Quadruplex–Drug Complexes

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Solution Structure of a G-quadruplex Bound to the Bisquinolinium Compound Phen-DC_3**

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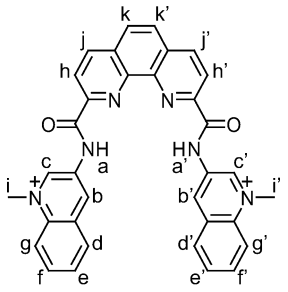
Abstract: *Phen-DC_3 is a highly promising compound that specifically targets G-quadruplexes, with potent biological effects observed in vivo. We used NMR spectroscopy to solve the structure of the complex formed between Phen-DC_3 and an intramolecular G-quadruplex derived from the c-myc promoter. Structural information revealed that Phen-DC_3 interacts with the quadruplex through extensive π-stacking with guanine bases of the top G-tetrad. On the basis of our structure, modifications are proposed for the development of this compound for selective targeting of a specific G-quadruplex conformation.*



Phen-DC_3

Scheme 1. Chemical structure of Phen-DC_3 with the proton numbering used herein (a–g and i/a′–g′ and i′ for the two quinolinium moieties; h, j, k and h′, j′, k′ for the phenanthroline moiety).

Besides the Watson–Crick double helix, DNA can adopt alternative secondary structures, such as G-quadruplexes, which are made up of guanine-rich sequences folded into four-stranded structures through stacking of consecutive guanine tetrads.[1] Cations coordinated to the carbonyl oxygen atoms of the guanine bases in the G-tetrad core further stabilize the structure.[2] Nucleic acid sequences capable of folding into G-quadruplexes are found in various regions of the human genome, such as the telomeres, promoter regions of oncogenes, and also the untranslated regions (UTRs) of mRNA.[3] The formation of G-quadruplexes in these regions has been shown to promote anticancer activity.[4–6] The possibility of targeting G-quadruplexes in anticancer therapy has motivated the development of numerous G-quadruplex-targeting ligands over the past decade.[7–18]

Among these small molecules, a bisquinolinium compound named Phen-DC_3 (Scheme 1) was shown to be a highly promising G-quadruplex ligand that greatly enhanced G-quadruplex thermal stability (ΔT_m up to 29.7 °C) and exhibited exceptional selectivity for G-quadruplexes over duplexes.[18,19] Consequently, it has been used in several assays to probe G-quadruplex formation in yeast and mammalian cells.[20–23] Moreover, Phen-DC_3 can be obtained by a rapid and readily scalable pathway, has a high chemical and metabolic stability, and is live-cell-permeant. It is therefore a highly suitable G-quadruplex-targeting anticancer drug candidate. However, despite the large number of studies performed with Phen-DC_3, detailed structural information on its interactions with G-quadruplexes has not yet been reported. Herein, we present the first structure of a G-quadruplex–Phen-DC_3 complex, as solved by NMR spectroscopy. Detailed structural information on the complex could be of utmost importance for the development of optimized analogues that target a specific G-quadruplex.[24] This information can be used to establish general molecular guidelines for the design of derivatives with improved druglike properties.

The binding of Phen-DC_3 to G-quadruplexes of diverse topologies was observed by NMR spectroscopy (see Figure S1 in the Supporting Information), in agreement with previous data showing it to be a universal G-quadruplex binder.[18–21,23,25,26] Our study focused on the interaction between Phen-DC_3 and a human *c-myc*-promoter G-quadruplex, as well-resolved NMR spectra suitable for high-resolution structural analysis were obtained for the 1:1 DNA–ligand complex. c-Myc is a transcription factor whose overexpression has been associated with human malignancies.[27] Down-regulation of *c-myc* transcription was shown to be possible through the ligand-induced stabilization of G-quadruplexes in the promoter region of this gene.[28] G-Quadruplex structures formed by different G-rich sequences derived from the *c-myc* promoter have been reported previously.[29–31] In this study, the 24 nt sequence Pu24T containing five guanine tracts (Figure 1 A) was selected as our model. Pu24T has been

