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**REVIEW ARTICLE** Daisuke Miyoshi *et al.* Three- and four-stranded nucleic acid structures and their ligands

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# Three- and four-stranded nucleic acid structures and their ligands

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DNA

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**Transcription** 

Nucleic acids have the potential to form not only duplexes, but also various non-canonical secondary structures in living cells. Non-canonical structures play regulatory functions mainly in the central dogma. Therefore, nucleic acid targeting molecules are potential novel therapeutic drugs that can target 'undruggable' proteins in various diseases. One of the concerns of small molecules targeting nucleic acids is selectivity, because nucleic acids have only four different building blocks. Three- and four-stranded non-canonical structures, triplexes and quadruplexes, respectively, are promising targets of small molecules because their three-dimensional structures are significantly different from the canonical duplexes, which are the most abundant in cells. Here, we describe some basic properties of the triplexes and quadruplexes and small molecules targeting the triplexes and tetraplexes.

Coding RNA (1.5%

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# Introduction

Most small-molecule therapeutics in clinical use currently target proteins. Human cells express about 20000 species of proteins, following the hypothesis of "one gene expresses one protein". Within these proteins, up to 15%, corresponding to 3000 species of proteins, are disease related (Fig. 1).<sup>1-3</sup> Within the disease-related proteins, many of the proteins are termed 'undruggable', which refers to proteins that are difficult to target pharmacologically. An undruggable protein often lacks a binding site for a small molecule. Intrinsically disordered proteins, lacking fixed or ordered structures, are also often undruggable. The most well-known examples of the former and the latter undruggable proteins are RAS<sup>4</sup> and MYC,<sup>5</sup> respectively, although these two proteins are important targets for cancer therapeutics. Moreover, recent interactome studies suggest that there are up to 650 000 protein-protein interactions (PPIs) in humans.<sup>6</sup> PPIs are also considered undruggable by small molecules because of the large, flat, and featureless surfaces of PPIs.<sup>7</sup> In fact, only a few percent of these PPIs can be targeted with small drugs.<sup>8,9</sup> Therefore, alternative strategies for the inactivation of these disease-related undruggable proteins are required.

The 20 000 species of proteins in a human cell are encoded by only 1.5% of the human genome, which contains  $3\,000\,000\,000$  base pairs in total (Fig. 1).<sup>10,11</sup> Among these proteins, fewer than 700 have been targeted by drugs. This corresponds

Fig. 1 Schematic diagram depicting protein expression in the central

dogma and the potential nucleic acid-targeted druggable genome.

Protein (~20000)

to only 0.05% of the genetic information encoded in the human genome. However, around 80% of the human genome is transcribed to RNA. As described above, DNA coding amino acid information corresponds to 1.5% of the genome, so the remaining part of the 80% is non-coding RNA (ncRNA),<sup>12</sup> which controls the central dogma at various steps, such as replication, transcription, processing and localization of mRNA, and translation. When compared with druggable proteins, there is far more opportunity to target RNAs to expand druggability and improve our understanding of biological processes governing the central dogma. If DNA and RNA can be targeted, protein products can be up- or down-regulated at the transcriptional and translational levels.

The canonical structures of DNA and RNA are B-form and Aform duplexes, respectively, with Watson–Crick base pairs. Canonical duplexes are suitable and highly adapted for their functions: storage, inheritance, and the transition of genetic information. However, both DNA and RNA can fold to form various non-canonical and transient structures (Fig. 2a–m), depending on the nucleotide sequence as well as the

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Disease-related proteins

Undruggab

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Druggable

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**Fig. 2** Schematic illustration of a canonical duplex (a) and non-canonical structures (b)–(m) of nucleic acids. (b) Mismatch, (c) parallel duplex, (d) hairpin loop, (e) bulge, (f) internal loop, (g) three-way junction, (h) four-way junction, (i) pseudoknot, (j) kissing loop, (k) triplex, (l) G-quadruplex, and (m) i-motif. Black arrows indicate strand orientation. Red and blue bars with circles represent canonical Watson–Crick and non-canonical base pairs, respectively. (n) Schematic illustration of proteins targeting non-canonical structures on the long canonical duplex of nucleic acids.

surrounding molecular environment. Similar to a knot providing a firm place to grip a rope (Fig. 2n), non-canonical structures can be recognition sites and controlling elements for a series of enzymes, including DNA and RNA polymerase, helicase, topoisomerase, nuclease, DNA and RNA modifiers, spliceosome, and ribosome, as well as transcriptional factors.<sup>13–15</sup> In fact, roughly 1500 proteins are predicted to bind to RNA in a sequence- and structure-selective manner.<sup>16</sup> Small molecules targeting DNA and RNA could regulate not only the expression of proteins but also the functions of the DNA- and RNA-binding proteins. Thus, small molecules targeting non-canonical structures have drawn attention as new therapeutic regimens.<sup>17–19</sup> Risdiplam, sold under the brand name Evrysdi, represents a milestone given its status as the first small molecule drug that targets RNA.<sup>20</sup>

One of the concerns of small molecules targeting nucleic acids is selectivity, because nucleic acids have only four different building blocks, whereas proteins are composed of 20 amino acids. In addition, it is generally considered that nucleic acid structures are static and monotonous. In contrast, it has been discussed that there are up to 10000 unique protein folds in nature.<sup>21-23</sup> From this point of specificity, three- and fourstranded non-canonical structures, triplexes and quadruplexes, respectively, are promising because their three-dimensional structures are significantly different from the canonical duplexes. Noteworthy, recent progresses in bioinformatics, DNA and RNA sequencing, and bioimaging have demonstrated the existence of the multiplexes and their biological roles in living cells of various organisms from virus to human. In this review, we briefly describe the triplex and quadruplex structures of nucleic acids and their potential biological roles. We then describe recent progress in the development of small molecules that target these non-canonical nucleic acid structures.

### Triplexes

#### Structure and biological role of triplexes

The canonical duplex has two grooves formed by the sugarphosphate backbones of the two strands. The grooves play a vital role in interactions with other molecules, such as protein, small molecules, metal ion, and water. The grooves further play an important role in the sequence-specific recognition of the duplex by single-stranded nucleic acids, either DNA or RNA. These interactions are mediated by the formation of Hoogsteen-type hydrogen bonds between the bases of the third strand, often called a triplex-forming oligonucleotide (TFO), and the purine bases of the duplex formed by a polypurinepolypyrimidine sequence (Fig. 3a). The first example of a triplex was reported by Felsenfeld et al. in 1967. They found the formation of an intermolecular RNA triplex by two strands of polyribo(uridylic acid) and one strand of polyribo(adenylic acid).<sup>24</sup> After the discovery of the triplex, many triplex structures under various conditions have been identified.<sup>25</sup> There are two classes of triplex motifs, distinguished by the orientation of the TFO, which binds to the major groove of the target duplex. A polypyrimidine TFO binds to the polypurine strand of the duplex via Hoogsteen hydrogen bonds in a parallel fashion (in the same orientation as the purine strand of the duplex). In contrast, a polypurine TFO binds in an antiparallel orientation to the polypurine strand of the duplex via reverse-Hoogsteen hydrogen bonds.<sup>26,27</sup> In an antiparallel triplex, the base pairs are G:G-C and A:A-T (":" and "-" indicate Hoogsteen and Watson-Crick base pairs, respectively). In a parallel triplex, the base pairs are C<sup>+</sup>:G-C and T:A-T (C<sup>+</sup> represents a protonated cytosine). Thus, the ideal target of a TFO is a duplex of a homopurine sequence in one strand and a homopyrimidine sequence in the complementary strand. For both parallel and antiparallel motifs, contiguous homopurine-homopyrimidine runs of at least 10 base pairs are required for thermodynamically



**Fig. 3** (a) Base pairs in parallel and antiparallel triplexes. (b) Schematic illustration of glycosidic conformation for nucleobases in TFO.

stable TFO binding.<sup>28</sup> In addition, all TFOs adopt *anti*-conformations (Fig. 3b),<sup>29</sup> although 8-oxoadenin in a TFO can adopt a *syn*-conformation.<sup>30</sup> The *anti*-conformation in a TFO is the same as that of nucleotides in B- and A-form duplexes.

