Biochemistry

Article

Cite This: Biochemistry 2017, 56, 4879-4883

pubs.acs.org/biochemistry

Substitution of Cytosine with Guanylurea Decreases the Stability of i-Motif DNA

Elisé P. Wright,[†] Katarzyna Lamparska,[‡] Steven S. Smith,[‡] and Zoë A. E. Waller*[,†,§]

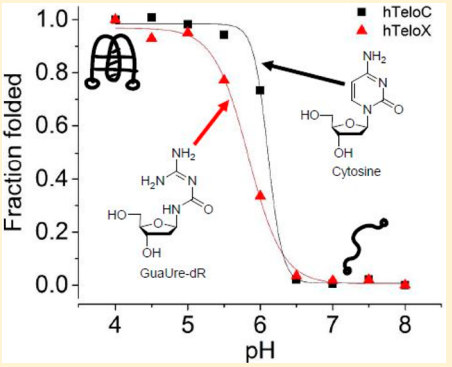
[†]School of Pharmacy, University of East Anglia, Norwich Research Park, Norwich NR4 7TJ, U.K.

[‡]Beckman Research Institute and Division of Urology, City of Hope, 1500 East Duarte Road, Duarte, California 91010-3000, United States

[§]Centre for Molecular and Structural Biochemistry, University of East Anglia, Norwich Research Park, Norwich NR4 7TJ, U.K.

Supporting Information

ABSTRACT: Both 5-aza-2′-deoxycytidine (decitabine) and its primary breakdown product, 2′-deoxyriboguanylurea (GuaUre-dR), have been shown to act as mutagens and epimutagens that cause replication stress and alter both DNA methylation and gene expression patterns. As cytosine analogues, both are expected to be preferentially incorporated into regions of GC skew where runs of cytosine residues are sequestered on one strand and guanine residues on the other. Given that such regions have been identified as sites with the potential for effects on gene expression and replication stress linked to formation of alternative DNA secondary structures, it is of interest to determine the influence that these base analogues might have on the stability of structures of this kind. Here we report that incorporation of GuaUre-dR into an i-motif-forming sequence decreases both the thermal and pH stability of an i-motif despite the apparent ability of GuaUre-dR to base pair with cytosine.



The nucleoside drug decitabine, 5-aza-2′-deoxycytidine, has been identified as an epimutagen and a chemotherapeutic.[1,2] It has been found to induce fragile sites in DNA, inhibit DNA methylation, and interact with human DNA methyltransferase I (DNMT1) possibly through the formation of a complex at DNA sites containing 5-aza-2′-deoxycytidine (5azaC-dR) and 2′-deoxyriboguanylurea (GuaUre-dR).[3] As 5azaC-dR is unstable in aqueous environments, there has been interest in the activity of its breakdown products; 5azaC-dR readily hydrolyzes to GuaUre-dR[2,4] (Figure 1). It has been shown that GuaUre-dR induces the same effects that are seen with the parent drug,[3] suggesting it may be responsible for much of the activity observed with 5azaC-dR. GuaUre-dR contributes to replication stress by sequestering methyltransferases, inducing hypomethylation and encouraging DNA instability, much like its precursor.[3,5]

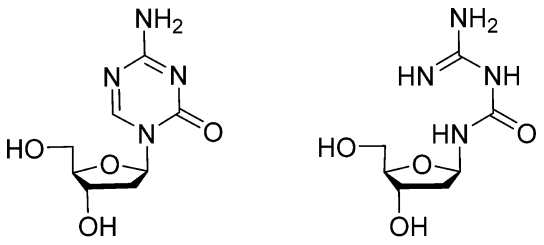


Figure 1. Structure of the β-form of 5azaC-dR and its hydrolysis product, the β-form of GuaUre-dR.

5azaC-dR has been shown to induce the proteolytic degradation of DNMT1 but not of DNMT3a or DNMT3b. While the formation of a covalent bond between DNMT1 and 5azaC-dR-treated DNA could be ruled out as a cause of proteolysis, the mechanism by which DNMT1 is targeted for degradation is not yet well understood.[6] However, it is known that noncanonical DNA structures rich in cytosine are targeted by DNMT1.[7] This interaction results in methylated CG dinucleotides at sites of abnormal base pairing that bind to and trap the DNMT1 enzyme in tight DNA complexes.[8−11] An interaction with a noncanonical DNA structure, such as a hairpin or an i-motif (C-quadruplex), could trap the enzyme and provide an additional trigger for the proteolysis of DNMT1. Cytosine-rich i-motif and hairpin structures are dependent on hemiprotonated cytosine:cytosine base pairing,[12] and a single sequence can give rise to either morphology or both in equilibrium depending on the environment.[13−15] It has been proposed that GuaUre-dR can base pair with cytosine in the absence of protonation;[16] this suggests that the presence of GuaUre-dR in a cytosine-rich sequence may influence the stability and type of secondary structure that may form. The human telomeric i-motif sequence is a well-characterized model oligonucleotide that has been extensively used as a typical i-

