

Evaluation of the Stability of DNA i-Motifs in the Nuclei of Living Mammalian Cells

Simon Dzatko⁺, Michaela Krafcikova⁺, Robert Hänsel-Hertsch, Tomas Fessl, Radovan Fiala, Tomas Loja, Daniel Krafcik, Jean-Louis Mergny, Silvie Foldynova-Trantirkova,^{*} and Lukas Trantirek^{*}

Abstract: C-rich DNA has the capacity to form a tetra-stranded structure known as an i-motif. The i-motifs within genomic DNA have been proposed to contribute to the regulation of DNA transcription. However, direct experimental evidence for the existence of these structures in vivo has been missing. Whether i-motif structures form in complex environment of living cells is not currently known. Herein, using state-of-the-art in-cell NMR spectroscopy, we evaluate the stabilities of i-motif structures in the complex cellular environment. We show that i-motifs formed from naturally occurring C-rich sequences in the human genome are stable and persist in the nuclei of living human cells. Our data show that i-motif stabilities in vivo are generally distinct from those in vitro. Our results are the first to interlink the stability of DNA i-motifs in vitro with their stability in vivo and provide essential information for the design and development of i-motif-based DNA biosensors for intracellular applications.

DNA sequences with stretches of cytidines are abundant in eukaryotic genomes and are overrepresented in biologically

important genomic regions such as centromeres, telomeres, and/or the promoter regions of (onco)genes.^[1] Under specific in vitro conditions, these sequences can form the so-called i-motif, a four-stranded structure consisting of two parallel DNA duplexes zipped together by the intercalation of protonated cytosine–cytosine (C.C⁺) base pairs (Figure 1).^[2]

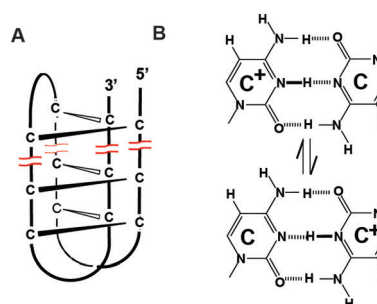


Figure 1. A) Schematic of an intramolecular i-motif structure and B) C.C⁺ base pair according to Lieblein et al.^[2c].

Only recently, the i-motifs in promoter and telomeric regions were proposed to be implicated in the regulation of transcription^[3] and the integrity of telomeric DNA.^[4] However, despite all available indications of i-motif function in vivo, the biological relevance of i-motifs has remained a matter of dispute for the following reasons: 1) The lack of direct experimental evidence for the existence of i-motifs in vivo and 2) the very strong dependence of i-motif stability on chemical and physical environmental factors such as pH that generally appear to be incompatible with the parameters of the intracellular space. While i-motifs are rather stable in acidic pH, low ionic strength, and dehydrating conditions, the propensity for i-motifs is generally lower under simulated physiological conditions comprising relatively high ionic strength (ca. 200 mM), physiological temperatures (35° to 37°C for human cells), and near-neutral pH (ca. 7.0).^[5] Whether i-motif formation in genomic C-rich DNA is only a peculiarity of its conformational space under specific environmental conditions in vitro and whether the i-motif structure can be formed and remain stable in the presence of the numerous environmental factors present in the complex intracellular milieu of living cells is not currently known.

To evaluate the stability of i-motifs formed from naturally occurring C-rich sequences from the human genome in a complex intracellular environment, we employed in-cell NMR spectroscopy^[6] of DNA in living human cells. Four

[*] S. Dzatko,^[a] M. Krafcikova,^[a] Prof. R. Fiala, Dr. T. Loja, D. Krafcik, Dr. S. Foldynova-Trantirkova, Prof. L. Trantirek
CEITEC-Central European Institute of Technology, Masaryk University
Brno 625 00 (Czech Republic)
E-mail: silvie.trantirkova@ceitec.muni.cz
lukas.trantirek@ceitec.muni.cz