Large numbers of potential triplex forming sites have been identified in the human genome.<sup>31</sup> Most annotated genes in the human genome contain at least one triplex-forming site, and these sites are enriched in the promoter regions,<sup>32</sup> indicating a potential role of triplex in controlling gene expression. These TFO-binding sites have attracted much attention because of their therapeutic potential to inhibit the expression of disease-related genes. Evidence for the existence of triplexes in cells comes from triplex-binding antibodies.<sup>33,34</sup> However, small molecules probing the triplex are still required to observe the direct dynamic behavior of the triplex over time in living cells.

#### **RNA triplexes**

Triplexes have been observed in transcripts such as tRNA<sup>Phe</sup>, telomerase RNA, self-splicing group II introns, U2-U6 snRNAs, riboswitches, virus RNAs, and RNA stability elements.<sup>35</sup> These RNA triplexes play roles in telomere synthesis, RNA splicing,

binding to ligands and ions in metabolite-sensing riboswitches, and protecting RNA from degradation.

Telomerase RNAs in various eukaryotes have a conserved pseudoknot in which a triplex exists.<sup>36,37</sup> The triplex structure is essential for telomerase catalytic activity,<sup>38</sup> demonstrating that the triplex structure rather than the nucleotide sequence is conserved and required for the ribonucleoprotein function. Triplexes observed in riboswitches are also important as binding sites of a series of ligands. For example, SAM-II riboswitch has triplex-forming sites that serve as structural platforms for ligand binding.<sup>39</sup> RNA triplexes have also been proposed for various viruses, such as Kaposi's sarcoma-associated herpesvirus (KSHV).<sup>40</sup> The triplexes protect the viral RNAs and inhibit decay by host nuclear RNA degradation to accumulate in KSHVinfected cells.41,42 Human metastasis-associated lung adenocarcinoma transcript 1 (MALAT1), a long noncoding RNA (lncRNA), folds to form a triplex (Fig. 4). MALAT1 is localized in nuclear speckles and is involved in the regulation of gene expression.43,44 MALAT1 is upregulated in multiple cancer types, depending on the triplex formation, making it a potential drug target.<sup>45,46</sup> Unlike many lncRNAs that are rapidly degraded and thus expressed at near-undetectable levels, MALAT1, as well as MEN  $\beta$  and PAN, which are also known to form triplexes (Fig. 4), are stable transcripts with long half-lives.<sup>47</sup> It has been proposed that the triplexes formed by these lncRNAs inhibit nuclear RNA decay.48 Although the biological roles of RNA stability elements remain unclear, the triplex supports the transport, stability, and translation of an RNA, allowing efficient repression by microRNAs.



Fig. 4 Secondary structures of MALAT1, PAN, and MENβ RNAs.

Moreover, it was recently reported that lncRNAs bind target DNAs in a sequence-specific manner by forming RNA–DNA triplexes.<sup>49,50</sup> This process allows lncRNAs to recruit proteins to specific genomic regions and to regulate gene expression. LncRNAs such as Fendrr,<sup>51</sup> MEG3,<sup>52</sup> KHPS1,<sup>53</sup> PARTICLE,<sup>54,55</sup> and HOTAIR<sup>56</sup> bind to their target DNAs in a sequence-specific manner, utilizing the RNA–DNA triplexes. It has further been proposed by computational analysis that there are many triplex target sites of lncRNAs in the genome.<sup>57</sup> Interestingly, such bioinformatic analysis has revealed a group of genomic regions that may have a very high propensity for triplex formation with a wide range of different RNAs.<sup>58</sup> Excellent reviews have discussed the biological roles of RNA–DNA triplexes.<sup>59,60</sup>

#### **Triplex-binding proteins**

It has been reported that some proteins such as histones H1 and H2A, and topoisomerase II, bind to AT-rich sequences.<sup>61–63</sup> A protein that binds preferentially to TAT DNA triplexes was reported for the first time in 1991, although its identification was not possible at that time.<sup>64</sup> Proteins that specifically recognize certain DNA triplex motifs have been reported.<sup>65,66</sup> Since the 2000s, many proteins have been identified as DNA triplex-binding proteins<sup>67–83</sup> (Table 1). Although the biological roles of these DNA triplex-binding proteins are highly diverse, it is reasonable that some helicases are triplex-binding proteins. In addition, nuclear proteins are known to bind DNA/RNA triplexes, but their biological functions are unexplored.<sup>84</sup>

#### Small molecules that target triplexes

The Hoogsteen base pairs between the TFO and the target duplex are generally weaker than the Watson–Crick base pairs in the duplex.<sup>85</sup> The instability of the triplex under physiological conditions limits its biological roles and applications. Of note, a parallel triplex is stable only at acidic pH, because the protonation of cytosines is required for Hoogsteen base pairing, although the stability of antiparallel triplexes does not depend on solution pH.<sup>86</sup> Moreover, multivalent cations such as Mg<sup>2+</sup> are required to neutralize the electrostatic repulsion between the three nucleic acid strands. The triplex but not the

duplex is stabilized under molecular crowding conditions that mimic a cellular environment.<sup>87</sup> To overcome the limited thermal stability, small molecules as well as chemical modifications of TFOs have been applied.

Hélène and co-workers demonstrated the enhancement of triplex thermal stability by attaching small molecules such as acridine, ellipticine, and psoralen to the TFO.<sup>88-90</sup> Small molecules that bind strongly and selectively to the triplex can also stabilize the triplex without covalent modification. Some triplex-binding small molecules are shown in Fig. 5. Ethidium bromide stabilizes a triplex containing only TAT base pairs,<sup>91</sup> whereas it destabilizes a triplex containing both TAT and C<sup>+</sup>GC base pairs because of electrostatic repulsion with C<sup>+</sup>.<sup>92</sup> Some of the earliest discovered small molecules that stabilize the TAT and C<sup>+</sup>GC of triplexes are benzopyroindole derivatives (BePI and BgPI) and coralyne.93-96 BePI stabilizes TAT-rich triplexes, whereas coralyne does not show sequence selectivity. BePI enhances the sequence-specific inhibition of transcription initiation of a specific gene by RNA polymerase by a TFO. Quinoxaline derivatives BfPQ and BQQ were further developed as analogues of BPI derivatives.<sup>97–99</sup> These molecules have been shown to significantly stabilize triplexes under physiological conditions, whereas they bind poorly to duplexes. Notably, BQQ promotes a short TFO to bind to its target site in a plasmid and enhances the inhibition of restriction enzyme cleavage of the target site.

Wilson and co-workers proposed that the structural properties of a triplex intercalator include the following: (i) the compounds should be cationic because of the high negative charge density of the triplex. (ii) The compounds should have an aromatic surface to form stacking interactions with the three bases in the triplex. (iii) The aromatic compounds should have torsional flexibility, because the three bases in a triplex have torsional freedom.<sup>100</sup> Based on these properties, they synthesized a series of quinoline derivatives and found that a 2-naphthyl derivative could bind to poly(dA)·2poly(dT) to a much greater extent than to the corresponding duplex.<sup>100</sup> Other triplex intercalators, such as fisetin,<sup>101</sup> luteolin,<sup>102</sup> palmatine,<sup>103</sup> sanguinarine,<sup>104</sup> and ruthenium(II) complexes,<sup>105</sup> are also shown in Fig. 5.

