Pergamon

SCIENCE DIRECT®

Bioorganic & Medicinal Chemistry Letters 13 (2003) 3801–3804

BIOORGANIC & MEDICINAL CHEMISTRY LETTERS

Determination of Binding Affinities of Triplex Forming Oligonucleotides Using a Fluorescent Intercalator Displacement (FID) Assay

Bryan K. S. Yeung, Winston C. Tse and Dale L. Boger*

Department of Chemistry and The Skaggs Institute for Chemical Biology, The Scripps Research Institute, 10550 North Torrey Pines Road, La Jolla, CA 92037, USA

Received 6 June 2003; accepted 28 July 2003

Abstract—The binding affinities of several triplex forming oligonucleotides were determined using a fluorescent intercalator displacement (FID) assay.

Since the discovery of triplex DNA in 1957,[1] its formation has been widely used as a means for sequence selective DNA cleavage,[2] gene targeting,[3] and gene repression.[4] The canonical DNA triplex is generally formed between a homopyrimidine oligonucleotide and a homopurine–homopyrimidine duplex. The triplex forming oligonucleotide (TFO) aligns parallel to the purine strand and binds within the major groove of the DNA by Hoogsteen base pairing (Fig. 1).

A second triple helix motif is possible when the third TFO strand is rich in purine residues. This results in an anti-parallel arrangement with respect to the purine strand and consists of reverse Hoogsteen hydrogen bonding (Fig. 2).[5]

There have been many studies carried out in which the binding affinity of triplex formation with various TFOs have been established. Some of the methods employed in the study of the kinetics or quantitative assessments of triplex formation include UV and CD melting,[6] differential scanning calorimetry,[7] affinity cleavage,[8] footprinting,[8a,9] restriction endonuclease protection assay,[10] filter-binding assay,[11] isothermal titration calorimetry,[12] and FRET assays.[13] We have recently introduced a technically non-demanding and non-destructive fluorescent intercalator displacement (FID) assay for characterizing small molecule,[14–16] hairpin polyamide,[17] or protein[18] DNA binding affinity, selectivity, and stoichiometry.[19] The method may be utilized for the high throughput screening of libraries of compounds against a single sequence to select high-affinity binders,[15–17] or to screen a single compound against a library of DNA sequences to establish DNA binding selectivity.[14,15,17] Scatchard analysis of quantitative titrations of molecules of interest against any single sequence embedded in a hairpin oligonucleotide provides accurate association constants and the stoichiometry of binding.[14] Herein, we report the use of this FID assay to establish the stoichiometry and binding affinity of TFOs. This method is based on the loss of fluorescence derived from the titration displacement of ethidium bromide from a hairpin DNA containing the sequence of interest.

Results and Discussion

Below, we report the binding affinities of several triplex forming oligonucleotides with homopurine homopyrimidine hairpins consisting of either a pure T·AT and C^+·GC triad (hairpins 1 and 2, 9-mer TFOs) or incorporating both T·AT and C+·GC triads (hairpins 3 and 4, 13-mer TFOs) (Fig. 3).

The results are summarized in Table 1. In each case of triplex formation, a well defined titration curve was observed exhibiting the expected 1:1 stoichiometry of binding and providing association constants consistent

*Corresponding author. Tel.: +1-858-784-7522; fax: +1-858-784-7550; e-mail: boger@scripps.edu

0960-894X/$ - see front matter © 2003 Elsevier Ltd. All rights reserved.
doi:10.1016/j.bmcl.2003.07.005

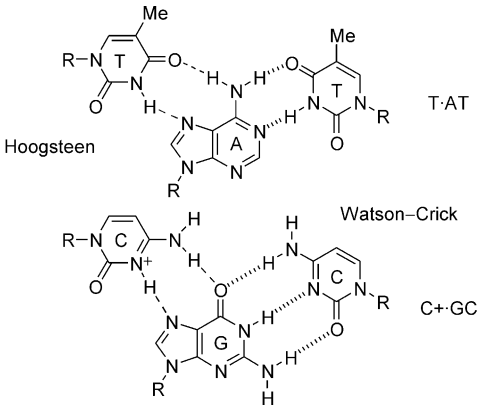

Figure 1. Representation of the T·AT (top) and C^+·GC (bottom) triplets formed by Hoogsteen base pairing to the corresponding Watson–Crick base pair of duplex DNA (parallel). The GC triplex requires protonation of the N3 of cytosine TFO (pH < 7.0).

