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Effect of strand orientation on the interaction of berenil with DNA triple helices

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Abstract Using circular dichroism spectroscopy the ability of berenil, a minor groove binding drug, to induce triple helix formation was investigated with two oligonucleotides designed to form two intramolecular triplexes containing T*A:T and G*G:C triplets, which differ only by the orientation of their third strand: 5′-d($G_4A_4G_4$-[T_4]-$C_4T_4C_4$-[T_4]-$G_4T_4G_4$), and 5′-d($G_4T_4G_4$-[T_4]-$G_4A_4G_4$-[T_4]-$C_4T_4C_4$), where [T_4] represents a stretch of four thymine residues. We demonstrate that when added to the duplex form of these oligonucleotides, berenil induces triplex structure formation only if the orientation of third strand is antiparallel to the purine strand.

Keywords Triple helix · Berenil · Circular dichroism

Introduction

Interest in the triple helical structure of DNA is due to potential biological applications. Thus triple helix formation through binding of a nucleotide probe to a double-stranded DNA target has been proposed as a possible means of controlling gene expression at the transcriptional level (Le Doan et al. 1987; Maher et al. 1992; Moser and Dervan 1987). Besides, triple helix formation has also been exploited to achieve cuts and excisions at specific sequences by attaching DNA cleaving agents to a triplex-forming oligonucleotide (Helene and Toulme 1990; Moser and Dervan 1987). In the past few years the ability of several DNA binding drugs to modulate the affinity and specificity of the third strand for their duplex target has been investigated.

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Berenil is classified as a diarylamidine compound with mild cytotoxic and antiviral properties (Clercq and Dann 1980) that is used extensively for the treatment of bovine trypanosomiasis. This compound, which interacts non-covalently with DNA, has been shown by several methods to bind preferentially to AT sequences (Abu-Daya et al. 1995; Bailly et al. 1992; Brown and Fox 1996; Colson et al. 1996; Laughton et al. 1990). A body of relevant information accumulated from X-ray, NMR and molecular modeling studies tends to demonstrate that, like netropsin, the concave surface of berenil is accommodated by the minor groove of DNA by virtue of specific hydrogen bonding with appropriate base atoms (Barcelo and Portugal 1993; Brown et al. 1990, 1992; Conte et al. 1995; Gabelica et al. 1999; Gresh and Pullman 1984; Hu et al. 1992; Lane et al. 1991; Pearl et al. 1987; Pilch et al. 1995; Portugal and Waring 1986; Reinert 1999; Schmitz et al. 1995; Yoshida et al. 1990; Zakrzewska et al. 1984). In contrast to the AT sequences, the 2-amino group of guanosine represents a steric hindrance to the entry of the drug in the minor groove of GC sequences. However, several studies have shown that, despite a strong selectivity for AT sites, berenil can exhibit, under appropriate conditions, in the GC and AT sequences an intercalative contribution to the binding event (Colson et al. 1996; Pilch et al. 1995).

In this study we compared the interaction of berenil with two oligonucleotides designed to form two intramolecular triplexes which differ only by the orientation of their third strand. We have previously studied these two oligonucleotides, 5′-d($G_4A_4G_4$-[T_4]-$C_4T_4C_4$-[T_4]-$G_4T_4G_4$) (sequence 1) and 5′-d($G_4T_4G_4$-[T_4]-$G_4A_4G_4$-[T_4]-$C_4T_4C_4$) (sequence 2), and demonstrated that (1) in the absence of $MgCl_2$ salt, both adopt a hairpin duplex structure with the dangling tail d([T_4]-$G_4T_4G_4$); (2) in the presence of $MgCl_2$ salt, each oligonucleotide folds back on itself twice to give a triple helix via a double hairpin formation with a [T4] single-strand loop (Gondeau et al. 1998a, 1998b) (Fig. 1). These triplexes have the particularity to involve non-isomorphic stretches of G*G:C and T*A:T triplets. Furthermore,

