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Triple helices formed at oligopyrimidine•oligopurine sequences with base pair inversions: effect of a triplex-specific ligand on stability and selectivity

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ABSTRACT

Oligonucleotide-directed triple helix formation is mostly restricted to oligopyrimidine•oligopurine sequences of double helical DNA. An interruption of one or two pyrimidines in the oligopurine target strand leads to a strong triplex destabilisation. We have investigated the effect of nucleotide analogues introduced in the third strand at the site opposite the base pair inversion(s). We show that a 3-nitropyrrole derivative (M) discriminates G•C from C•G, A•T and T•A in the presence of a triplex-specific ligand (a benzo[e]pyridoindole derivative, BePI). N6-methoxy-2,6-diaminopurine (K) binds to an A•T base pair better than a T•A, G•C or C•G base pair. Some discrimination is still observed in the presence of BePI and triplex stability is markedly increased. These findings should help in designing BePI–oligonucleotide conjugates to extend the range of DNA sequences available for triplex formation.

INTRODUCTION

The sequence-specific recognition of double-helical DNA (dsDNA) through oligonucleotide-directed triple helix formation, also called 'antigene strategy', is a topic of considerable interest in developing oligonucleotide-based tools in molecular biology and therapeutics (1,2). Triple helix-forming oligonucleotides (TFOs) can compete with the binding of proteins (3,4) and impede transcription of a specific gene (5–9). Recognition of DNA by TFOs is achieved by hydrogen bonding interactions in the major groove of target dsDNA between the bases in the oligonucleotide and the purines already engaged in Watson–Crick hydrogen bonding with the complementary pyrimidine bases (10–12). On the basis of base sequence composition and the relative orientation of the phosphate–deoxyribose backbone of the third strand, the triple helices can be broadly categorised into three motifs (see 12 for a review): (i) in (T,C)- or pyrimidine-motif, the oligonucleotide binds parallel to the purine strand of the duplex, forming T•A*T and C•G*C$^+$ canonical Hoogsteen base triplets (10–14); (ii) in the (G,A)- or purine-motif, the oligonucleotide binds to the oligopurine strand of the duplex through reverse Hoogsteen hydrogen bonds, giving rise to C•G*G and T•A*A base triplets (15,16); iii) in the (G,T)- or purine/pyrimidine mixed-motif, the third strand binds to the oligopurine strand in the duplex by forming C•G*G and T•A*T base triplets through either Hoogsteen (parallel) or reverse Hoogsteen (antiparallel) hydrogen bond formation (5,17,18). The orientation of the (G,T)-containing oligonucleotide is directed by the number of 5′-GpA-3′ and 5′-ApG-3′ steps within the target sequence and length of G and A tracts (17,18). It is worth noticing that an oligonucleotide containing T, C and G has been shown to bind in a parallel orientation with respect to the target oligopurine strand (19).

It was reported that a well known DNA intercalator, ethidium bromide, could stabilise poly(dA)•2poly(dT) (20). However, it destabilised a (T,C)-motif triplex (21). A family of tetracyclic aromatic compounds, benzo[e]pyridoindole (BePI) derivatives, exhibited a preferential stabilisation of (T,C)-motif triplexes rather than the underlying duplex (22). Other ligands, such as benzo[g]pyridoindole (BgPI) (23), coralyne (24), quinacrine (25), amidoanthraquinone derivatives (26) and naphtylquinoline derivative (27) have been shown to provide significant and selective stabilisation of triple helices. A structure–function analysis of several series of benzopyridoindole derivatives indicated that both the shape of the heterocycle and the position of positively charged aminoalkyl side chains play an important role in triplex stabilisation (28). In addition, it turns out that BePI not only stabilises the (T,C)-motif triple helices but also induces the formation of triple helices with an antiparallel (G,T)-containing third strand that were not formed in the absence of ligand (29).

Several studies have been carried out to assess the destabilising effect of a base pair inversion in the target sequence (30–34). The use of non-natural nucleotide analogue allows stable triplex formation when the target oligopurine strand is interrupted by one pyrimidine (35). We have previously reported that an acridine

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derivative incorporated within an oligopyrimidine TFO can strongly stabilise triple helices formed at non-perfect oligopyrimidine•oligopurine sequences (36,37).