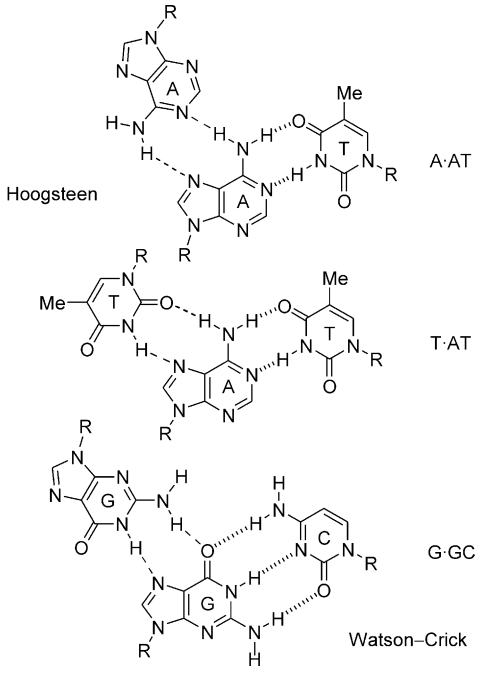

Figure 2. Representation of the A·AT, T·AT and G·GC triplets formed by reverse Hoogsteen base pairing to the corresponding Watson–Crick base pair of duplex DNA (antiparallel).

with expectations. Thus, although the viability of the technique for monitoring and qualitatively or quantitatively assessing triplex formation was not obvious at the initiation of our studies and numerous reasons for its potential failure could be imagined, the FID assay provides an effective and simple method for assaying triplex formation. This may be attributed to in large measure to the weak and rapidly equilibrating intercalative binding of ethidium bromide that is not effectively competing with triplex formation.

A 9-mer poly[C] TFO (TFO 1) was titrated into a solution of the hairpin 1 forming triplex A and a loss of fluorescence was observed as the triplex formed resulting from the displacement of prebound ethidium bromide. As anticipated, formation of the C^+·GC triplex

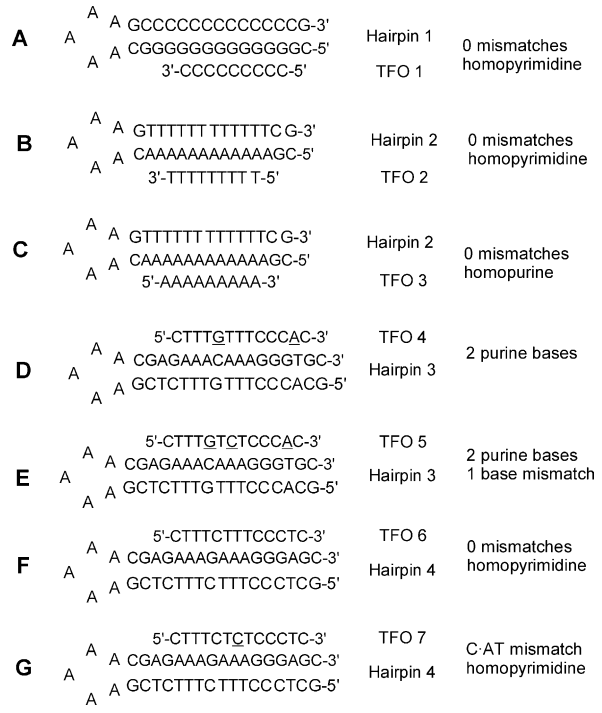

Figure 3. The four hairpin DNAs used in the experiments. The first two triplex incorporate a homopyrimidine TFO and a homopurine–homopyrimidine hairpin containing the poly C^+·GC, poly T·AT triads (triplex A and B). Triplex C incorporates a polypurine TFO with reversed Hoogsteen base pairing (A·TA triad). Hairpin 3 contains a sequence that requires a pyrimidine TFO containing two purine bases (G and A, triplex D). Hairpin 3 was also treated with a TFO incorporating the two purine bases and an additional base mismatch (C·AT, triplex E). Hairpin 4 was treated with two homopyrimidine TFOs. Triplex F incorporates only matching cytosine and thymine bases, while triplex G included a C·AT mismatch.

