Ligand-induced formation of Hoogsteen-paired parallel DNA

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zIntroduction: Based on molecular modeling studies, a model has been proposed for intercalation of triple-helix-specific ligands (benzopyridoindole (BPI) derivatives) into triple helices, in which the intercalating compounds interact mainly with the Hoogsteen-paired strands of the triple helix. We set out to test this model experimentally using DNA duplexes capable of forming parallel Hoogsteen base-paired structures.

Results: We have investigated the possible formation of a parallel DNA structure involving Hoogsteen hydrogen bonds by thermal denaturation, FTIR spectroscopy and gel-shift experiments. We show that BPI derivatives bind to Hoogsteen base-paired duplexes and stabilize them. The compounds induce a reorganization from a non-perfectly matched antiparallel Watson–Crick duplex into a perfectly matched parallel Hoogsteen-paired duplex.

Conclusions: These results suggest that preferential intercalation of BPI derivatives in triple helices is due to their ability to interact specifically with the Hoogsteen-paired bases. The results are consistent with a model proposed on the basis of molecular modeling studies using energy minimization, and they open a new field of investigations regarding the biological relevance of Hoogsteen base-pairing.

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Introduction

Hoogsteen-type hydrogen bonds were first postulated on the basis of X-ray crystallographic studies of co-crystals of adenine and thymine derivatives [1]. They are formed between N_3 and O_4 of thymine and N_7 and N_6 of adenine, respectively (Fig. 1), and were proposed to be present in triple helices made from two strands of polyU and one strand of polyA [2]. Triple helices made by poly(dTdC).poly(dAdG).poly(dTdC) were later observed [3] and shown to contain Hoogsteen-type hydrogen bonds between protonated cytosine and guanine. These hydrogen bonding schemes were confirmed by NMR spectroscopy in 1989 [4,5]. Hoogsteen-type base pairing was extensively studied in triple helices made by polynucleotides during the 1970's. Interest in triple helices was renewed at the end of the 1980's because of the discovery that intramolecular triple helices could be formed in supercoiled plasmids [6] and that short oligonucleotides can bind to a duplex [7,8], forming a triple helix. Oligonucleotides can thus be used to selectively recognize sequences of double-stranded DNA and may prove useful in this capacity as tools for molecular biology [9] and as therapeutic agents [10].

One important characteristic of Hoogsteen base pairing in triple helices is that it is generally less stable than Watson–Crick base pairing. Double-helical polynucleotides usually adopt Watson–Crick base pairing rather than Hoogsteen pairing. The latter type of base pairing can be observed, however, using bulky groups attached to bases, which prevent Watson–Crick base pairing [11]. More recently, Sasisekharan and collaborators [12] showed, for the first time, that a parallel Hoogsteen-paired oligonucleotide DNA duplex could exist independently of the triple helix of which it is a component part. They built a stereochemically acceptable model, which showed that the structure of a Hoogsteen-paired duplex resembles that of the Hoogsteen-paired strands in a triple helix [13].

Triple-stranded complexes can be stabilized by a high ionic strength or by the presence of cations like magnesium [14]. We have previously shown that benzopyridoindole (BPI) derivatives **3e** (3-methoxy-7H-8-methyl-11-amino-benzo[e]pyrido[4,3-b]indole), **9e** (3-methoxy-7H-8-methyl-11-[(3'-amino)propylamino]-benzo-[e]pyrido[4,3-b]indole) and **14g** (3-methoxy-7-[((3'-diethyl-amino)propyl)-amino]-10-methyl-11H-benzo[g]pyrido[4,3-b]indole) (Fig. 1) are triple helix specific ligands. These agents stabilize triple helices [15,16] and intercalate in triple-helical complexes between T.AxT base triplets (where the '.' represents Watson–Crick base pairing and the 'x' represents Hoogsteen base pairing) [17,18]. The detailed structure of the complex between a triple helix and an intercalator is not yet solved. While investigating the mode of binding of benzopyridoindole derivatives to triple helices, we wondered whether these compounds were able to bind to Hoogsteen-paired

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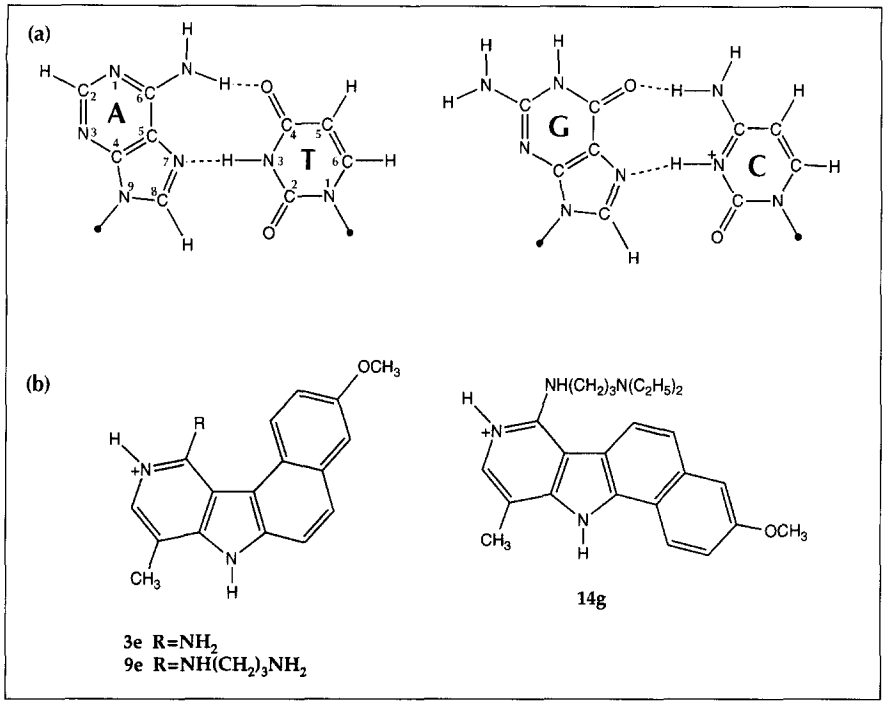

