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A Review

Chromatin higher order structure and possible therapeutic target

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Canonical form of chromatin structure was unveiled by James Watson and Francis Crick which unravels the better understanding of their structural and functional significance. Experimental observations disclose that non-canonical DNA structures like hairpin, cruciform, Z-DNA, multi-stranded structures such as DNA triplex, G-quadruplex, i-motif forms by depending on specific sequence. Non-canonical structure specifically G-quadruplex stability depends on various factors such as base sequence, ions, super helical stress, and ligands, which ultimately regulates different processes like replication, transcription, translation, and recombination. However, chromatin higher order structure also modifies the gene expression level by modulating nucleosome and also recruited different chromatin remodeler protein to the transcription site to maintain an epigenetic landscape in the genome. Though G-quadruplex regulates multiple cellular processes, but their presence accelerated the mutagenicity and genome instability. The present review is an overview of higher order chromatin structure particularly focussed on G- quadruplex, which is summarized from recent literature, demonstrated the structural form and stability through some specific binding ligand and their role in cellular level. This would provide a mechanistic view to derive the structure-function relation of these non-canonical forms of DNA and their identification as potential therapeutic targets for diverse genetic diseases and cancer.

Keywords: G-quadruplex structure, Ligand, Non canonical DNA, Targeted therapy

Introduction

The three-dimensional representation of genetic material in cell presents a higher order of intricacy. The canonical form of DNA is wrapped with nucleosome forming chromatin. It is embraced with nucleosomes the repeating unit containing histone molecules form octamer comprised of two H3-H4 and two H2A-H2B histone dimers, which is covered by a stretch of DNA (~145-147 bp)[1-2]. Nucleosomes are associated with short (10-80 bp) DNA segments known as ‘linker DNA’ into nucleosomal arrays, which ensure transient interactions with neighbouring nucleosomes to form chromosome fibres. These interactions influence high degree of association of condensed chromosome[3]. This DNA-nucleosome complex shapes a 10 nm diameter fibre resembling ‘beads on a string’ structure[4,5]. It has been shown *in vitro* to form 30 nm helical fibre containing 6-11 nucleosomes per turn[6-7] which form higher order complex chromatin fibres in interphase. Thus the most pronounced chromatin higher-order structure is formed with 200-300 nm chromonema in a mitotic chromosome where the DNA is compressed with 10,000 to 20,000 fold[8]. At the different stages of chromatin arrangement epigenetic events play a major role in biological processes. These events involved into covalent histone modifications through DNA methylation. Additionally, different non-covalent mechanisms such as histone repositioning, chromatin remodeler and non-coding RNAs including microRNAs (miRNAs) are also involved in these epigenetic events. These events influence the local structural changes of chromatin, its openness and compactness. These modifications recreates a dynamic ‘epigenetic landscape’ that regulates genome in different cells and disease states[9-14]. These epigenetic modulations often involve dynamic chemical shifts to DNA or the histones, which entail enzymatic methylation, acetylation, phosphorylation, ubiquitination, and sumoylation[15]. The extension of an acetyl group on a histone is accomplished by lysine acetyltransferases (KATs) since its disposal is achieved by histone deacetylases (HDACs). Histone acetylation permits an “open” state of chromatin, known as euchromatin. It is uncovered by DNA to permit transcription on. On the contrary methylation may act either activation or repressive mark. Intriguingly, there is a “cross-talk” among the histone

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modifications and DNA methylation/de-methylation machinery, which is mediated by proteins that embroiled in these modifications. Recent studies have highlighted the critical role of the chromatin structure in gene regulation[16-22] with different techniques like chromosome conformation capture (3C). Such variations enlighten topologically associating domains (TADs) as elementary units of the nuclear architecture[23]. These domains participate in gene regulation, and their abnormalities are most of the time linked to different human diseases including cancers[17-18,20,24]. Different new techniques including DNA fluorescence in situ hybridization (FISH) have been developed to reduce cost and enhanced proficiencies of high-throughput sequencing offering high-resolution sensitivity at the single-cell level[24].

Polymorphic structure of chromatin

The most common canonical form of nucleic acid is the DNA double helix that emphasizes the structural form for two DNA strands connected through antiparallel Watson-Crick (W–C) base pairs[25]. However DNA is structurally dynamic and capable of employing alternative noncanonical secondary structures like triplexes[26-27], Z-DNA, cruciform, G quadruplexes[28], i-motif[29], and junctions (Fig.1). Structure of DNA duplexes can be categorized into three generically different forms A, B, and Z based on the nucleotide, salt concentration etc. In this context A and B types endorse a double-helix twisted in right handed with Watson-Crick base pairing, while the unusual Z-form is being a left-handed helical structure[30]. Triplex formation occurs by the definite binding of the triplex-forming oligonucleotide (TFO) *via* Hoogsteen (H) base pairs. Here a pyrimidine and a purine base linked to the duplex by parallel and antiparallel conformation[31-32]. DNA triplexes are majorly found in disease-related genes and involved in disease onset and progression. Such triplexes found in the *c-MYC* gene intervenes transcription and showsanti-proliferation in breast cancer cells[33-34]. Recently, it is reported that certain long non-coding RNAs (lncRNAs) like metastasis-associated lung adenocarcinoma transcript 1 and multiple endocrine neoplasia-β RNAs[35-36], possesses intramolecular RNA triplex in their 3′ ends are stable in cancer. Helical junctions are another non-canonical form. In this structure two or more double-helices converge at a junction with discontinued axis. Strands are interchanged between the helices where all bases paired with Watson-Crick bond or mismatched or unpaired at the branch point. Although helicaljunctions are more prevalent in RNA, but also present in DNA four-way junctions in the form of cruciform structure[37]. Recently, three-way junctions have been found in HIV-1 viral genomes, where it acts as recognition sites for proteins important for viral survivability and infectivity[38]. These structures are also ascertained to play important roles in ribosome function and telomerase activity[39]. The four-way junctions are also coordinating in recombination process perceived by proteins that promote branch movement. Human telomeric DNA with 5′-TTAGGG-3′ and 5′-CCCTAA-3′ repeats are involved in formation of four-way junction. When active telomerase is absent, Holliday intermediate can form to protect the telomere through recombination process. The maintenance of telomeres through this alternative pathway (ALT) promotes immortalized cells to lengthen theirtelomeres[40]. I-motifs (iMs) are unusual knot-like DNA conformation in the human chromosome to furnish critical functions. They are repeatedly created in the genome and also in genes in G0/G1 phase. Their presence and redistribution in genome have been substantiated and established by immunofluorescence imaging and more recently NMR and CUT & Tag. Recent literature reported that, approx. 53,000 iMs are found among three human cell lines (MCF7, U2OS, HEK293T)[41]. Among all major known DNA non-canonical secondary conformation, the most important is the four-stranded G-quadruplex

