Bioorganic & Medicinal Chemistry Letters 20 (2010) 4346–4349

Contents lists available at ScienceDirect

Bioorganic & Medicinal Chemistry Letters

journal homepage: www.elsevier.com/locate/bmcl

Furan based cyclic homo-oligopeptides bind G-quadruplex selectively and repress c-*MYC* transcription

Tani Agarwal [a], Saumya Roy [b], Tushar Kanti Chakraborty [b,c,*], Souvik Maiti [a,*]

[a] *Proteomics and Structural Biology Unit, Institute of Genomics and Integrative Biology, CSIR, Mall Road, New Delhi 110 007, India*
[b] *Indian Institute of Chemical Technology, CSIR, Hyderabad 500 007, India*
[c] *Central Drug Research Institute, CSIR, Lucknow 226 001, India*

ARTICLE INFO

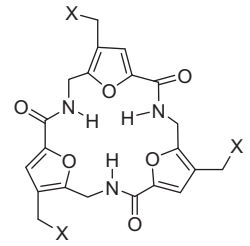
Article history:
Received 19 March 2010
Revised 24 May 2010
Accepted 14 June 2010
Available online 17 June 2010

Keywords:
Quadruplex
Cancer
c-*MYC*
Macrocyclic compounds
Oligopeptides

ABSTRACT

We demonstrate the synthesis and selective binding of two novel furan based tricyclic homo-oligopeptides to G-quadruplex and using Real Time PCR show its repressive effect on c-*MYC* transcription. CD spectroscopy and FRET melting studies show that these ligands can induce G-quadruplex structures in the G rich 22 mer c-*MYC* DNA sequence and further stabilize the structure. Using real time polymerase chain reaction we observed that up to 70% downregulation of c-*MYC* transcripts upon ligand treatment in HeLa cells.

G-Quadruplex forming motifs have been found throughout the genome with distinct predominance in the promoters of most protooncogenes like c-*MYC*, c-*KIT*, c-*FOS*, *KRAS*, and c-*ABL*.[1] Quadruplexes in these promoter regions are suggested to alter molecular recognition and hence control disease phenotypes.[1] Moreover, stabilization of G-quadruplexes by small molecules has recently been shown to inhibit the transcriptional activity of some oncogenes.[2] Several ligands including derivatives of acridines,[3] cationic Porphyrin,[4] ethidium,[5] quindoline,[6] and telomestatin[7] have been studied for their quadruplex binding ability and thereby resulting in altered gene product or function affecting the legitimate biological functions. Thus, the G-quadruplex motif has emerged as a promising target for the design of selective antitumor therapeutics. Yet it has been a challenging task to identify suitable ligands that can selectively interact with G-quadruplex DNA. Attempts at identifying suitable molecules to target G-quadruplexes have failed to combine all the desirable features viz. high affinity, high selectivity and low toxicity. Hence new scaffolds encompassing these features must be identified. In this league we employed two novel ligands; furan amino acid based cationic cyclic homo-oligopeptides (Scheme 1). In a previous study by our group we showed that the parent molecules of the same league were efficient quadruplex binders and showed high selectivity for quadruplex over duplex.[9] In the present study, we have



Scheme 1. Chemical structures of furan based cyclic homo-oligopeptides.

synthesised two new cationic cyclic homo-oligopeptides, 1 and **2**, prepared from the trisubstituted furan amino acid, H–Faa(CH_2N_3)–OH and show the selective binding of these two molecules with c-*MYC* G-quadruplex. The expression of c-*MYC* protooncogene implicated in various cellular processes like growth, proliferation, loss of differentiation and cell death (apoptosis), is known to be controlled by a G-quadruplex motif located in the promoter.[10] Hence, the 22 mer quadruplex forming sequence from c-*MYC* promoter serves as an appropriately well established model system to investigate the efficient targeting of quadruplex motifs by these molecules. We have used CD spectroscopy and FRET assay to explore the binding, stabilization and selectivity for quadruplex, along with molecular assays to understand their effect on c-*MYC* gene expression and cell survival (Supplementary data).

* Corresponding authors. Tel./fax: +91 52 22623405 (T.K.C.); tel.: +91 11 27666156; fax: +91 27667471 (S.M.).
E-mail addresses: chakraborty@cdri.res.in (T.K. Chakraborty), souvik@igib.res.in (S. Maiti).