Received: July 4, 2017
Revised: August 8, 2017
Published: August 30, 2017

DOI: 10.1021/acs.biochem.7b00628
Biochemistry 2017, 56, 4879−4883

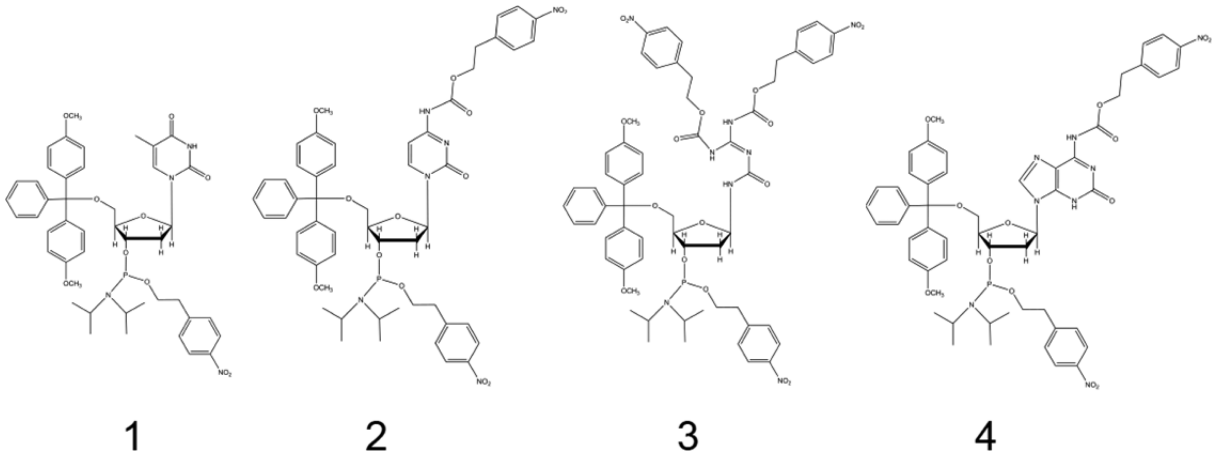


Figure 2. Structures of phosphoramidites prepared for use in the synthesis of the GuaUre-dR-containing oligodeoxynucleotide: 1, 5′-*O*-dimethoxytrityl-3′-*O*-[2-(4-nitrophenyl)ethoxy-*N*,*N*-diisopropyl]-2′-deoxythymidine phosphoramidite; 2, 5′-*O*-dimethoxytrityl-3′-*O*-[2-(4-nitrophenyl)ethoxy-*N*,*N*-diisopropyl]-2′-deoxy-N4-[2-(4-nitrophenyl)ethoxycarbonyl]cytidine phosphoramidite; 3, 5′-*O*-dimethoxytrityl-3′-*O*-[2-(4-nitrophenyl)ethoxy-*N*,*N*-diisopropyl]-2′-deoxyribofuranosyl-3-guanyl-*N*,*N*-bis[2-(4-nitrophenyl)ethoxycarbonyl]urea phosphoramidite; 4, 5′-*O*-dimethoxytrityl-3′-*O*-[2-(4-nitrophenyl)ethoxy-*N*,*N*-diisopropyl]-2′-deoxy-N6-[2-(4-nitrophenyl)ethoxycarbonyl]adenosine phosphoramidite. Each phosphoramidite was characterized prior to use by chromatography, ^{31}P nuclear magnetic resonance, and mass spectrometry as described previously.[3]

motif structure.[17] This sequence was modified with GuaUre-dR to determine its effect on the stability of the resultant secondary structure. Ultraviolet (UV) thermal melting, thermal difference spectra, and circular dichroism spectroscopy were used to characterize the pH and thermal stability of the human telomeric i-motif sequence with and without GuaUre-dR modification.

