DNA triple helix stabilisation by a naphthylquinoline dimer

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Received 18 January 1999; received in revised form 22 February 1999

Abstract We have used DNase I footprinting to examine the effect of a novel naphthylquinoline dimer, designed as a triplex-specific bis-intercalator, on the stability of intermolecular DNA triplexes. We find that this compound efficiently promotes triplex formation between the 9-mer oligonucleotide 5′-TTTTTTCTT and its oligopurine duplex target at concentrations as low as 0.1 μM, enhancing the triplex stability by at least 1000-fold. This compound, which is the first reported example of a triplex bis-intercalator, is about 30 times more potent than the simple monofunctional ligand.

Key words: Triplex ligand; Footprinting; DNA triplex; Bis-intercalator; Naphthylquinoline

1. Introduction

Intermolecular triple helices, which are formed by the sequence-specific binding of an oligonucleotide in the major groove of duplex DNA, have a potential use as antigen agents for treating diseases such as cancer or viral infections [1–4]. The third strand bases form hydrogen bonds with substituents on the purine bases of the duplex [5,6]. Pyrimidine-containing third strands bind parallel to the duplex purine strand forming C^+·GC and T·AT triplets [7,8].

Although third strand oligonucleotides bind to duplex DNA with a high sequence specificity, the binding may not be strong and is limited by charge repulsion between the three poly-anions. Several strategies have been suggested for increasing the triplex affinity, including modification of the backbone [9,10], bases [11–13] and sugar residues [14,15]. The binding affinity can also be improved by tethering a non-specific DNA-binding agent such as acridine or psoralen to either end of the third strand oligonucleotide [16–19]. An alternative strategy is to develop compounds which bind specifically to triplex, but not duplex, DNA. Several of such agents have been described (see Fig. 1A) including the benzopyridoindoles BePI (1) and BgPI [20,21], benzopyridoquinoxaline [22], benzoquinoquinoxaline [23] (2), dibenzophenanthrolines [24] (3), coralyne (4) [25,26] and bis-substituted anthraquinones (5) [27]. A further series of triplex-binding ligands consists of substituted naphthylquinolines (6) which also appear to bind by intercalation [28–30]. These compounds possess a large aromatic area which can stack with the three bases in the triplex, yet since the aromatic portions are not fused they possess torsional flexibility and can accommodate the propeller twist of the triplets, in which the three bases may not be coplanar.

The affinity of duplex intercalators, such as acridine, has been previously increased by tethering two or more together using flexible linkers generating poly-intercalators [31]. In this paper we have used DNase I footprinting to examine the ability of a novel naphthylquinoline dimer (7) to stabilise DNA triplexes and have compared its activity with a monofunctional derivative (6).

2. Materials and methods

2.1. Chemicals and enzymes

Oligonucleotides were purchased from Oswel DNA service and were stored at −20°C in water. DNase I was purchased from Sigma and stored at −20°C at a concentration of 7200 U/ml. Reverse transcriptase was purchased from Promega, restriction enzymes were purchased from Promega, Pharmacia or New England Biolabs. The naphthylquinoline triplex-binding ligand (6) (Fig. 1A) was prepared as previously described [28]. Synthesis of the bis-naphthylquinoline (7) will be described elsewhere. These compounds were stored as 20 mM stock solutions in dimethylsulfoxide at −20°C and diluted to working concentrations in the appropriate buffer prior to use.

2.2. DNA fragments

The sequence of the 160 base pair *tyr*T fragment was modified by PCR-mediated site-directed mutagenesis introducing a 17 base homopurine tract between positions 43 and 59 [32]. The sequence of the oligopurine tract is shown in Fig. 1B. The 160 base pair fragment *tyr*T(43-59) was obtained by digesting the plasmid with *Eco*R1 and *Sma*1 and was labelled at the 3′ end of the *Eco*R1 site with [α^{32}P]dATP using reverse transcriptase. The labelled fragment of interest was separated from the remainder of the plasmid DNA on a 6% non-denaturing polyacrylamide gel, eluted and dissolved in 10 mM Tris-HCl, pH 7.5, containing 0.1 mM EDTA at a concentration of about 10 cps/μl (approximately 10 nM). This procedure labelled the purine-containing strand of the triplex target site.

