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Mini Review

Hoogsteen base-pairing revisited: Resolving a role in normal biological processes and human diseases

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Abstract

For a long time since the discovery of an alternative type of hydrogen bonding between adenine and thymidine, termed Hoogsteen base-pairing, its biological role remained elusive. Recent experiments provide compelling evidence that Hoogsteen base pairs manifest in a gamut of nuclear processes encompassing gene expression, replication, recombination, and telomere length maintenance. An increasing number of proteins that have been shown to bind, unwind or cleave G-quadruplexes or triplexes with high specificity underscore their biological significance. In humans, the absence of these cellular factors or their dysfunction leads to a wide spectrum of genetic diseases including cancer, neurodegenerative syndromes, and a myriad of other disorders. Thus, development of clinically useful compounds that target G-quadruplexes or triplexes, and interfere with specific cellular processes, provides considerable promise for successful and improved treatment of human diseases.

Keywords: Hoogsteen base-pairing; DNA transactions; G-quadruplexes; RecQ helicases; Triplexes; Genetic diseases

In double-stranded DNA, whether in A-, B- or Z-form, the complementary strands are held together solely through Watson–Crick (W-C) base-pairing geometry. The hydrogen bonds orchestrate base-pairing between dA·dT or dG·dC and maintain the geometric structure of regular double-helical DNA. Over 40 years ago, K. Hoogsteen discovered a novel type of hydrogen bonding between adenine and thymidine, termed Hoogsteen base-pairing geometry [1]. These pairings involve either purines or pyrimidines interacting with the sites on purine bases that are not involved in W-C hydrogen bonding (N7 and O6 for guanine, N7 and N6 for adenine) [1,2]. Hoogsteen base pairs have quite different properties from W-C base pairs. The angle between the two glycosylic bonds (ca. 80° in the A·T pair) is larger and the C1′–C1′ distance (ca. 8.6 Å) is smaller than in the regular geometry. In some cases, called reversed Hoogsteen base pairs, one base is rotated 180° with respect to the other [1–3]. Since the discovery of Hoogsteen base-pairing, forms of the DNA double helix containing both inter- as well as intra-strand Hoogsteen and reverse Hoogsteen base pairs have been discovered in crystal structures of undistorted B-DNA [4–6], DNA complexed with intercalating drugs [7], protein/DNA complexes [5,8,9], and RNA [10]. The Hoogsteen base pairs are frequently engaged by proteins and ligands to interact with DNA. With the recent discovery of the formation of Hoogsteen base pairs during DNA replication and pairing of double-stranded DNA helices, it is an exciting time for those interested in the biochemistry of specialized DNA structures (see below).

Hoogsteen base-pairing in DNA replication

DNA polymerases, in addition to their key role in DNA replication, also play a prominent role in DNA repair and recombination. The main replicative DNA polymerase recognizes its substrate with exceptionally high specificity and incorporates the correct nucleotide that forms the W-C base pair with the normal template base (Fig. 1A). But this