MATERIALS AND METHODS

Oligonucleotide Synthesis. The methods used in the synthesis of oligodeoxynucleotides and associated phosphoramidites have been described in detail.[3] All syntheses were performed on a 1 μmol scale using a Cyclone Plus DNA Synthesizer (MilliGen Bioresearch, Novato, CA). In brief, the syntheses were performed as follows.

Human Telomeric Sequence (hTeloC). Synthesis of the control sequence was performed using phosphoramidites purchased from Glen Research (Sterling, VA). Deprotection was performed with 32% ammonium hydroxide overnight at 55 °C, followed by purification using high-performance liquid chromatography (HPLC) as described previously.[3] The resulting pure fractions were characterized by polyacrylamide gel electrophoresis and mass spectrometry. The control 24-mer [5′-TAA-CCC-TAA-CCC-TAA-CCC-TAA-CCC-3′ (hTeloC)] had a predicted monoisotopic mass (MS) of 7127.246. The observed MS was 7127.274, and the predicted MW was 7130.723 g mol^{-1}. The yield of the pure oligodeoxynucleotide was 2.12 mg (297.3 nmol).

Modified Human Telomeric Sequence (hTeloX, X = GuaUre). As previously noted,[3] GuaUre-dR-containing oligodeoxynucleotides are not stable when deprotection is performed with standard ammonium hydroxide. Consequently, we adopted an alternative approach using *p*-nitrophenylethoxycarbonyl protecting groups for this purpose.[3,18] The required phosphoramidites were synthesized and characterized as previously described.[3] Each phosphoramidite carried a dimethoxytrityl group at the 5′ coupling site and a bis(*N*,*N*-didisopropylamino)-2-(4-nitrophenyl) ethoxyphosphane group at the 3′ coupling site. Primary amines were protected with *p*-nitrophenylethoxycarbonyl groups (Figure 2).

Synthesis of the homologous GuaUre-dR-containing sequence with GuaUre-dR = X at position 6 was performed using phosphoramidites depicted in Figure 2. This position was selected as previous work[19] has shown that epigenetic modification of the cytosines adjacent to a loop changed the stability of the structure. Deprotection was performed with a solution of 0.5 M 1,8-diazabicyclo[5.4.0]undec-7-ene in anhydrous pyridine containing 5 mg/mL thymine for 16 h at room temperature[20] as previously described.[3] Deprotection was followed by purification using HPLC as previously described.[3] The resulting pure fractions were characterized by polyacrylamide gel electrophoresis and mass spectrometry. The GuaUre-dR-containing 24-mer [5′-TAA-CCX-TAA-CCC-TAA-CCC-TAA-CCC-3′ (hTeloX)] had a predicted monoisotopic mass (MS) of 7118.257. The observed MS was 7118.268, and the predicted MW was 7121.713 g mol^{-1}. The yield of the pure oligodeoxynucleotide was 513.5 μg (72.1 nmol).

The unmodified (hTeloC) and modified (hTeloX) oligonucleotides (ODNs) were dissolved in Milli-Q water to give final concentrations of 100 μM and confirmed using a Nanodrop Spectrophotometer (LabTech, Heathfield, UK). The extinction coefficient for the unmodified control sequence (hTeloC) was used to calculate the concentration for both sequences. For all biophysical characterization experiments, ODNs were diluted in buffer containing 10 mM sodium cacodylate and 100 mM sodium chloride at the pH as detailed, thermally annealed by heating in a heat block at 95 °C for 5 min, and slowly cooled to room temperature overnight. To allow comparison, all experiments were performed in the same buffer type, though some pHs examined were outside the buffering capacity of sodium cacodylate (pH 4.0, 4.5, and 5.0), and control experiments that examined the pH under the conditions of the experiment showed little difference in the overall change in pH (see Figure S1).

Circular Dichroism (CD). CD spectra were recorded on a Jasco J-810 spectropolarimeter using a 1 mm path length quartz cuvette as described previously.[21] ODNs were diluted to 10 μM

DOI: 10.1021/acs.biochem.7b00628
Biochemistry 2017, 56, 4879−4883

(total volume of 200 μL) in buffer at pH increments of 0.5 pH unit from pH 4.0 to 8.0. Additional details are included in the Supporting Information. The transitional pH (pH_T) for the i-motif was calculated from the inflection point of the fitted ellipticity at 288 nm. Final analysis and manipulation of the data were performed using Origin 2015 (OriginLab, Northhampton, MA).