Dr. R. Hänsel-Hertsch
Cancer Research (UK) Cambridge Institute, University of Cambridge
Li Ka Shing Centre, Cambridge CB2 0RE (UK)

Dr. T. Fessl
Faculty of Science, University of South Bohemia
370 05 Ceske Budejovice (Czech Republic)

Dr. J.-L. Mergny
Université de Bordeaux, INSERM U1212, CNRS UMR 5320, ARNA
Laboratory, IECB
Pessac (France)

Dr. J.-L. Mergny, Dr. S. Foldynova-Trantirkova
Institute of Biophysics, Academy of Sciences of the Czech Republic
612 65 Brno (Czech Republic)

[†] These authors contributed equally to this work.

Supporting information, including experimental details, and the ORCID identification number(s) for the author(s) of this article can be found under:
<https://doi.org/10.1002/anie.201712284>.

© 2018 The Authors. Published by Wiley-VCH Verlag GmbH & Co. KGaA. This is an open access article under the terms of the Creative Commons Attribution-NonCommercial License, which permits use, distribution and reproduction in any medium, provided the original work is properly cited and is not used for commercial purposes.

Table 1: Sequences of the model DNA oligonucleotides used in this study.

Name	Sequence [5'→3']
DAP	(C ₅ G) ₄ C ₅
HIF-1 α	(CG) ₂ CTC ₃ GC ₅ TCTC ₄ TC ₄ GCGC
PDGF-A	C ₂ GCGC ₄ T(C ₅ G) ₃ C ₁₃
JAZF1	C ₈ G(C ₅ G) ₂ C ₃ TC ₆

oligonucleotide constructs, corresponding to the i-motif forming sequences from DAP, HIF-1 α , PDGF-A, and JAZF1 promoter regions^[7,8] from the human genome (see Table 1) and displaying the capacity to form i-motif structures in vitro under near-physiological conditions including ionic strength and near-neutral pH,^[7,8] were selected for the in-cell NMR study.

CD spectra of individual constructs acquired as a function of pH in high-ionic strength (T-) buffer are displayed in the Supporting Information, Figure S1. Under these conditions and at pH > 7, the CD spectra of all constructs are marked by a positive peak at circa 290 nm, which is a characteristic spectral marker of i-motif formation (Figure S1).^[7-9] Formation of the i-motif structures in the T-buffer of pH 7.0 for all the constructs was further confirmed by the observation of NMR imino signals at 15–16 ppm, which are specific to imino protons involved in C.C⁺ base pairs^[9] (Figures 2 and 3).

Individual constructs in the T-buffer were mixed in a ratio of 40:1 with covalently modified analogs bearing a fluorophore (FAM) at their 5'-terminus (FAM-DAP, FAM-HIF-1 α , FAM-PDGF-A, and FAM-JAZF1) and separately transfected into living (human) HeLa cells (for experimental details see the Supporting Information). The FAM-modified oligonucleotides were to serve as probes to monitor the transfection efficiency and localization of transfected DNA constructs in cells using flow cytometry (FCM) and confocal microscopy, respectively. For all constructs, transfection was highly effective, generally more than 97% of the cells were transfected (Figure 2). Following the transfection, the viability of the cells was assessed by staining with propidium iodide (PI), which specifically stains dead cells or cells with compromised membrane integrity. Generally, over 85% of the transfected cells were negative for PI uptake (Figure 2). This indicated that the transfections did not compromise cell viability. Control experiments based either on in-cell NMR readout or on the use of pH-sensitive fluorescent probes showed that the transfection of DNA did not compromise the homeostasis of the intracellular pH (pH_i) and that the pH_i in the transfected cells is stable over at least 3–4 h (Supporting Information, Figure S2). As evidenced by confocal microscopy images, the model DAP, HIF-1 α , PDGF-A, and JAZF1 oligonucleotides entered the cells and spontaneously and quantitatively localized to the cell nucleus (Figure 2).