Fig. 1. **(a)** Hoogsteen AxT and GxC$^+$ base pairs. The latter base pair includes a protonated cytosine. **(b)** Structure of the three BPI derivatives used in this study: compounds **3e** and **9e** are benzo[e]pyrido[4,3-b]indole derivatives (BePI), whereas compound **14g** is a benzo[g]pyrido[4,3 b]indole derivative (BgPI).

duplexes. Here we show that formation of parallel-stranded Hoogsteen-paired DNA is stabilized by magnesium and BPI derivatives, and that these compounds are able to induce a reorganization of a Watson–Crick duplex into a Hoogsteen duplex.

Results

A pH-dependent structure involving protonated cytosines

The oligonucleotides shown in Figure 2a were designed to form triple helices upon Hoogsteen base pairing of the strand called H to the Watson–Crick duplex made by strand Y, containing pyrimidines, and strand R, containing purines. The triple helix is thus depicted as Y.RxH. Two types of sequences were investigated, which will be referred to by the numbers 3 and 5 to indicate the number of cytosines in the pyrimidine strand (or of guanines in the purine strand). We had previously studied the formation of triple helices by these sequences and the stabilization of this structure by specific ligands [15,16]. Purine-containing oligonucleotides form Watson–Crick duplexes (Y3.R3 and Y5.R5) with their complementary pyrimidine-containing oligonucleotides. The pyrimidine oligonucleotides in the reverse orientation can also form partial Watson–Crick duplexes (e.g., H3.R3 and H5.R5). Possible structures for these duplexes are shown in Figure 2b. Oligonucleotides R3 and H3 might form a 10-base-pair (bp) antiparallel Watson–Crick duplex, whereas H5 and R5 cannot form this structure with more than four contiguous base pairs. The structure shown in Figure 2b contains 12 base pairs with two bulged bases. A parallel 14-bp Hoogsteen complex should be possible for both sequences and is noted as R3xH3 and R5xH5. When a mixture of strands R3 and H3 or R5 and H5 is described before characterizing its precise structure, the notations R3H3 and R5H5 will be used.

We investigated whether R3H3 or R5H5 formed structures involving Hoogsteen base pairs. Melting curves of equimolar mixtures of strands Y and R or R and H were determined by absorption spectroscopy as a function of the pH for both types of sequences. A pH-dependent melting profile was observed when oligonucleotides R5

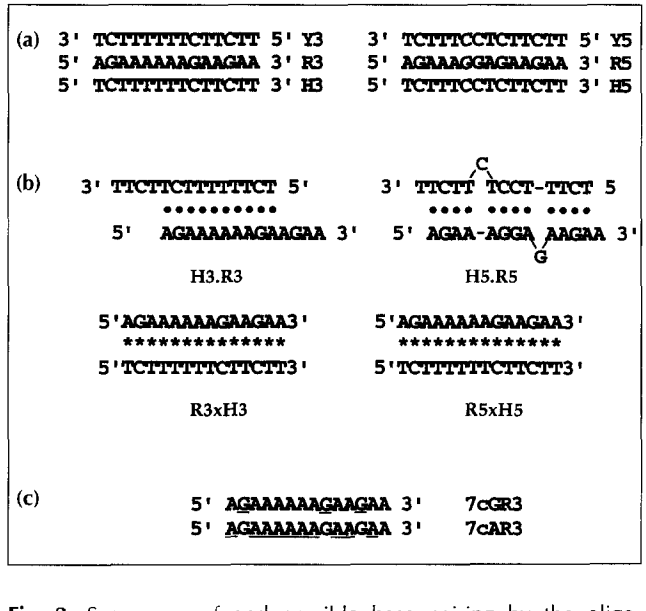

Fig. 2. Sequences of and possible base pairing by the oligonucleotides used in this study. **(a)** Sequences of the oligonucleotides used in this study. Strands R (purine) and Y (pyrimidine) are complementary in the Watson–Crick sense (antiparallel) whereas strand H (pyrimidine) is the Hoogsteen complement of R (parallel). Oligonucleotides in the H3 sequence contain a stretch of six T nucleotides. In the H5 sequence, two contiguous T nucleotides were changed to C. **(b)** Possible structures for complexes between R and H oligonucleotides: partial Watson–Crick duplex (H.R) or 14-bp parallel Hoogsteen duplex (RxH). ● indicates Watson–Crick base pairing, whereas * indicates Hoogsteen base pairing. **(c)** Sequence of oligonucleotides containing 7-deaza-adenines or 7-deaza-guanines (the 7-deaza-purines are underlined).

and H5 were mixed together. At pH 7, a transition of weak amplitude was observed at very low temperature (< 10 °C), whereas at pH 6.5 and below, transitions could be observed at higher temperatures, and the melting profiles were not reversible (Fig. 3a). This phenomenon was previously observed for triple-helices [19] and other structures where cytosines need to be protonated such as i-DNA [20]. The hysteresis of the melting curves in this case was attributed to slow kinetics of association and dissociation due to cytosine protonation.

Subtraction of the absorbance at 0 °C from that at 50 °C for the Watson–Crick (Y5.R5) and the Hoogsteen (R5xH5) duplexes at pH 5.6 shows a weak but reproducible negative band around 295 nm for the Hoogsteen duplex, but not for the Watson–Crick duplex (Fig. 3b). This hyperchromism presents the same hysteresis phenomenon as that at 260 nm (Fig. 3b). Such a hyperchromism, which has been previously observed in triple helices [21] and in i-DNA motifs [20], results from a red-shift of the absorption of cytosines upon protonation, and is consistent with the formation of Hoogsteen-type hydrogen bonds.