UV Absorption Spectroscopy. UV spectroscopy experiments were performed as described previously.[21] Briefly, a Cary 60 UV−visible spectrometer (Agilent Technologies) equipped with a TC1 Temperature Controller (Quantum Northwest) was used to measure the absorbance of a 2.5 μM ODN sample at a specific pH in a small-volume masked quartz cuvette (1 cm path length). The absorbance of the ODN was measured at 295 nm as the temperature of the sample was held for 10 min at 4 °C and then increased to 95 °C at a rate of 0.5 °C/min and held at 95 °C for 10 min before the process was reversed; each melting/annealing process was repeated three times. Data were recorded every 1 °C during both melting and annealing, and each point was the average of three scans. Melting (T_m) and annealing (T_a) temperatures were determined using the first-derivative method. Additional details are available in the Supporting Information. Thermal difference spectra were calculated by subtracting the spectrum between 220 and 320 nm of the folded structure at 4 °C from that of the unfolded structure at 95 °C. The data were normalized, and the maximum change in absorption was set to +1 as previously described.[22]

RESULTS AND DISCUSSION

The pH and thermal stability of two ODN sequences based on the human telomeric i-motif were examined to determine the effect of GuaUre-dR modification on i-motif formation. The control sequence (hTeloC) remained unmodified, while the modified sequence (hTeloX) included GuaUre-dR instead of cytosine at the last position of the first cytosine tract. We used biophysical techniques to characterize the control and modified sequences; CD was used to determine the pH_T and UV spectroscopy to identify the T_m and T_a values under different environmental conditions.

The pH stabilities of hTeloC and hTeloX sequences were assessed using CD spectroscopy. The spectra were measured at pH values ranging from 4 to 8. At acidic pH (pH 4.0−5.5), the modified and unmodified sequences showed spectra with classic i-motif characteristics: a positive peak at ∼288 nm and a negative peak at ∼255 nm (Figure 3).[23] However, the behavior of the sequences differed at pH 6.0; the hTeloX sequence spectra shifted to more closely resemble that of a random coil with a positive peak at 273 nm and a negative peak at 250 nm, while the hTeloC spectra remained more similar to those of the i-motif (Figure 3). By pH 6.5, both sequences showed spectra characteristic of random coil,[23] and the calculated pH_T values for hTeloX and hTeloC were 5.8 and 6.1, respectively (Figure 3). This indicated that the substitution of GuaUre-dR for a cytosine reduced the pH stability of the human telomeric i-motif-forming sequence.

UV spectroscopy was used to further characterize hTeloC and hTeloX. The thermal stability of each ODN was assessed from pH 4.0 to 7.0. Beyond this pH, CD had shown that the sequences would be unfolded; indeed, the pH 7.0 melting and annealing curves could not be normalized for either sequence as they showed no differences in absorbance between high and low temperatures (Figures S2B and S3B and Table S1). At pH

