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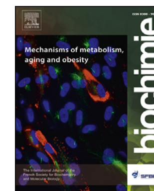

Short communication

Exploring the binding of d(GGGT)$_4$ to the HIV-1 integrase: An approach to investigate G-quadruplex aptamer/target protein interactions

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ABSTRACT

The aptamer d(GGGT)$_4$ (T30923 or T30695) forms a 5′-5′ dimer of two stacked parallel G-quadruplexes, each characterized by three G-tetrads and three single-thymidine reversed-chain loops. This aptamer has been reported to exhibit anti-HIV activity by targeting the HIV integrase, a viral enzyme responsible for the integration of viral DNA into the host-cell genome. However, information concerning the aptamer/target interaction is still rather limited. In this communication we report microscale thermophoresis investigations on the interaction between the HIV-1 integrase and d(GGGT)$_4$ aptamer analogues containing abasic sites singly replacing thymidines in the original sequence. This approach has allowed the identification of which part of the aptamer G-quadruplex structure is mainly involved in the interaction with the protein.

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1. Introduction

Aptamers are folded oligonucleotides (ON) that bind to a molecular target (both macro- and small organic molecules) with high affinity and specificity. They can be generated by a combinatorial technique termed SELEX (Systematic Evolution of Ligands by Exponential Enrichment) [1] that, starting from a random sequence ON library, includes cycles of selection, amplification and enrichment of the ON pool. Due to their favourable properties, aptamers are an emerging class of molecules with many potential applications in therapeutics, diagnostics and analytics [2]. Although the selection of these molecules for several functions is relatively straightforward, the adaptation of aptamers for therapeutic use *in vivo* requires further research. Particularly, post-SELEX modifications are needed with the aim of improving their pharmacokinetic and pharmacodynamic properties, reducing the serum degradation and decreasing the short half-life. However, the design of suitable modified analogues requires accurate information concerning the main features of the aptamer/target interaction, in order to avoid the drop of the high affinity and specificity obtained by the SELEX process.

Among aptamers endowed with promising biological properties, that formed by the sequence d(GGGT)$_4$ (T30923 or T30695) is particularly interesting due to its ability to bind with a high affinity two different not related target proteins, namely the HIV-1 integrase (HIV-IN) [3,4] and the interleukin-6 receptor (IL-6R) [5]. Earlier investigations on the structure adopted by d(GGGT)$_4$ suggested the formation of a chair-like antiparallel G-quadruplex characterized by two stacked G-tetrads and three lateral loops [6,7]. However, more recent thermodynamic and NMR studies have proven that the aptamer sequence forms a 5′-5′ dimer of two stacked parallel G-quadruplexes, each characterized by three G-tetrads and three single-thymidine reversed-chain loops (Fig. 1) [8,9].

In a molecular dynamics study of HIV-IN in complex with 93del, which is a different anti-HIV aptamer also adopting a dimeric G-quadruplex structure, the authors show that the aptamer is positioned in the catalytic cavity of the protein thus preventing its activity [10]. The authors also compare the docking complexes of HIV-IN with 93del and T30695, thus accounting for the higher

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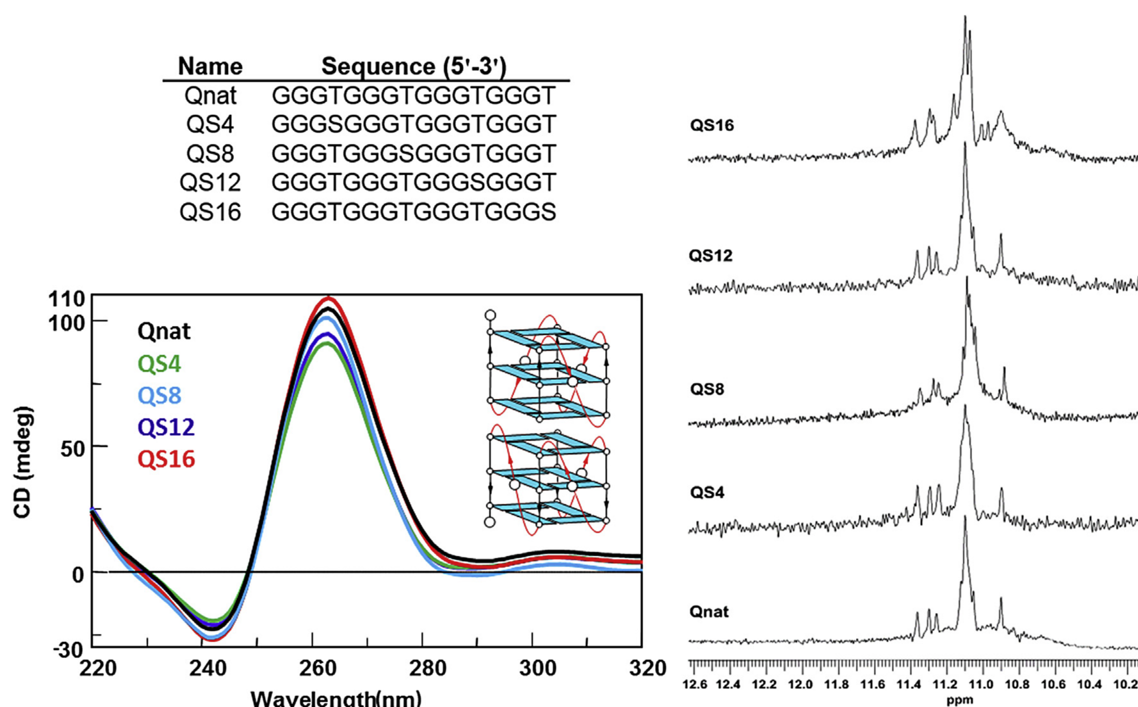