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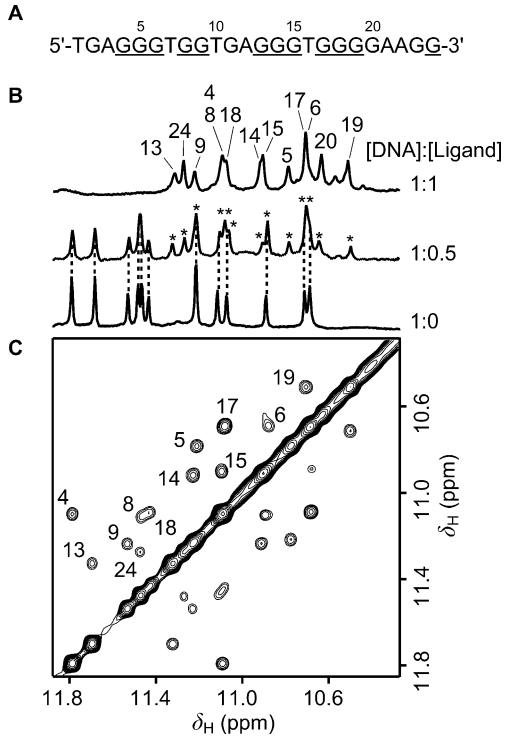

Figure 1. Interaction between Pu24T and Phen-DC_3, as monitored by NMR spectroscopy at 25 °C. A) DNA sequence of Pu24T with guanine residues participating in the G-tetrad core underlined. B) NMR titration of the ligand to Pu24T. The DNA/ligand ratio is indicated on the right of the spectrum. For the DNA/ligand ratio of 1:0.5, signals due to bound Pu24T are indicated with asterisks. For the DNA/ligand ratio of 1:1, imino protons of Pu24T in the complex are labeled with the corresponding residue numbers. C) NOESY spectrum (mixing time: 200 ms) of the complex at the DNA/ligand ratio of 1:0.5. Exchange cross-peaks between imino protons of free and bound Pu24T are labeled with the corresponding residue numbers.

Resonances of the bound drug were identified by through-bond (TOCSY) and through-space (NOESY) connectivities (see Figures S5 and S6). *N*-Methyl protons of the quinolinium moieties were identified by the use of site specifically ^{13}C labeled Phen-DC_3 (see Figure S7). Exchange cross-peaks were observed for some pairs of protons of Phen-DC_3 (e.g. between protons b and b′ or g and g′; see Figure S5) and were attributed to the flipping motion of Phen-DC_3 as it moved in and out of the binding site, thereby leading to an exchange of the chemical environment between protons in the two symmetrical halves of the molecule.

The assignment of resonances of the bound DNA and the drug enabled the identification of 36 intermolecular NOEs (Figure 2; see also Figures S8 and S9). The structure of the 1:1 DNA–ligand complex (Figure 3; see Table S1) was calculated

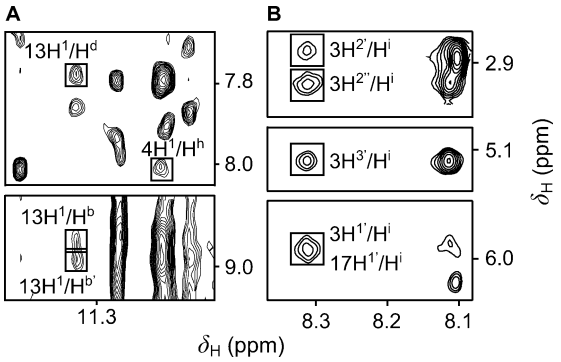

Figure 2. Intermolecular NOE cross-peaks between Pu24T and Phen-DC_3. A) NOESY spectrum in H_2O (mixing time: 350 ms). B) NOESY spectrum in D_2O (mixing time: 700 ms). Intermolecular cross-peaks are framed and labeled with the DNA and Phen-DC_3 proton in the first and second position, respectively.

shown to form a well-defined parallel-stranded G-quadruplex.[29] A particular feature of this structure is a snapback motif adopted by the 3′-end GAAGG segment, which forms a stable diagonal loop that contains a G·(A-G) triad and caps the 3′ side of the G-tetrad core.[29]