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        Table 1
        Triplex-binding proteins
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Protein	Function/target	Ref.
GAGA factor	Gene regulation and alteration of chromatin structure/pyrimidine motif	67
Stm1	Involved in mitosis/purine motif	68
CDP1	Chromosome segregation and histone displacement/purine motif	69
type III intermediate filaments	Gene regulation, DNA repair, and chromatin remodelling/pyrimidine motif	70
Loricrin	Keratinocyte cornification/purine motif	71
Orc4	Origin organization during DNA replication/purine motif	72
hnRNPs	DNA and RNA binding/synthetic oligo DNAs	73
Tn7	Transposon insertion/pyrimidine motif	74,75
XPC-hHR23B, XPA-RPA	DNA damage recognition/TFO-directed psoralen-interstrand crosslink	76
HMGB1	Chromatin structure, transcriptional regulation/triplex-directed psoralen interstrand crosslinks	77
U2AF65, PSF, p54nrb	Splicing factors/purine motif	78
RecQ, BLM, WRN,	$3^7 \rightarrow 5^7$ helicase/pyrimidine motif with a free 3' tail	79
FANCJ	$5' \rightarrow 3'$ helicase/pyrimidine motif with a free 3' tail	80
DHX9	$3' \rightarrow 5'$ helicase/purine motif with a free 3' tail	81
SV40 large T-antigen	$3' \rightarrow 5'$ helicase/pyrimidine motif with a free 3' tail	82
p53	Tumor suppressor/intra and intermolecular pyrimidine motif	83



Groove binders have also been demonstrated to have affinity with DNA triplexes. It has been shown that netropsin, a drug that interacts with the minor groove of a DNA duplex, binds the minor groove of a triplex and destabilizes it, but stabilizes the duplex.<sup>106,107</sup> Umemoto et al. showed by using distamycin 2, an analogue of netropsin, that the drug does not destabilize.<sup>108</sup> Breslauer and coworkers showed that berenil, DAPI, ethidium, and netropsin induce the formation of a TAT DNA/RNA/DNA triplex.<sup>109</sup> In addition, a series of small molecules such as methyl green and mitharamycin, which bind and destabilize triplexes, have been reported.<sup>110-112</sup> Aminoglycosides, especially neomycin, stabilize a triplex, while they do not alter the stability of a duplex.<sup>113</sup> Triplex stabilization by neomycin is saltand pH-dependent, and increasing the concentrations of K<sup>+</sup> and Mg<sup>2+</sup> and the solution pH decreases the stabilization effects of neomycin.<sup>114,115</sup> These aminoglycosides bind with not only DNA triplexes, but also RNA and DNA/RNA triplexes.<sup>115</sup> A combination of intercalation and groove binding has also been reported. Intercalator-neomycin conjugates have been studied to enhance binding affinity with DNA triplexes. BQQneomycin enhances the binding affinity with a TAT DNA triplex up to almost 1000-fold more than that of neomycin through a dual recognition mode: groove binding and intercalation of neomycin and BQQ, respectively.<sup>116</sup>

As described above, helix-forming MALAT1 lncRNA is a potential anticancer target due to its overexpression in cancer cells, and its knockdown reduces tumor growth. Deletion and mutation studies in the triplex-forming region have shown that alterations in the stability of the triplex lead to significant changes in transcript levels.<sup>117,118</sup> Several small molecules, such as diphenylfuran derivatives, have been reported as lncRNA triplex-targeting molecules.<sup>119</sup> Small molecules targeting the MALAT1 triplex with high selectivity have also been reported, of which a representative example is compound 5 (Fig. 5).<sup>120</sup> Compound 5 was obtained by small molecule microarray screening of a library comprising approximately 26 000 compounds. Compound 5 regulates MALAT expression. Since NEAT1 has a triplex structure, these results confirm that compound 5 selectively binds to the MALAT1 triplex in living cells. NMR experiments and MD simulations demonstrated that compound 5 binds within the deep and narrow major groove of the MALAT1 triplex through several van der Waals interactions with the phosphate and nucleobase groups of the Hoogsteen strand and the Watson-Crick strands. An RT-qPCR experiment showed that the MALAT1 expression level in mouse model mammary tumor organoids was suppressed by 51% following treatment with 1 µM of compound 5 whereas NEAT1 expression was unaffected,120 demonstrating the high sensitivity of compound 5. Molecules such as compound 5, which specifically target the MALAT1 triplex, are a promising new class of anticancer therapeutics and molecular probes for the treatment and investigation, respectively, of cancers driven by MALAT1.

# **G**-quadruplexes

A G-quadruplex (G4) structure of guanylic acid was reported for the first time by Gellert *et al.* in 1962.<sup>121</sup> The G4 structure of a

guanine-rich telomeric sequence was then revealed by Sen and Gilbert in 1988.<sup>122</sup> These studies made us realize that guaninerich sequences can form G4s (Fig. 6a). Putative G4-forming sequences are generally considered to have the following consensus:  $G_{2 \leq} L_{1 \leq} G_{2 \leq} L_{1 \leq} G_{2 \leq} L_{1 \leq} G_{2 \leq}$ , in which guanine stretches composing more than two guanines are connected by linker loop sequences (L) including at least one nucleotide.<sup>123</sup> These putative G4-forming motifs are observed ubiquitously in diverse organisms' genomes. Among the various putative G4forming sequences, the most well-studied G4 is that of telomere DNA. Human telomere DNA comprises repetitive sequences of the hexanucleotide  $d(TTAGGG)_n$  repeat unit. In normal human somatic cells, telomere DNA ranges from 5-15 kb in length with an extended single-stranded 3'-overhang of a few hundred bases (Fig. 6b).<sup>124-126</sup> In each cell division, the telomere DNA is shortened because of the end replication problem, which leads to apoptosis.<sup>127</sup> In contrast, the telomere DNA length is maintained in cancer cells. In most cancers, telomerase, a ribonucleoprotein, extends the telomere 3' overhang by its reverse transcription activity.<sup>128,129</sup> Thus, telomerase inhibitors provide a new approach to cancer chemotherapy.<sup>130</sup> The hybridization between the telomere DNA and its complementary cytosine-rich sequence in the telomerase RNA is essential for the reverse transcription activity.<sup>131</sup> Formation and stabilization of the G4 in the telomere overhang by a small molecule can inhibit telomerase activity, and thus cancer cell proliferation.  $^{\rm 132,133}$ 

A G4 exhibits various topologies, parallel, antiparallel, and hybrid, in an intermolecular and intramolecular manner (Fig. 6c).<sup>134–137</sup> G4s show such highly structural polymorphisms depending on the nucleotide sequence as well as molecular environmental factors.<sup>138</sup> Moreover, G4 guanines can adopt either an anti- or syn-conformation, resulting in different B-form duplexes. The resulting pronounced variations in the glycosidic torsion angles result in four grooves of different sizes, which could be important for the specificity of G4 ligands binding to target G4s. Parallel G4s comprised exclusively of guanines adopt the *anti*-glycosidic conformation (Fig. 6c)<sup>139</sup> whereas in an antiparallel G4, guanines adopt both anti- and syn-conformations and at least one of the four strands must be oriented antiparallel to the other strands (Fig. 6c).<sup>139</sup> In this chapter, factors affecting the structure and stability of G4 will be briefly explained.

# Factors affecting the structure and stability of G4 originating from the nucleotide sequence

**Hydrogen bonding**. Like the canonical duplex, hydrogen bonding interactions within a G-quartet are critical for the structural stability of G4. The four guanine bases in a coplanar arrangement form eight Hoogsteen-type hydrogen bonds



**Fig. 6** (a) Schematic illustration of the folding of a guanine-rich (G-rich) sequence. The G-rich sequence folds into a G-quadruplex (G4) *via* a G-quartet. The three-dimensional structure of G4 is adapted from PDB ID: 2HY9. Guanine bases are highlighted in red. (b) Schematic illustration of a telomeric end with a double-stranded G-rich strand (red) and cytosine-rich (C-rich) strand (blue) and with a single-stranded G-rich region. Guanine and cytosine bases are highlighted in red and blue, respectively. (c) Three-dimensional structure of parallel (PDB ID: 2M27, ref. 134), antiparallel (PDB ID: 6ZX7, ref. 135), and hybrid (PDB ID: 2HY9, ref. 136) G4s.