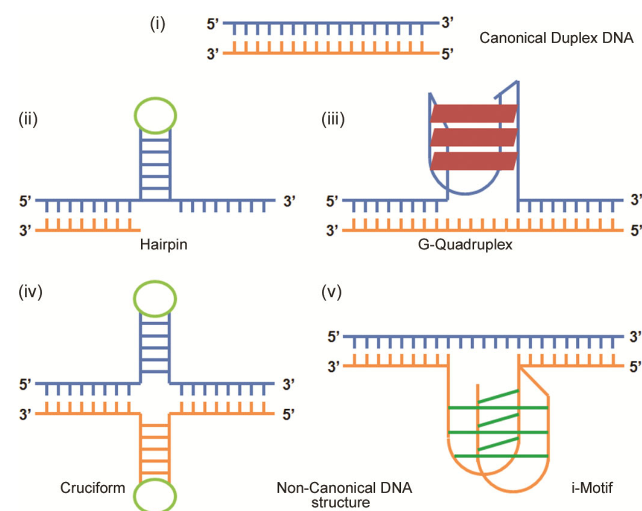

Fig. 1 — Canonical and Non-canonical structure of DNA. Duplex DNA (i) is the canonical form with Watson-Crick hydrogen bond. Non-canonical DNA secondary structures; (ii) Hairpin; (iii) G-quadruplex; (iv) Cruciform; and (v) i-motif forms by Watson-Crick and Hoogsteen hydrogen bond

(G4). Genomewide about 10^5 to 10^6 putative G-quadruplex-forming sequences are distributed in the human genome[42]. It is rich in particular regions of genes like; telomeres, oncogene-promoter locations, 5′- and 3′-untranslated region (UTR) of mRNA, and transcription factor binding sites as well as in other genomes. Strong evidence reinforces the function of G4s in the regulation of many physiological processes and their involvement in pathological conditions[43]. These conformations in specific regions maintain gene integrity, mRNA processing, protein translation, DNA replication, telomere function, as well as cancer progression, and degenerative disorders[44]. G4 is the non-canonical DNA structure, consists of three or more G-tetradwhich contain four guanine and a monovalent cation (especially K^+) at its centre[45-48] (Fig. 2). Four guanine residues are connected through Hoogsteen hydrogen bond. G4s form intramolecular or intermolecular with one or more than one nucleic acid molecule respectively. Rationally, parallel, hybrid/mixed or antiparallel G-quadruplexes can form based on the arrangement of all adjacent G-strands[49]. Conspicuously, physiological pH and ionic condition is very crucial for folding intoquadruplexes[50].

Extrinsic factors influencing chromatin structure stability

The intracellular environment comprises of distinct biomolecules about 20-40% that are commingled. In the nucleus, DNA is wrapped by chromatin with high density while the cytoplasm is occupied by cytoskeletal proteins, cell organelles, metabolites, and molecules that regulate osmosis. In parallel to analyse the demeanour of biomolecules in cells 100-150 nM KCl or NaCl solution is used. To assess the effect of the intracellular environment in the cell, polyethylene glycol, dextran, ficoll, bovine serum albumin, and nucleic acids such as tRNA are accustomed as cell mimicking crowding agents. Such high-molecular-weight crowding agents lowered the steric hindrance between the structure[51]. In generalhydrogen bond, base, conformational entropy, hydration, and cation binding are very strong factors in maintaining nucleic acid structure and stability[52-55].

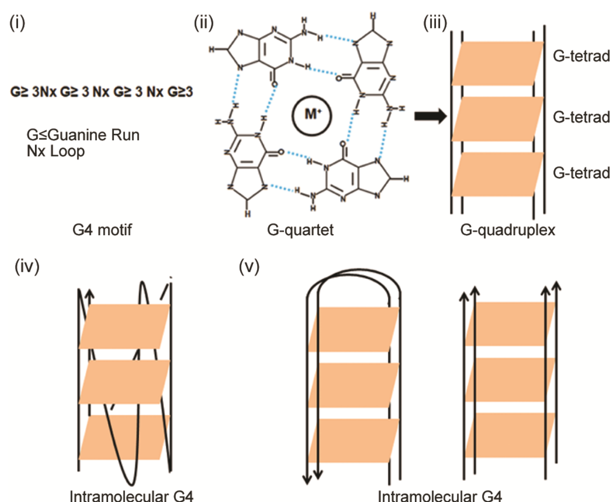

Fig. 2 — Different forms of G-quadruplex structure of DNA. (i) Putative G-quadruplex forming sequence containing three or more Guanine consecutively; (ii) Four G-quadruplex motifs form G-quartet or G-tetrad; (iii) Three G-tetrad stack to each other and form G-quadruplex structure; (iv) Monomeric G-quartets linked by three loops form intramolecular G-quadruplex; and (v) Intermolecular G-quadruplexes form by connecting multimeric G-quartets from multiple strands of DNA

Biological importance of noncanonical G-quadruplex structure in genome and other regions of chromatin