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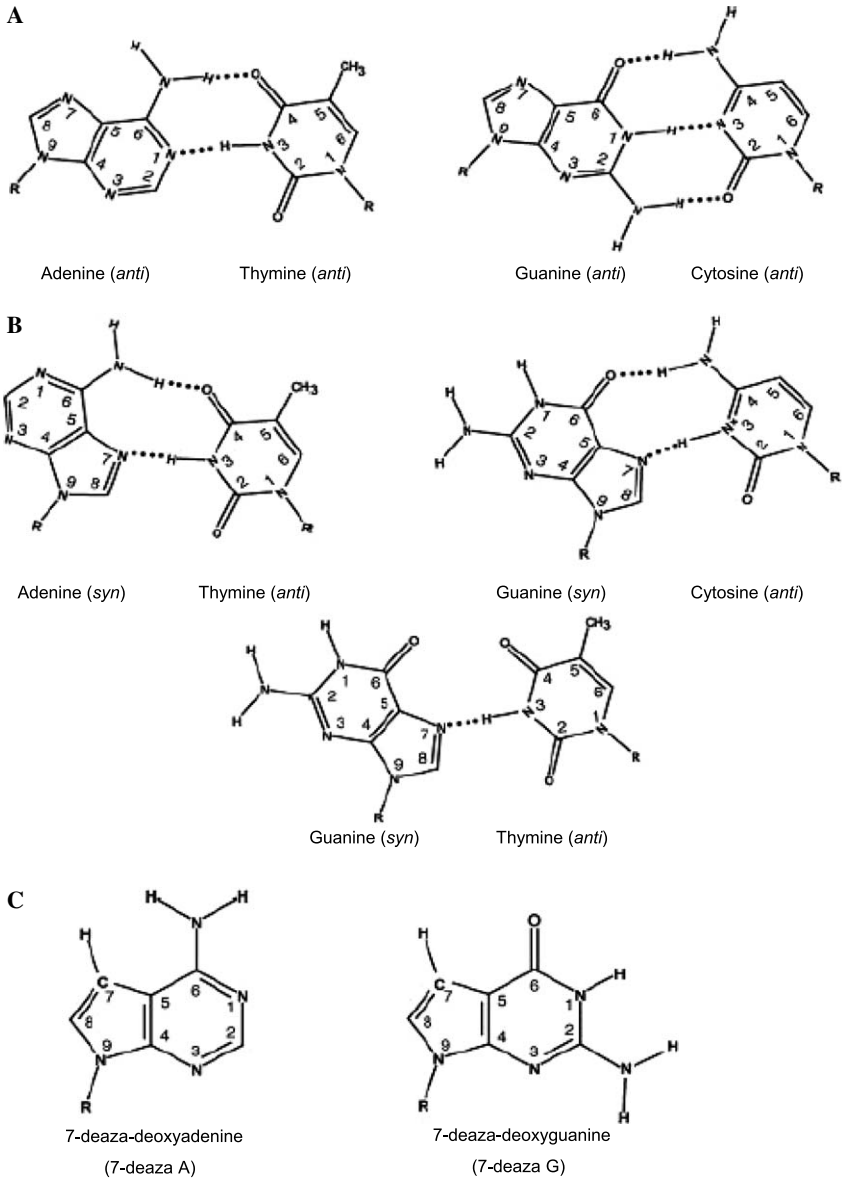

Fig. 1. Hydrogen bonding in W-C and Hoogsteen base pairs. (A) W-C A · T and G · C base pairs. (B) Hoogsteen base pair formation between adenine and thymine, guanine and cytosine, and guanine and thymine. (C) 7-Deaza purine analogs 7-deaza-A and 7-deaza-G. The R indicates the sugar moiety (adapted from Johnson et al., Proc. Natl. Acad. Sci. USA 102 (2005) 10466–10471).

capability also makes it very poor at dealing with damaged DNA, where it very often stalls at the point of damage [11]. How do polymerases replicate DNA through damaged or mutated regions of the templates? Several novel Y-family of DNA polymerases, which display low fidelity and poor processivity, can efficiently bypass DNA lesions during replication. However, the mechanism remained obscure and thought to vary with each of the members of the Y family of polymerases. Using structural and functional approaches, three papers have uncovered evidence that Hoogsteen and not W-C base-pairing geometry is adopted by DNA during replication by human DNA polymerase ι (hPolι) (Fig. 1B) [12,13].

The issue was the lack of precedence for the existence of an alternative base-pairing scheme other than W-C base pairs in newly synthesized DNA. Human Polι, a member of the Y-family DNA polymerases, displays the ability to replicate through DNA lesions [11]. Cells lacking Polι display pleiotropic effects, including an unusual mutator phenotype [14]. Recent studies from the A. Aggarwal, L. Prakash, and S. Prakash groups provided the structural evidence for the formation of Hoogsteen base pairs during DNA replication [12,13]. The crystal structure of hPolι with the template–primer junction and an incoming dTTP showed that hPolι correctly incorporates T opposite template A via Hoogsteen base-pairing geometry [12]. As is the case with many original findings, doubts have been raised about this striking observation [15]. Additionally, it is presumed that G-C base pair has no potential to form Hoogsteen base pair at physiological pH; therefore, it is

unlikely to be used in DNA replication. Now, even that small doubt has been dispelled. In a follow-up study, these authors have shown Hoogsteen base-pairing between template guanine and incoming dCTP by hPolι [13]. Although the significance of Hoogsteen base pairs in DNA replication is obscure, it is possible that Hoogsteen base pair is thermodynamically more stable than W-C base pair in the context of abnormal and detrimental DNA secondary structures and replication intermediates.