After transfection, the cells were evaluated using 1D ¹H in-cell NMR spectroscopy. Notably, for all oligonucleotides, the imino regions of the resulting in-cell NMR spectra, obtained circa 40 min after transfection, featured evident signals in regions characteristic of imino protons involved in C.C⁺ base pairs, which are indicative of the presence of an i-motif structure in vivo (Figure 2). The 1D spectra of extra-

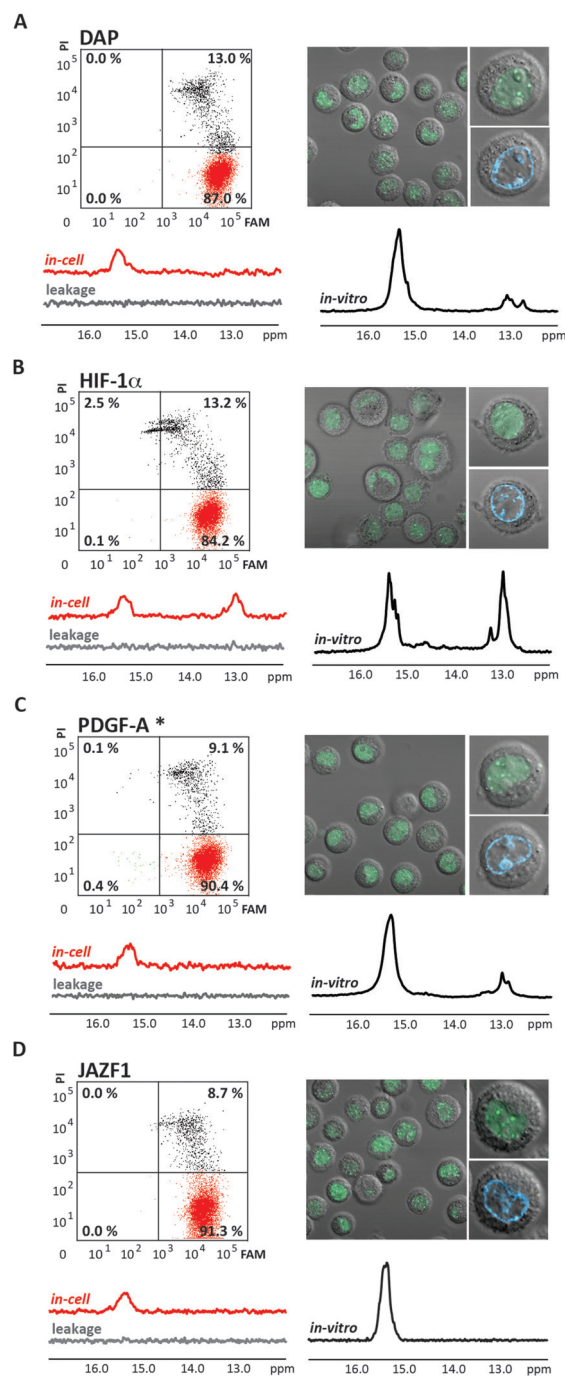


Figure 2. A) Double-staining (PI/FAM) FCM analysis of transfected HeLa cells with the (FAM)-DAP construct (upper left corner). Percentages of viable DNA non-transfected cells, viable DNA-containing cells, non-transfected dead/compromised cells, and transfected dead/compromised cells with DNA are indicated in left-bottom, right-bottom, left-top, and right-top quadrants, respectively. Confocal microscope images of cells transfected with (FAM)-DAP (upper right corner). The green color indicates the localization of (FAM)-DAP. The blue color corresponds to a cell nucleus stained by Hoechst 33342. Imino region of 1D ¹H NMR spectra of DAP in vitro in T-buffer (140 mM sodium phosphate, 5 mM KCl, 10 mM MgCl₂, pH 7.0) (black) and in-cell (red). Imino region of 1D ¹H NMR spectrum of extracellular fluid (Leibovitz L15 medium) taken from the in-cell NMR samples after completion of the spectra acquisition (gray). The (in-cell) NMR spectra were acquired at 20°C. B–D) Analogous data for HIF-1 α , PDGF-A, and JAZF1. For the extended version of Figure 2 including all controls, see Figure S4.