0960-894X/$ - see front matter © 2010 Elsevier Ltd. All rights reserved.
doi:10.1016/j.bmcl.2010.06.080

The syntheses of the cyclic peptides (**1**, **2**) are given in details in the Supplementary data. The deprotected monomer TFA. H–Faa(CH_2N_3)–OH, was cyclooligomerized in a single step by using pentafluorophenyldiphenylphosphinate (FDPP) in DMF under dilute conditions (10^{-2} M) to give both cyclic products, *cyclo*-[Faa(CH_2N_3)]$_3$ and *cyclo*-[Faa(CH_2N_3)]$_4$ in 30% and 11% yields, respectively. The azide groups of the major cyclized product *cyclo*-[Faa(CH_2N_3)]$_3$ were reduced to primary amines by catalytic hydrogenation, followed by in situ protection with Boc_2O. The product was purified at the Boc-protected stage by standard silica gel column chromatography. Then Boc-deprotection using TFA–CH_2Cl_2 provided the ligand **1**. Coupling of **1** with Boc-β-Ala-OH was again followed by Boc-deprotection which provided the other product **2**. The final products were fully characterized by spectroscopic methods before using them in the binding studies (see Supplementary data).

CD spectroscopy was employed to investigate the induction of G-quadruplex signature in the c-*MYC* sequence: $(G_4AG_3T)_2G_4$. The sequence adopts the predominant parallel quadruplex structure in presence of 100 mM KCl, as observed by a strong positive peak at 265 nm and a negative peak at 240 nm, while in absence of any monovalent cation, the peak at 265 nm was weak. But with addition of ligand (**1** and **2**) in absence of any monovalent cation there was enhancement in the positive peak at 265 nm signifying induction of quadruplex folding. Ligand **2** led to greater induction into folded parallel topology comparable to the intensity obtained in potassium containing buffer. In case of ligand **2** the signal showed a greater increase suggesting greater effectiveness of ligand to induce quadruplex folding, preferentially for parallel topology. Up to 2 equiv of ligand was added to understand the concentration driven folding which eventually showed some increase in signal intensity especially for ligand **2** (Fig. 1). Further increase in molar excess of ligand did not lead to significant increase in the folded population.

Next we conducted FRET melting assay to assess the ligand mediated thermal stabilization c-*MYC* quadruplex in presence of 25 mM KCl in absence and presence of increasing amounts of ligands. The melting temperature (T_m) of the c-*MYC* quadruplex in absence and presence of increasing amounts of ligand was monitored by FRET techniques and analyzed accordingly. The T_m of the sequence in 10 mM sodium cacodylate with 25 mM KCl in the absence of any ligand was observed to be 59 °C, while change

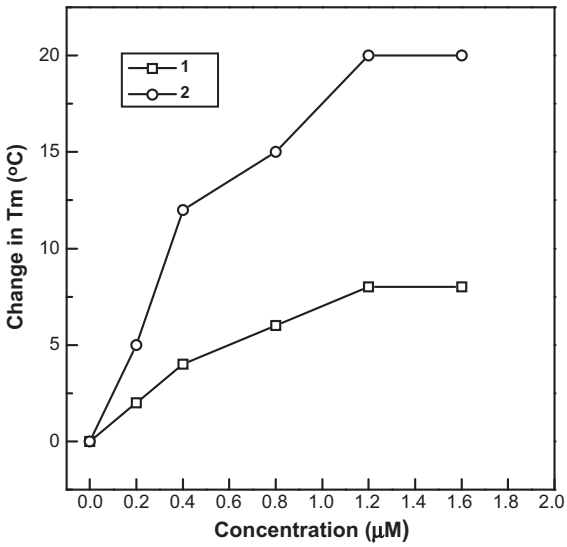


Figure 2. Increase in T_m of 0.2 μM 5′fluorescein- 3′dabsyl labeled c-*MYC* 22 mer quadruplex as a function of concentration of ligands in presence of 25 mM KCl.

in T_m (ΔT_m) in presence of 0.2, 0.4, 0.8, and 1.2 μM of ligand **1** was 2 °C, 4 °C, 6 °C, and 8 °C respectively (Fig. 2). Further addition of ligand resulted in only a nominal change in ΔT_m. Higher stabilization was obtained for ligand **2** leading to ΔT_m increment of 5 °C, 12 °C, 15 °C, and 20 °C at 0.2, 0.4, 0.8, and 1.2 μM concentrations, respectively. There was not much increase in ΔT_m beyond 1.2 μM ligand concentration (Supplementary Fig. 1a). These ΔT_m values obtained are quite encouraging as they are in a similar range of ΔT_m reported for other well studied quadruplex ligands like telomestatin (ΔT_m = 24 °C), the cationic porphyrin TmPyP4 (ΔT_m = 17 °C) and the other metal based compounds like Ni(II) porphyrin (ΔT_m = 33 °C).[4,7] Oxazole based peptide macrocyclic compounds have also been shown to stabilize c-*KIT* quadruplex by 10 °C using FRET assay.[8]