To validate the above conclusions, the L. Prakash and S. Prakash groups provided complementary biochemical evidence [16]. They used N7-modified purine analogues as the templating residues, where the N7-modification disrupts Hoogsteen but not W-C base pair formation (Fig. 1C). These experiments demonstrated that, unlike other DNA polymerases belonging to the A, B, or Y family, DNA synthesis by hPolι was severely inhibited by the N7-modified bases. The data from mutational studies with Leu-62, which lies in close proximity to the templating residue in polymerase ι ternary complex [16], support the notion that both the steric constraints within the active site and the stability provided by the hydrogen bonds in the Hoogsteen base pair contribute to the efficiency and fidelity of DNA replication by hPolι. Equally fascinating is the observation that human DNA Polη and *Solfolobus solfactaricus* Dpo4 both members of Y-family of DNA polymerases promote replication through cyclobutane pyrimidine dimers via W-C base-pairing and not by Hoogsteen base-pairing configuration [17,18]. These findings are extremely unambiguous and support the notion that DNA polymerases utilize alternative base-pairing schemes during DNA synthesis.

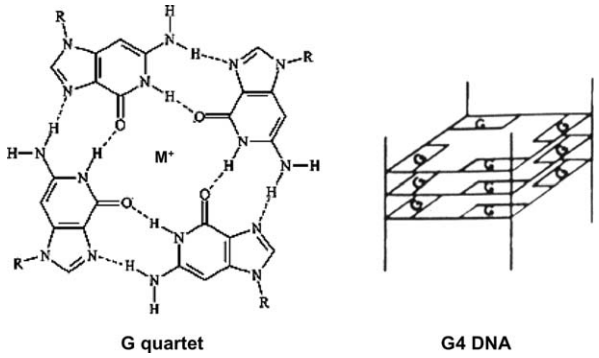

Fig. 2. The structure of G-quartet DNA. Each G-quartet consists of a cyclic array of four guanine residues, which are connected by Hoogsteen hydrogen bonding (dotted lines). The guanosine nucleotides can be either in syn- or anti-conformation about the glycosidic bonds. The center of the G-quartet forms a pocket for metal ion binding (Me+). Stacks of G-quartets produce G4 DNA configuration (right).

Hoogsteen-bonded G-quadruplexes

Molecular genetic analysis of telomeres provided the impetus for understanding the biological significance of G-quadruplexes. These structures, also referred to as G-quartets, G-tetraplex or G4 DNA, are composed of arrays of guanine bases with the phosphodiester backbones of the four participating strands in parallel or anti-parallel orientation (Fig. 2). Each guanine residue serves as a donor as well as acceptor of two Hoogsteen hydrogen bonds. The formation of G-quartets in vitro requires the presence of cations that bind specifically to guanine O6 carbonyl groups between the planes of the G-quartets [19,20].

Telomeres are composed of several thousand repeats of the nucleotide sequence TTAGGG, ending in a single-stranded segment that overhangs at the end of the double-stranded DNA. Oligonucleotides corresponding to the G-rich strand of telomeric DNA of a variety of organisms have been shown to fold into G4 DNA configurations in vitro. High-resolution structures of the human telomeric sequence $d(T_2AG_3)_n$ have been solved using NMR spectroscopy and X-ray crystallography. The complementary runs of cytosine can fold into i-motif structures [19,20]. G-quadruplexes, which form readily under physiological conditions, have been studied more extensively than i-motifs, since the formation of i-motif structures requires acidic pH, which makes their physiological relevance less likely in the in vivo context. The structure of G-quadruplexes varies in a number of different ways, including strand stoichiometric ratio's, strand orientation, and the loop, consisting of any of the four bases with a size of 2–6 bases. The interconversion between double- or single-stranded DNA and G-quadruplex in cells is dependent upon a number of cellular proteins. Some of these proteins include the β-subunit of the *Oxytricha* telomere-binding protein [21], *Saccharomyces cerevisiae* Rap1 [22], Sgs1 [23], Hop1 [24], Mre11 [25], and Kem1 [26] proteins. Further, isolation and characterization of a human nuclease with G4 DNA specificity [27] and the ability of hPot1p to resolve telomeric G-quadruplexes into unmodified single-stranded DNA suggest that these structures might exist under in vivo conditions in humans [28].