The NMR spectrum of free Pu24T in aqueous solution in the presence of K^+ ions is characterized by thirteen peaks in the imino-proton region.[29] As Phen-DC_3 was gradually titrated into the solution of Pu24T, a new set of distinct peaks appeared (Figure 1B). At an equimolar DNA/ligand ratio, only peaks for the G-quadruplex–ligand complex were observed (Figure 1B). Unambiguous assignments of the imino and aromatic protons of the bound Pu24T were made on the basis of exchange cross-peaks at a DNA/ligand ratio of 1:0.5 (Figure 1; see also Figure S2).[29] As most of the regions of aromatic and sugar protons of the complex showed similar NOE cross-peak patterns to those of free Pu24T, spectral assignment of the free DNA also aided the spectral assignment of the bound DNA. Protons of several adenine bases were independently identified through site-specific deuterium labeling[32] (see Figure S3). Both NMR and CD spectra of the bound DNA showed that the general folding of Pu24T remained unchanged (see Figure S4).

on basis of NMR restraints (see the Supporting Information). Like most reported G-quadruplex ligands,[29,33–39] Phen-DC_3 was found to interact with Pu24T through π-stacking on the top G-tetrad at the 5′ end. Binding of Phen-DC_3 disrupted the A3·A12 base pair[40] formed above the top G-tetrad of the free Pu24T.[29] Despite this disruption, a higher thermal stability was observed with an increase of approximately 12 °C in the melting temperature (see Figure S10). Although the present structural study focused only on the highest-affinity binding site (above the top G-tetrad at the 5′ end), our NMR titration (see Figure S11) showed a second binding event (possibly the stacking of a second Phen-DC_3 molecule onto the bottom G-tetrad at the 3′ end) at a 1:2 DNA/ligand ratio, in agreement with the original report on the possibility of two Phen-DC_3 molecules binding to a G-quadruplex.[18]

In the 1:1 Pu24T–Phen-DC_3 complex, the ligand is oriented in such a way as to establish an optimal interaction with the top G-tetrad, with maximal overlap observed between the quinolinium moiety and residue G13 (Figure 3B). The quinolinium moieties are directed away from the flexible pendent 5′ end and face the propeller loops. This positioning might be adopted to minimize steric clashes with the flexible pendent 5′ end. The two *N*-methyl groups are positioned above the grooves and have minimal contact with

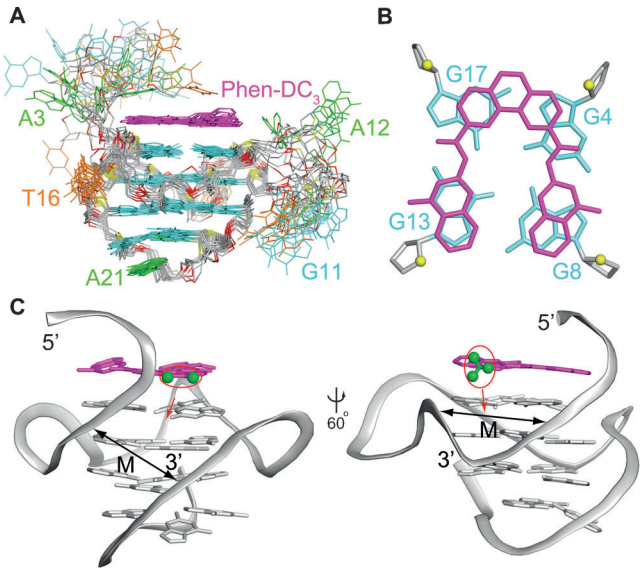

Figure 3. Solution structures of the Pu24T–Phen-DC_3 complex (PDB code: 2MGN). A) Ten superimposed refined structures. Guanine bases are colored in cyan; adenine bases, green; thymine bases, orange; backbone and sugar moieties, gray; O4′ atoms, yellow; P atoms, red; Phen-DC_3, magenta. B) Top view showing the stacking of Phen-DC_3 on the top G-tetrad of Pu24T. C) Suggested modifications of Phen-DC_3 to enable groove binding. Left: Addition of side-chain substituents to the k and k′ positions of the phenanthroline ring. Right: Extension of the *N*-methyl substituent positioned above a groove. The positions to be modified are highlighted in green and circled in red. Pu24T is shown in gray.