(Fig. 6a). The hydrogen bonds are formed between the N1 on one guanine with the O6 on the neighbor guanine, and the N2 on the first guanine with the N7 on the second guanine, resulting in eight cyclic hydrogen bonds in each G-quartet. By using a series of modified bases, it has been found that the central part of the G-quartet, including the central hydrogen bonds, contributes more than the outer hydrogen bonds to stabilize G4.<sup>140</sup>

 $\pi$ - $\pi$  stacking interactions. Not only hydrogen bonds, but also the  $\pi$ - $\pi$  stacking interactions between G-quartets, which may be one of the largest planer aromatic surfaces of biomolecules, are central to the structure and stability of G4 (Fig. 7).<sup>141-144</sup> A greater number of G-quartets leads to more stable G4s, although the G4 structures reported so far contain two to four G-quartets. The stacking interactions among intermolecular G-quartets can induce multimerization of G4s. Phan *et al.* reported the dimerization of G4s of (GGGT)<sub>4</sub>.<sup>145-147</sup> Multimerization of G4s further induces a high-order structure, known as a G-wire.<sup>36,148-153</sup> A G-wire-like formation has been proposed as a possible structure of the long telomere DNA, though other high-order structures, such as a bead-on-a-string structure, have also been proposed.<sup>154-156</sup>

Loop composition. An intramolecular G4 has three loops connecting the four guanine stretches. An intermolecular G4 of two strands has two loops. Some interactions, such as stacking and hydrogen bonding interactions between bases in the loops and between the bases in the loops and the neighboring G-quartets, are also important for the stability and topology of G4 (Fig. 7). It has been reported that loop length plays a role in the structure and stability of G4.<sup>123,157-160</sup> Generally, longer loops result in a more unstable G4. Mergny and co-workers demonstrated that each added base in a loop destabilizes it by 2 °C of melting temperature or by 0.3 kcal mol<sup>-1</sup> of  $\Delta G^{\circ}$  in the presence of potassium ions.<sup>161</sup> Moreover, the same group showed that both the conformation and thermal stability are greatly dependent on loop permutation.<sup>123</sup> The G4 conformation depending on the loop length has also been observed in other studies. Hazel et al. found that a parallel intramolecular

G4 is folded when three loops with a single base are present.<sup>158</sup> When single thymine loops are present in combination with longer length loops, or when all loops compose more than two bases, both parallel and antiparallel G4s can be formed.<sup>158</sup>

Flanking sequences. The flanking sequences next to the first and last guanine stretches also play a role in the structure and stability of G4s. The flanking nucleotides at the 5' and 3' ends can form additional stacking and hydrogen bonding interactions with the neighboring G-quartet or the coalescing loop (Fig. 7). For example, the capping structures formed by the extended flanking nucleotides have been shown to contribute to the stabilization of the c-MYC promoter G4 and the G4 aptamer to the hemagglutinin of influenza a virus.<sup>162,163</sup> The flanking sequences further affect the whole G4 structure. The flanking bases contribute to a particular type of G4 by the specific capping structures formed by the flanking and loop bases.<sup>136,164,165</sup> In addition, completely truncated sequences without any flanking residues are often prone to form higherorder intermolecular structures.<sup>162</sup> These studies lead to the conclusion that each G4 in DNA and RNA has a unique capping structure formed by its specific flanking and loop sequence, and it contributes to the stability and overall structure of a G4.166

**G4-duplex hybrid.** Phan and coworkers structurally demonstrated by NMR that thermally stable G4-duplex junction can be formed in various topological combinations (Fig. 8a).<sup>167–169</sup> The G4-duplex hybrid can be identified not only by NMR but also by a fluorescent probe and an intrinsic fluorescence of G4.<sup>170,171</sup> Importantly, a combined genomic and structural study of the G4-duplex hybrids identified around 80 000 G4-duplex hybrids-forming sequences in the human genome. About 60% of these sequences were strand-specifically located in genic/ promoter regions.<sup>172</sup> These G4-duplex hybrids have been reported to play multiple roles in determination of topology, folding and unfolding kinetics, and cation binding of G4.<sup>173–176</sup> Moreover, G4s involving the duplex showed more efficient



**Fig. 7** Factors affecting the structure and stability of G4. Note that not all factors are shown for clarity. For example, many more hydrogen bonds and stacking interactions are formed in these structures.



**Fig. 8** (a) Schematic structure of the G4-duplex hybrids. G-quartet and Watson–Crick base pair are shown in gray and yellow. Green shows non-canonical base pairs. (grey), (b) three-dimensional G4-duplex hybrid structure in PIM1 gene (PDB: 7CV3). The duplex region is highlighted in yellow.

transcriptional inhibition.<sup>170,177</sup> Since the G4-duplex hybridforming DNA sequences were identified in the sense and antisense strands,172 corresponding RNA structure can exist in naturally occurring RNAs such as PIM1 mRNA (Fig. 8b)<sup>178</sup> as well as synthetic RNA aptamer such as SC1<sup>179</sup> and Spinach.<sup>180,181</sup> The G4-duplex hybrid can be a promising target in diverse biological and pathological processes, because the junction between the G4 and the duplex gives distinct structure from the "canonical" G4, which has shorter loop length. Moreover, the duplex region could be useful for sequence selective binding for a target G4.

#### Factors affecting the structure and stability of G4 originating from the surrounding environment

Cations. A cation coordinates the O6 carbonyl from the guanines in a G-quartet, resulting in reduced electrostatic repulsion of the central oxygen atoms, enhancing hydrogen bonding interactions, and stabilizing the  $\pi$ - $\pi$  stacking interactions.<sup>182</sup> Therefore, cations are critical for the structure and stability of G4s. The stabilization effects of monovalent and divalent cations on the thrombin binding aptamer G4 are on the order of  $Sr^{2^+}>\,Ba^{2^+}>\,K^{\scriptscriptstyle +}>\,Rb^{\scriptscriptstyle +}>\,NH_4^{\,\,+}\,\sim\,Na^{\scriptscriptstyle +}>\,Cs^{\scriptscriptstyle +}$  $(\sim Li^{+})$  (Fig. 9a).<sup>183-187</sup> In particular, Sr<sup>2+</sup> and K<sup>+</sup> have a large stabilization effect on G4s.188 These cation effects are common for DNA and RNA G4s with different nucleotide sequences. Thus, the dependency of structural stability on a coexisting cation is often utilized as supporting evidence of G4 formation of a nucleotide sequence of interest. In contrast to the stabilization effects of Sr<sup>2+</sup> and Ba<sup>2+</sup>, in the presence of the physiologically relevant monovalent cations  $K^+$  and  $Na^+$ , low



Fig. 9 (a) Schematic structure of the thrombin binding aptamer (TBA) (left). Melting temperature ( $T_m$ ) of the TBA in the presence of various cations (right). Adapted from ref. 186. Copyright © 2001 American Chemical Society. (b) Schematic structure of  $d(G_3T_4G_3)$  (left) and the relationship between the dehydration energy of the cations and the thermodynamic stability of G4 (right). Adapted from ref. 192. Copyright © 1996 American Chemical Society

concentrations of divalent cations destabilize G4 in the following order:  $Zn^{2+} > Co^{2+} > Mn^{2+} > Mg^{2+} > Ca^{2+}$ .<sup>189–191</sup> Moreover, divalent metal ions at higher concentrations induce a structural transition from an antiparallel G4 to a parallel G4.<sup>191</sup>

The ion radius of a cation is critical for binding with G4. It is generally considered that this is because there is an optimal size that fits into the cavity of the G-quartet(s). It is also evident that dehydration is required for cation coordination. Thus, the hydration energy is important to determine the structural stability of G4s.<sup>144,186</sup> Hud et al. studied by NMR the competition between Na<sup>+</sup> and K<sup>+</sup> for coordination with G-quartets using the oligonucleotide  $d(G_3T_4G_3)$ , which forms an antiparallel G4 in the presence of  $Na^+$  and  $K^+$  (Fig. 9b).<sup>192</sup> They found that the more favored binding of K<sup>+</sup> than Na<sup>+</sup> is driven by the greater dehydration energy of Na<sup>+</sup> than K<sup>+</sup>. Hydration from cations is also important for the structure and stability of G4s under cellmimicking molecular crowding conditions, as explain in the next section.