The G4s form in genome play vital roles in cancer regulation. Normally the regions distant from the start of transcription are affluent in G4-motifs rather than the other parts of genome[56]. However, pG4s are also formed in end terminal of chromosome and between introns and exons gap. Contemporary studies divulged that origin of replication of genome are overlapped with pG4 motifs around 90% and coordinated with the 5′-untranslated region (UTR) of the mRNAs[57]. It is very apparent that G4 structures strongly regulate the functional mechanism of the genome by correcting error in replication machinery. G4s are also known to disrupt transcription and chromatin remodelling, in addition to translation from mRNA[44]. The diagrammatic representation of regulatory functions of G4 structure in different locations of the genome is depicted in (Fig.3). Many G4 motifs are located in KRAS[58], VEGF[59], PDGF-A[60] and BCL-2[61] promoters stimulating the cancer progression, and also play role in regulation of gene. Surprisingly, activator and repressor interacting sequences are overlapped in G4 motifs. For example, the *MYC* promoter G4 has been occupied by the nonmetastatic factor NM23-H2. Pull-down and chromatin immunoprecipitation assays describe that upstream of KRAS promoter where G4 motifs are present occupied by transcription factors Myc-associated zinc finger (MAZ) and poly (ADP-ribose) polymerase 1 (PARP-1)[62]. This non canonical G4 form is strongly allied with epigenetic disruption. Another factor Pif1 is engaged in homologous recombination (HR) to repair DNA double strand

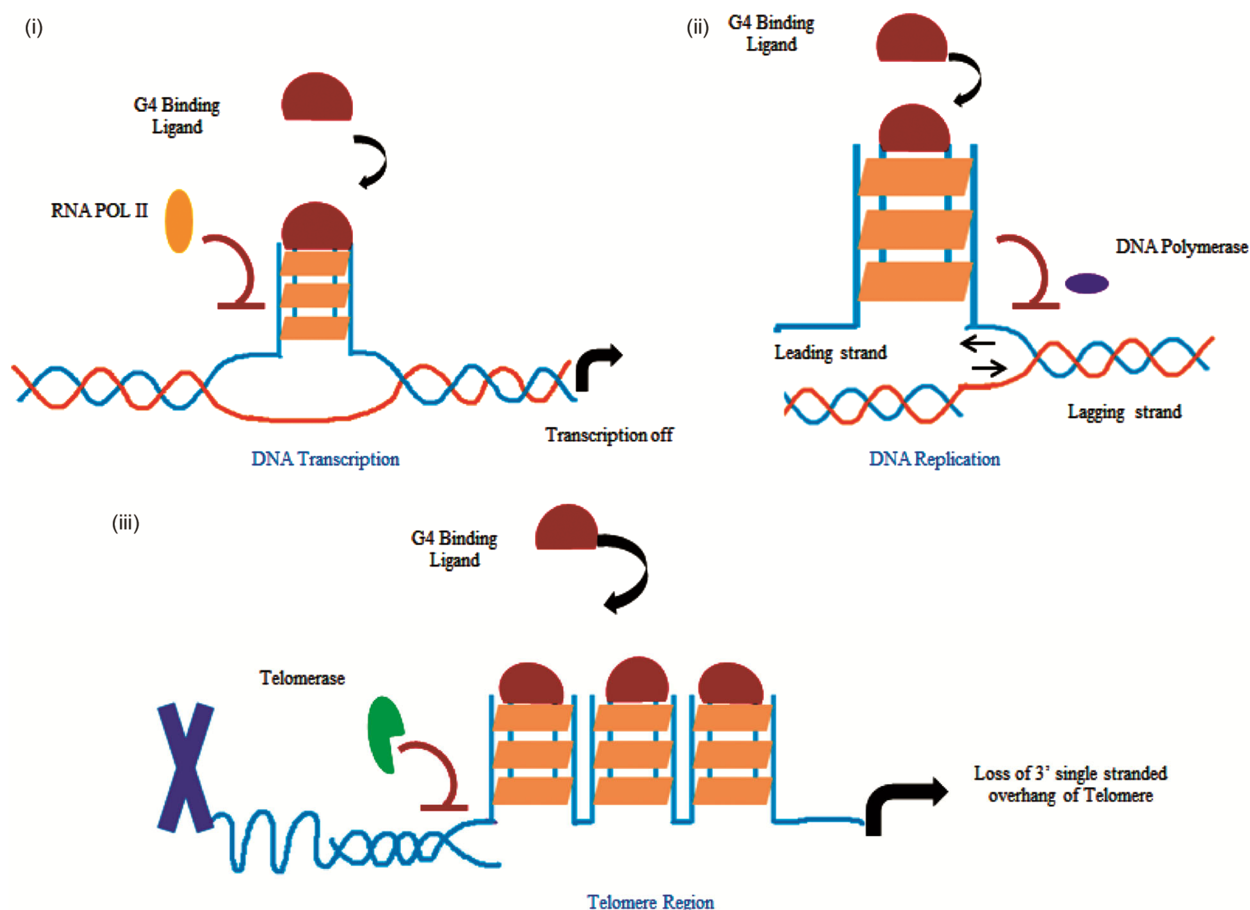

Fig. 3 — Functional overview of G-quadruplex structure and ligand in genome. (i) Formation of G-quadruplex structure and their stabilization through ligand molecule in promoter region stops transcription by inhibiting the binding of RNA POL II; (ii) Replication fork is stalled due to G-quadruplex formation in leading strand and stops replication fork movement by inhibiting binding of DNA polymerase; and (iii) Telomeric 3′ single stranded overhang sequence forms multiple G-quadruplex structure upon stabilization by ligand and stops Telomerase to synthesis the complementary C strand

breaks through releasing G4 structure[63]. The presence of the G4 in the insulin-binding polymorphic region, regulate its transcription[64]. Normally most G4s hinder transcription activity. For example, oncogene *c-MYC* expression heightened cell proliferation[65], and *c-KIT* which encrypts a tyrosine kinase receptor, triggers signal to promote cell proliferation through activation[66]. G4 formation has been shown to inhibit transcription of *c-MYC* and *c-KIT* simultaneously through their promoters[67-68].