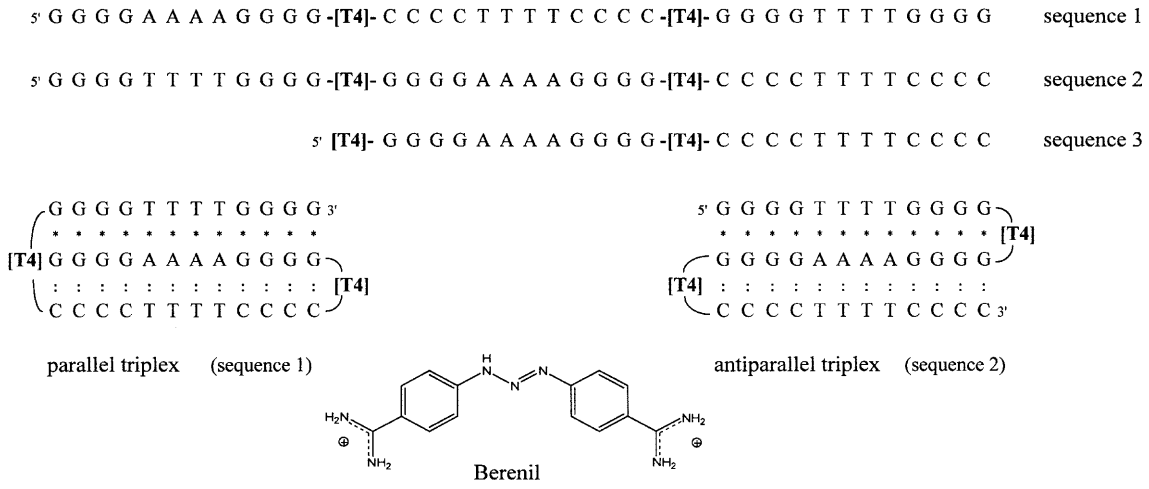

Fig. 1 Sequences of the oligonucleotides used in this study

the design of sequence 1 provides evidence that, when the triplex structure is adopted, the third strand binds to the purine strand of the underlying duplex with parallel orientation, in contrast to sequence 2 where the third strand is in antiparallel orientation (Fig. 1). Using circular dichroism (CD) spectroscopy and UV thermal denaturation experiments, we have investigated the ability of berenil to induce the formation of triple helices and evaluated the influence of the orientation of the third strand relative to the purine strand on this process. For comparison, similar experiments were performed with a reference hairpin duplex formed by the oligonucleotide 5′-d([T_4]-$G_4A_4G_4$-[T_4]-$C_4T_4C_4$) (sequence 3) involved in the sequences 1 and 2.

Materials and methods

Solution preparation

The synthetic DNA oligonucleotides purified by ion exchange HPLC were purchased from Appligene Oncor. They were used after further purification on 19% polyacrylamide gels containing 6 M urea and 20% formamide. Oligonucleotide solutions were prepared in a buffer containing 10 mM sodium cacodylate and 0.2 mM disodium EDTA (pH 7.0). Before use, the solutions were heated in a water bath at 95 °C for 10 min and allowed to slowly return to ambient temperature before storing at 4 °C overnight.

Berenil was obtained from Sigma. Working solutions were prepared daily by dissolving berenil in the buffer. In all experiments the concentration was determined at 20 °C using the molar extinction coefficient $\epsilon_{380} = 29{,}800\ M^{-1}\ cm^{-1}$.

UV thermal denaturation experiments

The UV absorbance and melting studies were carried out on a Uvikon KONTRON 941 spectrophotometer. The temperature of cell holder was regulated by a Bioblock cryothermostat. The measurements were initiated approximately near 3 °C and the temperature was increased up to 95 °C at a rate of 0.5 °C/min. Melting temperatures (T_m) were taken as the temperature of half-dissociation of the complex and were obtained from the maximum of the first derivative dA/dT plots (where A is absorbance and T is temperature). Precision in T_m values, estimated from variance in three or four repeated experiments, was ±0.5 °C.

CD spectroscopy

CD measurements were carried out on a Jobin-Yvon mark IV dichrograph. The temperature of the cell was adjusted with a circulating refrigerated water bath and held constant to ±0.5 °C. Spectral titrations were carried out at 3 °C. Each CD spectrum was an average of at least two scans.