For the mixture of R3 and H3, a single pH-independent transition occurs at 23 °C at pH values above 5.6, which can be attributed to the 10-bp R3.H3 duplex described in Figure 2b (see below). A T_m increase similar to that of the H5xR5 mixture was observed only at more acidic pH values (data not shown).

Interactions of compound 3e with Hoogsteen-paired duplex DNA

The half-dissociation temperatures of the R5H5 complex are plotted as a function of the pH in Figure 3c, either in the buffer alone or in the presence of $MgCl_2$ (10 mM) or compound **3e** (15 μM). T_m values are higher in the presence of compound **3e** or $MgCl_2$ and increase further when the pH is lowered. These results suggest that the parallel duplex is stabilized by magnesium ions and by compound **3e**.

The pH dependence of the melting temperature of the Y3.R3 and R3H3 duplex is shown in Figure 4 under various conditions. For the perfectly matched Watson–Crick duplex (Y3.R3), no significant change in the denaturation temperature could be observed while decreasing the pH from 7.0 to 5.2, either in a buffer containing NaCl (0.1 M) or after addition of compound **3e** (15 μM) (Fig. 4a). When the oligonucleotides R3 and H3 were mixed together at pH above 5.6, a pH-independent, reversible transition with a T_m of 23 °C was observed (Fig. 4b) which can be attributed to the melting of the 10-bp Watson–Crick duplex H3.R3 (see Fig. 2b). We obtained a pH-dependent T_m at higher temperature, however, by adding compound **3e**. This ligand probably induced a reorganization of the 10-bp H3.R3 Watson–Crick duplex into a 14-bp R3xH3 Hoogsteen duplex at acidic pH. The same phenomenon could be observed in the presence of $MgCl_2$, (10 mM) which is known to stabilize triple helices. When ethidium bromide

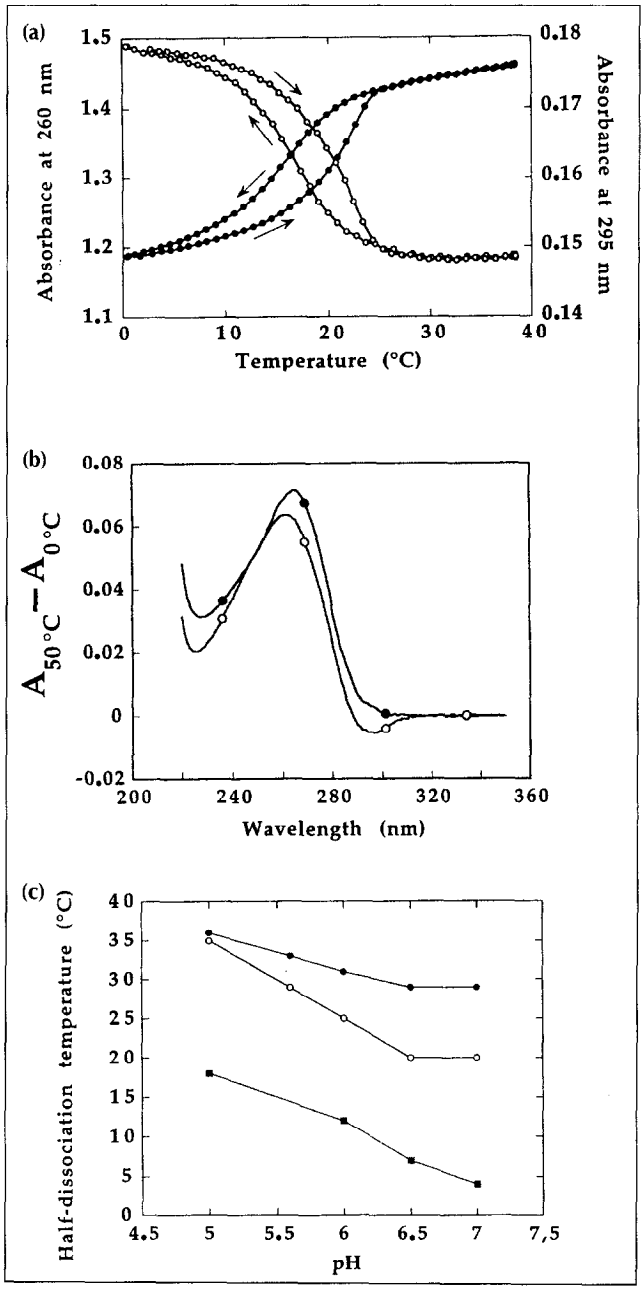

Fig. 3. Evidence for formation of a complex between R5 and H5 containing Hoogsteen base pairs. **(a)** Denaturation profile of the complex formed by R5 and H5 at pH 5.6, measured at 260 nm (●) and 295 nm (O) (for conditions, see Materials and methods). The two curves in each series were obtained upon heating and cooling as indicated by the arrows. **(b)** Difference between absorption spectra at 0 °C and 50 °C for the Y5.R5 Watson–Crick duplex (●) and the R5xH5 complex (O). **(c)** Plot of melting temperatures of the complex formed by R5 and H5 as a function of pH, in a cacodylate buffer (10 mM) containing 0.1 M NaCl (■) or after addition of 15 μM compound **3e** (O) or 10 mM $MgCl_2$ (●).

was added instead of compound **3e**, the H3.R3 complex dissociated at 32 °C, independently of the pH. Ethidium bromide thus stabilizes only the Watson–Crick duplex.