Role of G-quadruplex in Telomere

Telomere sequences are double-stranded short protected region located in chromosome terminal. Normally length of telomere extends several kilobases, ending with an overhang of the single stranded G-rich sequence, shapes into G4 structures, where four guanines form a planar stack joined by Hoogsteen Hydrogen bond[69]. Multiple G-quartets stack on each other to shape a stable G4 structure at the telomere region[70]. The dexterity of telomeric single-stranded overhang sequences those form into G4 *in vitro* is highly conserved in diverse species, including other unicellular eukaryotes. Interestingly *Plasmodium*[71], *Giardia*[72] and some other organisms including human contains telomeric repeat sequence TTAGGG[73]. Whereas Plants like *Arabidopsis*[74] and other organisms including the silkworm *Bombyx mori*[75] also contain the similar hexameric single stranded telomeric overhang. In human cells, G-quadruplex-binding ligands evidenced the initial visual demonstrations of G4 structure upon stabilization. A radiolabeled G4 ligand 360A was used to detect the ends of metaphase chromosome of human cell by autoradiography[76]. Additionally

another biotin labeled ligand Pyridostatin was used topull-down telomeric G4s from human genomic DNA[77]. G4 in telomere influences the dislocation of proteins POT1 and TRF2 of shelterin complex from chromosome ends[78-79]. Gradual loss of the telomeric G-rich overhang[80], and increase the DNA damage mediated signals at telomeres, ultimately leads to defective replication of telomeres[81]. The promoter of the gene hTERT, also harbors G4 structure, where the G4-stabilizing small ligand molecule bind strongly and decrease the levels of hTERT expression to promote deprotection of telomere[82].

Identification and structure-function relationship of genomic G-quadruplexes in cancer cell

Structural studies using crystallography and NMR, provides detail overview into the DNA G4s. This is identified by repeat sequences of telomere and the promoter regions of certain human genes like *MYC* or *KIT*[83-85]. DNA polymerase-stop assay in whole genome followed by sequencing (G4-seq), chromatin immunoprecipitation with BG4 and parallel sequencing (G4 ChIP-seq), or the use of a small synthetic protein as G4 probe (G4P) have been developed in recent years. Reverse transcriptase stalling with next-generation sequencing (rG4-seq) and G4-RNA-specific precipitation (G4RP) with sequencing (G4RP-seq) are used rigorously to detect RNA G4 structures[86188]. Recently, the function of G4 is illustrated in live cancer cells in different experimental conditions. G4 structure shows stability through the surrounding K^+ concentration[89]. In malignant cells, due to overexpression of the K^+ channel, K^+ concentration is decreased when compared with that in normal cells[90-91]. On the contrary, in normal cells, the transcript from template DNA with G4 is lowered because the G4s are stable. In highly metastatic breast cancer cells G4s are enriched produce more transcripts rather than in noncancerous cells. It is noted that in normal cells, K^+ ions inhibit the transcription of oncogenes by stabilizing the G4 structure[92]. Additionally, in malignant cells 5′ untranslated region (UTR) of mRNAs form G4 structure are occupied by tRNAs and promoting translation[93].

G-quadruplex binding proteins influencing gene functions:

The redistribution of G4s in the genome puts a rational overview for further investigation on the functional mechanism of G4s. Proteins playvery crucial role for the formation of G4 and their accomplishment in functions. These proteins could be grouped into two ways according to their functional regulation with G4s. Majority of G4-folding protein influences on G4 structures. However, there are some other proteins show activity upon recruitment on G4 (Fig. 4). G4 binding protein can also be categorised based on the binding on RNA and DNA. Specifically, these proteins favour to fold and unfold G4 structure by modulating their function[94-95] (Table 1). G4BPs are mainly detected through mass spectrometry, affinity chromatography and fluorescence energy resonance transfer (FRET). Proteins with G4 motif are separated through chromatography and combination of mass spectrometry[96]. Proteins binding to G4s in the 5′ UTR of tumor-associated mRNA are usually identified by this technique. Several techniques including FRET have developed to detect G-quadruplexes and their interacted proteins[97]. Since protein purification did not consider the native state of chromatin, Shankar Balasubramanian *et al.* developed a co-binding-mediated protein profiling (CMPP) approach to detect the G4BPs in living cells[98]. In this technique small-molecule ligands are taken specifically to target DNA G4 in cells with minimal obstruction of G4-protein interactions.

Methods

Many experimental techniques are currently available to locate G-quadruplexes (G4s) throughout the human genome. These methods are mainly based on the isolation of chromatin fragments containing G4s*in vivo* conditions. Some others are based on structural study of *in vitro* conditions.

ChIP-Seq

Chromatin is cross-linked by formaldehyde, followed by sonication to obtain DNA fragments of ∼100-500 bp. The fixation is necessary prior to fragmentation to preserve chromatin architecture, including histones and accessible sites. Both BG4 and SG4 (nanobody) are fused to a FLAG-tag and are used as recombinant antibodies to bind and enrich chromatin fragments containing G4s by immuno-precipitation. Such fragments are precipitated using magnetic beads bind with an anti-FLAG antibody and unbound fragments are washed off. After reverse cross-linking by heating at 55°C with proteinase K treatment