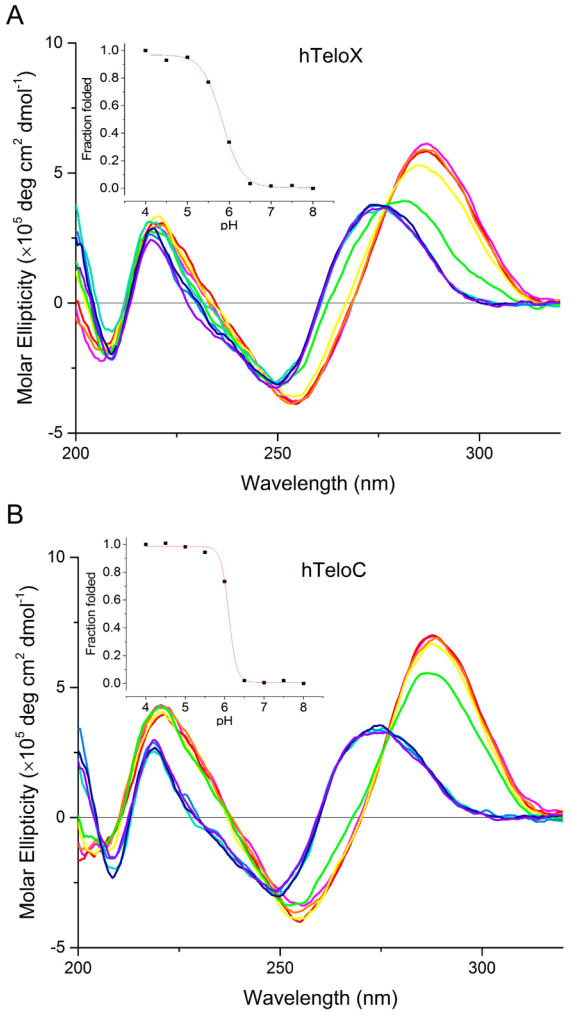


Figure 3. Circular dichroism of (A) the GuaUre-dR-modified hTeloC sequence [the inset shows the fraction folded at 288 nm, used to determine the pH_T of hTeloX of 5.83 (adjusted R^2 = 0.997)] and (B) the unmodified hTeloC sequence [the inset shows the fraction folded at 288 nm, used to determine the pH_T of hTeloC of 6.11 (adjusted R^2 = 0.998)]: (magenta) pH 4.0, (red) pH 4.5, (orange) pH 5.0, (yellow) pH 5.5, (light green) pH 6.0, (dark green) pH 6.5, (blue) pH 7.0, (black) pH 7.5, and (purple) pH 8.0. All oligonucleotide concentrations were 10 μM in 10 mM sodium cacodylate buffer with 100 mM sodium chloride buffer at the required pH.

4.0−6.0, both ODNs showed two-state (folded and unfolded) melting and annealing curves (Figures 4, S2, and S3). This was also true of the modified hTeloX at pH 6.5. However, the unmodified sequence hTeloC showed a three-state UV melting curve at pH 6.5, resulting in two melting transitions (Figure 4A). This indicated the possibility of two distinct secondary structures at pH 6.5 and was interesting because the CD results indicated that at this pH the secondary structure would be unfolded. A separate study that evaluated the pH stability of the hTeloC sequence also showed an unfolded CD spectra at pH 6.4 and 6.6,[24] which was consistent with the pH stability reported here. However, only a single value was reported for the T_m, suggesting only a single transition in the UV melt at a similar pH.[24] We have observed double transitions in i-motif sequences previously,[21] and others have attributed these to an equilibrium mixture composed of the i-motif and hairpin.[14,15] Here, this isolated phenomenon opens an opportunity for further investigation but does not impact the primary objective

DOI: 10.1021/acs.biochem.7b00628
Biochemistry 2017, 56, 4879−4883

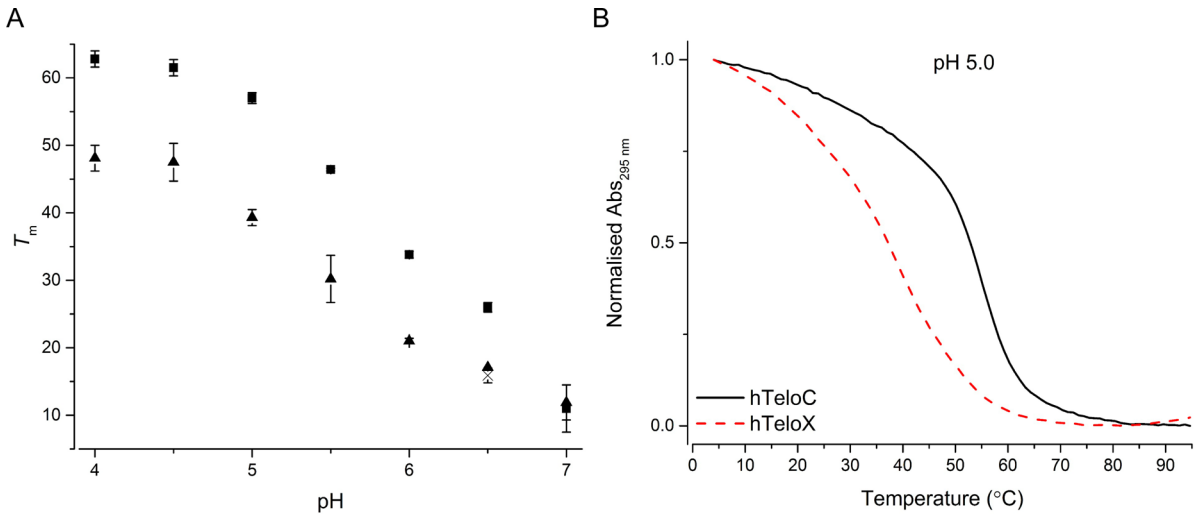


Figure 4. (A) Relationship between the pH and the T_m of hTeloC and hTeloX. T_m values were determined using the first-derivative method: (■) hTeloC T_m and (▲) hTeloX T_m. At pH 6.5, the UV melting curve of hTeloC showed two transitions indicating two melting curves. The times sign indicates the secondary melting temperature, T_{m2}, of hTeloC at this pH. (B) Normalized UV melting curves of GuaUre-dR-modified hTeloX and unmodified hTeloC sequence at pH 5.0. All oligonucleotide concentrations were 2.5 μM in 10 mM sodium cacodylate buffer with 100 mM sodium chloride buffer (pH 5.0).

