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Research paper

Exploring multiple binding sites of an indoloquinoline in triple-helical DNA: A paradigm for DNA triplex-selective intercalators

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ABSTRACT

Employing NMR spectroscopic methods preferred binding sites of a triplex-selective indoloquinoline drug were examined with three DNA triplex targets. To directly derive and evaluate number and type of the different sites of interaction, studies were performed on short triple-helical constructs specifically labeled with 3-^{15}N thymidine probes. The detection and assignment of several coexisting species was enabled through the observation of slow exchange on the chemical shift timescale between complexes and free triplex. In general, the 5′-triplex–duplex junction constitutes the most favorable intercalation site, in particular when flanked by a TAT base triad. NMR data also revealed two different orientations for the intercalating indoloquinoline drug. Binding affinity significantly decreases with a C$^+$GC triad bordering the junction but junction binding is still preferred over intercalation between TAT base triads within the triplex stem. In addition to the intercalation between two uncharged TAT triplets, intercalation between a TAT and a 3′-terminal C$^+$GC triplet was also observed, indicating a non-protonated third strand cytosine at the triplex end position.

1. Introduction

In addition to the canonical Watson–Crick double-helical DNA with its prominent role in storing genetic information, a number of alternative DNA secondary structures have been found to exist under *in vivo* and *in vitro* conditions. Such non-canonical DNA species may form on tracts of repeat sequences and also include three- as well as tetra-stranded helices. Although the biology of these special DNA structures is far from fully understood, there is ample evidence that some of them play an important biological role and are involved in critical DNA metabolic processes.

Double-helical regions with a mirror repeat symmetry can fold into an intramolecular three-stranded triplex called H-DNA [1,2]. In fact, mirror repeat sequences capable of adopting H-DNA structures are abundant in mammalian genomes [3] and studies have suggested their regulatory role in transcription [4] but also their potential contribution to genetic instabilities [5,6]. In addition to intramolecular triplexes, the sequence-specific recognition of a DNA duplex by synthetic third strand oligonucleotides (TFO) results in the formation of intermolecular triplexes. In the most widely used triplex motif, a homopyrimidine TFO binds through Hoogsteen hydrogen bonds parallel to a homopurine tract of the target duplex within the major groove, forming TAT and C$^+$GC base triplets [7]. Because cytosines of the TFO must be protonated, binding of a homopyrimidine third strand is pH dependent. Such TFO-mediated triplex formation has caused a lot of attention due to various potential applications in therapy, diagnostics and molecular biology. Thus, triplexes may directly be used to control the transcription of specific genes, e.g. through blocking the binding of transcription factors or through the inhibition of RNA polymerase [8]. On the other hand, tethering the TFO with a signal transducer or a reactive moiety allows for the detection of double-stranded sequences without prior denaturation [9,10] and for site-directed mutagenesis, DNA strand cleavage or base modifications, respectively [11,12].

In attempts to expand triplex-based methodologies and to directly detect or further stabilize triplexes with their inherently low thermodynamic stability at neutral pH, a large number of ligands have been developed and tested for a high-affinity binding to triple-helical structures in the past [13]. Whereas some of these natural and synthetic ligands represent typical duplex binding agents, others strongly discriminate between double- and triple-

Abbreviations: PIQ, 11-phenyl-10*H*-indolo[3,2-*b*]quinoline; TFO, triplex-forming oligonucleotide; DQF-COSY, double-quantum-filtered correlated spectroscopy; EASY ROESY, efficient adiabatic symmetrized ROESY; NOESY, nuclear Overhauser enhancement spectroscopy; TOCSY, total correlation spectroscopy; ROESY, rotating-frame Overhauser enhancement spectroscopy.

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helical structures and exhibit true triplex-selective binding behavior. The latter include annelated quinolizinium salts [14], benzopyridoindole (BPI) and benzopyridoquinoxalines (BPQ) [15,16], 2-naphthyl substituted quinolines [17], and disubstituted amidoanthraquinones [18]. In line with their extended aromatic ring systems and supported by indirect evidence from low-resolution structural studies, thermodynamic data or model calculations, intercalation within the triplex stem is generally regarded as their major mode of binding. In contrast, aminoglycosides and in particular neomycin have been found to selectively recognize a triplex over a duplex through non-intercalative groove binding [19]. Expanding on these findings, the potency of triplex ligands have been enhanced by the design of intercalator-neomycin conjugates, which exhibit superior binding when compared to their individual components due to their dual mode of triplex recognition [20,21].

Indoloquinolines being either of natural or artificial origin have attracted considerable interest due to their broad spectrum of biological activities and their binding to various types of nucleic acids [22]. Recent spectroscopic and calorimetric measurements on the thermodynamics of binding have revealed that the synthetic phenyl-indolo[3,2-*b*]quinoline derivative PIQ (Fig. 1) binds triple-helical DNA with dissociation constants in the submicromolar range depending on conditions and base sequence [23]. The observed favorable entropic contribution upon binding may at least partially be ascribed to the PIQ 11-phenyl substituent. In addition to its high triplex affinity, a strong discrimination between triplex and duplex structures makes PIQ a promising triplex-selective ligand that compares favorably with other triplex-targeting small molecules [23,24]. The PIQ ligand shows a strong preference for triplexes rich in TAT base triads as commonly observed for other polyaromatic triplex binders [23]. Clearly, the indoloquinoline scaffold of PIQ shares structural features with many potential triplex intercalators. In fact, indoloquinolines may be viewed as structural isomers of the tetracyclic benzopyridoindole family of compounds comprising some of the most potent triplex-selective ligands. However, no high-resolution structure of any non-covalent triplex-ligand complex either from NMR or X-ray diffraction has been reported to date. As a result, a solid structural basis of triplex binding from experimental data is largely missing and the discrimination and detailed characterization of different binding modes and specific binding sites remains surprisingly vague.