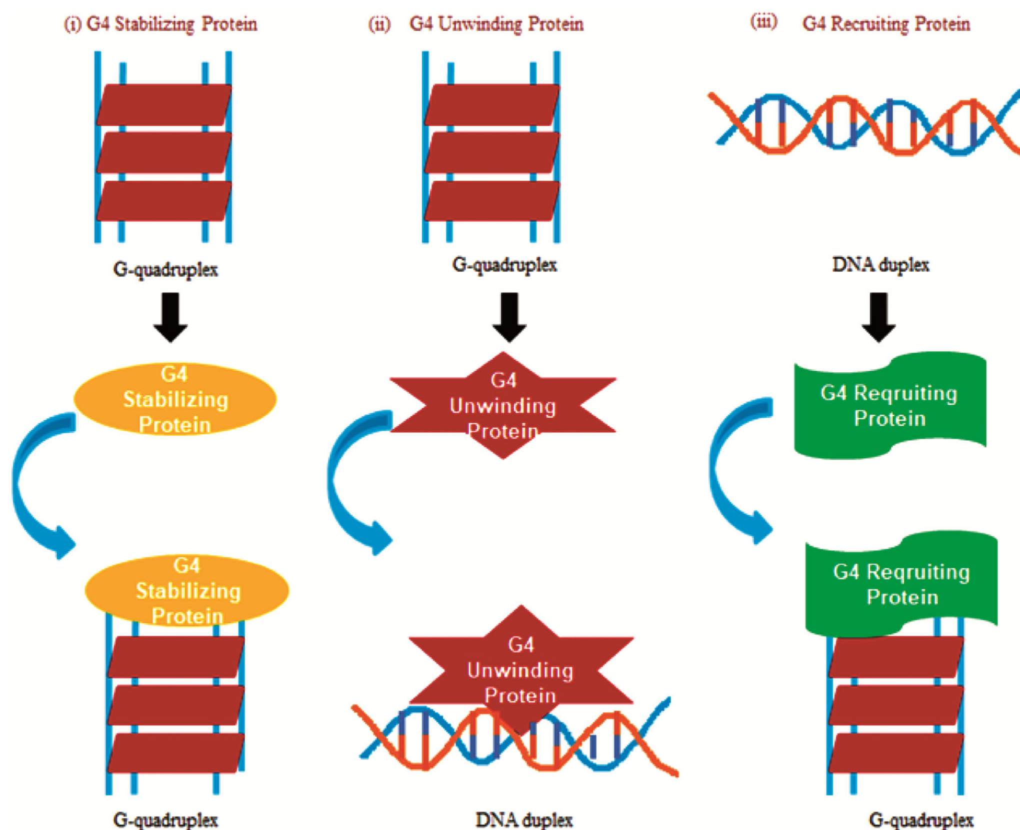

Fig. 4 — Mechanism of function of G-quadruplex Binding proteins. (i) G4 stabilizing; (ii) G4 unwinding; (iii) G4 recruiting proteins facilitate G-quadruplex structure stabilization, unwinding and formation by binding in genome, respectively

Table 1 — G4 Binding Protein, recognition domain and their biological functions

Mechanism Of Function	Biological Function	Protein Name	Recognition Domain (DNA/RNA)
G4 recruited	Telomere protection	Shelterin Complex Proteins (TPP1,POT1,TRF1,TRF2,RAP1,TIN2)	OB-fold, DNA
G4 recruited	Telomere maintenance	CST Complex Proteins (CTC1, STN1, TEN1)	OB-fold, DNA
G4 folding	G4 resolves and genome stability	WRN, BLM, FANCJ	Unknown
G4 recruited	Transcriptional regulation	NCL, NM23-H2,p53	RGG, RRM, DNA
G4 recruited	Chromatin remodelling and Histone modification	ATRX, DNMT1	Unknown
G4 recruited	Translational regulation	DDX3X, FMRP	RGG, RNA

for protein digestion. The precipitated DNA fragments are sequenced using standard Illumina sequencing. It is very fruitful to assess the quality of G4-ChIP enrichment prior to sequencing using qPCR[87].

Fluorescence energy resonance transfer (FRET)

FRET is a spectroscopic technique that provides information about the conformation and dynamics of biomolecules. In FRET experiments, G4 oligonucleotides are labelled at the 5′- and 3′-end with a donor and acceptor fluorophore, respectively. Briefly, the excited state energy is transferred from the donor to the acceptor via dipole–dipole interactions. The distance of the two labelled sites are the measurement of energy transfer. The most common FRET pairs include 6-carboxyfluorescein

(FAM) as a donor and 6-carboxy tetramethylrhodamine (TAMRA) or Black Hole Quencher 1 (BHQ1) as acceptors[97].

Nuclear magnetic resonance (NMR)

NMR has proved to study the structural, kinetics and dynamics of G4/ligand complexes. The technique provides information about G4/ligand binding under in vitro conditions. In specific cases, the conditions were set to mimic cellular media with the addition of molecular crowding agents such as PEG and Ficoll. Recently the closer conditions observed in the living cells using cytoplasmic extractions or directly inside living cells such as *Xenopus* oocytes influence strongly the environmental conditions and stability of DNA/ligand complexes. NMR study proved the suitability of the technique to validate ligands, after an in vitro assessment of promising ligands[83].