2.3. DNase I footprinting

Radiolabelled DNA (1.5 μl) was mixed with 1.5 μl oligonucleotide, dissolved in an appropriate buffer and 1.5 μl of triplex-binding ligand or buffer. This mixture was left to equilibrate at 20°C for at least 1 h. Experiments were performed in 50 mM sodium acetate, pH 5.0, containing 10 mM $MgCl_2$. The mixture was then digested by adding 2 μl DNase I, diluted in 20 mM NaCl containing 2 mM $MgCl_2$ and 2 mM $MnCl_2$, and stopped after 1 min by adding 3.5 μl of a solution containing 80% formamide, 10 mM EDTA and 0.1% (w/v) bromophenol blue. Samples were boiled for 3 min before electrophoresis.

2.4. Gel electrophoresis

Products of DNase I digestion were separated on 10% (w/v) polyacrylamide gels containing 8 M urea which were run for about 2 h at 1500 V. Samples were heated to 100°C for 3 min prior to electrophoresis. After about 2 h electrophoresis, the gels were fixed in 10% (v/v) acetic acid, transferred onto Whatman 3 MM paper, dried under vacuum at 80°C for 1 h and subjected to autoradiography at −70°C with an intensifying screen. Bands in the digests were assigned by comparison with Maxam-Gilbert dimethyl sulfate-piperidine markers specific for guanine.

2.5. Quantitative analysis

Autoradiographs of DNase I digestion patterns were scanned using a Hoefer GS365 microdensitometer or analysed using a Molecular