Oligonucleotides containing 7-deazapurines

Formation of Hoogsteen-type hydrogen bonds involves the N7 atom of purines. 7-Deaza-adenines and 7-deaza-guanines were incorporated into oligonucleotides 7cAR3

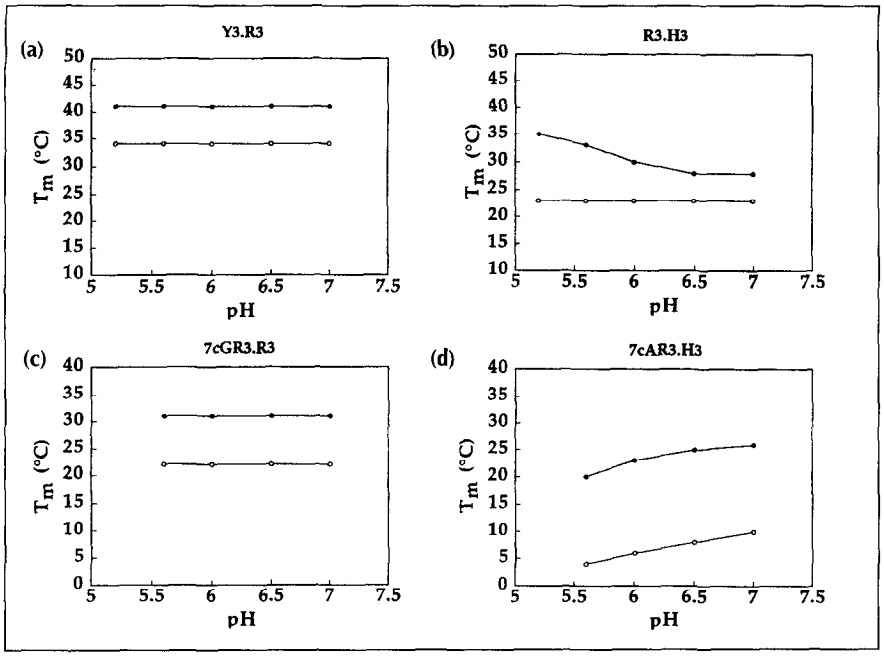

Fig. 4. Compound **3e** induces a shift in the T_m of R3H3 at acidic pH indicating a reorganization of base pairing. Plots of the melting temperatures (T_m) of various duplexes as a function of pH, in the absence (O) or in the presence (●) of ligand **3e**. **(a)** Y3.R3 Watson–Crick duplex. **(b)** R3H3 duplex. **(c)** 7cGR3.H3 duplex. **(d)** 7cAR3.H3 duplex.

and 7cGR3, respectively. These oligonucleotides should not be able to form Hoogsteen hydrogen bonds. The melting temperature of the complex formed between 7cGR3 and H3 was independent of pH, either in the absence or the presence of compound **3e** or magnesium. The melting temperature of the complex formed between 7cAR3 and H3 decreased at acidic pH, probably due to the protonation of 7-deaza-adenine. In the presence of compound **3e**, the T_m values of 7cGR3.H3 and 7cAR3.H3 were shifted upwards by 9 °C and 16 °C, respectively (Figs. 4c,d), but no additional significant stabilization at acidic pH could be detected. Thus, oligonucleotides containing 7-deaza adenines or 7-deaza-guanines are unable to adopt the same structure as natural oligonucleotides in the presence of compound **3e** at acidic pH.

Gel-shift experiments

The electrophoretic mobility of both the Watson–Crick and Hoogsteen base-paired duplexes and of the triple helix was studied at 4 °C in the presence of magnesium (10 mM) (Fig. 5). The Hoogsteen duplex migrates more slowly than the Watson–Crick duplex, as previously reported [12]. This behavior probably reflects the protonation of the cytosines as well as a different helical structure compared to the Watson–Crick double helix. The triple helix migrates more slowly than the Hoogsteen duplex due to the presence of an additional pyrimidine strand. The species migrating as a Hoogsteen duplex involved one strand of each of the oligonucleotides R3 and H3 as shown by studies at different concentrations of the two oligomers. When the labeled oligopurine strand was replaced by oligonucleotides containing 7-deaza-adenines or 7-deaza-guanines, the complex formed with the Hoogsteen complementary strand migrated like the Watson–Crick duplex, and no triple helix could be observed. Thus, as observed by thermal denaturation experiments, oligonucleotides containing 7-deaza-A or 7-deaza-G were unable to form a Hoogsteen duplex, but could still form a 10-bp Watson–Crick duplex.

Fourier transform infrared (FTIR) spectroscopy

Compound **3e** could not be used for FTIR spectroscopy experiments because of its poor solubility. Compounds **9e** and **14g** are also efficient triple helix-stabilizing ligands, but were shown to have an increased affinity for duplexes when compared to compound **3e** [16]. It should thus be more difficult to induce the Watson–Crick to Hoogsteen transition with these compounds. Thermal denaturation experiments showed that this transition occurred at more acidic pH levels than

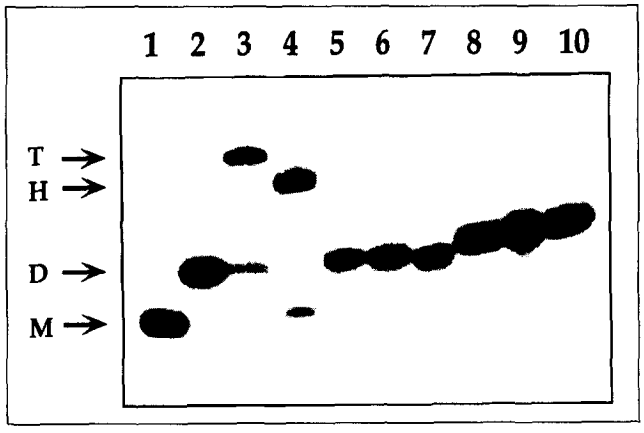

Fig. 5. Electrophoretic mobility shift analysis of nucleic acid structures. Gel-shift experiments at pH 6 and 4 °C in a buffer containing 50 mM MES, 10 mM $MgCl_2$ are shown. Each oligonucleotide was at a concentration of 2 μM. Lane 1: single-stranded ^{32}P-labeled R3 oligonucleotide. Lane 2: Watson–Crick Y3.R3 duplex. Lane 3: Triple helix Y3.R3xH3. Lane 4: Hoogsteen-paired R3xH3 duplex. Lanes 5, 6, 7: as lanes 2, 3, 4 but replacing R3 with 7cAR3. Lanes 8, 9 10: as lanes 2, 3, 4 but replacing R3 with 7cGR3. T, triple helix; H, Hoogsteen duplex; D, Watson–Crick duplex; M, monomer.