of this work. Increasing the pH resulted in a decreased thermal stability for both hTeloC and hTeloX (Figure 4A and Table S1). However, hTeloX was less thermally stable than hTeloC; at pH 4.0, the T_m of hTeloC is 15 °C higher than the T_m for hTeloX (Figure 4A). This difference between the T_m of hTeloC and hTeloX is largest when the absorbance is measured under pH 5.0 conditions [18 °C (Figure 4B)].

The measurement of thermal difference spectra (TDS) was also used to supplement the characterization of the hTeloC and hTeloX secondary structures. DNA secondary structures absorb UV light differently when they are folded and unfolded, and taking the difference between these spectra gives an indicative spectrum that can be used for characterization. TDS were determined to indicate the dominant folded species of each ODN at each pH. The resultant difference spectra are characteristic of the folded DNA structure present at the lower temperature.[22] At pH 4.0−5.0, the TDS of hTeloX showed a positive peak at 240 nm and a negative peak at 295 nm (Figure S4). This was consistent with the TDS of the previously characterized folded human telomeric i-motif.[22] This indicated that the GuaUre-dR-modified sequence (hTeloX) was folded into the i-motif structure between pH 4.0 and 5.5. At higher pHs (6.0−7.0), the TDS were more indicative of random coil, consistent with the observations using CD spectroscopy.

Similarly, the TDS of the unmodified hTeloC indicated a folded i-motif. As seen in the UV melting experiment, evidence of a folded i-motif structure (the characteristic positive 240 nm peak and negative 295 nm peak) was indicated at all the measured pH values between 4.0 and 6.5. Only at pH 7.0 does the TDS for hTeloC indicate a random coil structure (Figure S4).

The biophysical evidence presented here indicates that the dominant secondary structure assumed by both the unmodified (hTeloC) and the GuaUre-dR-modified (hTeloX) sequence is an i-motif. The addition of GuaUre-dR instead of a cytosine in the first tract decreases the thermal and pH stability of the structure, despite its suggested ability to base pair with cytosine.[16] This degree (10 °C) of thermal destabilization (change in T_m) is consistent with what others have observed upon mutation of a cytosine, which is known to form C-C base pairs, to a thymine.[25] This suggests that GuaUre may be interacting with cytosine in a way that does not contribute to i-motif stability. It may well be that GuaUre can base pair with a cytosine, just not in the context of an i-motif, but further work would be required to determine whether this is the case.

While GuaUre-dR has been implicated in the overall activity of 5azaC-dR through multiple possible mechanisms, an interaction based on formation of the i-motif is likely more complicated than first thought. DNA secondary structures have been associated with the regulation of genes, and the i-motif in particular has been described as a regulatory switch.[14,15] Moreover, the cytosine-rich sequences in i-motif-forming regions have been shown to be preferred sites for R-loop formation.[26] These structures arise when the RNA transcript base pairs with the template strand during transcription, dislodging its DNA complement to form a RNA−DNA hybrid;[27] the affected regions on the template have no complement on the RNA strand and so form single-stranded bulges. R-Loop formation has been associated with DNA damage and may contribute to genomic instability.[28] Although the presence of GuaUre-dR in DNA does not inhibit DNA synthesis *in vitro*,[3] its effects on RNA synthesis and RNA:DNA hybrid stability are unknown. Even so, it is clear that R-loop formation is confined to regions of GC skew where 5azaC-dR and GuaUre-dR are most likely to be found during drug treatment. Our findings clearly show that GuaUre-dR destabilizes i-motif structures; however, biological consequences of altering the stability of the many structures that can form in cytosine-rich sequences may have additional, and perhaps unforeseen, ramifications in a system as complex as the interaction between 5azaC-dR and biological molecules.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.biochem.7b00628.

DOI: 10.1021/acs.biochem.7b00628
Biochemistry 2017, 56, 4879−4883

Full methodological details, data, and control experiments (PDF)

AUTHOR INFORMATION

Corresponding Author

*School of Pharmacy, University of East Anglia, Norwich Research Park, Norwich NR4 7TJ, U.K. E-mail: z.waller@uea.ac.uk. Telephone: +44 (0) 1603 59 1972. Fax: +44 (0) 1603 59 2003.