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PII: S0014-5793(99)00284-7

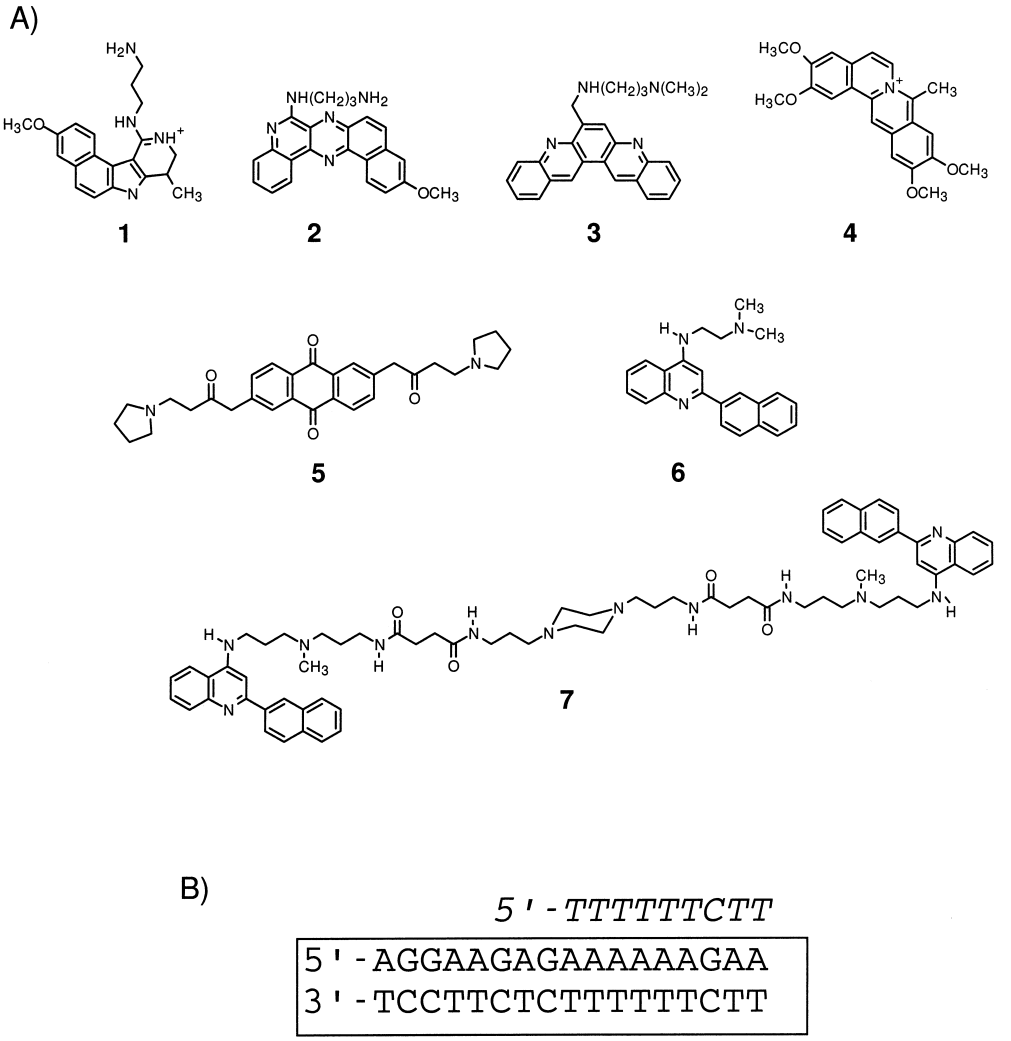

Fig. 1. (A) Structures of several triplex-binding ligands. (1) BePI [20,21], (2) benzoquinoquinoxaline [23], (3) dibenzophenanthroline [24], (4) coralyne [25,26], (5) bis-substituted anthraquinones, (6) monofunctional naphthylquinoline derivative [28–30] and (7) bifunctional naphthylquinoline derivative. (B) Sequence of the 17 base pair oligopurine tract in *tyr*T(43-5) (boxed) together with the third strand 9-mer oligonucleotide.

Dynamics STORM phosphorimager. For the densitometric analysis, we chose a band in each site which was well resolved and cut well in the control (ApG, position 53). The intensity of this band was estimated using the manufacturers software and normalised with respect to the intensity of two bands outside the target site (positions 38 and 61 or 69). For phosphorimage analysis we calculated the intensity of bands within the entire footprint. It should be noted that in all conditions the concentration of the third strand oligonucleotide is much greater than that of the DNA target. As a consequence, the amount of bound oligonucleotide will be determined by the equilibrium dissociation constant, rather than the stoichiometric ratio of third strand to target. Footprinting plots [33] were constructed from these data and C_{50} values, indicating the oligonucleotide concentration which reduced the band intensity by 50%, were derived by fitting a simple binding curve to plots of the band intensity against the oligonucleotide concentration using FigP for Windows (Biosoft). These were fitted to the equation $I_c=I_0\times(C_{50}/(L+C_{50})$ where I_c is the band intensity in the presence of the ligand, I_0 is the band intensity in the control and L is the oligonucleotide concentration.

3. Results

We have previously shown that the 9-mer triplex-forming oligonucleotide 5′-TTTTTTCTT binds very weakly to its target site in *tyr*T(43-59) (Fig. 1B) [34]. This weak interaction is attributed to its short length and the large number of T·AT triplets, with only one C^+·GC triplet [34,35]. The interaction with this oligonucleotide alone is shown in the first panel of Fig. 2 in which it can be seen that there are no oligonucleotide-induced changes in the cleavage pattern at concentrations as high as 30 μM, even at this low pH (5.0) in the presence of 10 mM $MgCl_2$. As expected, the binding of this oligonucleotide is potentiated by addition of the mono-naphthylquinoline triplex-binding ligand (6). The fourth panel of this Figure shows the effect of varying oligonucleotide concentrations in the presence of 3 μM of the monofunctional ligand (6). A footprint, which is most clearly seen from the inhibition of cleavage of the band at position 53, is now evident at the target site, which persists to an oligonucleotide concentration of about 0.4 μM. The footprinting plot shown in Fig. 3A was generated from these data revealing that, in the presence of 3 μM ligand the C_{50} value (which approximates to the apparent dissociation constant of the oligonucleotide) is 0.31 μM. This is comparable with a value of 0.19 μM in the presence of 10 μM of this ligand [34]. In the presence of lower