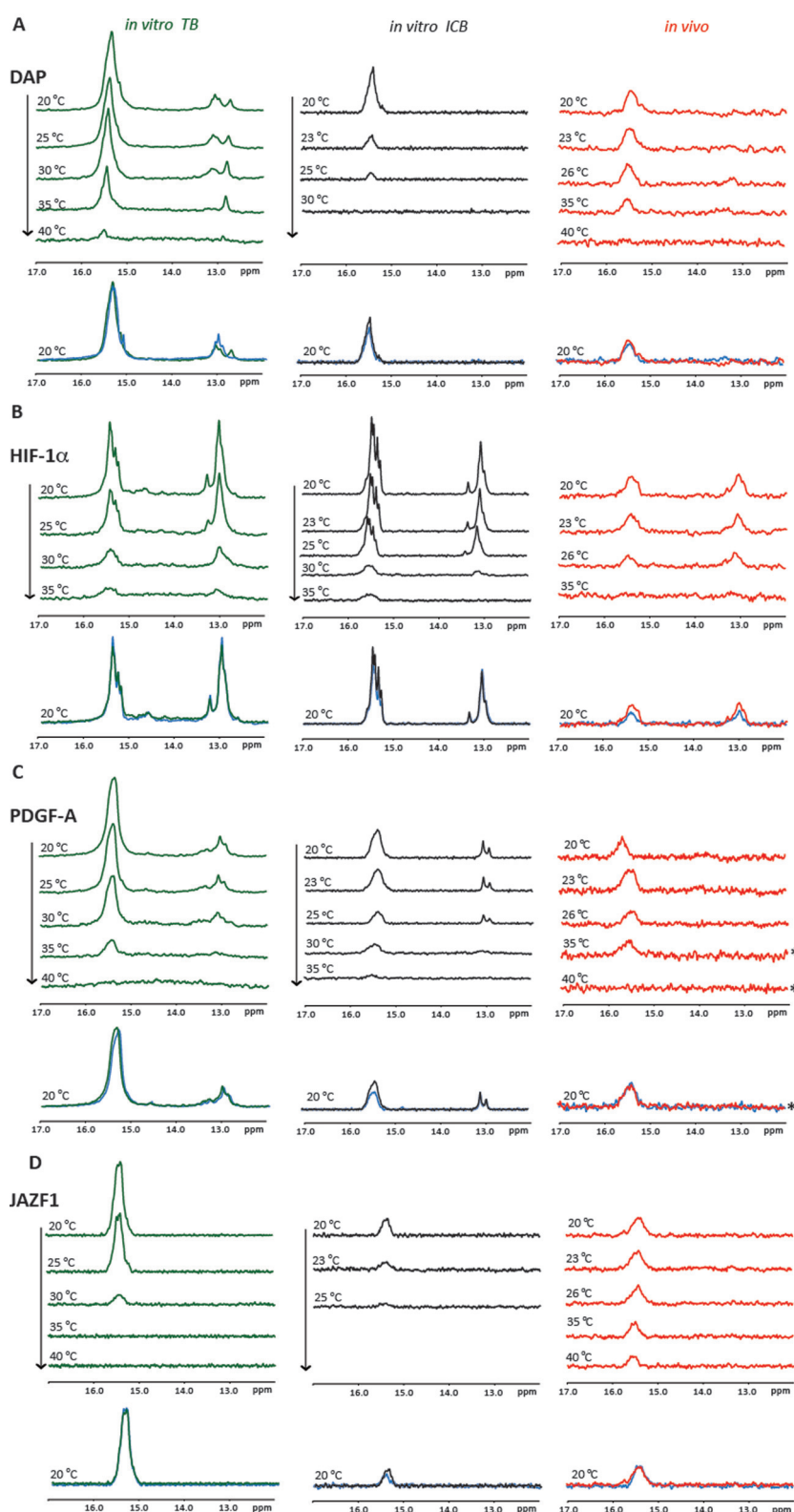


Figure 3. Imino region of 1D ¹H NMR spectra acquired at various temperatures under *in vitro* conditions in T-buffer (TB: 140 mM sodium phosphate, 5 mM KCl, 10 mM MgCl₂, pH 7.0) in green, in IC buffer (ICB: 25 mM potassium phosphate, 85 mM KCl, 10 mM NaCl, 1 mM MgCl₂, 130 nM CaCl₂, pH 7.2) in black, and under *in vivo* conditions (red) for A) DAP, B) HIF-1α, C) PDGF-A, and D) JAZF1. Blue spectra were acquired at 20 °C, 30 min after the exposure of *in vitro*/*in vivo* samples to 40 °C. The PDGF-A data are from two independent experiments. The data from second experiment are marked with asterisks. For controls relevant to temperature jump experiment see Figure S4.

cellular fluids (Leibovitz L15 medium) taken from individual in-cell NMR samples after completion of spectra acquisition showed that the imino signals came from DNA localized in cells and were not artifacts of oligonucleotide leakage from cells during the course of the in-cell NMR experiments (Figure 2, grey spectra “leakage”). A comparison of the line-width of imino signals in buffered solutions and in the in-cell NMR spectra indicated that the in-cell NMR signals originated from unbound i-motif DNA (Supporting Information, Figure S3).

Taken together, the in-cell NMR spectra indicated that the individual i-motifs introduced into the cells were stable and persisted in the complex environment of the nuclei of living HeLa cells. However, it must be stressed that in the present experimental setup (see above), preformed i-motifs were introduced into the cells. Therefore, while the observation of imino signals for C.C⁺ base pairs in in-cell spectra indicates that the i-motif structure is compatible with (and not actively destabilized by) a complex intracellular environment, it provides no information on the ability of model sequences to form i-motifs in the intracellular space *de novo*. It must also be noted that the temperature employed during the acquisition of the in-cell NMR spectra (20 °C) was well below physiological temperatures (35 to 37 °C). To evaluate the stability of i-motif structures at physiological temperature *in vivo* and assess *de novo* i-motif formation in an intracellular space, we acquired a set of in-cell NMR spectra for individual constructs at elevated temperatures (up to 40 °C) and performed temperature-jump experiments by first gradually increasing the temperature from 20 to 40 °C to destabilize the i-motif structure *in vivo* and then lowering the temperature back to 20 °C to initiate its refolding (for details, see below). The work-flow of the temperature-jump in-cell NMR experiment is schematically presented in the Supporting Information, Figure S5. As shown in Figure 3, imino signals at 15–16 ppm, indicative of the presence of i-motif structures, were present in in-cell NMR spectra for DAP, PDGF-A, and JAZF1 even at 35 °C, suggesting that a significant population of the folded species (i-motif) is present even at physiological temperature. In contrast, no i-motif-specific signals were detected for the HIF-1α construct. Notably, the i-motif-specific signals

were present in the in-cell NMR spectrum for JAZF1 at 40 °C (Figure 3). Most importantly, the intensity of the signals characteristic of i-motif formation for DAP, HIF-1 α , PDGF-A, and JAZF1 were restored in the corresponding in-cell NMR spectra upon lowering of the temperature of the in-cell sample back to 20 °C (Figure 3). Although the time required to acquire the in-cell spectra (30 min or more) does not allow quantitative assessment of i-motif folding kinetics in vivo, the observation that the i-motif-specific signals had the same intensity (at 20 °C) observed prior to the temperature unfolding event within 30 min of initiation of the temperature jump (from 40 to 20 °C) indicates that the i-motif unfolding/folding cycle in vivo is reversible. Altogether, our data, for the first time, directly demonstrate that DNA i-motifs formed from natively occurring C-rich sequences in the human genome are stable and persist in the native complex environment of live human cells.