In this report, we have investigated the stabilising effect of a triplex-specific ligand, BePI, on triplexes formed by TFOs which may contain non-natural bases, with target oligopyrimidine•oligopurine sequences containing base pair inversion(s), in order to explore the possibility of extending the range of DNA sequences for triplex formation. The non-natural bases used in this study include the 5-propynyl-pyrimidines (dU and dC), 5-nitroindole and 3-nitropyrrole, as well as a bicyclic pyrimidine analogue of N4-oxy-2′-deoxycytosine (P), a purine analogue, N6-methoxy-2,6-diaminopurine (K). The analogue P shows both T and C character, but is somewhat more T-like. It hydrogen bonds with A and G giving duplexes of similar stabilities (38,39). The tautomeric constant (K_T) is not known but the closely related N4-methoxyC has a value 10–30 in favour of the oximino-form (Fig. 1) and has a pK_a of 2.3 (40 and references quoted therein); thus P is unlikely to form protonated base pairs or triplets. The analogue K has a K_T of 9 (in DMSO) in favour of the oximino-form (Fig. 1) (41,42). Thus in this case, the K_T should be near to unity in aqueous solution. The analogues, therefore, provide a means of testing the effect of ambivalent hydrogen-bonding bases in stabilising triple helices.

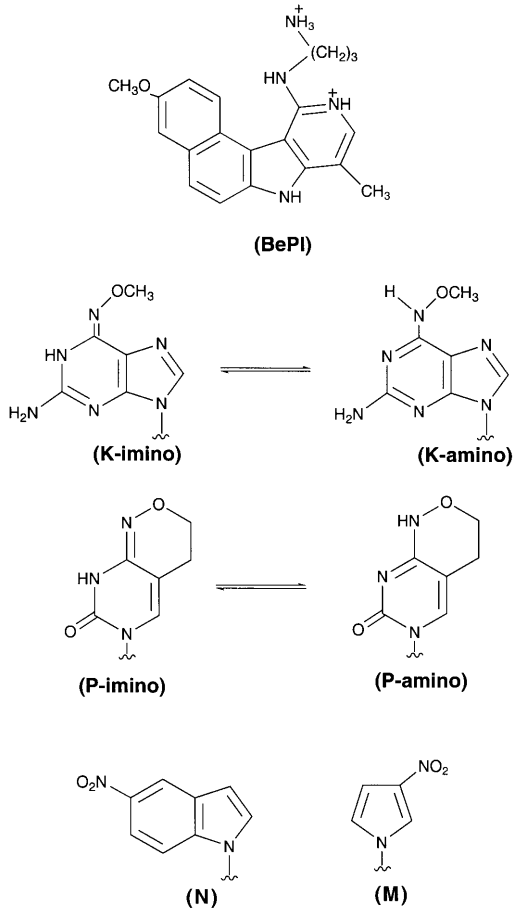

Figure 1. Top: chemical structure of the BePI derivative, 3-methoxy-7H-8-methyl-11-((3′-amino-propyl)amino)benzo(e)pyrido(4,3-b)indole. Middle: the N6-methoxy-2,6-diaminopurine (K) in the *anti*-configuration and the N4-oxy-cytosine (P), both shown in their imino- and amino-forms. Bottom: the 5-nitroindole (N) and the 3-nitropyrrole (M).

MATERIALS AND METHODS

Nomenclature

The following convention for TFOs and base triplets are used. The duplexes are written as 26YX•26RY, with the third strand as 14YZ. The letters X and Y stand for the bases involved at the base pair inversion site in the oligopyrimidine and oligopurine target sequences, respectively. Z is the corresponding base in TFOs. A base triplet is designated X•Y*Z, where the symbols • and * indicate Watson–Crick and Hoogsteen hydrogen bonds, respectively. The C5-modified pyrimidine, propynyl-dC and propynyl-dU, are designated as C and U respectively. Other non-natural bases were also used, such as a bicyclic pyrimidine analogue of N4-oxy-2′-deoxycytosine (P), a purine analogue, N6-methoxy-2,6-diaminopurine (K), as well as 5-nitroindole (N) and 3-nitropyrrole (M) (Fig. 1). A propanediol linker (L) which mimics the natural internucleotidic distance but without sugar or any base was also introduced in some oligonucleotides.