Molecular crowding. The molecular environment inside of cells is surprisingly crowded with many biomolecules.87,193-197 The total concentration of biomolecules reaches 400 g  $L^{-1}$ , and about 40% of the cell volume is occupied by them. These biomolecules inside cells result in molecular crowding conditions that are totally different from those in a test tube, where most biochemical experiments are carried out. Some key interactions such as hydration and counterion condensation, which are required to form nucleic acid structures, are dependent on the surrounding molecular conditions. Moreover, biochemical reactions are temporally, spatially, and specifically controlled under such complex molecular crowding conditions. Thus, it is necessary to study biomolecules under cell-mimicking conditions to unveil their characteristics inside of cells.

In many cases, molecular crowding conditions induced by the addition of neutral cosolute molecules increase the thermodynamic stability and folding kinetics of G4s.198-205 Stabilization effects of molecular crowding have been reported for other non-canonical structures such as triplexes, junctions, dangling ends, and loops.<sup>156,206,207</sup> In contrast to the stabilization effects on non-canonical structures, molecular crowding destabilizes the canonical duplex.<sup>198,208</sup> These opposite effects on non-canonical and canonical structures indicate that G4s and other non-canonical structures are thermodynamically more stable under molecularly crowded cellular conditions than has been considered based on test-tube experiments. Thus, G4s as well as other non-canonical structures of nucleic acids are promising candidates as bidding sites for proteins and small molecules. These molecular crowding effects are attributed to the osmotic pressure that stabilizes the less hydrated G4 and other non-canonical structures.200,201,209-212 In contrast, positive hydration changes through duplex formation led to destabilization under molecular crowding conditions, where the concentration and activity of water molecules decrease.213,214

In addition, structural polymorphisms of G4 are induced and regulated by molecular crowding. For example, molecular crowding induces a structural transition from a duplex to a G4 in a mixture of telomere G-rich and complementary C-rich strands.<sup>215</sup> Molecular crowding further alters the strand orientation of G4s from antiparallel and mixed to parallel,<sup>202,216–219</sup> which regulates the activity of enzymes such as telomerase and affects protein and ligand binding to G4s.<sup>220–222</sup>

**G4 binding proteins.** Many G4 binding proteins have been identified by affinity proteomics and computational analysis (Table 2).<sup>223–230</sup> A database of G4 binding proteins is open to the public (https://bsbe.iiti.ac.in/bsbe/ipdb/).<sup>229,231</sup> Here, we briefly introduce proteins that bind to DNA and RNA G4s.

DNA G4 binding proteins. The best studied G4 binding proteins are those that bind to telomere DNA and maintain the telomere length by regulating telomerase activity.<sup>248</sup> Shelterin, which is important for homoeostasis of telomeric length, also binds to G4 in the c-MYC promoter.<sup>249</sup> Because of the importance of c-MYC as a transcription factor and thus as a promising target for anticancer drugs,<sup>250</sup> proteins targeting c-MYC G4s have been focused on. Nucleolin binds to the c-MYC promoter G4, promotes the formation of the G4, and inhibits c-MYC promoter activity.<sup>232</sup> Contrary to these studies, promoter G4s are often found in active genes.<sup>251,252</sup> Recently, it was reported that transcription factors can selectively bind G4s, and that G4s and the i-motif, which is another four-stranded non-canonical structure of the complementary cytosine-rich strand as will be discussed later, recruit transcription factors, leading to active transcriptional output.<sup>253</sup> Although further studies are required to confirm whether there is a universal upregulatory or down-regulatory role for G4s in transcription,<sup>254</sup> it is now evident that G4s largely affect transcription. DNA methyltransferase enzymes, which catalyze cytosine methylation at specific locations of the genome, show high affinity with G4s derived from c-MYC as well as CDKN1C and MEST.<sup>233</sup> Other c-MYC G4-binding proteins include Pif1 helicase;<sup>241</sup> nucleophosmin, which is a nucleocytoplasmic shuttling protein;<sup>234</sup> ADAR (double-stranded RNA-specific adenosine deaminase), which has a Z-DNA-binding domain;<sup>235,255</sup> and DNA polymerase eta.<sup>256</sup> G4-forming promotor regions of other genes, such as *BCL-2*, *KRAS*, *MYB*, *KIT*, and *VEGF*, are also recognized by proteins including PARP1, hnRNP A1, and MAZ, many of which play a role in transcriptional regulation, chromatin remodeling, and DNA repair.<sup>257</sup>

Although many G4-binding proteins have been identified, there are still a very limited number of three-dimensional structures of G4-protein complexes. Three proteins, RHAU,<sup>258,259</sup> RAP1,<sup>260</sup> and a designed ankyrin protein utilize an  $\alpha$ -helix to recognize a G-quartet (Fig. 10).<sup>261</sup> A single  $\alpha$ -helix of RHAU and RAP1 stacks on an exposed G-quartet. In contrast, ankyrin derivative uses a bundle of helices and loops to bind an exposed G-quartet. These binding modes lead to the specific recognition of the parallel G4. In these complexes, amino acids such as Arg, Ala, Tyr, Leu, and Met of the proteins form  $\pi$ - $\pi$  and CH- $\pi$ interactions with the bases and the sugar ring of the parallel G4. In addition, electrostatic and hydrogen bonding interactions between DNA and polar/basic residues contribute to stabilize the interactions.

Because G4s in the genome inhibit replication and transcription, DNA G4s must be unfolded to proceed with these reactions. Thus, helicases are another category of G4-binding protein.<sup>262–264</sup> A combination of G4 helicases and DNA replication proteins have therapeutic potential against cancer, because G4 helicases are upregulated in cancer cells.<sup>265</sup> In fact, cancer cells deficient in a G4 helicase, FANCJ, have higher sensitivity to the G4 ligand telomestatin (Fig. 12).<sup>240</sup> A series of 5' to 3' and 3' to 5' helicases proposed to bind G4s is listed in Table 2.<sup>236–238,245–247,266</sup> Recently, Balasubramanian's group reported a co-binding-mediated protein profiling strategy with their own developed G4 ligand, pyridostatin (PDS, Fig. 12), to investigate the interactome of DNA G4s in native chromatin.<sup>230</sup>

Table 2         Cellular functions of G4-binding proteins			
Protein	Function/Target	Ref.	
Nucleolin	Regulation of transcript/c-MYC	232	
DNA methyltransferase	Methylation of cytosine at C-5/c-MYC, CDKN1C and MEST	233	
Nucleophosmin	Nucleolar phosphoprotein/c-MYC	234	
ADAR	Convert adenosine to inosine/c-MYC	235	
PARP1	Gene regulation and DNA repair/BCL-2	236	
hnRNP1	Gene regulation, DNA repair, and chromatin remodelling/KRAS	237	
MAZ	DNA repair and chromatin remodelling/KRAS	238,239	
FANCJ	$5' \rightarrow 3'$ Helicase/Telomeric DNA	240	
Pif1	$5' \rightarrow 3'$ Helicase/c-MYC	241	
SMARCA4	Chromatin remodelling/c-KIT	230	
UHRF1	Ubiquitin ligase/c-MYC and c-KIT	230	
RBM22	pre-mRNA-splicing factor/c-MYC and c-KIT	230	
TTF2	Transcription termination factor/c-MYC and c-KIT	230	
DDX24	Helicase/c- <i>KIT</i>	230	
DDX1	Helicase/c- <i>MYC</i> and c- <i>KIT</i>	230	
CIRBP	cold-inducible RNA-binding protein/c-MYC	228	
DHX36	Helicase/c-MYC	242	
DDX21	Helicase/ <i>C</i> 9 <i>orf</i> 72	243	
DHX9	$3' \rightarrow 5'$ Helicase/TP-G4	244	
FUS	DNA repair and regulation of splicing/Telomeric DNA	245	
TDP-43	Regulation of transcription and splicing/Expanded C9orf72 gene	246	
α-synuclein	DNA repair and rescuing neurons/Telomeric DNA	247	



Fig. 10 G4 recognition modes of (a) RHAU, (b) RAP1, and (c) ankyrin derivative. Adapted from ref. 261. Copyright  $\textcircled{}{}$  2024 American Chemical Society.