with compound **3e** (data not shown), and the FTIR experiments described below confirm this result.

The two duplexes H3.R3 and H5.R5 were formed by mixing equimolar concentrations of the respective single-strand solutions at neutral pH. To study the effect of BPI derivatives, we mixed the appropriate amounts of drug (solutions of compound **14g** or **9e** at acidic pH) with the duplex (at pH = 6.0) at an input ratio of 0.2 mole of drug per mole of base pairs. To study the effect of Mg^{2+} ions, we added the appropriate amounts of $MgCl_2$ to the duplex solution (at pH = 4) at an input ratio of 0.5 mole of Mg^{2+} per mole of base pairs.

The spectra of the complexes of R3H3 and R5H5 with the drugs and those obtained in the presence of Mg^{2+} under acidic conditions, showed striking differences when compared with those recorded in the absence of drug or Mg^{2+}. Figure 6a shows the spectrum in the region of in-plane double bond vibrations of the bases (1750–1600 cm^{-1}) for the D_2O solution of H3.R3 at neutral pH. The bands located at 1696 and 1661 cm^{-1} are assigned to the vibrations of the $C_2{=}O_2$ and $C_4{=}O_4$ groups of thymine, respectively [22]. The band located at 1641 cm^{-1} is a characteristic band of thymine engaged in Watson–Crick base pairing. The band located at 1625 cm^{-1} is assigned to vibrations of the adenine ring (C=C and C=N double bond stretching) coupled to an ND_2 vibration. The expected bands of guanine and cytosine at 1650 and 1685 cm^{-1} are probably overlapping the intense band of thymine at 1661 cm^{-1}.

The spectra of the H3.R3 1:1 mixture with compound **14g** or with Mg^{2+} at two different pH values are shown in Figures 6b–d. The vibration band at 1661 cm^{-1} ($C_4{=}O_4$ of thymine) in the R3H3 spectrum shifted to 1657 cm^{-1} in the presence of drug or Mg^{2+}. No significant shift was observed for the absorption located at

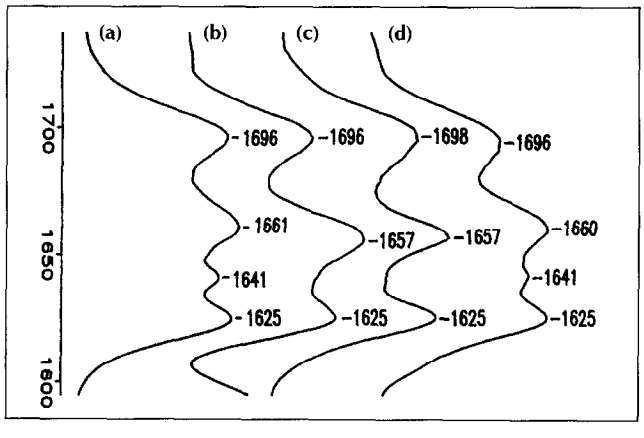

Fig. 6. FTIR spectra (1750–1600 cm^{-1}) of R3H3 complexes, recorded in D_2O solution, indicate that Mg^{2+} or compounds **14g** or **9e** induce formation of Hoogsteen-paired parallel DNA at acidic pH. **(a)** R3H3 pH = 7.8. **(b)** R3H3 in the presence of one mole of compound **14g** per five moles of base pairs, pH = 5.7. **(c)** R3H3 in the presence of one mole of Mg^{2+} per two moles of base pairs, pH = 5.7. **(d)** R3H3 in the presence of one mole of Mg^{2+} per two moles of base pairs, pH = 7.

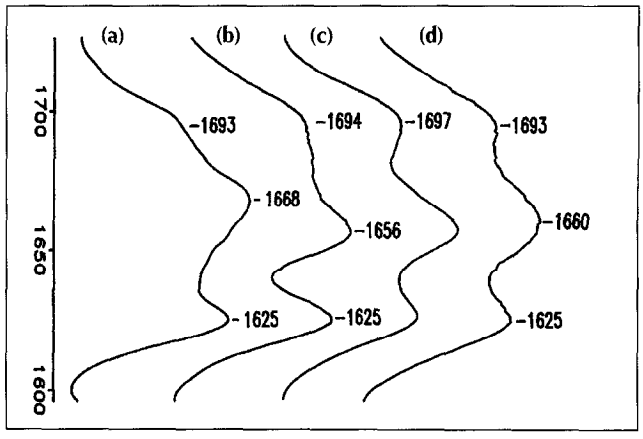

Fig. 7. FTIR spectra (1750–1600 cm^{-1}) of R5H5 complexes, recorded in D_2O solution, indicate that Mg^{2+} or compounds **14g** or **9e** induce formation of Hoogsteen-paired parallel DNA at acidic pH. **(a)** R5H5, pH = 7.8. **(b)** R5H5 in the presence of one mole of compound **14g** per five moles of base pairs, pH = 5.7. **(c)** R5H5 in the presence of one mole of Mg^{2+} per two moles of base pairs, pH = 5.7. **(d)** R5H5 in the presence of one mole of Mg^{2+} per two moles of base pairs, pH = 6.8.