Oligonucleotides and chemical ligand

The TFOs containing P, K, N and M base analogues were synthesised as described earlier (40,41,43,44) and the rest of the oligonucleotides (OliGold grade) were purchased from Eurogentec (Belgium). Oligonucleotides were ethanol-precipitated in the presence of 0.3 M sodium acetate, washed with ethanol and used without further purification. The concentrations of all oligonucleotides were determined spectrophotometrically using the extinction coefficients calculated by a nearest-neighbour method (45). The BePI derivative (Fig. 1), 3-methoxy-7H-8-methyl-11-((3′-amino-propyl)amino)benzo[e]pyrido[4,3-b]indole, was synthesised according to the method previously described (46).

UV absorption spectrometry

Triple helix stability was measured by UV spectrophotometer. All the DNA thermal denaturation experiments were performed on a UVIKON 940 Spectrophotometer using quartz cuvettes of 1 cm optical pathlength. The spectrophotometer was interfaced to an IBM-AT computer for data collection and analysis. The cell holder was thermostated with a circulating liquid (80% water/20% ethyleneglycol) in a Haake P2 water bath. The temperature of the water bath was decreased from 80 to 0°C and then increased up to 80°C at a rate of 0.15°C/min with a Haake PG 20 thermoprogrammer. The absorbance at 260 was recorded every 8 min. The sample temperature was measured by a teflon-coated temperature probe immersed directly in a control cuvette. All the oligonucleotide samples were prepared in 20 mM sodium cacodylate buffer at pH 6.0 containing 100 mM sodium chloride and 10 mM magnesium chloride, in the presence or in the absence of 10 μM BePI derivative. The triplexes were formed by first mixing the two strands (26YX and 26RY) of the Watson–Crick duplex, each at 1 μM concentration, and then adding 1.5 μM of the third strand. The triplex melting temperatures (T_m) were

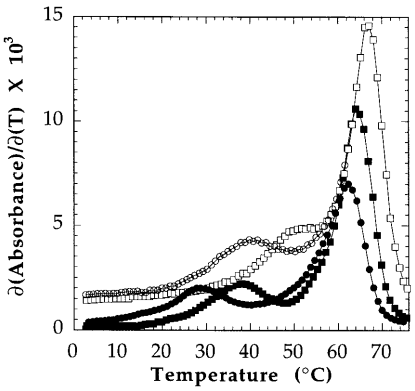

Figure 2. First derivative of the thermal denaturation profiles showing the effect of BePI on triplexes (see sequences on top of Table 1). The following triplexes are shown: X•Y*Z = T•A*T in the absence (filled squares) and in the presence (open squares) of BePI; X•Y*Z = A•T*G in the absence (filled circles) and in the presence (open circles) of BePI.

evaluated as the maximum of the first derivative of the melting profiles. Based on multiple experiments, the uncertainty in T_m was estimated at ±1°C.

Table 1. Melting temperature values (±1°C) of the triplex-to-duplex transition in the absence (–) and in the presence (+) of 10 µM BePI

3' TGTCAA TTCTTCTT **X** TTTCT AACTCG 5' 26YX
5' ACAGTT AAGAAGAA **Y** AAAGA TTGAGC 3' 26RY
5' TTCTTCTT **Z** TTTCT 3' 14YZ

Tm (±1°C)

X•Y =	T•A		A•T		G•C		C•G	
BePI	–	+	–	+	–	+	–	+
Z = A	18	36	18	35	15	37	30	36
C	10	45	14	40	24	41	**45**	**48**
G	08	38	**28**	**41**	19	40	22	44
T	**37**	**50**	10	35	**26**	40	24	35
L	11	36	20	37	20	33	10	29
U	**38**	**52**	12	35	**26**	41	27	38
C	05	42	11	36	14	35	32	37
M	14	36	14	28	15	**42**	07	30
P	33	48	12	35	**27**	**41**	26	36
K	10	32	**28**	**41**	17	36	07	32
N	11	32	15	39	16	35	21	31
None	22	42	< 5	27	13	35	09	26

The sequences are described at the top of the table. T_m values of the best combinations are highlighted in bold. 'Z = None' indicates the absence of any nucleotide or base analogue at the Z position in the third strand. Experimental conditions are described in Materials and Methods.