Aiming at structural details of a triplex interacting with a ligand, a PIQ analog tethered to the third strand oligonucleotide (Fig. 1) [25–27] was recently studied by NMR spectroscopic techniques after triplex formation with a target duplex [28]. These studies considerably benefited from the restricted and thus more specific ligand-triplex interactions as a consequence of the drug attachment to the TFO 5′-terminus and revealed the geometry of the intercalated ligand at the 5′-triplex–duplex junction of the triple-helical construct. Unfortunately, the generality of these results may be limited because the tethered PIQ ligand is guided to the corresponding intercalation site by the sequence-specific binding of the TFO in the duplex major groove and other putative but more remote binding sites are effectively excluded by this approach.

In this paper we address the binding of a free triplex-selective PIQ ligand. Unlike a TFO-bound ligand, free ligands in solution will sample the entire surface of the triplex and multiple binding sites with populations depending on the free energies of binding are conceivable. Employing NMR spectroscopic methods various coexisting species with PIQ ligands interacting at different sites are detected through their chemical exchange with the parent triplex and characterized through chemical shift footprints and intermolecular NOE data.

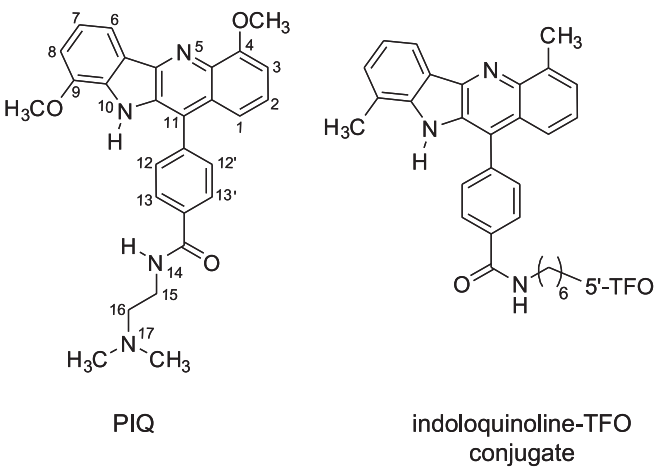

Fig. 1. Structure of indoloquinolines. 11-Phenyl indoloquinoline derivative PIQ with atom numbering (left) and an indoloquinoline-TFO conjugate (right).

2. Materials and methods

2.1. Materials

The NHS-ester of 4,9-dimethoxy-11-(4-carboxyphenyl)-10*H*-indolo[3,2-*b*]quinoline was reacted with 2-dimethylamino ethylamine in DMF to yield the PIQ derivative as reported previously [24]. [3-^{15}N]-thymidine was prepared as described [29] and used as its phosphoramidite for the oligonucleotide synthesis. DNA oligonucleotides were purchased from *TIB MOLBIOL* (Berlin, Germany). Before use, oligonucleotides were ethanol precipitated and the concentrations were determined spectrophotometrically by measuring the absorbance at 260 nm. NMR samples with triplex concentrations of 0.7 or 1.2 mM were obtained by dissolving the corresponding oligonucleotides in 90% H_2O/10% D_2O, pH 5.0, containing 50 mM NaCl and 1 mM NaN_3. Complexes were formed by the addition of a concentrated solution of the PIQ ligand in DMSO-d_6 to the DNA triplex solution. After titration the pH was readjusted with HCl to pH 5. It should be noted, that due to its poor solubility in the buffer solution a fraction of added ligand was possibly lost through precipitates and/or adsorption to the glass wall of the NMR tube. Thus, not being relevant for the present structural studies we refrained in the following from quantifying the effective ligand concentration.

2.2. NMR spectroscopy

NMR spectra were recorded on a *Bruker Avance* 600 MHz spectrometer equipped with an inverse ^{1}H/^{13}C/^{15}N/^{31}P quadruple resonance cryoprobe head and *z*-field gradients. The data were processed using Topspin 3.1 (Bruker) and analyzed with the Topspin or CcpNmr Analysis software [30]. The water signal of samples in 90% H_2O/10% D_2O was suppressed by a WATERGATE sequence with a w5 element. Taking into account the temperature dependence of the H_2O chemical shift, ^{1}H spectra were referenced relative to the water signal and ^{15}N chemical shifts relative to nitromethane by using the indirect referencing method.

Typically, NOESY as well as off-resonance ROESY and EASY ROESY [31] experiments were acquired at 283 or 298 K with a spectral width of 15 kHz and several mixing times 30 ms $\leq \tau_m \leq$ 200 ms. For off-resonance ROESY experiments in 90% H_2O/10% D_2O a spin lock pulse of 4.16 kHz was applied 3.6 kHz off-resonance for 50 ms. The angle θ for the tilted rotating frame in EASY ROESY experiments was fixed to 50°. In general, 2 K × 800

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