Fig. 1. Modified ODNs T30695 analogues investigated; S indicates an abasic site mimic (*Table*). CD spectra (*left*) and imino proton regions (*right*) of the ^{1}H NMR spectra (500 MHz) of the T30695 analogues. Schematic structure of the parallel-stranded G-quadruplex dimer of T30695 (see Ref. [6]) (*CD inset*).

interaction energy observed for the latter by considering the additional contribution of the 6 single nucleotide loops of the T30695 dimer against the 4 single nucleotide loops of the 93del dimer. Despite these considerations highlighting the importance of the loops for the complex formation, the interaction between HIV-IN and T30695 has not been described in detail.

2. Results and discussion

In order to acquire further data about the interaction between T30695 and HIV-IN, in this communication we propose a new approach in which d(GGGT)$_4$ analogues containing abasic-sites (Fig. S1) replacing, one at a time, loop thymidines have been prepared (Fig. 1) and investigated for their binding properties to the protein target by microscale thermophoresis (MST) [11,12].

A crucial point of this approach is the careful preliminary verification that the modified sequences preserve the ability to fold in the parallel G-quadruplex structure characteristic of the natural sequence, in order to avoid any misinterpretation of the data concerning the interaction with the target, due to the presence of alternative conformations differing from the original one. For these reasons, the modified ODNs d(GGGT)$_4$ have been preliminarily investigated by CD and ^{1}H NMR techniques. Fig. 1 shows the CD spectra of the modified sequences and their natural counterpart.

Despite small differences in intensity, all profiles are almost superimposable on each other, showing a minor negative band at 242 nm and a major positive band at 263 nm, which are characteristic of parallel G-quadruplex structures in which all guanosines adopt an *anti* glycosidic conformation. Furthermore, as in the case of the original structure, the CD melting profiles of the modified G-quadruplex structures (Fig. S2) indicate melting temperatures (T_m) above 75 °C, thus suggesting that abasic sites do not significantly affect the conformation adopted by the original sequence and its stability in the experimental conditions used.

In order to corroborate the CD results, ^{1}H NMR spectra of the modified aptamers have been acquired, as well.

Fig. 1 shows the imino proton regions diagnostic of the presence of G-quadruplex structures (10.5–12.0 ppm) in which the strong similarity between most of the NMR profiles of the modified aptamer and their unmodified version is quite evident. The major differences observed in the case of QS16 should not be particularly surprising taking into account the fact that, as suggested by the molecular model of the dimer [8], the T16 stacks on the 3'-end G-tetrad thus probably preventing the 3′-3′ interaction. Therefore, in the case of the QS16, the lack of T16 and then, the possible occurrence of higher order structures, could account for the differences observed in the ^{1}H NMR spectrum.

Taken together, the CD and NMR data strongly suggest that the modified sequences adopt G-quadruplex structures strictly resembling that of the original aptamer, thus clearly indicating that, regardless of the position in the sequence, an abasic site replacing a thymidine is not able to affect significantly the conformation and stability of the original structure.

In order to investigate the ability of the modified sequences to interact with HIV-IN, which is the main protein target of their natural counterpart, MST experiments have been carried out. The labelled HIV-IN (DYE NT-647-NanoTemper Technologies) at a constant concentration of 10 μM, has been titrated with the ODNs (increasing concentrations ranging from 18 nM to 600 μM) and subjected to thermophoresis experiments. MST measurements with an unspecific double stranded oligo, used as negative control, have also carried out. The thermophoresis signal has been plotted against the ligand concentration to obtain a dose-response curve, from which the binding affinity could be evaluated (Fig. 2, S3 and S4). The binding curves achieved for the natural sequence (Qnat) and its analogues show a clear association with HIV-IN, as demonstrated by the thermophoretic traces (Fig. S3). Taking into account that the apparent K_D values for QS8, QS12 and QS16 are comparable with those calculated for their parent sequence Qnat (0.5 μM), these data suggest that thymidines in positions 8, 12 and