The plot of ΔT_m versus molar concentration of ligand (Fig. 2) shows the gradual increase in ΔT_m with increasing concentration of ligand in the system thus suggesting increased binding and hence stabilization of the quadruplex. We extended the assay to

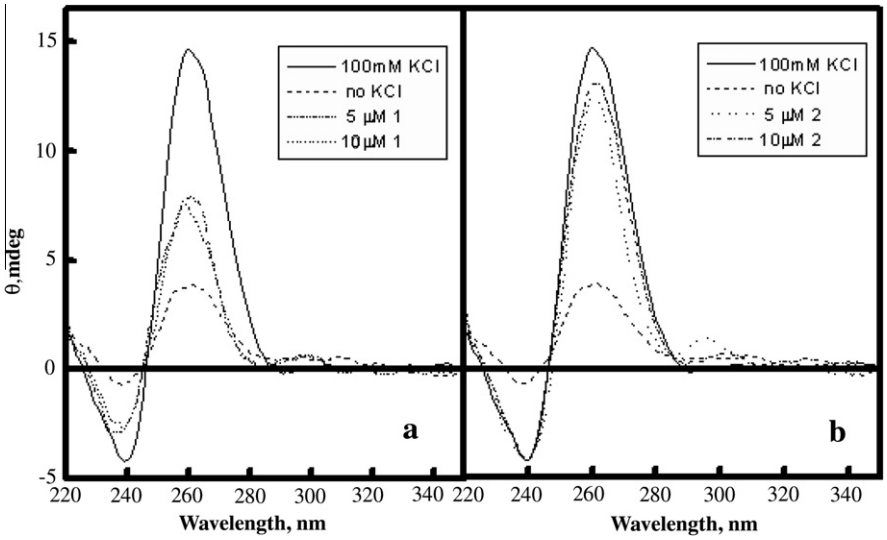


Figure 1. CD spectroscopy shows induction of the quadruplex structure upon addition of 1 and 2 equiv of each ligand **1** (a) and **2** (b). Ligand **2** shows greater enhancement of quadruplex signature. 5 μM oligonucleotide was taken in 10 mM sodium cacodylate buffer.

include Calf Thymus (CT) DNA to understand competitive binding to duplex too. Hence, in a ligand saturated system (such that no more change in ΔT_m was recorded) we added increasing amounts of CT DNA up to a molar ratio of 50. Interestingly, there was no change in the melting temperatures despite increasing amounts of duplex even at the highest molar ratios considered (Supplementary Fig. 1b). This is a strong exhibition of selectivity of ligands to bind with only quadruplex structures and exclude duplexes.

We have also conducted ITC experiments to measure the binding parameters for the ligands with c-*MYC* quadruplex. It was observed that there was a single binding mode with a stoichiometry of 3 each for ligand **1** and **2**. The binding affinities in case of both ligands were of the order of 10.[5] The ITC thermograms and binding parameters have been shown in Supplementary data (Supplementary Fig. 2 and Table 1).

The cellular effect of these ligands through quadruplex stabilization was also checked by Real Time PCR. As c-*MYC* quadruplex is structurally and functionally well characterized, and is suggested to be involved in gene regulation, therefore, any perturbation in the quadruplex stability and molecular recognition will affect the downstream expression. We performed Real Time PCR to assess the transcript level of c-*MYC* gene upon pharmacological perturbation by quadruplex binding ligands **1** and **2** in HeLa cells. The relative fold change was calculated by normalizing against β-2 microglobin transcript. Figure 3 shows the respective fold changes in c-*MYC* expression upon ligand treatment. There was an evident overall decrease in the abundance of c-*MYC* transcript and was found to be down regulated with the increasing concentration (10, 50, 100, and 200 μM of each ligand was used to treat the cells). There was almost 50% down regulation of c-*MYC* transcription with 200 μM of ligand **1** while the same amount of down regulation was obtained with only 100 μM of ligand **2** suggesting its greater effectiveness (Fig. 3). Similar reports are known where porphyrin treatment caused down regulation of various genes including c-*MYC*, hTERT.[11] While other reports also show pharmacologic perturbation resulting in downregulation of other protooncogenes like *KRAS*, *VEGF*, and *PDGF*.[12] These reports impinge that stabilization of quadruplexes in the genome can be effectively utilized to modulate gene expression. Additionally we have also performed cell viability assay to determine the cytotoxicity of the ligands on HeLa cells over a period of 7 days and have found that the ligands have only a limited toxicity (Supplementary Fig. 3). We have also conducted apoptosis measuring assays and have conclusively shown that the ligands are effective at inducing apoptosis after 24 h treatment in HeLa cells (Supplementary Fig. 4) as compared to untreated cells, up to 18% and 22% cell populations were observed to be apoptotic after treatment with 200 μM ligand **1** and **2** for 24 h. This apoptosis induction is relevant here due to c-*MYC* down regulation as c-*MYC* is an important molecular partner which determines the cellular choice between cell death or proliferation.[13] Though other factors can not be ruled out, but it is intuitive here that c-*MYC* down regulation could have led to these cellular effects.