Results and discussion

In the 300–450 nm region, both free berenil and DNA do not exhibit any CD signal. For DNA it is due to the fact that it does not absorb in this wavelength range and for the free berenil is due to the presence of a planar symmetry which makes it optically inactive. In this wavelength range, the binding of berenil to the duplex structure can be detected and monitored by the appearance of a binding-induced Cotton effect arising from the interaction of the berenil chromophore with the dissymmetric environment of the DNA (Durand et al. 1994; Lane et al. 1991; Pilch et al. 1995).

Figure 2a shows the CD change observed upon incremental addition of berenil to the reference oligonucleotide 5′-d([T_4]-$G_4A_4G_4$-[T_4]-$C_4T_4C_4$) (sequence 3) at 3 °C. In these experimental conditions the oligonucleotide forms a stable double helix since its denaturation occurs at 58 °C (Gondeau et al. 1998b). With increasing concentrations of berenil, we notice the progressive appearance of two positive bands in the near-UV region of the CD spectrum, which confirms complex formation between the drug and the oligonucleotide. In this wavelength range a similar induced spectrum was previously observed using the *Eco*RI dodecamer $d(CGCGAATTCGCG)_2$ (Lane et al. 1991) and the $(dA)_{12}(dT)_{12}$ sequence (Durand et al. 1994). When the berenil concentration increases, a shift of the more intense band to higher wavelengths occurs. Figure 2b shows a CD titration curve using the signal integrated between 360 and 375 nm for more precision. This curve presents a change of slope close to $R = 2$ (where R = drug to oligonucleotide ratio), suggesting that the berenil interaction with the reference sequence involves more than one mode of binding. Based on previous studies,

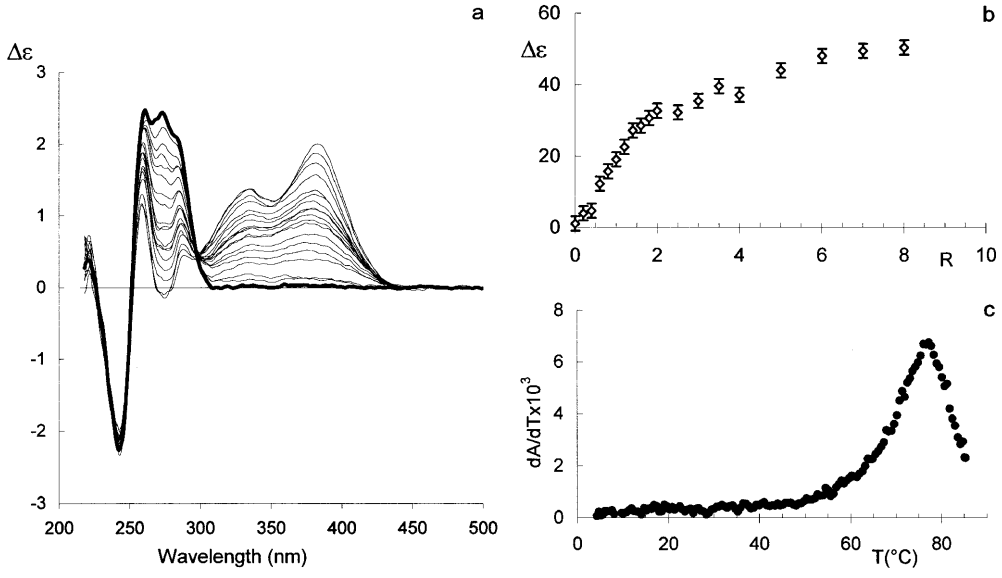

Fig. 2 **a** CD spectra of the reference oligonucleotide 5′-d([T_4]-$G_4A_4G_4$-[T_4]-$C_4T_4C_4$) (sequence 3) in the absence (*bold solid line*) and in the presence of increasing concentrations of berenil at 3 °C, in a buffer containing 10 mM sodium cacodylate, 0.2 mM EDTA, pH 7.0. The ratio berenil/oligonucleotide (R) varied from 0 to 8. Nucleotide concentration was 7×10^{-5} M. **b** CD titration curve using the signal integrated between 360 and 375 nm. The concentration used to calculate the CD intensity was that of the oligonucleotide unit. **c** First derivative UV profile of the melting of the berenil-oligonucleotide complex for $R=8$

one can attribute the first step of this curve to the minor groove interaction of berenil with the central A_4T_4 site of the oligonucleotide. The second step might be due to the minor groove binding of berenil to the G_4C_4 sites or to intercalative binding either in the AT or GC sites, since such a type of binding has been suggested by several studies (Colson et al. 1996; Lane et al. 1991).