As shown in Figure 3, the intensities of the imino signals in the in-cell NMR spectra for the DAP and JAZF1 constructs are perturbed by increasing temperature to a notably lower extent than under the simplistic conditions of high ionic-strength and near-physiological pH, herein represented by the IC buffer. Under the simplifying assumption that temperature-induced changes in the intensities of the imino NMR signals are primarily determined by the changes in the populations of the C.C⁺ base pairs in the i-motif structure, this observation indicates higher stabilities of the DAP and JAZF1 i-motifs in vivo compared to their stabilities in the IC buffer (for experiments validating this assumption see the Supporting Information, Figures S6 and S7 and Commentary S1). While our data do not allow direct identification of the source of this stabilizing effect, it needs to be noted that the stabilization of C.C⁺ base pairs have been previously observed in i-motif DNA under conditions simulating the effect of the excluded volume, that is, under the conditions expected in the intracellular space.^[10] To assess the behavior of the DAP and JAZF1 i-motif constructs under excluded-volume conditions, we acquired CD melting curves and temperature-resolved NMR spectra for the DAP and JAZF1 i-motif constructs in the IC buffer of pH 7.0 supplemented

with either with 20% glycerol (to emulate volume exclusion by osmotic stress) or 20% Ficoll 70 (to emulate volume exclusion by molecular crowding). The CD and NMR data jointly showed that Ficoll 70, in contrast to the glycerol, stabilized the DAP and JAZF1 i-motif structures (Figure 4 and the Supporting Information, Figure S6). In parallel, a comparison of the temperature-resolved NMR spectra for the DAP and JAZF1 constructs acquired in the absence and in the presence of the low-molecular-weight cellular (metabolic) fraction showed that the cellular metabolites did not increase the stability of the i-motif DNA (Supporting Information, Figure S8). These observations suggest that one of the factors responsible for stabilization of i-motif structures in vivo might be molecular crowding of the high-molecular-weight co-solutes present in the intracellular space.^[10]

To conclude, our data suggest that C-rich sequences, such as those recently identified by the groups of Waller^[8] and Burrows,^[11] displaying the capacity to form i-motifs with only moderate differences in their association and dissociation kinetics under neutral pH and near-physiological ionic strength are likely to form stable i-motif structures in vivo. These observations open an avenue for the identification of genomic C-rich sequences capable of forming stable i-motifs in vivo and provide essential information for the design and development of i-motif-based DNA nanodevices/biosensors for intracellular applications.^[12] In biological terms, our results support the concept that i-motif structures may act as active regulators of genomic DNA and potential drug targets.^[3,4,13]

Acknowledgements

This project was funded by grants from the Czech Science Foundation (16-10504S), European Regional Development Fund (CZ.02.1.01/0.0/0.0/15_003/0000477), European Molecular Biology Organization (IG2535), and Horizon 2020 Programme of the EU (iNEXT: grant agreement 653706). Access to the research infrastructure was supported by grants