There is increasing evidence linking eukaryotic RecQ helicases to the maintenance of telomeres [29,30]. *S. cerevisiae SGS1* encodes a DNA helicase with homology to the human Bloom's syndrome gene *BLM* and the Werner's syndrome gene *WRN* [30]. *EST2* encodes the telomerase catalytic subunit in yeast. Deletion of *EST2* in *sgs1* strains gives rise to a twofold increase in the rate of telomere loss and a ~30% reduction in division capacity compared to *est2 SGS1* strains [31]. Triple *sgs1 est2 rad52* mutants senesce extremely rapidly and no survivors are generated due to the absolute requirement of Rad52 in recombinational telomere lengthening [31]. Expression of WRN in *est2 sgs1* strains can partially rescue the defect in telomere lengthening, suggesting WRN and Sgs1 may have a similar telomeric function. WRN, BLM, and Sgs1 can unwind G4 DNA and other secondary structures in telomeric DNA raising the possibility that WRN may resolve such structures in vivo [32].

Several biochemical and structural studies have demonstrated the formation of G-quadruplexes in vitro, however

direct evidence supporting their in vivo existence remained elusive [19,20,33]. Two reports have provided persuasive evidence for the existence of G-quadruplexes in the ciliate *Stylonychia lemnae*. The first evidence stems from immunofluorescence staining of *S. lemnae* macronuclei by antibodies raised against G4 DNA [34]. Now elegant new evidence based on RNAi and methylation protection approaches not only confirm the existence of G4 DNA, but also have revealed that telomere-binding proteins control its formation in vivo [35]. Furthermore, genetic and electron microscopic studies in bacteria provide unequivocal evidence for the formation and stable existence of G-quadruplexes in vivo [36]. Other approaches that have been used to uncover the existence of G-quadruplexes in vivo include treatment of cells with G-quadruplex-specific agents such as acridine derivatives, quinines, fluorenone-based molecules, telomestatin and cationic porphyrin, TMPyP4 and alteration of various nuclear processes [37,38].

G-quadruplexes regulate gene expression

Several lines of evidence implicate G-quadruplex as an important source of regulation of gene expression and genome stability [33]. The polypurine/polypyrimidine tracts embedded in the promoter regions of a number of eukaryotic genes have been shown to undergo structural transitions in transcriptional regulation [39,40]. In general, DNA unwinding at the promoter region of both prokaryotic and eukaryotic genes is believed to enhance open promoter complex formation with transcriptional machineries leading to transcriptional activation of genes [39,40]. A polypurine/polypyrimidine tract found proximal to the promoter region of the VEGF gene contains multiple binding sites for Sp1 and Egr-1 transcription factors, which is frequently elevated in many types of cancer. The VEGF promoter contains a guanine-rich sequence consisting of four arrays of 3–5 contiguous guanines separated by one or more bases. Using a variety of approaches, evidence has been provided that specific G-quadruplex structures can naturally be formed by the G-rich sequence of the VEGF promoter region, raising the possibility that the transcription of this gene can be controlled by ligand-mediated G-quadruplex stabilization [41].

A growing body of evidence points to a function of G-quadruplex DNA in the regulation of gene expression and anti-neoplastic activity [42,43]. Expression of the c-*Myc* oncogene is linked to potentiation of cellular proliferation and to inhibition of differentiation, leading to its association with a number of human and animal malignancies, including carcinomas of the breast, colon, and cervix, as well as small-cell lung cancer, osteosarcomas, glioblastomas, and myeloid leukemias [44]. Oligonucleotide sequences corresponding to the coding and non-coding strands of the c-myc promoter can adopt i-motif and G-quadruplex structures, respectively, and function as transcriptional repressors [45,46]. Similarly, intracellular transcription of plasmids containing G-rich sequences in the non-transcribed strand produced G-loops, containing G4 DNA on the non-transcribed strand and a stable RNA/DNA hybrid on the other [36]. Interestingly, G-loop formation was suppressed in *Escherichia coli recQ*$^{+}$ strains, indicating a role for RecQ in their processing. The inverse correlation is consistent with the proposal that G-rich sequences can adopt intra- and intermolecular G-quadruplex structures in vivo.