After titration recorded by CD and at the upper drug to oligonucleotide ratio reached in this experiment ($R=8$), the denaturation process of the berenil-oligonucleotide complex was followed using UV absorbance at 260 nm (Fig. 2c). The denaturation occurs at a temperature higher ($T_m=77$ °C) than that measured in the absence of berenil ($T_m=58$ °C). As expected, the berenil stabilizes the duplex.

Figure 3 shows similar experiments performed with sequence 1, which is susceptible to form a parallel triple helix. In the experimental conditions mentioned in Materials and methods, this oligonucleotide forms a hairpin duplex with a dangling extremity (Gondeau et al. 1998a). When berenil is added to the oligonucleotide solution, we observe in the 300–440 nm region an induced CD signal. The significant observation is that for R values smaller than 2 the induced signal in the 300–440 nm region is identical to that detected when the reference molecule (sequence 3) serves as the host structure (inset of Fig. 3a). For R values larger than 2, the induced signal is slightly different from that observed when sequence 3 was used. Particularly one can notice that the 335 nm band and the 385 nm band are of almost equal intensity (Fig. 3a). As with the reference duplex, we note that the titration curve presents a change of slope (Fig. 3b) close to an R value of 2. After titration, the denaturation process, followed using UV

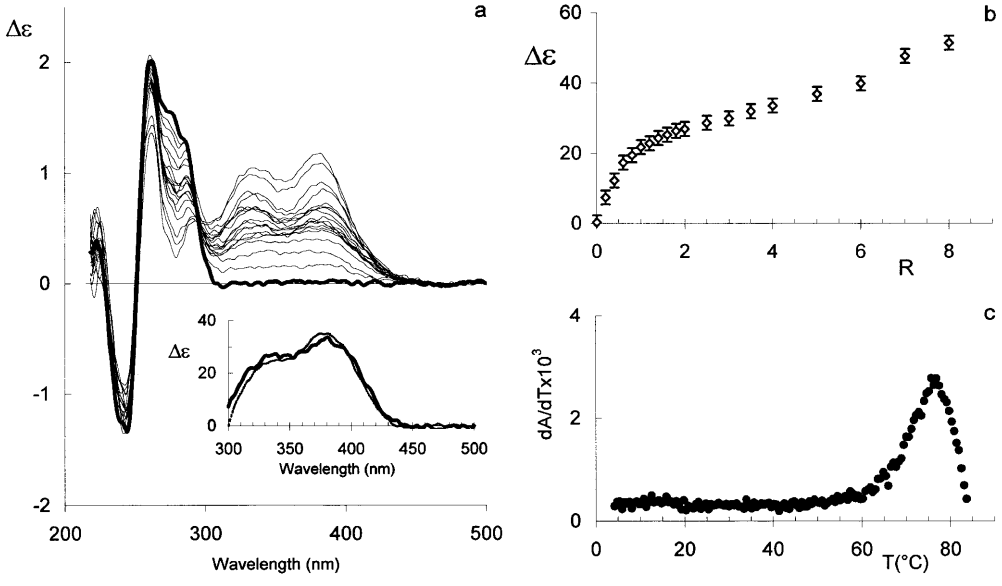

Fig. 3 **a** CD spectra of the oligonucleotide 5′-d($G_4A_4G_4$-[T_4]-$C_4T_4C_4$-[T_4]-$G_4T_4G_4$) (sequence 1) in the absence (*bold solid line*) and in the presence of increasing concentrations of berenil in the same experimental conditions as in Fig. 2. The R ratio varied from 0 to 8. Nucleotide concentration was 9.5×10^{-5} M. *Inset*: CD spectra in the 300–500 nm region of the oligonucleotide-berenil complexes for the sequences 2 (*solid line*) and 3 (*broken line*). The R ratios were respectively equal to 1.5 and 1.7. **b** CD titration curve as described in Fig. 2. **c** First derivative UV profile of the melting of the oligonucleotide-berenil complexes recorded at 260 nm ($R=8$)