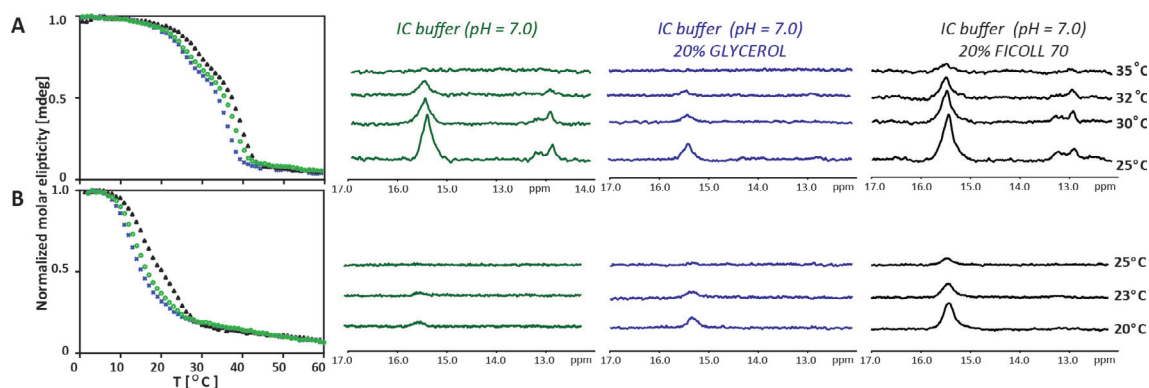


Figure 4. Normalized molar ellipticities (left panel) extracted from the CD spectra for the A) DAP and B) JAZF1 i-motif constructs recorded at signal maximum in the range between 285–290 nm as a function of temperature in IC buffer (25 mM potassium phosphate, 85 mM KCl, 10 mM NaCl, 1 mM MgCl₂, 130 nM CaCl₂, pH 7.0) in the absence (green) and the presence of either 20% glycerol (blue) or 20% Ficoll 70 (black). Imino region of 1D ¹H NMR spectra (three panels on the right) recorded for the A) DAP and B) JAZF1 i-motif constructs as a function of the temperature under identical conditions.

from the MEYS CR (CEITEC 2020 LQ1601; CIISB-LM2015043; Czech-BioImaging LM2015062; EATRIS-CZ LM2015064), and by a grant from the European Regional Development Fund (CZ.02.1.01/0.0/0.0/15_003/0000441).

Conflict of interest

The authors declare no conflict of interest.

Keywords: DNA · i-motifs · in-cell NMR spectroscopy · structural biology

How to cite: *Angew. Chem. Int. Ed.* **2018**, *57*, 2165–2169
Angew. Chem. **2018**, *130*, 2187–2191