ORCID

Zoë A. E. Waller: 0000-0001-8538-0484

Funding

E.P.W. is supported by the Biotechnology and Biological Sciences Research Council (BB/L02229X/1).

Notes

The authors declare no competing financial interest.

ACKNOWLEDGMENTS

We thank Dr. Myles Cheesman of the Henry Wellcome Laboratories for Biological Chemistry, University of East Anglia, for the use of the CD spectrometer.

ABBREVIATIONS

5azaC-dR, 5-aza-2′-deoxycytidine; GuaUre-dR, 2′-deoxyriboguanylurea; hTeloC, human telomeric sequence (5′-TAA-CCC-TAA-CCC-TAA-CCC-TAA-CCC-3′); hTeloX, GuaUre-dR (X)-modified human telomeric sequence (5′-TAA-CCX-TAA-CCC-TAA-CCC-TAA-CCC-3′); ODNs, oligonucleotides; pH_T, transitional pH; T_a, annealing temperature; TDS, thermal difference spectra; T_m, melting temperature.

REFERENCES

(1) Christman, J. K. (2002) 5-Azacytidine and 5-aza-2′-deoxycytidine as inhibitors of DNA methylation: mechanistic studies and their implications for cancer therapy. *Oncogene 21*, 5483−5495.

(2) Beisler, J. A., Abbasi, M. M., Kelley, J. A., and Driscoll, J. S. (1977) Synthesis and antitumor activity of dihydro-5-azacytidine, a hydrolytically stable analogue of 5-azacytidine. *J. Med. Chem. 20*, 806−812.

(3) Lamparska, K., Clark, J., Babilonia, G., Bedell, V., Yip, W., and Smith, S. S. (2012) 2′-Deoxyriboguanylurea, the primary breakdown product of 5-aza-2′-deoxyribocytidine, is a mutagen, an epimutagen, an inhibitor of DNA methyltransferases and an inducer of 5-azacytidine-type fragile sites. *Nucleic Acids Res. 40*, 9788−9801.

(4) Rogstad, D. K., Herring, J. L., Theruvathu, J. A., Burdzy, A., Perry, C. C., Neidigh, J. W., and Sowers, L. C. (2009) Chemical decomposition of 5-aza-2′-deoxycytidine (Decitabine): kinetic analyses and identification of products by NMR, HPLC, and mass spectrometry. *Chem. Res. Toxicol. 22*, 1194−1204.

(5) Lamparska, K., and Smith, S. S. (2015) The genetic and epigenetic effects of 5-azacytidine and its major breakdown product guanylurea. *Epigenetic Diagnosis & Therapy 1*, 28−36.

(6) Ghoshal, K., Datta, J., Majumder, S., Bai, S., Kutay, H., Motiwala, T., and Jacob, S. T. (2005) 5-Aza-deoxycytidine induces selective degradation of DNA methyltransferase 1 by a proteasomal pathway that requires the KEN box, bromo-adjacent homology domain, and nuclear localization signal. *Mol. Cell. Biol. 25*, 4727−4741.

(7) Smith, S. S. (2013) Maintaining the unmethylated state. *Clin. Epigenet. 5*, 17−17.

(8) Smith, S. S., Kan, J. L., Baker, D. J., Kaplan, B. E., and Dembek, P. (1991) Recognition of unusual DNA structures by human DNA (cytosine-5)methyltransferase. *J. Mol. Biol. 217*, 39−51.

(9) Kho, M. R., Baker, D. J., Laayoun, A., and Smith, S. S. (1998) Stalling of human DNA (cytosine-5) methyltransferase at single-strand conformers from a site of dynamic mutation. *J. Mol. Biol. 275*, 67−79.

(10) Laayoun, A., and Smith, S. S. (1995) Methylation of slipped duplexes, snapbacks and cruciforms by human DNA(cytosine-5)methyltransferase. *Nucleic Acids Res. 23*, 1584−1589.

(11) Chen, X., Mariappan, S. V., Catasti, P., Ratliff, R., Moyzis, R. K., Laayoun, A., Smith, S. S., Bradbury, E. M., and Gupta, G. (1995) Hairpins are formed by the single DNA strands of the fragile X triplet repeats: structure and biological implications. *Proc. Natl. Acad. Sci. U. S. A. 92*, 5199−5203.