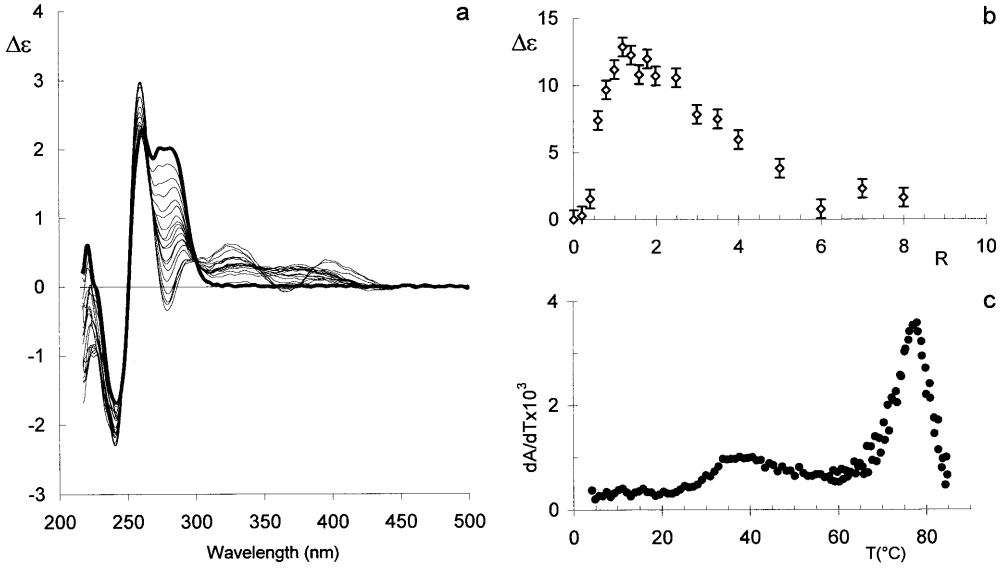

Fig. 4 **a** CD spectra of the oligonucleotide 5′-d($G_4T_4G_4$-$[T_4]$-$G_4A_4G_4$-$[T_4]$-$C_4T_4C_4$) (sequence 2) in the absence (*bold solid line*) and in the presence of increasing concentrations of berenil in the same experimental conditions as in Fig. 2. The *R* ratio varied from 0 to 8. Nucleotide concentration was 9.5×10^{-5} M. **b** CD titration curve as described in Fig. 2. **c** First derivative UV profile of the melting of the berenil-oligonucleotide complex ($R=8$)

absorbance, presents only one transition with $T_m = 77$ °C (Fig. 3c). Similar results were obtained using CD measurements (data not shown). We conclude that: (1) the conformation duplex-dangling end of sequence 1 is conserved up to the upper *R* value reached in our experiment; (2) as with the reference duplex, the first mode of interaction is a minor groove binding in the A_4T_4 site of the oligonucleotide.