- [1] a) T. A. Brooks, S. Kendrick, L. H. Hurley, *FEBS J.* **2010**, *277*, 3459–3469; b) J. Amato, N. Iaccarino, A. Randazzo, E. Novelino, B. Pagano, *ChemMedChem* **2014**, *9*, 2026–2030.
- [2] a) K. Gehring, J.-L. Leroy, M. Guéron, *Nature* **1993**, *363*, 561–565; b) J.-L. Leroy, M. Guéron, J.-L. Mergny, C. Hélène, *Nucleic Acids Res.* **1994**, *22*, 1600–1606; c) A. L. Lieblein, M. Krämer, A. Dreuw, B. Fürtig, H. Schwalbe, *Angew. Chem. Int. Ed.* **2012**, *51*, 4067–4070; *Angew. Chem.* **2012**, *124*, 4143–4146.
- [3] a) H. J. Kang, S. Kendrick, S. M. Hecht, L. H. Hurley, *J. Am. Chem. Soc.* **2014**, *136*, 4172–4185; b) S. Kendrick, H. J. Kang, M. P. Alam, M. M. Madathil, P. Agrawal, V. Gokhale, D. Yang, S. M. Hecht, L. H. Hurley, *J. Am. Chem. Soc.* **2014**, *136*, 4161–4171; c) R. V. Brown, T. Wang, V. R. Chappeta, G. Wu, B. Onel, R. Chawla, H. Quijada, S. M. Camp, E. T. Chiang, Q. R. Lassiter, C. Lee, S. Phanse, M. A. Turnidge, P. Zhao, J. G. N. Garcia, V. Gokhale, D. Yang, L. H. Hurley, *J. Am. Chem. Soc.* **2017**, *139*, 7456–7475; d) B. Roy, P. Talukder, H. J. Kang, S. S. Tsuen, M. P. Alam, L. H. Hurley, S. M. Hecht, *J. Am. Chem. Soc.* **2016**, *138*, 10950–10962; e) C. Sutherland, Y. Cui, H. Mao, L. H. Hurley, *J. Am. Chem. Soc.* **2016**, *138*, 14138–14151; f) C. E. Kaiser, N. A. Van Ert, P. Agrawal, R. Chawla, D. Yang, L. H. Hurley, *J. Am. Chem. Soc.* **2017**, *139*, 8522–8536; g) S. Kendrick, A. Muranyi, V. Gokhale, L. H. Hurley, L. M. Rimsza, *J. Med. Chem.* **2017**, *60*, 6587–6597; h) S. Takahashi, J. A. Brazier, N. Sugimoto, *Proc. Natl. Acad. Sci. USA* **2017**, *114*, 9605–9610.
- [4] a) Y. Chen, K. Qu, C. Zhao, L. Wu, J. Ren, J. Wang, X. Qu, *Nat. Commun.* **2012**, *3*, 1074; b) P. Školáková, S. Foldynová-Trantírková, K. Bednářová, R. Fiala, M. Vorlíčková, L. Trantírek, *Nucleic Acids Res.* **2015**, *43*, 4733–4745.
- [5] a) J.-L. Mergny, J. Lacroix, X. Han, J.-L. Leroy, C. Hélène, *J. Am. Chem. Soc.* **1995**, *117*, 8887–8898; b) J.-L. Mergny, L. Lacroix, *Nucleic Acids Res.* **1998**, *26*, 4797–4803.
- [6] R. Hänsel, S. Foldynová-Trantírková, F. Löhr, J. Buck, E. Bongartz, E. Bamberg, H. Schwalbe, V. Dötsch, L. Trantírek, *J. Am. Chem. Soc.* **2009**, *131*, 15761–15768.
- [7] J. A. Brazier, A. Shah, G. D. Brown, *Chem. Commun.* **2012**, *48*, 10739–10741.
- [8] E. P. Wright, J.-L. Huppert, Z. A. E. Waller, *Nucleic Acids Res.* **2017**, *45*, 2951–2959.
- [9] A.-T. Phan, J.-L. Mergny, *Nucleic Acids Res.* **2002**, *30*, 4618–4625.
- [10] a) A. Rajendran, S. Nakano, N. Sugimoto, *Chem. Commun.* **2010**, *46*, 1299–1301; b) S. Pramanik, S. Nagatoishi, N. Sugimoto, *Chem. Commun.* **2012**, *48*, 4815–4817; c) D. Miyoshi, K. Nakamura, H. Tateishi-Karimata, T. Ohmichi, N. Sugimoto, *J. Am. Chem. Soc.* **2009**, *131*, 3522–3531.
- [11] A. M. Fleming, Y. Ding, R. A. Rogers, J. Zhu, J. Zhu, A. D. Burton, C. B. Carlisle, C. J. Burrows, *J. Am. Chem. Soc.* **2017**, *139*, 4682–4689.
- [12] a) L. A. Yatsunyk, O. Mendoza, J.-L. Mergny, *Acc. Chem. Res.* **2014**, *47*, 1836–1844; b) F. Wang, X. Liu, I. Willner, *Angew. Chem. Int. Ed.* **2015**, *54*, 1098–1129; *Angew. Chem.* **2015**, *127*, 1112–1144; c) J. J. Alba, A. Sadurní, R. Gargallo, *Crit. Rev. Anal. Chem.* **2016**, *46*, 443–454.
- [13] H. A. Day, P. Pavlou, Z. A. E. Waller, *Bioorg. Med. Chem.* **2014**, *22*, 4407–4418.

Manuscript received: November 30, 2017

Accepted manuscript online: December 20, 2017

Version of record online: January 29, 2018