(12) Gehring, K., Leroy, J.-L., and Gueron, M. (1993) A tetrameric DNA structure with protonated cytosine-cytosine base pairs. *Nature 363*, 561−565.

(13) Day, H. A., Wright, E. P., MacDonald, C. J., Gates, A. J., and Waller, Z. A. E. (2015) Reversible DNA i-motif to hairpin switching induced by copper(II) cations. *Chem. Commun. 51*, 14099−14102.

(14) Kendrick, S., Kang, H. J., Alam, M. P., Madathil, M. M., Agrawal, P., Gokhale, V., Yang, D., Hecht, S. M., and Hurley, L. H. (2014) The dynamic character of the BCL2 promoter i-motif provides a mechanism for modulation of gene expression by compounds that bind selectively to the alternative DNA hairpin structure. *J. Am. Chem. Soc. 136*, 4161−4171.

(15) Kang, H. J., Kendrick, S., Hecht, S. M., and Hurley, L. H. (2014) The transcriptional complex between the BCL2 i-motif and hnRNP LL is a molecular switch for control of gene expression that can be modulated by small molecules. *J. Am. Chem. Soc. 136*, 4172−4185.

(16) Jackson-Grusby, L., Laird, P. W., Magge, S. N., Moeller, B. J., and Jaenisch, R. (1997) Mutagenicity of 5-aza-2′-deoxycytidine is mediated by the mammalian DNA methyltransferase. *Proc. Natl. Acad. Sci. U. S. A. 94*, 4681−4685.

(17) Phan, A. T., and Mergny, J.-L. (2002) Human telomeric DNA: G-quadruplex, i-motif and Watson-Crick double helix. *Nucleic Acids Res. 30*, 4618−4625.

(18) Pfleiderer, W. (2007) Universal 2-(4-nitrophenyl)ethyl and 2-(4-nitrophenyl)ethoxycarbonyl protecting groups for nucleosides and nucleotides. *Current Protocols in Nucleic Acid Chemistry*, Chapter 2, Unit 2, 13, Wiley, New York.

(19) Xu, B., Devi, G., and Shao, F. (2015) Regulation of telomeric i-motif stability by 5-methylcytosine and 5-hydroxymethylcytosine modification. *Org. Biomol. Chem. 13*, 5646−5651.

(20) Guimil Garcia, R., Brank, A. S., Christman, J. K., Marquez, V. E., and Eritja, R. (2001) Synthesis of oligonucleotide inhibitors of DNA (cytosine-C5) methyltransferase containing 5-azacytosine residues at specific sites. *Antisense Nucleic Acid Drug Dev. 11*, 369−378.

(21) Wright, E. P., Huppert, J. L., and Waller, Z. A. E. (2017) Identification of multiple genomic DNA sequences which form i-motif structures at neutral pH. *Nucleic Acids Res. 45*, 2951−2959.

(22) Mergny, J. L., Li, J., Lacroix, L., Amrane, S., and Chaires, J. B. (2005) Thermal difference spectra: a specific signature for nucleic acid structures. *Nucleic Acids Res. 33*, e138.

(23) Kypr, J., Kejnovská, I., Renčiuk, D., and Vorlíčková, M. (2009) Circular dichroism and conformational polymorphism of DNA. *Nucleic Acids Res. 37*, 1713−1725.

(24) Lannes, L., Halder, S., Krishnan, Y., and Schwalbe, H. (2015) Tuning the pH response of i-motif DNA oligonucleotides. *ChemBioChem 16*, 1647−1656.

(25) Kendrick, S., Akiyama, Y., Hecht, S. M., and Hurley, L. H. (2009) The i-Motif in the bcl-2 P1 Promoter Forms an Unexpectedly Stable Structure with a Unique 8:5:7 Loop Folding Pattern. *J. Am. Chem. Soc. 131*, 17667−17676.

(26) Ginno, P. A., Lott, P. L., Christensen, H. C., Korf, I., and Chédin, F. (2012) R-Loop formation is a distinctive characteristic of unmethylated human CpG island promoters. *Mol. Cell 45*, 814−825.

(27) Groh, M., and Gromak, N. (2014) Out of balance: R-loops in human disease. *PLoS Genet. 10*, e1004630.

(28) Zeman, M. K., and Cimprich, K. A. (2014) Causes and consequences of replication stress. *Nat. Cell Biol. 16*, 2−9.

DOI: 10.1021/acs.biochem.7b00628
Biochemistry 2017, 56, 4879−4883