Role of promoter specific G4 binding proteins

The more prominent G4 forming region is promoter of gene where G4BPs recruit successively. In this regard, the well-studied is *c-MYC* promoter. *cMYC* and related oncogenes are considered as master regulators in cancer progression. Overexpression of these oncogenes influences cellular growth and metabolism[99]. Specificity protein 1 (Sp1) and a member of the Krüppel-like family protein are the most frequent factors able to confine both duplex and quadruplex within the promoter of the *c-KIT*, *HRAS*, and *VEGF*[100-104]. Sp1 binding to G4 forming *HRAS* promoter functions as transcriptional repressor and MAZ (another G4-binding zinc finger protein) is required for its activity[104]. Interestingly, a recent study revealed it also binds to the G4 forming *ZEB1* promoter and activates transcription of *ZEB1*, promoting breast cancer progression[105]. Another most prevalent transcription factor, Yin and Yang 1 (YY1) binding to G4 structure facilitated its dimerization andlong-range DNA looping[106]. Nucleolin, another chromatin remodelling phosphoprotein found throughout the cell, but abundant highly in the nucleolus. Apart from its functions in ribosomal biogenesis, chromatin remodelling, transcription, and apoptosis[107], nucleolin binds to *c-MYC* promoter through stabilization of the G4 structure and resulting transcriptional repression[108]. Similarly, it also binds to the G4 forming *ZEB1* promoter that overlaps to the Sp1 binding element activating transcription of *ZEB1*[105]. *In vitro*, nucleolin is showing to bind with G4s of the *VEGF*, *RET*, *PDGF-A*, bcl2, hTERT and *c-KIT* promoters. However the functional significance of these binding yet to be unravelled[109]. Another G4 binding protein, XPD helicase part of the human transcription factor II (TFIIH) complex relaxes the G4 structure in a 5′-3′ direction in an ATP-dependent manner. However another component of TFIIH, XPB helicase acts in an 3′-5′ direction to binds to G4, without unwinding it[109]. Like promoter, some G4 binding proteins can bind to the telomere region also. They can target to the non-canonical G4s of telomere and unfurl these structures by maintaining telomere length and integrity. Many proteins are characterized as G4BPs, acting on the telomeric region by attachment, stabilization and relaxing of the G4s[110]. Among them, Protection of Telomeres 1 (POT1) strongly protects the single stranded overhang from being sensitive to chromosomal damage. Single-molecule fluorescence resonance energy transfer (smFRET) reveals that a monomer of POT1 binds the G4 followed by unfolding from the 3′-5′ direction[111]. Similarly binding of the Tripeptidyl-Peptidase 1 (TPP1) to POT1 acts as enhancer that unfolds and then refolds the G-quadruplex consequently[112]. Another most abundant single-stranded DNA (ssDNA) binding protein Replication Protein A (RPA) is involved in DNA damage repair processes. It also binds to the human telomere[113]. Like POT1, RPA unfolds telomeric G4s from the 5′-3′ direction and relaxing both parallel and antiparallel G4s[114]. Other telomeric protective protein CST (CTC1–STN1–TEN1), largely involved in replication fork, but also employs critical roles at the telomere maintenance. CST binds to telomeric G4s, unfold them, effecting of the filling process of C-rich strand[115].

Involvement of G4 binding protein in DNA replication

Efficient DNA replication through relaxation of G4s is mediated by interacting with G4BPs. In this regard, Bloom protein (BLM) plays a crucial role on the leading strand replication by unfolding G4. While Werner's syndrome protein (WRN) shows its helicase function on the lagging strand replication by binding and unwinding G4s through RQC and HDRC domains. Furthermore, Preimplantation factor-1 (Pif1) is a highly conserved helicase that plays a role in G4 unwinding facilitates to suppress of genome instability[116]. Another DNA helicase Fanconi anaemia complementation group J (FANCJ) releases G4 structures for efficient DNA replication. In absence of

FANCJ, DNA replication is perturbed[117]. Recently, another tumor suppressor protein breast cancer type 1 susceptibility protein (BRCA1) has been implicated in binding of G4 and modulating in gene functions[118].

Structural properties of G4BPs

Structural analysis of G4BPs enacted that the binding regions of these proteins have certain shared domains or motifs, which are strongly devoted to interacting with G4s[119]. The RGG (Arginine-Glycine-Glycine) domain containing proteins bind to G4s. They are highly conserved and known to modulate transcription, precursor mRNA splicing, DNA damage, mRNA translation, and apoptosis[120]. Similarly, hnRNPs, nucleolin, and CIRBP that shared RGG motif also shown to have G4 binding affinities[121]. The RNA-binding domain (RBD) or ribonucleoprotein domain (RNP) is another conserved domain that occurs in approximately 0.5-1% of human genes, also known as RRM domain. Likewise proteins with RRM domains bind to G4 implicated in the regulation of transcription, translation, RNA stability, RNA processing and RNA export[122]. Similarly, Oligonucleotide/Oligosaccharide binding (OB) fold domain is formed with a β-barrel structure contains five-stranded antiparallel β-sheet and an α-helix[123]. In this context Replication protein A (RPA) with three subunits relaxes the G4s. Similarly other telomeric binding protein POT1, TPP1, the CST also bind to the G4 through multiple OB-fold domain[124-126].

G4BPs participate in chromatin remodelling and Transcription

DNA and histone modifications can cooperatively modulate the binding of regulatory factors. Several epigenetic and chromatin remodeler have been reported to bind selectively to G4s[127]. For instance, DNA (cytosine-5)-methyltransferase 1 (DNMT1) like other DNMTs is orchestrated at G4 sites of proximal promoters, and inhibit transcription[128]. Additionally, G4s facilitate gene repression through repelling H3K4me1 and H3K4me2 by allowing the recruitment of RE1-silencing transcription factor-lysine-specific histone demethylase 1A (REST-LSD1)[129]. Similarly, the recruitment of BRD3, and SWI/SNF family protein ATRX are also found to bind with G4s *in vitro*[130-131]. SMARCA4, another protein of the SWI/SNF family and CCCTC-binding factor (CTCF), a chromatin remodelling factor are recruited to the endogenous promoter at G4 sites and facilitate nucleosome repositioning[132-133]. The biological role of G4 structures formed by RNA sequences (RNA-G4) have also major role in transcription, translation, epigenetic regulation[132].