Table 1. TFO binding affinities

Hairpin	TFO		Mismatches	K ($\times 10^6$ M^{-1})
1[a]	5′-CCCCCCCCC-3′	(**1**)	0 homopyrimidine	15
2[b]	5′-TTTTTTTTT-3′	(**2**)	0 homopyrimidine	5.1
2[b]	5′-AAAAAAAAA-3′	(**3**)	0 homopurine	No binding
3[a]	5′-CTTTGTTTCCCAC-3′	(**4**)	2 purine	34
3[a]	5′-CTTTGTCTCCCTC-3′	(**5**)	1 base, 2 purine	No binding
4[a]	5′-CTTTCTTTCCCTC-3′	(**6**)	0 homopyrimidine	224
4[a]	5′-CTTTCTCTCCCTC-3′	(**7**)	1 base	17

[a]10 mM NaOAc, 10 mM cacodylate, 0.2 M NaCl and 20 mM $MgCl_2$, pH = 4.8.
[b]10 mM NaOAc, 10 mM cacodylate, 0.2 M NaCl and 20 mM $MgCl_2$, pH = 7.5.

was pH dependent and only observed at pH 4.8. As in all examples where TFO binding was observed, the TFO complexed to the hairpin with 1:1 stoichiometry. Similarly, triplex formation of a 9-mer poly[T] TFO (triplex B) with hairpin 2 could be monitored with the FID assay conducted at pH 7.5. The binding constant for the T·AT (triplex B) was comparable, but 3 times lower than that observed for triplex A. When a polypurine TFO (poly[A]) was used with hairpin 2, no binding was observed (triplex C).

Triplex D was formed with a homopyrimidine 13-mer TFO containing two purine bases. The purine bases in the TFO require an antiparallel (vs parallel) configuration to maximize hydrogen bonding not afforded in the pyrimidine-rich TFO 4 and results in the decreased binding affinity observed relative to triplex F. Moreover, further incorporation a base pair C·AT mismatch (triplex E) resulted in no observable triplex formation. Thus, the combination of two non-productive parallel purine residues and a base mismatch preclude triplex formation.

The highest binding affinities observed were those for a 13-mer homopyrimidine TFO and a homopurine–homopyrimidine duplex containing cytosine and thymine bases (triplex F). The titration curve shows a stoichiometry of binding of 1.00 which was experimentally determined by the intersection of the pre- and postsaturation curves (Fig. 4). Scatchard analysis of the titration curve provided the negative slope and the binding constant of 22.4×10^7 M^{-1} (Fig. 5) Incorporating a single C·AT mismatch into this homopyrimidine TFO (TFO 7), decreased the binding affinity more than an order of magnitude. The magnitude of this decrease is consistent with results obtained with a similar 15-mer TFO incorporating the same C·AT base mismatch.[8a]

Thus, the studies above establish that the FID assay provides a technically simple method for qualitatively or quantitatively assessing triplex formation. Most important of the findings is the ability of the assay and technique to quantitatively measure the effects of single base-pair mismatches by a simple titration.

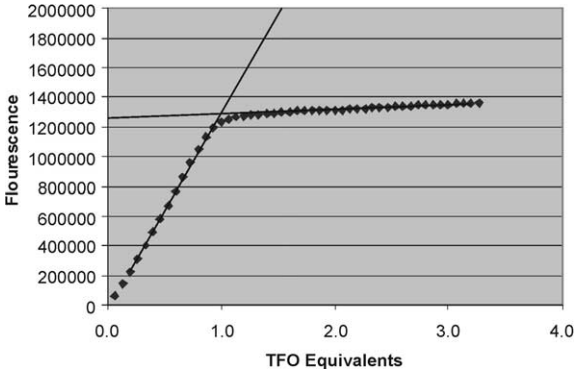

Figure 4. Titration of hairpin **4** with 5′-CTTCTTTCCCTC-3′ (homopyrimidine TFO 6). Experiments were performed at 25 °C in cacodylate buffer, pH 4.8.