1696 cm^{-1} ($C_2{=}O_2$ of thymine). In addition, the band at 1641 cm^{-1}, characteristic of a thymine involved in Watson–Crick base pairing, was no longer observed. It should be noted that upon increasing the pH to 7 in the presence of Mg^{2+}, the band at 1657 cm^{-1} gradually shifted back to 1661 cm^{-1} and the band at 1641 cm^{-1} reappeared (Fig. 6d). An identical effect was observed in the presence of compound **9e** (Table 1). The frequency shift of the band assigned to the stretching vibration of the $C_4{=}O_4$ group of thymine reflects the presence of an interaction at this site.

The spectra of R5H5 complexes prepared in the presence of compound **14g** or **9e** or of Mg^{2+} at acidic pH exhibited a similar frequency shift of the vibration band assigned to the $C_4{=}O_4$ stretching vibration (located at 1668 cm^{-1} in the H5.R5 spectrum in the absence of drug or Mg^{2+} and at 1657 cm^{-1} in the presence of drug or Mg^{2+}) (Fig. 7, Table 1). The slight frequency shift of the band located at 1693 cm^{-1}, observed in the spectrum of the H5.R5 complex with Mg^{2+} at acidic pH, is probably due to the overlapping contributions of the protonated cytosine ring (absorption at 1702 cm^{-1}) [23] with the $C_2{=}O_2$ carbonyl band of thymine.

The spectra of both the R3H3 and R5H5 complexes recorded at acidic pH in the absence of compound **14g** or **9e** or of Mg^{2+} show a band at 1699 cm^{-1}, characteristic for the protonation of cytosines, and a decrease of the band at 1642 cm^{-1}, showing that Watson–Crick base pairs are disrupted (data not shown). For both sequences, no band shift characteristic of Hoogsteen hydrogen bond formation could be detected.

These results are consistent with a transition from an antiparallel duplex with Watson–Crick base pairing to a Hoogsteen base-paired parallel-stranded DNA duplex

Table 1. Wavenumber of chosen observed infrared bands of *H3R3,H5R5* and their complexes with compound **14g** or **9e** or with Mg^{2+} (at different pH levels).

Sample	Wavenumber (cm^{-1})
H3.R3, pH = 7.0	1696;1661;1641;1625;1490
R3xH3 plus **14g**, pH = 5.7	1696;1657;1625;1484
R3xH3 plus **9e**, pH = 5.7	1696.6;1658;1625;1484
R3xH3 plus Mg^{2+}, pH = 5.7	1698;1657;1625;1484
R3xH3 plus Mg^{2+}, pH = 6	1698;1658;1625;1484
H3.R3 plus Mg^{2+}, pH = 7	1696;1660;1641;1625;1488
H5.R5, pH = 7.0	1693;1668;1625;1491
R5xH5 plus **14g**, pH = 5.7	1694;1656;1625;1486
R5xH5 plus **9e**, pH = 5.7	1695;1657;1625;1486
R5xH5 plus Mg^{2+}, pH = 5.7	1697;1657;1625;1486
R5xH5 plus Mg^{2+}, pH = 6	1695;1659;1625;1487
H5.R5 plus Mg^{2+}, pH = 6.8	1693;1660;1625;1488

induced at acidic pH by the presence of compound **14g** or **9e** or of Mg^{2+} ions. The proposed structures are shown in Figure 2b. A similar pattern of infrared absorptions was previously observed for a parallel double helix with Hoogsteen base pairing formed by a 20-base DNA independently of the triple helix of which it was a component part [12].

The Hoogsteen base pairing scheme in the R3xH3 and R5xH5 complexes was confirmed by studying another region of the spectra (spectra recorded in H_2O solution), containing an absorption band located around 1490 cm^{-1} assigned to a vibration of the $H\text{-}C_8\text{-}N_7$ group of adenine [24]. This absorption is extremely sensitive to interactions involving the N_7 site. In the spectra of all complexes prepared with either R3xH3 or R5xH5 we observed a frequency shift of this band when compared to its position in the spectra of H3.R3 or H5.R5 in the absence of ligand (Table 1). This frequency shift reflects an interaction involving the N_7 site of the adenine.

FTIR spectroscopy provided us with the opportunity to characterize the sugar conformations. The spectral domain between 1000 and 800 cm^{-1} contains absorption bands assigned to vibration modes of the deoxyribose and of the phosphodiester backbone. The presence of a band around 840 cm^{-1} is characteristic of the S-type deoxyribose sugar conformation (C_2, endo/anti, B family form), whereas the presence of a band around 860 cm^{-1} reflects the presence of the N-type deoxyribose sugar conformation (C_3, endo/anti, A family form) [25]. The FTIR spectra of R3H3 in the absence of added cations or in the presence of Mg^{2+} at two different pH values is shown in Figure 8. These spectra were recorded in H_2O solution. In all spectra, we observed a unique band at 840 cm^{-1}, which showed that the conformation of the sugars in R3xH3 is S-type in the presence of Mg^{2+}. The same result was found in the case of R5xH5 in the presence of Mg^{2+} (data not shown). Unfortunately, a similar analysis was impossible for the complexes prepared with compound **14g** or **9e**, as both drugs presented a strong absorption band located at 868 cm^{-1}.