The authors employed this approach in human cells and identified hundreds of putative G4-interacting proteins that comprised diverse functional classes, such as RNA binding, DNA binding, ATP binding, metal ion binding, ribonucleoprotein, hydrolase, repressor, helicase, activator, and chromatin regulator. Among them, SMARCA4, UHRF1, RBM22, TTF2, DDX24, and DDX1 were confirmed to bind to G4. Interestingly, the most frequently observed function of these proteins was RNA binding, even though the target was DNA G4 in these studies. However, this is consistent with the cold-inducible RNAbinding protein (CIRBP) being identified as a new G4 DNAbinding protein both *in vitro* and in cells.<sup>228</sup> These results indicate that G4s participate in more diverse biological processes in cells than previously considered *via* interactions with various proteins.

**RNA G4-binding proteins.** G4s in mRNAs play important roles in translation.<sup>267</sup> For example, a G-rich sequence found in the 5'-untranslated region (UTR) of NRAS mRNA folds into a stable parallel intramolecular G4 and inhibits translation.<sup>268</sup> RNA G4s in the cytoplasm are detected by using a G4 structure-specific antibody.<sup>269,270</sup> Moreover, over 13 000 loci where G4s form within the transcriptome in humans have been identified.<sup>271</sup> RNA G4s are enriched in functionally important regions, including 5'- and 3'-UTRs<sup>271–273</sup> and introns, although some G4s are observed in exons.

As shown for DNA G4 helicases, several helicases, including DHX36, DDX21, and DHX9, bind to and unwind RNA G4s,<sup>242–244</sup> because RNAs must be at least partly unfolded for their messenger function. Moreover, RNA G4-binding proteins observed in RNA modification reactions, viral pathogenesis, and mitochondria have been reviewed.<sup>274</sup> Other classes of RNA

G4-binding proteins are ribosomal proteins, the AFF family, and hnRNPs.<sup>257</sup> Ribosomal RNAs (rRNAs) have many G4-forming sequences with three and two G-quartets,<sup>275,276</sup> which exist as rRNA tentacles extending for hundreds of Å from ribosomal surfaces.<sup>277</sup> Thus, it is reasonable that ribosomal proteins bind to these RNA G4s. The AFF family of genes includes four members: AFF1/AF4, AFF2/FMR2, AFF3/LAF4, and AFF4/AF5q31. hnRNP proteins play critical roles in the packaging, transport, and splicing of mRNAs, and some hnRNP proteins, such as A1 and A2, have been reported to bind to RNA G4s.<sup>278,279</sup>

#### G4 in liquid-liquid phase separation

Interestingly, many of the RNA G4 binding proteins discussed above are involved in liquid-liquid phase separation (LLPS) to form membrane-less organelles (biomolecular condensates and liquid droplets) in cells, which play critical roles in cellular processes and diseases. LLPS is a hot topic in many research fields from cell biology to soft matter physics.<sup>280,281</sup> It is generally considered that LLPS is induced by intrinsically disordered proteins (IDPs) or that include intrinsic disorder regions (IDRs).<sup>282</sup> Nucleic acids (DNA and RNA) also undergo LLPS with binding to proteins to regulate transcription and translation at various stages coupled with other molecular regulatory mechanisms.<sup>283</sup> Multimolecular bindings among proteins and nucleic acids via electrostatic, hydrogen bonding,  $\pi$ - $\pi$  stacking, cation- $\pi$ , and CH- $\pi$  interactions are essentials for LLPS.<sup>284</sup> Notably, it has been proposed that DNA and RNA G4s are critical for LLPS, <sup>239,285–287</sup> despite the fact that IDP and IDR are essential for LLPS. Fig. 11 shows the LLPS model system with a G4-forming RNA oligonucleotide, derived from repeat expansion sequence in FMR1 mRNA, and Arg- and Gly-rich model peptide, derived from RGG motif of FMRP. It was demonstrated that the RNA-peptide mixture undergoes LLPS, depending on the formation of RNA G4. Moreover, aberrant LLPS may trigger protein aggregation in neurodegenerative diseases such as frontotemporal dementia associated with FUS, amyotrophic lateral sclerosis associated with TDP-43, Parkinson's disease associated with  $\alpha$ -synuclein, and Alzheimer's disease associated with tau.<sup>288</sup> In some of these neurodegenerative diseases, the expansion of G-rich repeat sequences, which may form G4s at the genomic and mRNA levels, participate in the onset mechanism.289



Fig. 11 LLPS model system, consisting of RNA G4 and Arg- and Gly-rich peptide. Adapted from ref. 289. Copyright  $\textcircled{}{}$  2022 Royal Society of Chemistry.

#### G-quadruplex-targeting molecules

The fact that stabilized DNA or RNA G4s inhibit series of enzymes, involving DNA and RNA polymerases, ribosome, and reverse transcriptase, has spurred a huge interest in the structure-based design and synthesis of ligands that bind and stabilize G4s (G4 ligands). As of November 2024, the G4 ligand database (G4LDB) opened.<sup>290</sup> G4LDB includes over 4800 ligands targeting quadruplexes (G4s and i-motifs of cytosinerich sequences) entries. Several G4 ligands are in clinical trials, as we discuss later.<sup>291,292</sup> The structures of some of these ligands are shown in Fig. 12 and are introduced here. Moreover, most G4 ligands possess cationic functional groups that form electrostatic interactions with anionic phosphates of nucleic acids and that make them water soluble. TMPvP4 is a typical and well-known G4 ligand. TMPyP4 shows high affinity for G4s due to its wide  $\pi$ -plane as well as its positive charges (Fig. 12).<sup>293</sup> TMPyP4 has been shown to inhibit human telomere elongation and gene expression by inhibiting telomerase and RNA polymerase, respectively.<sup>294</sup> However, the inhibitory effect on enzymatic activity is not structure selective. In telomerase reverse transcription assays in the presence of DNA duplexes, the inhibition effect on telomerase activity is significantly decreased.<sup>221,295</sup> This non-structure-selective binding of G4 ligands may decrease their inhibitory effects on enzymes of interest. At least partly due to their low selectivity, no G4 ligand has advanced beyond phase II trials yet. Quarfloxin (CX-3543, Fig. 12) is the ligand that has reached phase II (NCT00780663).<sup>296</sup> CX-3543 binds selectively to G4s formed in rDNA and disrupts rRNA interactions with nucleolin.<sup>297</sup> Another G4 ligand, QN-302 (Fig. 12), has most recently entered clinical phase I trials (NCT06086522).<sup>291</sup> We briefly discuss the development of ON-302 later in this manuscript.

Thus, structure-selectivity has also attracted attention in G4 ligand design. Telomestatin, which is isolated from Streptomyces anulatus 3533-SV4, selectively binds to and stabilizes telomeric DNA G4s, resulting in highly efficient telomerase inhibition (IC<sub>50</sub> = 5 nM).<sup>299</sup> Nagasawa and co-workers reported that L2H2-6OTD (Fig. 12), a macrocyclic hexa-oxazole, strongly stabilizes human telomere G4 and shows potent inhibition activity against telomerase.<sup>300</sup> Recently, a L2H2-6OTD derivate has also been used as a G4 imaging tool in living cells.<sup>301</sup> PDS was designed and synthesized by Balasubramanian in 2008 and has been used in various biological assays.<sup>230,302-305</sup> PDS was rationally designed with an expanded planar surface and hydrogen bond sites to efficiently bind to G4 structures. PDS binds and stabilizes telomeric G4 while having no effect on DNA duplexes, indicating high structure-selective binding.<sup>302</sup> Because of this property, a modified PDS analogue has also been used in intracellular G4 imaging applications. For example, Balasubramanian and co-workers reported that SiR-pyPDS, a fluorophore-modified PDS,<sup>305</sup> exhibits remarkable fluorescence turn-on properties upon binding to G4. PhenDC3, a bisquinolinium family compound, strongly selectively binds to and stabilizes G4s over DNA duplexes.<sup>295,306,307</sup> BRACO-19 is an aromatic molecule containing protonatable sites that was designed based on crystal structure and simulation studies to



**Fig. 12** Chemical structures of G4 ligands: TMPyP4, CX-3543, QN-302, telomestatin, L2H2-6OTD, pyridostatin, PhenDC3, telomestatin, BRACO-19, CuAPC, hemin, thioflavin T, *N*-methyl mesoporphyrin IX, ZnAPC, alkylated porphyrin.

bind to three G4 grooves. BRACO-19 exhibits 31-fold higher affinity for G4s than duplexes.