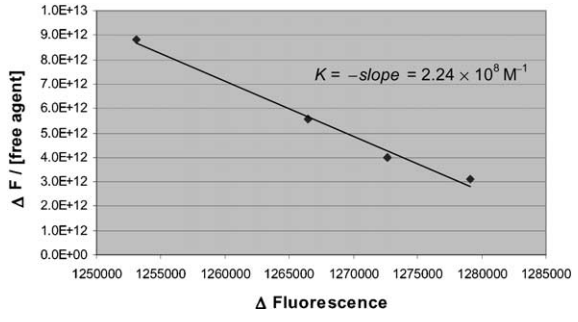

Figure 5. Scatchard plot of hairpin **4** with the 5′-CTTCTTTCCCTC-3′ TFO. The association constant (K) is equal to the negative slope, 2.24×10^8 M^{-1}.

Determination of DNA binding constants

A 3-mL quartz cuvette was loaded with cacodylate buffer (10 mM sodium acetate/10 mM cacodylic acid, 0.2 M NaCl, and 20 mM $MgCl_2$, pH 4.8) and ethidium bromide (1.2×10^{-5} M final concentration). The fluorescence was measured (excitation 545 nm, emission 595 nm, ethidium bromide) and normalized to 0% relative fluorescence. The hairpin deoxyoligonucleotide was added (1.5 μM final concentration), and the fluorescence measured again and normalized to 100% relative fluorescence. A solution of the TFO (2 μL, 0.1 mM in buffer) was added, and the fluorescence measured following 3 min of incubation at 23 °C. Subsequent addition of 2 μL aliquots of the agent was continued until the system reached saturation and the fluorescence remained constant with successive compound additions.

Scatchard analysis of the titration curve

The ΔF was plotted versus molar equivalents of agent and the ΔF_{sat} was determined mathematically by solving the simultaneous equations representing the pre- and postsaturation regions of the titration curve. Utilizing eqs 1–3, a Scatchard plot was generated where ΔF/[free agent] was plotted versus ΔF. The slope of the region immediately preceding complete saturation of the system provided $-K$.[14]

$$\left(\frac{\Delta F_x}{\Delta F_{sat}}\right)\frac{1}{X} = \text{fraction of DNA – agent complex} \quad (1)$$

$$\left[1-\left(\frac{\Delta F_x}{\Delta F_{sat}}\right)\frac{1}{X}\right] = \text{fraction of free agent} \quad (2)$$

$$[\text{DNA}]_T\left[X-\frac{\Delta F_x}{\Delta F_{sat}}\right] = [\text{free agent}] \quad (3)$$

Acknowledgements

We gratefully acknowledge the financial support of NIH (CA 41986, CA 78045) and the Skaggs Institute for Chemical Biology. W.C.T. is a Skaggs Fellow and recipient of an ACS Medicinal Chemistry Division Graduate Fellowship sponsored by Wyeth Ayerst (2002–2003).

References and Notes

1. Felsenfeld, G.; Davies, D. R.; Rich, A. *J. Am. Chem. Soc.* **1957**, *79*, 2023.
2. (a) Moser, H. E.; Dervan, P. B. *Science* **1987**, *238*, 645. (b) Singleton, S. F.; Dervan, P. B. *J. Am. Chem. Soc.* **1992**, *114*, 6957. (c) Maher, III, L. J.; Wold, B.; Dervan, P. B. *Science* **1989**, *245*, 725. (d) Pei, D.; Corey, D. R.; Schultz, P. G. *Proc. Natl. Acad. Sci. U.S.A.* **1990**, *87*, 9858.
3. (a) Vasquez, K. M.; Wilson, J. H. *Trends Biochem. Sci.* **1998**, *23*, 4. (b) Knauert, M. P.; Glazer, P. M. *Hum. Mol. Genet.* **2001**, *10*, 2243.