Discussion

Various types of parallel-stranded DNA structures have been described. The best studied one involves formation of reverse Watson–Crick hydrogen bonds and can be formed with appropriate sequences containing A and T nucleotides [26,27]. Such a duplex can incorporate G.C base pairs [28], but this pair leads to the formation of only one hydrogen bond and to a steric hindrance, resulting in a destabilizing effect. The stability of such a duplex does not require protonation of cytosines and should be formed with oligonucleotides containing 7-deazapurines. Therefore, the structure described in this study cannot be a reverse Watson–Crick parallel DNA. Parallel duplexes can also be formed between oligonucleotides containing alternating G-A sequences [29,30], and oligopyrimidines can also self associate to form i-DNA structures [20]. The structures observed in this study did not result from self-association of either strand.

Little is known regarding the interactions of drugs with nucleic acid parallel duplexes. Ethidium bromide was shown to intercalate into a reverse Watson–Crick parallel DNA duplex containing A and T, but the drug Hoechst-33258 was unable to bind to the groove of this structure [27]. BPI derivatives were shown to intercalate between DNA base pairs. They prefer, however, to intercalate between base triplets, especially T.AxT, in triple-helical structures [17,18]. It is unlikely that these compounds stabilize the Hoogsteen duplex by binding to one of the grooves, as the grooves of a Hoogsteen duplex do not possess a geometry that could accommodate the planar BPI molecule. One groove is very wide and shallow; the other is very narrow. Molecular modeling using energy

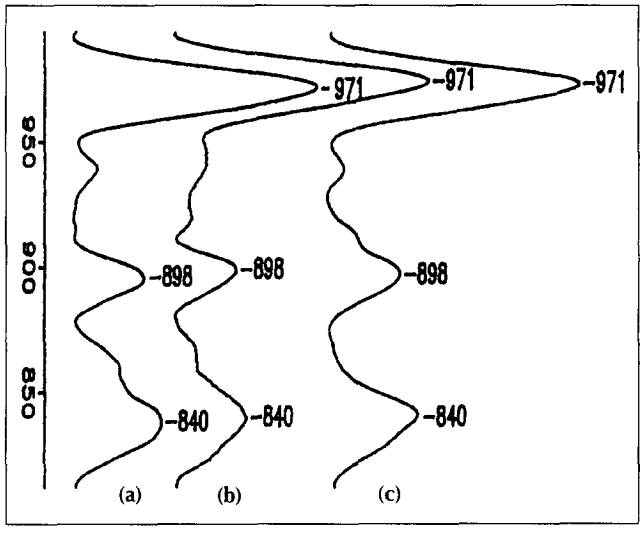

Fig. 8. FTIR spectra (1000–750 cm^{-1}) of R3H3 complexes, recorded in H_2O solution, indicate that the conformation of the sugars in R3xH3 is S-type in the presence of Mg^{2+}. **(a)** R3H3, pH = 7.8. **(b)** R3H3 in the presence of one mole of Mg^{2+} per two moles of base pairs, pH = 5.7. **(c)** R3H3 in the presence of one mole of Mg^{2+} per two moles of base pairs, pH = 7.0.

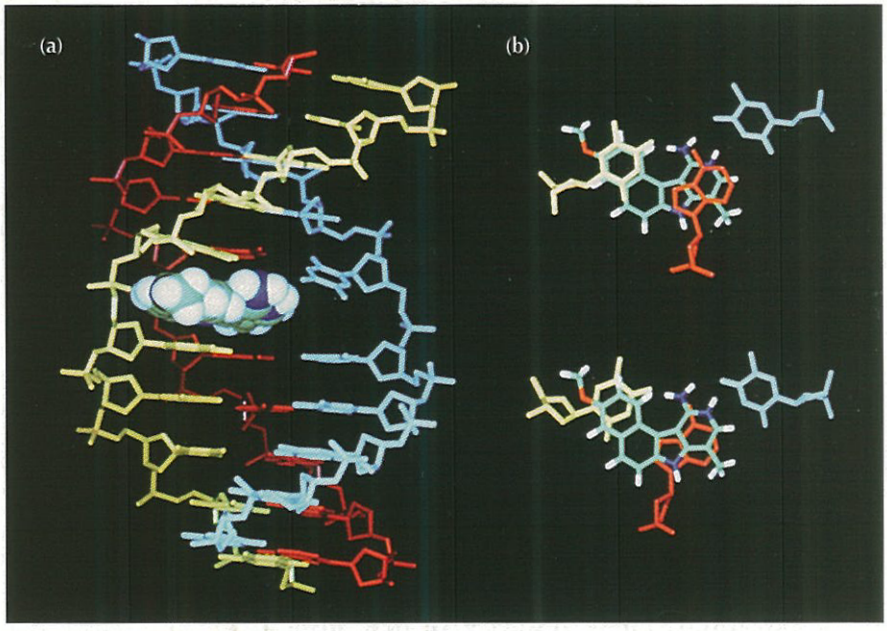

Fig. 9. Proposed model for intercalation of compound **3e** into a triple helix. A 10-base triplex T10.A10xT10 was built using coordinates derived from triple helix models. The molecule of compound **3e** was docked between the central base triplets or base pairs, which were separated to allow for intercalation. Then the complex was energy minimized using the JUMNA program (see [17]). **(a)** Structural model showing intercalation of compound **3e** in the triple helix. **(b)** The **3e** molecule is shown overlapping the base triplet at the 5' side (with respect to the purine strand) (top) and at the 3' side (bottom). Strands Y, R and H are colored in blue, red and yellow, respectively. The intercalated molecule is colored using standard colors for each atom (C in green, H in white, N in blue and O in red). For the sake of clarity, hydrogen atoms are not represented, except for the intercalated compounds.

minimization has been used previously to investigate the intercalation of compound **3e** in triple helices [16]. Energy minimized structures show that the planar molecule **3e** overlaps with the neighboring AxT Hoogsteen-paired bases (Fig. 9). The results presented here raise the interesting possibility that the stabilization of triple-helical structures by BPI derivatives is due to their specific intercalation between the Hoogsteen base pair part of the base triplets. Magnesium enhances the stability of both triple helices and Hoogsteen duplexes, whereas ethidium bromide, which is a well-known intercalator of double helices, binds poorly to triple helices [31]. The ability of a ligand to bind to triple helices could be related to its ability to bind to a Hoogsteen-paired duplex.