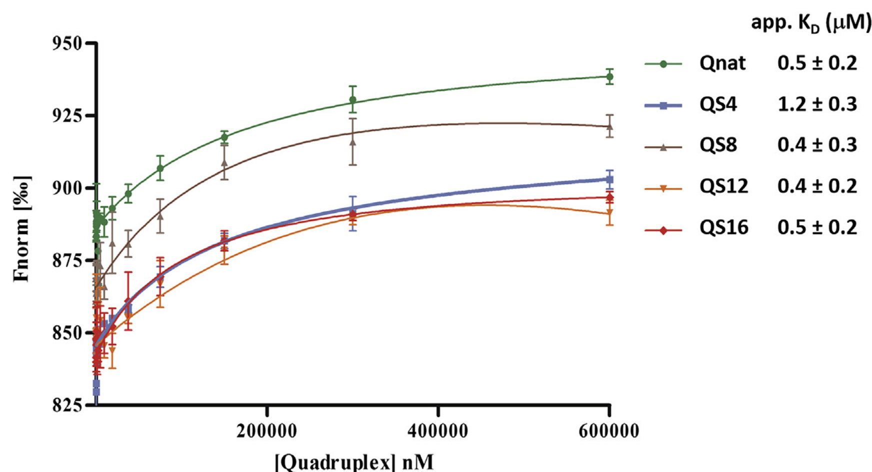

Fig. 2. Plot of normalized fluorescence obtained from each binding experiment *versus* ODNs at different concentrations (18 nM–600 μM). The experimental curves were fitted according to a one-site specific binding equation.

16 and their related single-residue loops play a negligible role in the aptamer/HIV-IN recognition. A different effect resulted in the case of QS4, for which a threefold higher value of the apparent K_D has been observed. Differently from the previous results, this datum suggests a more important role for thymidine in position 4 and its associated loop in the aptamer/protein recognition.

It should be noted that the apparent dissociation constants (K_D) estimated by MST do not show the same order of magnitude of the IC50 values (10–50 nM) for the inhibition reported in literature [6]. This discrepancy could be ascribed to a reduced activity of the integrase sample probably due to the labelled procedure of the protein required for the MST experiments or to the well-known ability of the protein to oligomerize [13]. However, taking into account that affinity of the various G-quadruplex structures with the integrase has been investigated by the same protein stock solution, such discrepancy should not affect conclusions deriving from the comparison of the dissociation constants of the modified aptamers with their natural counterpart.

Interestingly, in a SELEX experiment aimed at discovering aptamers against IL-6R [5], the same G-quadruplex-forming 16-mer DNA sequence d(GGGT)$_4$ was selected. Moreover, by studying single nucleotide variants in which each thymidine was individually replaced by adenosine, the analogue containing an adenine replacing T4 showed reduced affinities to the protein. This datum would suggest a critical role for T4 also in the case of the aptamer/IL-6R interaction. Surprisingly, in the same investigation and in other studies concerning a T30695 related aptamer (Zintevir) [14], these aptamers were proven to bind also the viral envelope protein gp120 and to inhibit HIV infection by interfering with the gp120-CD4 interaction.

Introduction of abasic site mimics in tetramolecular and monomolecular telomere G-quadruplex structures has already been investigated in a different context, since this alteration represents the most frequent lesion in DNA [15,16]. However, in the present study, the presence of an abasic site in the aptamer sequence has been exploited to investigate its interaction with the target protein.

From this point of view, aptamers forming G-quadruplex structures [17] could be particularly appropriate to be studied by this method, since it is reasonable to assume that most of the structural stability relies on the core formed by the stacked G-tetrads, while the external more accessible loop residues are potentially more prone to interact with the target and, then, to be replaced with abasic sites in order to obtain an insight about the aptamer/target interaction. It should be noted that despite the fact that methods to acquire detailed structural information on aptamer/target complexes are available (e.g. X-ray and NMR), the utility of these approaches is often limited by the difficulty in obtaining an appropriate crystal and the large molecular dimensions of the complex.

3. Conclusions

Although less informative than other techniques and limited to G-quadruplex aptamers, our approach has shown the following advantages in comparison with the above mentioned methods: 1) the required amounts of the aptamer are less; 2) the synthesis of the analogues containing abasic sites is straightforward and based on standard methods; 3) the analysis of the data is simpler and allows a faster acquisition of preliminary information concerning the parts of the aptamer structure crucial in the target recognition. Furthermore, the information obtained by this approach could be particularly useful in assisting molecular modelling docking studies by limiting the aptamer/target protein interaction interface to specific G-quadruplex regions.

Since literature data have shown that the G-quadruplex structure adopted by d(GGGT)$_4$ is able to bind three different proteins, namely HIV-IN [3–5], IL-6R [5] and gp120 [5,14], it would be interesting to investigate if also the interaction of T30695 with the other target proteins involves the same or different regions of the G-quadruplex structure. Furthermore, it should be noted that, similarly to T30695, several aptamers endowed with interesting biological properties (including 93del [18] and T30177 [19] both targeting HIV integrase, R12 targeting prion protein [20] and AS1411 targeting nucleolin [21]) adopt G-quadruplex structures showing one-residue loops. These structural characteristics have been proven particularly appropriate in investigating the interaction between the aptamer and its target through the proposed method. In this context, suitable experiments based on the described approach have been planned in our laboratory.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.biochi.2016.04.013.

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