4. (a) Cooney, M.; Czernuszewicz, G.; Postel, E. H.; Flint, S. J.; Hogan, M. E. *Science* **1988**, *241*, 456. (b) Maher, III, L. J.; Dervan, P. B.; Wold, B. *Biochemistry* **1992**, *31*, 70. (c) Duval-Valentin, G.; Thuong, N. T.; Helene, C. *Proc. Natl. Acad. Sci. U.S.A.* **1992**, *89*, 504.
5. (a) Vasquez, K. M.; Glazer, P. M. Q. *Rev. Biophys.* **2002**, *35*, 89. (b) Radhakrishnan, I.; Patel, D. J. *Biochemistry* **1994**, *33*, 11405. (c) Cheng, Y.-K.; Pettitt, B. M. *Prog. Biophys. Mol. Biol.* **1992**, *58*, 225. (d) Haner, R.; Dervan, P. B. *Biochemistry* **1990**, *29*, 9761.
6. (a) Rougée, M.; Faucon, B.; Mergny, J.-L.; Barcelo, F.; Giovannangeli, C.; Garestier, T.; Hélène, C. *Biochemistry* **1992**, *32*, 9269. (b) Mergny, J.-L.; Sun, J.-S.; Rougée, M.; Montenay-Garestier, T.; Barcelo, F.; Chomilier, J.; Hélène, C. *Biochemistry* **1991**, *30*, 9791. (c) Roberts, R. W.; Crothers, D. M. *Proc. Natl. Acad. Sci. U.S.A.* **1991**, *88*, 9397. (d) Völker, J.; Botes, D. P.; Lindsey, G. G.; Klump, H. H. *J. Mol. Biol.* **1993**, *230*, 1278.
7. (a) Plum, G. E.; Park, Y.-W.; Singleton, S. F.; Dervan, P. B.; Breslauer, K. J. *Proc. Natl. Acad. Sci. U.S.A.* **1990**, *87*, 9436. (b) Plum, G. E.; Park, Y.-W.; Singleton, S. F.; Dervan, P. B.; Breslauer, K. J. *J. Mol. Biol.* **1995**, *248*, 679. (c) Sugimoto, N.; Shintani, Y.; Tanaka, A.; Sasaki, M. *Bull. Chem. Soc. Jpn.* **1992**, *65*, 535.
8. (a) Singleton, S. F.; Dervan, P. B. *J. Am. Chem. Soc.* **1992**, *114*, 6957. (b) Singleton, S. F.; Dervan, P. B. *J. Am. Chem. Soc.* **1994**, *116*, 10376.
9. (a) Fox, K. R. *FEBS Lett.* **1995**, *357*, 312. (b) Protozonova, E.; Macgregor, R. B., Jr. *Anal. Biochem.* **1996**, *243*, 92.
10. Maher, III, L. J.; Dervan, P. B.; Wold, B. J. *Biochemistry* **1990**, *29*, 8820.
11. (a) Shindo, H.; Torigoe, H.; Sarai, A. *Biochemistry* **1993**, *32*, 8963. (b) Sarai, A.; Sugiura, S.; Torigoe, H.; Shindo, H. *J. Biomol. Struct. Dyn.* **1993**, *11*, 245.
12. Kamiya, M.; Torigoe, H.; Shindo, H.; Sarai, A. *J. Am. Chem. Soc.* **1996**, *118*, 4532.
13. Reither, S.; Jeltsch, A. *BMC Biochem.* **2002**, *3*, 27.
14. (a) Boger, D. L.; Fink, B. E.; Brunette, S. R.; Tse, W. C.; Hedrick, M. P. *J. Am. Chem. Soc.* **2001**, *123*, 5878. (b) Boger, D. L.; Tse, W. C. *Bioorg. Med. Chem.* **2001**, *9*, 2511.
15. Boger, D. L.; Fink, B. E.; Hedrick, M. P. *J. Am. Chem. Soc.* **2000**, *122*, 6382.
16. (a) Woods, C. R.; Faucher, N.; Eschgfaller, B.; Bair, K. W.; Boger, D. L. *Bioorg. Med. Chem. Lett.* **2002**, *12*, 1247. (b) Yeung, B. K. S.; Boger, D. L. *J. Org. Chem.* **2003**, *68*, 5249.
17. (a) Woods, C. R.; Ishii, T.; Wu, B.; Bair, K. W.; Boger, D. L. *J. Am. Chem. Soc.* **2002**, *124*, 2148. (b) Woods, C. R.; Ishii, T.; Boger, D. L. *J. Am. Chem. Soc.* **2002**, *124*, 10676. (c) Tse, W. C.; Ishii, T.; Boger, D. L. *Bioorg. Med. Chem.* In press.
18. Ham, Y.-W.; Tse, W. C.; Boger, D. L. *Bioorg. Med. Chem. Lett.* **2003**. doi: 10.1016/j.bmcl.2003.06.002.
19. Review: Tse, W. C.; Boger, D. L. *Acc. Chem. Res.* Submitted for publication.