It was previously suggested that local Hoogsteen base pairing with a *syn*-to-*anti* transition of the base could exist at echinomycin binding sites in an antiparallel DNA duplex [32]. Although it was later shown that such base pairing is not absolutely necessary for binding of this drug [33], Hoogsteen base pairing has been observed by NMR at low temperature in drug–DNA complexes [34]. Moreover, covalent binding of a drug to DNA can lead to Hoogsteen base pair formation [35]. Interactions of BPI derivatives with parallel duplexes should be considered in the light of these studies and of the results presented here. A local Hoogsteen base pairing could explain the specificity of BPI derivatives for oligopurine.oligopyrimidine tracts [36].

This is the first time that formation of a non-Watson–Crick parallel structure is shown to be induced by binding of a specific ligand. The relative stabilities of mismatched Watson–Crick and Hoogsteen-paired parallel duplexes depend both on the conditions (pH and the presence of cations) and on the respective affinity of BPI for both structures. BPI should be the prototype for the development of more specific Hoogsteen-stabilizing DNA ligands.

Spectroscopic evidence for the protonation of cytosines and higher stability at acidic pH are in good agreement with the proposed structure of a Hoogsteen-paired parallel DNA. Furthermore, the infrared spectra of the complexes of R3xH3 and R5xH5 with the intercalating drugs **14g** or **9e** or in the presence of Mg^{2+}, recorded at acidic pH, indicate that the $C_4{=}O_4$ carbonyl group of thymine as well as the N_7 site of adenine are involved in interactions whereas the $C_2{=}O_2$ group of thymine is not. These results are in agreement with a Hoogsteen-type base pairing scheme in a parallel duplex. Moreover, the sugars in this parallel structure have an S-type conformation. A slow electrophoretic mobility was observed for this new type of duplex. Substitution of the N_7 nitrogen with a carbon atom in adenines or guanines of the purine strand prevents formation of this structure.

The structure described here has several implications. First it is highly stabilized by magnesium. Some proteins might recognize this type of duplex and it could be involved in biological processes involving DNA strands, like topoisomerase-mediated strand passage or recombination. Oligopurine–oligopyrimidine sequences are over-represented in the genome of prokaryotes and eukaryotes. They may be able to adopt not only an intramolecular triple helix structure, but also Hoogsteen-paired structures. The discovery that benzopyridoindole derivatives, which are cytotoxic agents, can bind to this type of structure, opens a new field of investigation regarding the possible role of Hoogsteen hydrogen bond formation in drug–DNA interactions as well as in biological processes. Intoplicine, a BPI derivative, has been undergoing clinical trials [37] and has been shown to interfere with topoisomerases I and II. These results set the stage for structural as well as kinetic and thermodynamic studies of Hoogsteen base pairing in complex nucleic acids structures.

Significance

Stabilizing nucleic acid triple helices is of particular interest in the development of antigene strategies, in which an oligonucleotide specifically recognizes a double-stranded DNA sequence. Triple helices are known to be stabilized by magnesium as well as some intercalators like benzopyridoindole derivatives (BPI). Here we show by spectroscopic methods and gel shift experiments that BPI derivatives and magnesium can induce or stabilize nucleic-acid structures in which an oligopurine strand is bound to an oligopyrimidine strand of the same orientation through Hoogsteen-type hydrogen bonds. For the first time, formation of a non-Watson–Crick parallel structure is shown to be induced by binding of a specific ligand. This structure could be formed under near physiological conditions and have several biological implications. These results give new insights into interactions between ligands and triple helices, and confirm a previously proposed model for intercalation of BPI molecules in triple helices. They define a starting point for the design of new triple helix specific intercalators.

Materials and methods

Oligonucleotides and other materials

Oligonucleotides were synthesized by Eurogentec (Seraing, Belgium), including those containing 7-deaza-adenine and 7-deaza-guanine (Glen Research). Compounds **3e**, **9e** and **14g** were synthesized according to previously published procedures [16,38,39]. The numbering follows that of [16].

Thermal denaturation experiments

All thermal denaturation studies were carried out on a Uvikon 940 spectrophotometer, interfaced to an IBM-AT personal computer for data collection and analysis. Temperature control of the cell holder was maintained by a Haake P2 circulating water bath. The temperature of the water bath was decreased from 60 °C to 0 °C and then increased back to 60 °C, both at a rate of 0.15 °C min^{-1} by a Haake PG 20 thermoprogrammer. The absorbance at 260 nm (or 295 nm) was recorded every 10 min.

Gel shift experiments

Gel electrophoresis was run on a 10 % polyacrylamide/bisacrylamide (19:1) non-denaturing gel in a 50 mM 2-(N-morpholino)ethane-sulfonic acid (MES) buffer (pH 6) containing 10 mM $MgCl_2$. Each sample (2 μM for each strand) was incubated overnight at 4 °C in 50 mM MES buffer (pH 6) containing 10 mM $MgC1_2$, 50 mM NaCl, 10 % sucrose and 5 μg of tRNA, then loaded onto the gel and allowed to migrate for 3 hours at 5 W.

Fourier transform infrared spectroscopy

The spectra were recorded with a Fourier transform infrared spectrophotometer, model 1760 from Perkin Elmer. Spectral resolution was 1 cm^{-1}. Data treatment was performed by an interfaced PE 7700 microcomputer. The spectra were processed using the SpectraCalc program. Usually, a smoothing procedure was applied following the Savitzky-Golay algorithm with 7 or 9 points, and the baseline corrected for the solvent contribution.

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