Another strategy for obtaining structure-selective G4 ligands is the development of small molecules with anionic charges. Cationic ligands bind nucleic acids with non-structure-selectivity due to the negative charges on their backbones. Therefore, it is

useful to develop an anionic ligand that causes electrostatic repulsion with the phosphate groups of nucleic acids. Copper(II) phthalocyanine 3,4',4",4"'-tetrasulfonic acid (CuAPC, Fig. 12), an anionic ligand, has an IC50 of 1.6 µM against telomerase activity in the presence of excess and competitive DNA duplexes.<sup>221</sup> Hemin is also a typical negatively charged G4 ligand (Fig. 12).<sup>308</sup> Hemin suppresses xlr3b gene expression through its binding to a G4 formed at the CpG island in ATR-X model mice, leading to the recovery of cognitive deficits associated with ATR-X syndrome.<sup>309</sup> Although many developed G4 ligands aim to inhibit transcription or telomere elongation, ligands involved in other biological processes have been reported. Tetrandrine binds to G4 selectively and stabilizes it strongly.<sup>310</sup> Recently, it has been shown that stabilization of RNA G4 formed by an miR-23b/27b/24-1 cluster by using tetrandrine prevents the binding of Drosha to the miR cluster, resulting in the recovery of cardiac function.<sup>311</sup> Thioflavin T (ThT, Fig. 12) is also a selective fluorescent probe for G4, although it has a positive charge.<sup>312</sup> N-Methyl mesoporphyrin IX (NMM, Fig. 12) is another G4-selective ligand. Bolton et al. found that NMM binds to DNA G4 but not to duplexes by fluorescence spectroscopy.<sup>313</sup> NMM enhances fluorescence intensity upon binding to G4s, which is useful for their detection. Later, it was reported that NMM does not bind to other nucleic acid conformations<sup>314</sup> and is selective for the parallel topology. The  $K_{\rm d}$  value of NMM for the parallel topology is nearly 10 times less than for the mixed topology and 100 times less than for the antiparallel topology.<sup>315</sup>

Breaking down target RNA G4s is another therapeutic drug strategy. Our group reported that ZnAPC (Fig. 12) selectively binds to RNA G4 formed by the NRAS mRNA 5'-UTR. ZnAPC breaks down the target RNA G4 via reactive oxygen species upon photoirradiation, resulting in NRAS suppression.<sup>316</sup> Xodo and co-workers also reported that an alkylated porphyrin (Fig. 12) bind to KRAS mRNA G4 and cleaves it upon photoirradiation.<sup>317</sup> These results suggest that photodynamic therapy targeting mRNA G4s is promising as a new modality for cancer treatment. A remarkable recent progress in this field is ribonuclease targeting chimera (RIBOTAC), which can recruit the endogenous ribonuclease RNase L to a RNA target, resulting in the target RNA cleavage in living cells.318 This strategy has been applied to G4-binding proteins and RNA G4s.<sup>319-321</sup> However, selectivity of G4 ligand is the main issue to apply this technology for a practical and clinical use.<sup>322</sup>

Systematic comparisons of the three-dimensional structures of G4 ligand/G4 complexes are important for improving the affinity and selectivity of G4 ligands. Yang and colleagues reported the solution structures of c-MYC G4 with phenyl– ethenyl–quinoline (PEQ),<sup>298</sup> a specific c-MYC G4 binder, and of three other G4 ligands (Fig. 13).<sup>323</sup> This c-MYC G4 sequence contains G23-to-T and G14-to-T mutations in the 3'-flanking and second loop, respectively. This sequence was used as a model parallel G4 structure. Yang and colleagues found that the flexible flanking regions are recruited in a conserved and sequence-specific way, and that these regions have further potential for selective ligand–G4 hydrogen-bond interactions. For example, a combination of  $\pi$ - $\pi$  stacking interactions with a G-quartet core and hydrogen bonds with other regions such as the flanking and loop regions have been observed in other G4 ligands, including PDS and its derivatives.<sup>324</sup> Other systematic and detailed examples of G4 ligand/G4 complexes have been reported in the literature, 325-327 involving contributions of other binding modes, such as cation $-\pi$  and hydrophobic interactions. A quantitative study of nonselective interactions of G4 ligands with long DNA duplexes using a single molecule stretching technique was recently published.<sup>328</sup> Those results showed that five of eight G4 ligands commonly used in diverse studies bind the DNA duplex with a binding affinity similar to that of ethidium bromide, a typical DNA duplex binder. This structure-selectivity of G4 ligands therefore holds promise for practical applications in living cells, since there are many more DNA and RNA bases involved in duplex structures than in G4 structures.

### i-Motif

#### Structure, sequence, and biological role of the i-motif

The i-motif, another tetraplex, is formed by cytosine-rich (C-rich) sequences *via* cytosine-cytosine<sup>+</sup> base pairs (Fig. 14a). The first DNA i-motif was reported in 1993 for d(TCCCCC) (Fig. 14b and c).<sup>329</sup> Since protonation of cytosine is required, this structure is particularly stable in acidic conditions. Although the  $pK_a$  of free cytidine is 4.2, the  $pK_a$  is shifted in RNA and DNA.<sup>330,331</sup> The value is further shifted to be around 6.5 in the i-motif.<sup>332</sup> Moreover, the  $pK_a$  value increases as the number of cytosines per tract increases from two to seven.<sup>333</sup> Loop regions connecting cytosine stretches and capping nucleotides next to the stretches also affect the thermodynamics of i-motifs.<sup>334-336</sup>

The molecular environment also affects the structural stability of i-motifs. In contrast to G4, cation species do not affect i-motifs, but the cation concentration does. It has been reported that a higher cation concentration destabilizes i-motifs.337,338 A lower stability with higher cation concentrations is observed only for the i-motif, but not for other structures of DNA. In contrast to the opposing effects of the cation concentration, molecular crowding stabilizes i-motifs, as with G4s. It has been found that C-rich sequences adopt the i-motif structure at neutral pH under molecular crowding conditions.<sup>339,340</sup> Given the stabilization effects of molecular crowding on G4s, an intramolecular structure of an i-motif with a C-rich sequence + a G4 of a complementary G-rich sequence could be more stable than an intermolecular duplex of G- and C-rich sequences, which is generally thermodynamically more stable than the sum of the intramolecular structures (G4 and i-motif) in a physiological buffer.<sup>341</sup> In fact, it has been reported that molecular crowding induces a structural transition of telomeric G-rich and C-rich sequences from an intramolecular G4 and i-motif to an intermolecular duplex.<sup>215</sup> Another factor that affects thermal stability of i-motifs is negative superhelicity, which unwinds the duplex.<sup>342</sup> Various biological processes such as transcription, replication, recombination, and DNA damage repair produce negative supercoiling.<sup>343</sup> Thus, during



Fig. 13 (a) Chemical structures of phenyl-ethenyl-quinoline (PEQ), Quindoline-I (Qi), DC-34, and BMVC. (b) Comparisons of the high-resolution structure of c-MYC G4 with PEQ (PDB ID: 7KCX), Qi (PDB OD: 2L7V), DC-34 (PDB ID: 5W77), and BMVC (PDB ID: 6O2L) binding to the 5'-site and the 3'-site. (c) Three-dimensional structures of the PEQ, Qi, DC-34, and BMVC complexes with c-MYC G4 (PDB ID: 7KBX). Adapted from ref. 298. Copyright © 2021 Oxford University Press.

these processes, the formation of intramolecular structures, including i-motifs, could be accelerated. The C-rich sequence of the human telomere can form an i-motif in the S-phase of replicating human cells.<sup>344</sup> In addition, the stabilization of a G4 of a G-rich sequence further aids the complementary C-rich sequence to form an i-motif by reducing structural competition with the intermolecular duplex. Thus, it is possible that the i-motif exists in living cells. In fact, evidence for the existence of i-motifs in living cells has been provided by in-cell NMR and an i-motif antibody.<sup>345,346</sup>