Figure 4 shows experiments performed with sequence 2, which is susceptible to form an antiparallel triple helix. As for sequence 1, in the absence of berenil, and in the experimental conditions of the present study, this oligonucleotide forms a hairpin duplex with a dangling extremity (Gondeau et al. 1998b). For low values of *R* the CD spectra in the 300–450 nm region are similar to the CD spectra of the berenil-sequence 1 complex recorded under the same experimental conditions (Fig. 4a). However, from $R=0.7$, drastic changes are observed. The longer wavelength band progressively shifts to 395 nm and the shorter wavelength band shifts to about 330 nm (instead of 383 and 335 nm for sequence 1). Looking at the DNA wavelength region (below 300 nm), it is noteworthy that the band located around 225 nm from positive progressively becomes negative and that there is the appearance of a negative band centred at 278 nm, in contrast to what is observed in the case of sequence 1. We previously demonstrated that these changes in the CD spectrum are characteristic of the formation of an antiparallel triple helix (Gondeau et al. 1998b). Figure 4b shows that the titration curve is drastically different from those obtained with sequences 3 and 1. Until a value of *R* close to 1 is reached, the integrated CD signal increases, then it decreases until an *R* value close to 6 is reached. The denaturation process of the berenil-sequence 2 complex at a ratio of $R=8$ was followed using UV absorbance after CD titration. The melting profile presents two clear transitions (Fig. 4c): The first one has a T_m close to 38 °C, the second one at $T_m = 77$ °C. These results are confirmed when the melting is followed using the CD signal (Fig. 5). We conclude that the lower temperature transition corresponds to the antiparallel triplex-duplex melting and that the second transition corresponds to the duplex-coil melting. In order to determine precisely the *R* ratio from which there is formation of the antiparallel triple helix, we performed the denaturation of the berenil-sequence 2 complex at *R* values of 1, 2 and 4. The results are presented in Fig. 5. At a ratio of 4 we observe two well-resolved transitions, as for $R=8$: the first one with a T_m value of about 30 °C and the second one with T_m close to 73 °C. With a ratio of $R=2$ we also observed two sequential transitions. The higher temperature transition, corresponding to the duplex to single stranded melting, shows that the berenil induces a stabilization of the duplex by about 10 °C. The first transition is not well resolved, as we could not reach its lower temperature part. Nevertheless, we can estimate that the corresponding T_m is near 15 °C. With the ratio $R=1$, there is one clear transition at about 65 °C which cor-

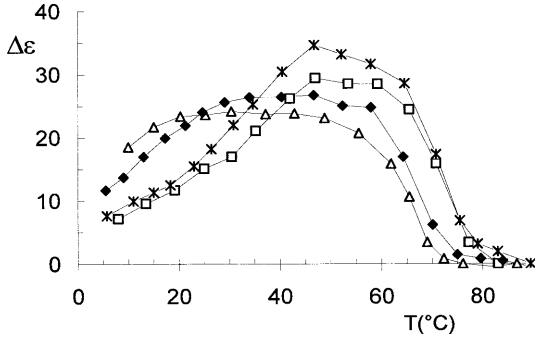

Fig. 5 CD melting of the berenil-oligonucleotide complexes for various *R* ratios: $R=1$ (*open triangles*), $R=2$ (*solid diamonds*), $R=4$ (*stars*), $R=8$ (*open squares*)

responds to the duplex to single stranded melting with a stabilization of 8 °C versus the oligonucleotide in the absence of berenil. One can clearly see in the lower temperature part of this curve, below 20 °C, a variation of the signal corresponding to the end of the triplex to duplex transition. For this ratio we cannot determine the T_m corresponding to this transition but it is clearly below 10 °C. All together, these observations suggest that the binding of only one berenil molecule is enough to induce the formation of the antiparallel triplex.

Conclusions

We have previously demonstrated, using an intramolecular oligonucleotide system, that in the low salt buffer used in the present study, berenil induces the formation of a parallel triple helix in a system containing only T*A:T triplets (Durand et al. 1994). The results reported here clearly demonstrate that in a system containing both potential T*A:T and G*G:C triplets, where two orientations of the third strand are possible, berenil induces the formation of only one of the two triplexes, the antiparallel one. In contrast, we demonstrated in previous studies that, in similar conditions, netropsin, a minor groove binding ligand, and ethidium bromide, the classical DNA intercalator, do not induce the parallel or the antiparallel triple helices (Gondeau et al. 1998a, 1998b, 2000). These studies have also shown that the binding of Mg^{2+} to these oligonucleotides induces both the parallel and the antiparallel triplexes, but they also have pointed out several differences of behaviour between both triplexes (monophasic versus biphasic melting, disruption by KCl, difference in the binding constant of netropsin, etc.), reflecting structural differences. These structural differences certainly explain the different effect of berenil on both sequences. The results presented here will be useful in defining the experimental conditions to orient the binding of the third strand to a sequence and to realize the potential usefulness of triple helices as artificial nucleases and regulators of gene expression in vitro and in vivo.

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