^{-7} M, in presence of 0.02 M NaCl. For the aggregation process a dimerisation model was considered to be a sufficient approximation,[32,33] thus the dimerisation constants K_D were calculated by using standard equations.[28] The binding constants (K) for the nucleotide–drug interaction were obtained by titration experiments, performed by adding increasing amounts of a 5.0×10^{-6} M solution in water of the drug to a 1.0×10^{-5} solution of the oligonucleotide. Then the binding constants K were calculated by including the dimerisation equilibrium. The system of non-linear equations[28] was solved by using MATLAB software (v. 5.1).

Molecular modeling and simulations

Molecular models were built using a Silicon Graphics 4D35GT workstation running the INSIGHT II & DISCOVER software. MM and MD simulations of $d(CGTACG)_2$ were carried out starting from B-DNA conformation generated by using standard bond lengths and angles contained in the INSIGHT library and in GROMOS96.[40] For the models generated by DISCOVER we used the CVFF force field supported by Biosym program. We included full charges on the phosphate groups and a neutralizing number of Na^+ counterions. The system was surrounded by a sphere of water molecules with radius of 25 Å. At the first step we performed a minimization by Discover applying 100 steps of steepest-descendent algorithm followed by conjugate gradient minimization, until the energy difference between successive minimization steps was less than 0.1 kcal mol^{-1}. Then, a 10 ps of simulation, at a constant temperature of 300 K was run. A separate study was performed by using the GROMOS96 package and force field version.[40] The oligonucleotide plus counterions was immersed in a rectangular box of 2644 water molecules (1.05 nm × 0.9 nm × 0.8 nm). Standard MM and MD simulations were performed at 300 K, including periodic boundary conditions using SHAKE[40] on all bonds. A total simulation time of 2 ns was performed. The atoms forming hydrogen bonds, except for the terminal base pairs, have been restrained through the simulation. For the MM and MD simulations (from 10 to 87 ps) of the complexes with Cpts we used the DISCOVER software. No other restraints were applied except for the NOE contacts and the hydrogen bonds between the base pairs (1.7–1.9), including or excluding those of the terminal base pairs C_1:G_6. The stability of the system was checked by plotting the total energy of the complexes *vs.* the time of MD simulation (plots of energy are supplied as electronic supplementary material). The CAP-model yielded the structure with the lowest energy (E = −24604 kcal mol^{-1}), SM1 and SM2 models being 191 and 504 kcal mol^{-1} higher in magnitude, respectively. The equilibrium is reached after 2 ps. The average structure for each complex was created in order to calculate the RMS deviation over all heavy atoms, relative to each single frame of the MD calculation; the RMS value converged to 0.9 ± 0.3 Å. Moreover we also calculated the RMS for each residue, as a measure of the internal stability; the values converged to 0.3 ± 0.1 Å for all residues, except for T_3 (0.7 ± 0.1 Å).

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