Current status of small molecule ligand binds to G-quadruplex structure

Small molecules, which especially interact with quadruplex structure of DNA, act as selective inhibitors of telomerase thereby representing as potential targets for anti-cancer therapeutics[133-134]. Several cationic porphyrins are known to efficiently interact to the G-quadruplex structure. Among these, the most prominently used ligand is the TMPyP4 (meso-5,10,15,20-Tetrakis-[N-methyl-4-pyridyl] porphine) that prohibit the telomerase to bind to the telomeric 3′ overhang by stabilizing G4 at telomeric end. *In vitro* experiments shown that TMPyP4 hinders the transcription of oncogene MYC by targeting a G4 motif in the nuclease hypersensitivity element (NHE) in the MYC promoter[135]. Though TMPyP4 also has little tendency to bind to the double helices, but its high affinity to bind to telomeric G4 may play a crucial role in cancer therapy. Another trisubstituted acridine compound BRACO-19 (N, N′-(9-(4-(Dimethylamino) phenylamino) acridine-3,6-diyl) bis(3-(pyrrolidine-1yl) propanamide) hydrochloride) shown antitumor activity[136]. In this context pyridine dicarboxamide derivative 360A/307A and bisquinolinium compounds Phen-DC(3)/Phen-DC(6) are more specific against G4 formed in the 5′-UTR of TRF2 and c-MYC promoter. Thus they inhibit the translation of TRF2 and transcription of c-MYC[137-138]. Pyridostatin (PDS) is a very potential compound that stabilizes G-quadruplex and unravel its DNA damage-related mechanisms[139-140]. The contributory role of G4 interactive compounds superfluously describes G-quadruplex function and their potential role as a therapeutic target and their generalized function is listed briefly in (Table 2).

Therapeutic relevance of G-quadruplex structure and diseases

G-quadruplex structures have been associated with several human diseases including neurological disorders[141], infectious diseases[142], and also cancer. Diverse length and loops of G-tetrad is very crucial to design G4-selective small molecules. Significant observation revealed that enriched G4 structure in regulatory regions, promoters and nucleosome free regions are selective target to G4s[143]. Many new approaches have been focused to enhance the potency of the G4-binder to kill cancer cell[144]. G4 ligands

Table 2 — G4 stabilizing ligand and their mode of functions

G4 Ligand	Chemical Name	Mode of Function	Source
TMPyP4	meso-5,10,15,20-Tetrakis-(N-methyl-4-pyridyl) porphyrin	Inhibit cell proliferation and promote cell death at high doses (>2 uM) Decreases cMYC, BCL2 and TERT protein level and reduces tumor growth	Chaschina, G. V. 2023 Yan J. 2017
BRACO-19	N,N′-(9-(4-(Dimethylamino)phenylamino)acridine-3,6-diyl)bis(3-(pyrrolidin-1-yl)propanamide) hydrochloride	Inhibit telomerase activity in cells and tumor xenografts	Burger, A. M. 2005
Pyridostatin (PDS)	4-(2-aminoethoxy)-N2,N6-bis(4-(2-aminoethoxy)quinolin-2-yl)pyridine-2,6-dicarboxamide	Arrest cell cycle and DNA DSBs induction Growth inhibition in BRCA deficient tumors	Rodriguez, R. 2012
360A/307A	2,6-pyridine-dicarboxamide	Inhibit telomeric and cMYC G-quadruplexes	Gomez, D. 2004
Phen-DC(3)/Phen-DC(6)	3,3'-((1,10-Phenanthroline-2,9-dicarbonyl)bis(azanediyl))bis(1-methylquinolin-1-ium) bis-triflate	Inhibit translation of TRF2 by binding to the G4 of 5'UTR of mRNA	Delemos, E. 2007
RHPS4	3,11-difluoro-6,8,13-trimethylquino[4,3,2-kl]acridinium methylsulfate	Induce DNA damage, inhibit telomerase activity by reducing cMYC, BCL2 and KRAS protein level	Phatak, P. 2007 Cookson, J. C. 2005 Salvati, E. 2007
QN-302	Napthalenediimides, NDIs	Inhibit tumor growth *in vivo*	Ahmed, A. A. 2023

strongly share definite structural features including (1) interactions between G-quartet through π-π stacking, (2) to introduce specific functional groups to aid the interaction with G4 loops or grooves[145-146]. Through this binding mechanism different innovative targeted therapeutic molecules are developed. These small molecules stabilize the G4 structures at telomeres, and inhibit telomerase activity. Another telomerase inhibitor G4 ligand is RHPS4 (pentacyclic acridine 3,11-difluoro-6,8,13-trimethyl-8H-quino [4,3,2-kl] acridinium methosulfate) that boost antitumoral activity in telomerase-negative (alternative length telomere; ALT) tumor cells[147]. Similarly, small cationic porphyrin ligand, TMPyP4 impairs c-Myc transcription by G4 stabilization within its promoter[148]. Therefore, different G4 ligands, such as 360A, BRACO-19, Pyridostatin (PDS), and CX-5461 exert gene repression towards *c-Myc* and several other proto-oncogenes, such as *Bcl-2*, *c-Kit* and *RET*[149]. Now, G4 is protracted as therapeutic target for regulation of gene expression by CX-5461 ligand to induce error in homologous recombination[150]. While PDS was corroborated to relieve supercoils on DNA by trapping topoisomerase II[151]. Apart from these functions, these ligands generate breaks in DNA double-strand by TOP2A through G4 formation[152]. All these findings underlie the application of G4 structures in the oncologic field and enact a significant expansion to the evolution of potential ligands that may be applied clinically.

G4 Ligands as a therapeutic target in Cancer: Novel strategies and future perspectives

While G4 structures distinguishing by large number of small ligand molecules, few ligands have poor qualities essential to reach the pre-clinical phase. Minimal cytotoxicity should be the main criteria for develop a probe rather than a G4 ligand. Low selectivity rate of G4 ligands are the major obstructions in the clinical progression of these molecules as anticancer agents due to presence of G4s in both normal as well as cancer cells. Based on this hypothesis, the rationale design of G4 ligands are indispensable to target the cancer cells more specifically. The development and design of efficient G4 binder relies on the available high-resolution NMR or crystallography, where G4 tetrad interacts through additional hydrogen bonds or electrostatic between loops[153]. Recently, some novel strategies have been explored, where G4 ligands are linked with oligonucleotides or peptide nucleic acid (PNA) and hit the specific targets by limiting the risk of side effects[154]. Another approach where nanoparticles or liposomes mediated interaction with cancer cells, would promote the authentic delivery of the G4 ligands and limit the risk of off-target effects[155]. The evaluation of the efficacy of the therapeutic

potentiality by combining these small molecules with other antitumoral drugs like, chemotherapeutic and immunomodulatory drugs are very essential for the clinical development of G4 ligands. Optimal concentration of these novel therapeutic candidates needs to be worked out to reduce toxic effects and better therapeutic efficacy.