RESULTS AND DISCUSSION

Several 26 bp synthetic dsDNA fragments (26YX•26RY) containing a tract of 14 bp oligopyrimidine•oligopurine target sequences for the binding of 14 nt long oligonucleotides (14YZ) were used (Table 1). Some of the duplexes contained one or two base pair inversion(s) at the central position (X•Y), thus interrupting the canonical oligopyrimidine•oligopurine sequences. A set of 14mer oligonucleotides (14YZ) was synthesised with different nucleotides at the Z position facing the (X•Y) base pair, including four natural bases A, G, C and T, as well as six base analogues (U, C, P, K, N, M) and a propanediol linker (L). One oligonucleotide had no nucleotide facing the (X•Y) base pair (indicated by 'None' in Tables 1 and 2). The thermal stabilities of these triplexes containing various base triplets (X•Y*Z) at the central position in the absence or in the presence of BePI were carried out by measuring the melting temperature (T_m) of the triplex-to-duplex transition.

Effect of BePI on the stability of a triple helix formed at an oligopyrimidine•oligopurine duplex site with a single base pair inversion

All the possible 48 combinations of the X•Y*Z base triplets were investigated in order to assess the effect of various Z nucleotides in the third strand (TFO) on the stability of triplexes. Each thermal denaturation profile showed a biphasic pattern (Fig. 2) which corresponds to the triplex-to-duplex and the duplex-to-single strands transitions. The choice of a longer DNA duplex fragment than the length of triplex allowed us to well separate these two transitions. Table 1 gives the temperature of half dissociation (T_m) of various triplexes derived from the triplex-to-duplex transition of the melting curves for all the combinations in the absence and in the presence of 10 µM BePI.

In the absence of BePI, (i) Z = T (also U) and C in the third strand form the most stable triplexes with the perfect oligopyrimidine•oligopurine target sequences (X•Y = T•A or C•G) as expected when the canonical T•A*T (T•A*U) and C•G*C^+ base triplets are formed; (ii) Z = G and K form the least destabilising triplexes at an A•T base pair inversion; (iii) Z = T, U or P are the best nucleotides to face a G•C base pair inversion. However, the presence of a single base pair inversion at the centre of the oligopyrimidine•oligopurine target sequences caused a loss in thermal stability of all triplexes with a ΔT_m of ~ 9°C at least. These results are consistent with previously published data for natural nucleotides (29–33). For the modified nucleotides (U, P, K), it is not surprising that the analogues U and P behave as T, whereas the guanine analogue K bearing a 2-NH_2 group which was shown to be engaged in a hydrogen bond with the O4(T) in an A•T*G triplet (30,47) could form an equally stable A•T*K triplet. In the absence of structural data, it is not possible to anticipate the tautomeric form of base analogues.

In the presence of BePI, all triplexes were stabilised but interestingly the stabilisation varied with the nature of the X•Y*Z triplet. This differential stabilisation of triple helices by BePI molecules indicates that the BePI molecule has different interactions with different base triplets X•Y*Z. For instance, the T_m values was increased in the presence of BePI by 13 and 3°C, for the perfect triple helices (X•Y*Z = T•A*T and C•G*C^+), respectively. Similar triplexes containing a C5-propynyl substitution of U and C (Z = U or C in the third strand) were also stabilised by 14 and 5°C respectively, in the presence of BePI. The weak stabilisation of the triplexes in which the central base triplet is C•G*C/C can be explained by the unfavourable electrostatic repulsion between the positively charged triplet and BePI molecules.

When a single base-pair inversion (X•Y = A•T or G•C) was introduced at the central position of the target sequence, the base triplets which afforded the least destabilised triplexes in the absence of BePI (A•T*G/K and G•C*T/U/P), also emerged among the most stable ones in the presence of BePI. However,

Table 2. Melting temperatures (±1°C) of the triplex-to-duplex transition in the presence of 10 μM BePI

3′ TGTCAA TTCTTCT **GA** TTTCT AACTCG 5′ 26GA
5′ ACAGTT AAGAAGA **CT** AAAGA TTGAGC 3′ 26CT
5′ TTCTTCT **TZ** TTTCT 3′ 14YZ

	Tm (±1°C)											
Z	A	C	G	T	L	U	C	M	P	K	N	None
Tm (±1°C)	26	29	27	29	27	29	27	29	27	25	26	22

The triplexes were formed upon binding of the various third strand oligonucleotides (14YZ), to a 26 bp oligopyrimidine•oligopurine DNA fragment in which a double base pair inversion was introduced (26GA•26CT). Experimental conditions are described in Materials and Methods.

some other non-canonical base triplets exhibited similar stability in the presence of BePI. The most striking example was Z = M (3-nitropyrrole) in the third strand facing a G•C base pair. Strong destabilisation was observed in the absence of BePI for all four X•Y target base pairs (T_m = 7–15°C) in agreement with recently reported data (48). However, the T_m of the triplex containing a G•C*M triplet was significantly increased from 15 to 42°C upon the addition of 10 μM BePI, whereas the stabilising effects were less pronounced for the triplexes containing other X•Y*M triplets. Since the non-discriminating 'universal' base M is presumed to stack but not hydrogen bond with canonical bases, the observed selective stabilisation of the triplex containing a G•C*M triplet might result from favourable local stacking interactions between the surrounding base triplets and BePI molecule.