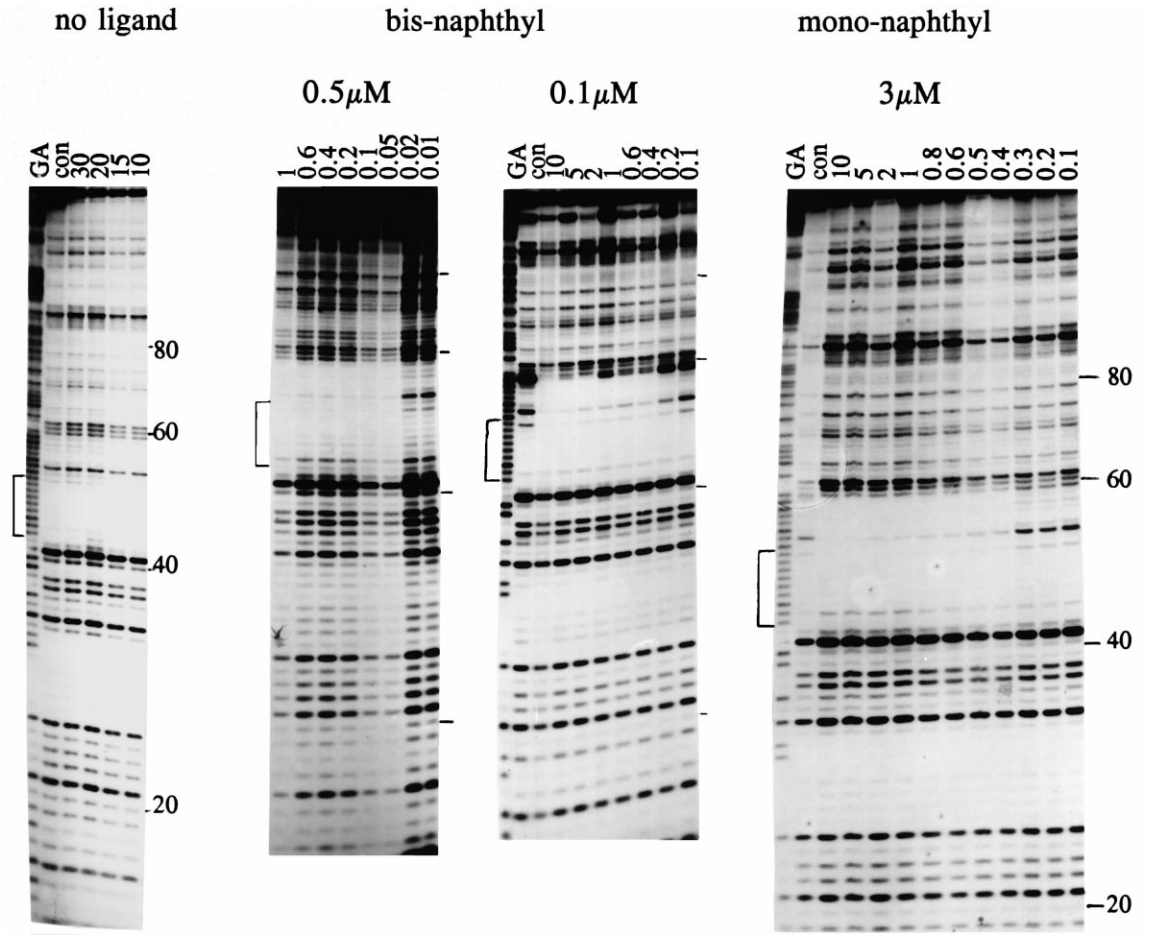

Fig. 2. DNase I cleavage patterns of *tyr*T(43-59) in the presence of varying concentrations of the 9-mer oligonucleotide 5′-TTTTTTCTT and the naphthylquinoline triplex-binding ligands. The second and third panels (bis-naphthyl) were performed in the presence of the bifunctional derivative (7), while the fourth panel (mono-naphthyl) included the monofunctional derivative (6). All reactions were performed in 50 mM sodium acetate, pH 5.0, containing 10 mM $MgCl_2$. The left hand panel shows digestion in the presence of the oligonucleotide without addition of the triplex-binding ligands. Oligonucleotide concentrations (μM) are indicated at the top of each lane. Tracks labelled 'con' show digestion of the DNA in the absence of added oligonucleotide or triplex-binding ligand. Tracks labelled 'GA' are Maxam-Gilbert markers specific for purines. The brackets indicate the position of the 9-mer target site. The bands are numbered according to the scheme used in previous publications [32,34].

concentrations of the ligand (not shown), larger oligonucleotide concentrations are required to produce DNase I footprints.