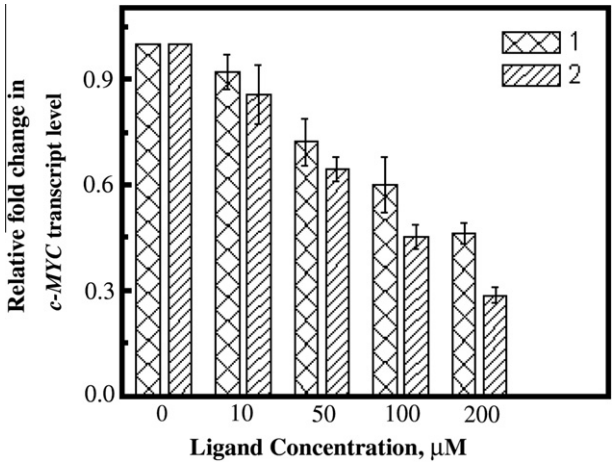


Figure 3. Downregulation of c-*MYC* transcripts upon treatment of ligands. RNA isolated from HeLa cells after 24 h treatment with different concentrations of ligand **1** and **2** was reverse transcribed and Real Time PCR was performed. Bar charts show relative fold change in the c-*MYC* transcripts upon ligand treatment showing downregulation in a concentration dependent manner. Normalization was done with respect to β-2 microglobin gene. The error bars indicate standard deviations from three independent experiments.

In summary, we have demonstrated that the ligands used in the study selectively bind to G-quadruplex and have also exhibited biological efficacy by lowering c-*MYC* transcription. Hence these ligands can act as good scaffolds to develop quadruplex selective drugs in future by combining biophysical and molecular properties to design ligands showing lower toxicity, and significant biological response and also have effective differentiation between different quadruplexes.

Acknowledgments

Financial support for this work from the Council of Scientific and Industrial Research (CSIR), Government of India, and Department of Science and Technology (DST), (Swarnajayanti project to S.M.) Government of India, are gratefully acknowledged.

Supplementary data

Supplementary data (experimental details for the synthesis of **1** and **2**, their spectroscopic characterization and associated biological studies) associated with this article can be found, in the online version, at doi:10.1016/j.bmcl.2010.06.080.