Hoogsteen base pairs in recombination

In meiosis, chromosome synapsis involves side-by-side pairing of homologous partners. The ultimate goal of meiosis is to produce four normal gametes carrying a copy of the full set of haploid chromosomes. Faulty chromosome pairing lead to aneuploidy and are a major cause of human birth defects such as Down syndrome. To ensure that homologs are aligned in a configuration that allows the formation of crossovers and proper segregation, they are attached physically to the synaptonemal complex (SC) [33]. SC is a tripartite proteinaceous structure that holds homologous chromosomes close together along their lengths during the pachytene stage of meiotic prophase I. Mutations in genes encoding structural components of the SC lead to homolog non-disjunction at meiosis I. Although the cytological nature of SC has been studied in yeast to humans for a number years, its composition, structure as well as function is poorly understood.

There is increasing evidence that G4 DNA plays an important role in many cellular processes [33]. They have been implicated in the pairing and alignment of meiotic chromosomes and the class switch recombination of IgG genes [47,48]. A distinct role for Hoogsteen base-pair configuration in the pairing of meiotic chromosomes has been brought to the forefront from data from our laboratory. *S. cerevisiae HOP1* encodes a component of the SC. Biochemical characterization of the Hop1 protein suggested it to be an oligomeric, G-quadruplex DNA-binding protein [24,49,50]. This observation revealed novel connection between Hoogsteen base pairs and homolog pairing. In accord, recent studies show that Hop1 promoted interstitial pairing of two duplex DNA molecules via the formation of guanine quartets, providing direct evidence for the involvement of Hoogsteen base pairs in the pathway of chromosome synapsis and genetic recombination [50]. Taken collectively, these findings suggest that G4 DNA plays a crucial role in the pathway of meiotic synapsis and recombination.

G-quadruplexes and human genetic diseases

Werner Syndrome (WS), Bloom Syndrome (BS), and Rothmund-Thomson syndromes are rare human autosomal recessive disorders characterized by chromosomal instability yet distinctly different clinical phenotypes. Cells from affected individuals show genomic instability, the hallmark feature being hyperrecombination between sister

chromatids and recombination repair defects especially at stalled replication forks [33,37,38]. BS, WS, and a subset of cases of Rothmund-Thomson's syndromes are caused by defects in the RecQ-like genes BLM, WRN, and RECQ4, respectively [51,52]. Other members of this class include the human helicases RecQL and RecQ5, *S. cerevisiae* Sgs1, *Schizosaccharomyces pombe* Rqh1, *Neurospora crassa* qde-3, and *Aspergillus nidulans* musN [52]. Biochemical studies show that the RecQ helicases preferentially unwind G4 DNA relative to B-DNA, but are also capable of resolution of aberrant DNA structures, in an ATP-dependent manner [53,54]. Trisubstituted acridines, which are ligands that stabilize G4 DNA, have been found to inhibit the helicase activity of BLM and WRN proteins on G4 DNA [37]. In addition, several heterogeneous nuclear ribonucleoproteins (hnRNP A1), which bind both single-stranded RNA and DNA, also unwind human telomeric G-quadruplex structures [55]. The biological significance of G-quadruplexes is further highlighted by the observation that elevated telomerase activity has been implicated in 85% of cancers [37,38]. Since the quadruplex DNA inhibits the telomerase activity in vitro [56]; therapeutic agents that stabilize the G-quadruplex structures could potentially be effective chemotherapeutic agents [37]. Also, a number of small molecules that inhibit RecQ function also arrest the growth of various cancer cells in vitro [33,38]. Importantly, the use of such compounds provides further validation for the concept of the existence of G-quadruplex structures in vivo.