The second and third panels of Fig. 2 show similar DNase I footprints performed in the presence of 0.5 μM and 0.1 μM of the bis-naphthylquinoline ligand (7). At these low concentrations, the ligand alone does not affect the DNase I cleavage pattern, though concentrations of 3 μM and above cause a general non-specific reduction in DNase I cleavage (not shown), presumably as a result of the interaction with duplex DNA. In the presence of 0.5 μM ligand (7), the footprint persists to an oligonucleotide concentration of about 0.05 μM. A footprint is still evident with concentrations of this ligand as low as 0.1 μM (third panel), persisting to an oligonucleotide concentration of about 0.2 μM. Quantitative analysis of these data produces the footprinting plots shown in Fig. 3B which yield C_{50} values of 0.026 and 0.12 μM with 0.5 and 0.1 μM ligand, respectively. Inspection of these C_{50} values reveals that 0.1 μM bifunctional ligand (7) stabilises triplexes to a greater extent than 3 μM of the monofunctional derivative (6), consistent with the suggestion that it acts as a triplex bis-intercalator.

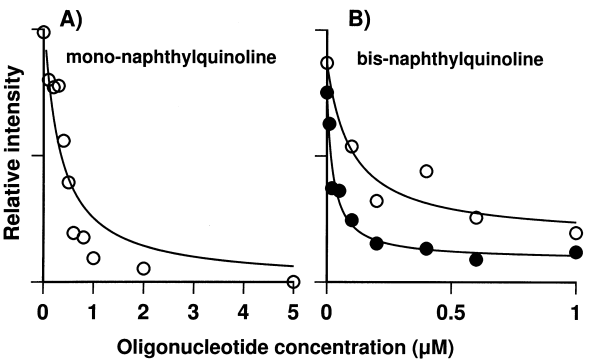

Fig. 3. Footprinting plots showing the interaction of the 9-mer oligonucleotide with its target site in the presence of (A) 3 μM mono-naphthylquinoline (6) (○), (B) 0.5 μM bis-naphthylquinoline (7) (○) or 0.1 μM bis-naphthylquinoline (7) (●). The intensity of the footprint was determined from analysis of the gels shown in Fig. 2. The ordinate shows the oligonucleotide concentration (μM), the abscissa shows the relative band intensity (arbitrary units). The lines drawn correspond to simple binding curves with C_{50} values of 0.31 μM for 3 μM mono-naphthylquinoline (6), 0.026 μM for 0.5 μM bis-naphthylquinoline and 0.12 μM for 0.1 μM bis-naphthylquinoline (7).

4. Discussion

The results presented in this paper demonstrate that the naphthylquinoline dimer (7) stabilises DNA triplexes at least 30 times more effectively than the monofunctional compound (6). The increased binding strength supports our suggestion that this compound is a triplex bis-intercalator and is the first report of such a compound. However, the present studies do not conclusively demonstrate the mechanism of binding. As well as simple bifunctional intercalation within the triplex, we cannot discount the possibility that one of the chromophores binds within the triplex grooves or interacts externally with the duplex or triplex. Bis-intercalators have two clear advantages over monofunctional derivatives. Firstly, as shown in this paper, bis-intercalators have an increased binding affinity and so can be used at lower concentrations to facilitate triplex formation, thereby reducing the incidence of unwanted side effects. In theory, the affinity of an ideal bis-intercalator should be the product of its monofunctional components, i.e. a monofunctional compound with a K_d of 10^6/M might produce a bifunctional compound with a K_d of 10^{12}/M. Such dramatic increases in binding are rarely achieved as there are additional steric and entropic constraints which limit the affinity of the bifunctional compound. For efficient bis-intercalation, the length and nature of the linker will need to be optimised and further studies on such compounds are in progress. Secondly, we would expect that bis-intercalators should be better able to discriminate between duplex and triple DNA. For example, a monofunctional compound which binds to triplexes 10 times better than to duplexes should yield a bifunctional derivative with 100-fold discrimination.

Acknowledgements: This work was supported by grants from the Cancer Research Campaign (K.R.F.) and the Petroleum Research Fund, administered by the American Chemical Society (L.S.).

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