Limitations of G4 ligand-based targeted therapies

Considering the tremendous side effect of chemotherapeutic drugs these novel target specific drugs are under trials. Despite the therapeutic potential endorsed by several of these molecules, few G4 ligands with suitable pharmacokinetic properties are enrolled for pre-clinical evaluation. In spite of their effectiveness at the biochemical and biophysical levels, it has been evident that few of G4 ligands show proper function when tested into cell based gene regulation. For example, it is incipiently reported as TMPyP4 a potential G4 ligand, recognizes both duplex and quadruplex DNA structures. It was first developed as a photosensitizer in photodynamic therapy, where photosensitizer was injectedinto the patient's blood and irradiate the diseased tissue with a high-intensity light[156]. It has anti telomerase activity and serve as a potential G-quadruplex stabilizer[157]. In absence of telomerase, cancer cells face to carry extremely short telomeres. In this situation, TMPyP4 enhances the risk of metastasis and not good as therapeutic candidate[158]. BRACO-19 is another G4 ligand that showing persuasiveness against telomerase, when tested *in vivo*. Particularly, the indigent permeability of membrane through this 3,6,9-trisubstituted acridine derivative fiercely hamper its therapeutic efficacy[159]. Although certain G4-stabilizing agents, have shown excellent exertion in cell-based assays, but poor functional activity was found to induce off-target effects. In this way, RHPS4 is represented as a sterling model because it ascertained severe cardiotoxic effects when tested in guinea pig[160].

From the above scenario, only a few molecules have entered clinical trials to date. Among these, one fluoroquinolone, Pidnaruex (CX-5461) was initially proved as a selective inhibitor of RNA synthesis and assigned to kill *BRCA1-/BRCA2* negative cells[161]. Another more promising G4 ligand is QN-302, a recently developed naphthalene diimide (NDI) derivative that strongly recognize, bind, and stabilize parallel G4 structures and deliberately shows the specificity for G4 structures confined outside the telomeres. Based on the mechanism, the anticancer properties of QN-302 ascribed that it binds to the S100P promoter G4 and hinders its expression which has a role in tumor proliferation and motility. Its pharmacological properties and overall results obtained both *in vitro* and *in vivo*, listed it into phase Ia clinical trial. In pancreatic ductal adenocarcinoma, sarcomas, gastrointestinal stromal tumors QN-302 is considered as therapeutic remedy[162]. Though the trial has started very recent, but we may have to wait for some time to know the future developments and further effectiveness of this drug.

Discussion

Majority of the G-quadruplex ligands have the potentiality to target a specific location of genome. So in recent years research has focus on them as potential therapeutic target specifically for the treatment of several types of cancer. Apart from their potentiality as good drug target, some of them also have several off-target effects, which become a major hurdle for development of drug. In this context the TMPyP4 is a good candidate for targeting both promoter and telomere G-quadruplexes. But, due to tendency to bind in duplex DNA, it may block transcription of some beneficial genes[163]. It also induces ROS in cells showing a global cell response along with changes in expression of a particular gene. It has not significant effect at low doses, thereby enhances the migration rate of cancer cells. It is obvious that cancer cell migration increase the metastasis, the ultimate cause of patient's death[164]. The pentacyclic acridium salt RHPS4 has the potentiality to target the telomere G-quadruplexes. But it also has off-target activity. It is showing some undesirable cardiotoxicity when treated in guinea pig. It ineracts with β2 adrenergic receptor and muscarinic receptors M1, M2, M3. In this way it acts as a potent inhibitor of the hERG (human Ether-a-go-go Related Gene) tail current[165]. Pyridostatin, another G-quadruplex-stabilizing small molecule showing several off-target effects like neurite retraction, synaptic loss, and dose-dependent neuronal death. PDS induces DNA double strand breaks, when cultured with primary neuron cells. It has adverse effects on neuronal death when treated in dose dependent manner[166].

Conclusion

Recent years have evidenced remarkable progress in our understanding of chromatin higher-order structure and show their importance in genomic integrity and telomere maintenance. G4 DNA-

mediated genome instability make the G4 as a significant regulator of transcription. In this perspective, future research seems especially unperturbed to significantly open new paths. Approaches that are likely to open new information include the application of G4 ligand as a therapeutic drug for disease recovery. At this stage, we like to focus on few examples that have excellent role in portraying small molecule-mediated G4 stabilization. Small molecules evaluated for their G4 stabilizing properties are innumerable and have been reported to wield a remarkable antiproliferative activity when used as therapeutic agents. Though small molecule ligands reach to certain development, many issues still need to be cleared for the design of more efficient G4 ligands. In this regard, the primary challenges include the proper identification of G4 ligands with more specific to distinguish quadruplex over duplex DNA. The preferential recognition of intermolecular and intramolecular G4 is more important in determining the biological effects exerted by some ligands. Different structural analysis like NMR, X-ray crystallography, UV/Visible spectroscopy, circular dichroism (CD), fluorescence resonance energy transfer (FRET), surface plasmon resonance (SPR), calorimetric titration, mass spectrometry have developed to identify the G4 target and the G4-ligand complex interacting residues. In this perspective the computational method and biophysical approaches have been declared as useful for the rational design of G4 ligands. Recently artificial intelligence based drug design also open a new path for researcher. This new approach can identify very efficiently the toxicity, bioavailability and solubility of drug based on their structure. It can modulate drug delivery system also by predicting potential risk.

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Conflict of interest

Both the authors declare no conflicts of interest.

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