References and notes

1. (a) Hurley, L. H.; Wheelhouse, R. T.; Sun, D.; Kerwin, S. M.; Salazar, M.; Fedoroff, O. Y.; Han, F. X.; Han, H.; Izbicka, E.; Von Hoff, D. D. *Pharmacol. Ther.* **2000**, *85*, 141; (b) Hurley, L. H. *Biochem. Soc. Trans.* **2001**, *29*, 692; (c) Simonsson, T.; Pribylova, M.; Vorlickova, M. *Biochem. Biophys. Res. Commun.* **2000**, *278*, 158; (d) Simonsson, T. *Biol. Chem.* **2001**, *382*, 621; (e) Huppert, J. L.; Balasubramanian, S. *Nucleic Acids Res.* **2007**, *35*, 406.
2. (a) Liu, W.; Sun, D.; Hurley, L. H. *Nucleosides Nucleotides Nucleic Acids* **2005**, *24*, 1801; (b) Siddiqui-Jain, A.; Grand, C. L.; Bearss, D. J.; Hurley, L. H. *Proc. Natl. Acad. Sci. U.S.A.* **2002**, *99*, 11593; (c) Sun, D.; Thompson, B.; Cathers, B. E.; Salazar, M.; Kerwin, S. M.; Trent, J. O.; Jenkins, T. C.; Neidle, S.; Hurley, L. H. *J. Med. Chem.* **1997**, *40*, 2113.
3. (a) Read, M.; Harrison, J. R.; Romagnoli, B.; Tanious, F. A.; Gowan, S. H.; Reszka, A. P.; Wilson, W. D.; Kelland, L. R.; Neidle, S. *Proc. Natl. Acad. Sci. U.S.A.* **2001**, *98*, 4844; (b) Harrison, R. J.; Cuesta, J.; Chessari, G.; Read, M. A.; Basra, S. K.; Reszka, A. P.; Morrell, J.; Gowan, S. M.; Incles, C. M.; Tanious, F. A.; Wilson, W. D.; Kelland, L. R.; Neidle, S. *J. Med. Chem.* **2003**, *46*, 4463; (c) Read, M. A.; Wood, A. A.; Harrison, J. R.; Gowan, S. M.; Kelland, L. R.; Dosanjh, H. S.; Neidle, S. *J. Med. Chem.* **1999**, *42*, 4538.
4. (a) Arora, A.; Maiti, S. *J. Phys. Chem. B* **2008**, *112*, 8151; (b) Erra, E.; Petraccone, L.; Esposito, V.; Randazzo, A.; Mayol, L.; Ladbury, J.; Barone, G.; Giancola, C. *Nucleosides Nucleotides Nucleic Acids.* **2005**, *24*, 753; (c) Freyer, M. W.; Buscaglia, R.; Kaplan, K.; Cashman, D.; Hurley, L. H.; Lewis, E. A. *Biophys. J.* **2007**, *92*, 2007; (d) Han, H.; Langley, D. R.; Rangan, A.; Hurley, L. H. *J. Am. Chem. Soc.* **2001**, *123*, 8902.
5. Rosu, F.; De, P. E.; Guittat, L.; Alberti, P.; Lacroix, L.; Mailliet, P.; Riou, J. F.; Mergny, J. L. *Biochemistry* **2003**, *42*, 10361.
6. (a) Zhou, J. L.; Lu, Y. J.; Ou, T. M.; Zhou, J. M.; Huang, Z. S.; Zhu, X. F.; Du, C. J.; Bu, X. Z.; Ma, L.; Gu, L. Q. *J. Med. Chem.* **2005**, *48*, 7315; (b) Ou, T. M.; Lu, Y. J.; Zhang, C.; Huang, Z. S.; Wang, X. D.; Tan, J. H.; Chen, Y.; Ma, D. L.; Wong, K. Y.; Tang, J. C. *J. Med. Chem.* **2007**, *50*, 1465.
7. (a) Rezler, E. M.; Seenisamy, J.; Bashyam, S.; Kim, M. Y.; White, E.; Wilson, W. D.; Hurley, L. H. *J. Am. Chem. Soc.* **2005**, *127*, 9439; (b) Agrawal, S.; Ojha, R. P.; Maiti, S. *J. Phys. Chem. B.* **2008**, *112*, 6828; (c) Gomez, D.; Paterski, R.; Lemarteleur, T.; Shin-ya, K.; Mergny, J. L.; Riou, J. F. *J. Biol. Chem.* **2004**, *279*, 41487; (d) Kim, M. Y.; Vankayalapati, H.; Shin-ya, K.; Wierzba, K.; Hurley, L. H.

J. Am. Chem. Soc. **2002**, *124*, 2098; (e) Rosu, F.; Gabelica, V.; Shin-ya, K.; De, P. E. *Chem. Commun. (Camb.)* **2003**, 2702.
8. Jantos, K.; Rodriguez, R.; Ladame, S.; Shirude, P. S.; Balasubramanian, S. *J. Am. Chem. Soc.* **2006**, *128*, 13662.
9. Chakraborty, T. K.; Arora, A.; Roy, S.; Kumar, N.; Maiti, S. *J. Med. Chem.* **2007**, *50*, 5539.
10. Ma, Y.; Ou, T. M.; Hou, J. Q.; Lu, Y. J.; Tan, J. H.; Gu, L. Q.; Huang, Z. S. *Bioorg. Med. Chem.* **2008**, *16*, 7582.
11. Sun, D.; Liu, W. J.; Guo, K.; Rusche, J. J.; Ebbinghaus, S.; Gokhale, V.; Hurley, L. H. *Mol. Cancer Ther.* **2008**, *7*, 880.
12. (a) Grand, C. L.; Han, H.; Munoz, R. M.; Weitman, S.; Von Hoff, D. D.; Hurley, L. H.; Bearss, D. J. *Mol. Cancer Ther.* **2002**, *1*, 565; (b) Cogoi, S.; Paramasivam, M.; Spolaore, B.; Xodo, L. E. *Nucleic Acids Res.* **2008**, *36*, 3765; (c) Qin, Y.; Rezler, E. M.; Gokhale, V.; Sun, D.; Hurley, L. H. *Nucleic Acids Res.* **2007**, *35*, 7698.
13. Kelly, K.; Siebenlist, U. *Annu. Rev. Immunol.* **1984**, *4*, 317.