G-quadruplexes in human genome

G-quadruplex structures have attracted considerable attention because of their putative occurrence and biological function in the genomes of a variety of model organisms. The abundance of G-quadruplex forming sequences found throughout eukaryotic genomes strongly indicates that this DNA configuration is biologically relevant. In humans, for example, G-quadruplex forming potential has been noted in human insulin gene [57,58], insulin-like growth factor II [59], fragile X-syndrome [60] mouse *Ms6-hm* hyper variable satellite repeat [61], and proto-oncogenes [38,42,62]. Further, bioinformatics and molecular modeling approaches indicate that sequences that can fold into G-quadruplex structures are widely dispersed in the human genome, and are abundant in regions of biological importance, for example, at telomeres, immunoglobin switch region, rDNA, in the promoters of many important genes, and at recombination hotspots [63,64].

G-quadruplexes and HIV

The genomes of several human viruses including HIV have the potential to form intramolecular hairpins and G-quadruplex structures. Although the stable association of HIV-1 RNA genome is facilitated by gag protein, RNA dimerizes by forming an interstrand quadruplex helix stabilized by guanine tetrads [65]. Recent studies have shown that the HIV-1 nucleocapsid protein preferentially recognizes and readily destabilizes and unfolds the intermolecular G-quadruplex structures [66]. Further, a number of studies have shown that G-quadruplex structure acts as a potent inhibitor of HIV-1 integrase, paving the way toward the potential design of improved HIV-1 integrase inhibitors. Consequently, efforts are being made to synthesize new molecules that stabilize the HIV RNA quadruplex and interfere with its proliferation [67].

Hoogsteen-bonded triplexes

Naturally occurring homopurine/homopyrimidine sequences can fold into triplex configuration by binding a third strand of DNA or RNA in the major groove of W-C duplex DNA through Hoogsteen or reversed Hoogsteen hydrogen bonds [39]. The purine strand of the W-C duplex engages the third strand through Hoogsteen hydrogen bonds in the major groove while maintaining the original duplex structure in a B-DNA like conformation. Triplexes are of interest both for their potential use as therapeutic agents to repress gene expression and their formation in vivo as H-DNA [33,38,40]. The latter is believed to be involved in diverse DNA transaction processes including DNA replication, gene transcription, recombination, and genome instability [39,40]. Interestingly, in silico analysis suggests that sequences capable of adopting H-DNA structures are very abundant in the human genome [68]. In contrast to other DNA secondary structure-forming sequences, which are typically located in the intergenic or intronic regions, H-DNA-forming sequences are found embedded most frequently in promoters and exons and have been shown to be involved in regulating the expression of several disease-linked as well as normal genes [39,40]. A number of neurological disorders are caused by the expansion of simple triplet repeat sequences in either coding or non-coding regions. Studies conducted in different prokaryotic and eukaryotic model systems suggest that, among several non-B DNA structures, triplex and/or sticky DNA conformations adopted by the expansion of triplet repeat sequences are likely to be involved in the etiology of >20 neurological disorders [39]. Together, these findings suggest that the H-DNA structures result in fragile sites or mutation hotspots leading to double-strand breaks thereby culminating in translocation of the gene [39].

Perspectives

The physico–chemical factors that govern folding of B-DNA into non-B DNA configurations in vivo are poorly understood. However, mounting evidence suggests that G-quadruplex and triplex DNA structures play important roles in a variety of nuclear processes. The mechanisms by which these non-B DNA conformations influence cellular functions are not well understood, but is thought

to introduce an additional measure of regulation. The biological relevance of Hoogsteen base pairs is highlighted by several new studies showing their involvement in normal DNA metabolism as well as human genetic and infectious diseases. In addition, these structures are relevant in the design of supramolecular assemblies as well as uncovering the nature and stability of biologically relevant genomic motifs [69,70]. Consequently, the architecture of G-quadruplexes, and their interactions with proteins and ligands, should continue to be an active area of research with broad therapeutic implications. The readership can be reassured that the pace of discovery will accelerate as newer approaches are brought to bear in order to characterize the structure, dynamics and energetics of G4 DNA-mediated processes and therapeutic intervention. Further investigations will lead a more comprehensive understanding of Hoogsteen base pairs in specialized DNA structures.

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