Pu24T. Further analysis revealed that the π-overlap between the Phen-DC_3 aromatic moieties and all four bases of the G-tetrad is more extensive than the interaction observed for most reported G-quadruplex–ligand complexes,[29,33–37] in which only one or two bases are overlapped by the ligand. This feature could be one of the underlying reasons for the strong binding affinity of Phen-DC_3 (the dissociation constant is in the submicromolar to nanomolar range) for many DNA and RNA G-quadruplexes.[25,41]

It was previously hypothesized that the crescent shape imposed by the central core (pyridine dicarboxamide or phenanthroline dicarboxamide) contributes to the exceptional G-quadruplex recognition properties of the bisquinolinium compounds.[18,42] This hypothesis was supported by the fact that G-quadruplex binding is considerably reduced for analogues exhibiting a more flexible core, such as a bipyridine core,[18] or even lost when the amide connectivity is inverted, thus leading to a linear conformation (see Figure S12 and Table S2). Follow-up studies involving analogues containing a different central aromatic core (see Figure S13) or additional cationic side chains (see Figure S14) also further emphasized the importance of the crescent shape of the central core (see Table S2).[25,26,41,43–47] The importance of the crescent-shaped core can be now rationalized in terms of a large π-overlap between Phen-DC_3 and the top G-tetrad.

From our structure, we can also conclude that the size of the quinolinium moieties, which affects the area of π-overlap, is another significant factor that contributes to the G-quadruplex-recognition properties. This hypothesis is supported by an early observation, whereby the replacement of the quinolinium with smaller aromatic moieties had a negative impact on G-quadruplex-binding ability and overall drug effectiveness.[47] A comparison of several recently reported analogues (see Figure S13) also showed a decrease in the thermal stability of the G-quadruplex–ligand complex when the quinolinium moieties were replaced with pyridinium rings.[41,44] Hence, it is highly desirable for further drug optimization to retain or incorporate quinolinium rings in the pendant arms, so as not to compromise the G-quadruplex-binding capacity of the scaffold and thus its biological activity.

Another factor that could be important for recognition is the flexibility of the ligand, as investigated in this study by molecular-dynamics (MD) simulations. We observed that the quinolinium moieties in free Phen-DC_3 displayed high flexibility along the N–C bonds connecting them to the central core (see Figure S15 A–D). These flexible movements were greatly reduced in the complex owing to the stacking interaction with the top G-tetrad (see Figure S15 E,F). During the MD simulations, rolling and tilting movements of the guanine bases in the G-tetrad core were observed, and we detected correlated movements between G13 and one of the quinolinium moieties: the two planes were parallel or formed an angle of less than 5° between one another (see Figure S16) for over 50 % of the simulation time. We propose that such in sync movements might help to maintain good π-overlap, thus emphasizing the importance of flexible compounds.

Finally, the structure reported herein suggests preferred positions on both the quinolinium and phenanthroline moieties that could be functionalized to enable additional interactions with the G-quadruplex. First, the two *N*-methyl groups present on the quinolinium units have no direct interaction with specific features of Pu24T. As they are positioned above two grooves, their replacement with long alkyl chains, possibly terminated by particular functional groups, would be a logical strategy for the creation of supplementary anchorage points in the grooves through hydrogen-bonding and electrostatic interactions. Likewise, the k and k′ positions of the phenanthroline ring are also positioned above a groove of Pu24T and could be used for derivatization. This strategy has been developed recently on a Phen-DC_3 platform[46] by the addition of cationic side chains (see Figure S14), which slightly improved the binding but at the expense of selectivity and cell permeation. It might be better to explore the effect of neutral or even anionic functions, as reported recently for pyridostatin.[48] The present structure determined by NMR spectroscopy points to the possibility of additional groove-binding interactions, which could result from a combination of the functionalization of the k and/or k′ position and the extension of the *N*-methyl groups to create a three-point anchor for a more specific binding of the bisquinolinium compound to the G-quadruplex.

In summary, we have shown that the *c-myc*-promoter G-quadruplex Pu24T binds to Phen-DC_3 in a 1:1 ratio through π-stacking on the top G-tetrad. The resolved structure allows us to better understand how bisquinolinium compounds interact with G-quadruplexes and should aid in the further develop-

ment of analogues with improved drug-like properties. More specifically, the structural data obtained is expected to aid in the optimization of Phen-DC_3 to maximize its selectivity and affinity for G-quadruplexes and thus its potency.

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