To sum up, BePI ligands can stabilise a triple helix even when the oligopyrimidine•oligopurine target sequences are interrupted by a single purine•pyrimidine base pair inversion.

Effect of BePI on the stability of a triple helix formed at an oligopyrimidine•oligopurine duplex site with a double base pair inversion

The study of the stabilisation of sequences with two base pairs inversions was carried out using the 14mer third strand (14YZ) and the corresponding duplex containing a double base pair inversion at the central position (26GA•26CT; Table 2). It should be pointed out that this was not an exhaustive study since the triplet on the 5′-side was fixed to G•C*T which was shown to be one of the least destabilising single mismatch triplet both in the absence and in the presence of BePI (see above), and only the effect of the 3′-side triplet (A•T*Z) was investigated. The presence of a double base pair inversion at the centre of the target sequence prevented triple helix formation with all 14mer oligonucleotides ($T_m \leq 5$°C). BePI was able to induce triplex formation with the T_m values raised up to 29°C. However, the extent of stabilisation does not seem to depend significantly on the nature of the Z nucleotide under the sequence context studied in the present work.

Base sequence specificity and nearest-neighbour effects

Examination of Table 1 shows that the range of triplex stabilities for a given base pair in the target sequence (each column in Table 1) is reduced in the presence of BePI, i.e. the triplex stability depends less on the nature of the nucleotide in the third strand in the presence than in the absence of BePI. This phenomenon was previously reported for naphtylquinoline derivatives (another triplex-stabilising ligand) (49,50). However, some discrimination of the inverted base pairs can be observed. For example, the triplex with a G•CxM base triplet discriminates G•C from T•A, C•G and A•T in the presence of but not in the absence of BePI. The triplex with an A•TxK base triplet discriminates A•T from T•A, C•G and G•C in the absence of BePI; in the presence of BePI some discrimination is preserved and stronger binding is achieved. It remains to be seen whether the covalent attachment of BePI at either the 5′- or the 3′-side of K/M within a TFO allows for sequence selectivity of triplex formation.

It is worth noticing that the nearest-neighbours of the base inversion site in the present study are T•AxT triplets. Previous studies (36,37) have shown that triplex stability of an acridine-containing TFO decreases when the base triplet on the 3′-side of the intercalation site changes from T•AxT to C•GxC$^+$, due to electrostatic repulsion between the charged base triplet and the charged ligand intercalated at the 5′-pyrimidine–purine-3′ step (which is a well established intercalation site for most monointercalators). Therefore, it can be anticipated that a similar nearest-neighbour effect could be observed for BePI derivatives.

Conclusions

BePI derivatives can be used to stabilise DNA triple helices when the oligopyrimidine•oligopurine target sequences contain a single or double base pair inversion. Based on the stability and the selectivity of the non-canonical base triplets with respect to all four Watson–Crick base pairs (Table 1), the use of modified nucleotides K and M incorporated in the TFOs facing the inverted A•T and G•C base pairs, respectively, cannot only achieve the highest stabilisation in the presence of BePI, but also retain the best sequence selectivity. This preliminary work paves the way for synthesising tailor-made TFO–BePI conjugates to further stabilise triplexes at base pair inversion sites. A synergistic effect can be obtained by combining the recognition element (base analogue) with a triplex-specific stabiliser (BePI), in a way similar to what was done with a dsDNA intercalator, such as acridine (36,37). Therefore, such a TFO–BePI conjugate might recognise base pair inversions, so that the range of DNA target sequences for triplex formation could be extended. Some encouraging results in this direction have recently been reported, showing that BePI derivatives and their analogues, benzo[f]pyrido[3,4-b]quinoxaline (BPQ) derivatives, form stable triple helices under physiological conditions, when conjugated at the 5′-end or at an internal position of TFOs (51,52).

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