As a complementary sequence to putative quadruplexforming sequences, putative i-motif-forming sequences (PIS) have been identified in various genes. The PIS in the c-MYC gene folds into several different i-motif structures with the stability being remarkable at pH 7.<sup>347</sup> There are more than 60 structures of i-motifs, with sequences including methylated and other modified cytosines, that are registered in the protein data bank (September 2021). As there are many PIS, there are also proteins that bind to i-motifs and PIS oligonucleotides. Poly-C binding protein is a ubiquitous oligonucleotide-binding protein in eukaryotic cells that regulates gene expression.<sup>348</sup> This family consists of the hnRNP K (heterogeneous nuclear ribonucleoprotein K) and isoforms of  $\alpha$ CP1, including  $\alpha$ CP1-4 and  $\alpha$ CP-KL. hnRNP K is known as a transcription factor that binds to CT elements in the promoter region of c-MYC.<sup>349</sup>  $\alpha$ CP1 also recognizes the C-rich sequence of human telomere DNA with high affinity.<sup>350</sup> Lacroix and coworkers studied the interaction of hnRNP K and ASF/SF2 with telomeric C-rich sequences under various pH conditions. Their results suggested that the protein binds to the unfolded sequence.<sup>351</sup>



**Fig. 14** Characteristics of the i-motif structure. (a) Chemical structure of a C:C+ base pair. (b) Illustration of the i-motif structure folded by a C-rich sequence, 5'-TCCCC-3'. (c) Crystal structure of the tetrameric i-motif (PDB ID: 2N89).

A later study indicated that hnRNA interacts with singlestranded DNAs.<sup>352</sup> In addition, hnRNP K and hnRNP LL, which are transcriptional factors, have been reported to bind to C-rich sequences of *VEGF* and *BCL-2*, respectively.<sup>353,354</sup> As the protein unfolds the i-motif structure and forms a stable single-stranded DNA–protein complex, the i-motif may play an important role in controlling gene expression. Moreover, i-motifs have been proposed to have various biological functions through their structure and protein binding. These biological roles include the inhibition of telomerase activity, a structural element that provides long-range interactions between laterally associated centromeric nucleosomes, transcriptional regulation, and stalling DNA polymerase and thus impeding DNA replication and repair. These biological roles of i-motifs have received thorough recent reviews.<sup>355-357</sup>

#### i-Motif targeting molecules

The first discovered i-motif ligand was TMPyP4, which was also known as a G4 ligand.<sup>358,359</sup> Since it is highly cationic, TMPyP4 binds to duplexes in a non-specific manner, and the binding to G4 was overestimated.<sup>295</sup> This original study stimulated many researchers to develop i-motif ligands.

As with the G4 ligands discussed above, many of i-motif ligands have a planar structure (Fig. 15). Such ligands include derivatives of phenanthroline,<sup>360</sup> acridine,<sup>361</sup> macrocyclic polyoxazole,<sup>362</sup> and metal complexes.<sup>363,364</sup> NMR studies have demonstrated that i-ligands such as L2H2-4OTD and IMC-48 form complexes through stacking interactions with C-C+ base pairs and interactions with loops.<sup>362,365</sup> Although the detailed binding modes (in addition to the stacking interactions) were not identified, these findings nonetheless suggest that interactions with the C-C+ base pairs and the loops are critical for stabilizing the i-motif. Phenanthroline derivatives show preferential binding to telomeric i-motifs over duplexes and G4. Acridine derivatives are based on BRACO-19, which is a wellknown G4 ligand. This compound has been demonstrated to suppress the proliferation of cancer cells.<sup>366</sup> One poly-oxazole derivative has been reported to interact with a telomeric i-motif, but not G4.362 Interestingly, carboxyl-modified single-walled carbon nanotubes (SWNTs) induce and stabilize human telomeric i-motifs. K562 and HeLa cells were treated with SWNTs, then the inhibition of telomere elongation was confirmed using a telomeric repeat amplification protocol assay. Colocalization of SWNTs and a telomere restriction fragment, a telomere



Fig. 15 Chemical structures of i-motif-targeting molecules phenanthroline, bisacridine, L2H2-4OTD, [Ru(bpy)<sub>2</sub>(dppz)]<sup>2+</sup>, c3, B19, a9, IMC-48, PBP1, mitoxantrone, tilorone, and tobramycin.

marker, suggested that SWNTs were located in the telomere region and stabilized telomeric i-motif DNA, causing folding of telomeric G4 and inhibiting telomere elongation.<sup>367,368</sup>

It has been shown that small molecules targeting i-motifs regulate the transcription of many genes. B19 and a9 bind to the c-MYC i-motif and downregulate transcription.369,370 Oleanolic acid derivatives interact with the VEGF i-motif and also downregulate transcription.<sup>371,372</sup> In contrast, IMC48 and PBP1 bind to the BCL2 i-motif and upregulate transcription.<sup>370,373</sup> Although these results suggest that i-motifs are important as cis-acting gene regulators, it is still unclear how genes are down- and up-regulated by i-motif formation and ligand binding. The fluorescent intercalator displacement assay is a powerful method to screen ligands that target i-motifs as well as other structures of nucleic acids. Waller and co-workers have discovered i-motif ligands, such as the antitumor agent mitoxantrone and antiviral agents tilorone and tobramycin, by fluorescent intercalator displacement assays utilizing thiazole orange.374,375 However, thiazole orange emits fluorescence not only with i-motifs but also other structures, including G4s and duplexes. Thus, an i-motif fluorescent probe will be useful to develop a more simplified and high-throughput screening system for i-motif-specific ligands.

# Targeting three- and four-stranded nucleic acids molecules toward clinical trials

#### Nucleic acid-targeting small molecule as approved drug

It is essential to consider clinical applications even at the earliest stages of drug development. For example, risdiplam (Evrysdi<sup>®</sup>) is an approved small molecule therapeutic that targets nucleic acids to treat spinal muscular atrophy, and it is the first small molecule to be identified as a splicing modifier.<sup>376</sup> Risdiplam was identified by phenotypic screening rather than by structure-based screening.<sup>377</sup> Screening was performed using a human embryonic kidney cell line harboring an SMN2 minigene to increase the inclusion of exon 7 during SMN2 pre-mRNA splicing. Various functional groups were introduced to enhance oral availability.<sup>378</sup> For example, the insertion of a pyridopyrimidinone moiety reduced the phototoxicity of the compound and a cyclic N-alkyl group prevented nonspecific protein binding. These design steps and modifications allow risdiplam to strongly promote SMN protein expression with low cytotoxicity.379-381 The biological activity of the compounds was confirmed using animal models of SMA. Pharmacokinetic studies showed that the compounds penetrated the brains of adult mice. Finally, the efficiency and safety of risdiplam was confirmed in a clinical development program with SMA patients.

#### G4 ligands for clinical applications

Similar problems need to be solved by chemical modifications for small molecules targeting non-canonical structures of nucleic acids. After screening or structure-based rational design the lead compounds must be optimized, as shown by the

example of G4 ligands being developed for clinical use. BRACO-19 (Fig. 12), developed by Neidle and coworkers, has been modified, with one derivative exhibiting high selectivity against duplexes and strong inhibition of telomerase activity.<sup>366</sup> This compound persistently arrested the growth of a breast carcinoma cell line<sup>366</sup> and is the first G4 ligand showing an anticancer effect and proven anticancer activity in human tumor xenograft models. Neidle and colleagues also developed and optimized compounds with a naphthalene diimide (NDI) core using structure-based design, medicinal chemistry and pharmacology. QN-302, an NDI derivative with a benzyl-pyrrolidine group, significantly inhibits the growth of pancreatic ductal adenocarcinoma cells in animal models, with GI<sub>50</sub> values of 1-2 nM and superior potency compared to derivatives with a methoxy group replacing the benzyl-pyrrolidine.382 QN-302 binds tightly to various parallel G4s and is highly specific for G4 compared to duplexes.<sup>382</sup> QN-302 began clinical trials in 2023.<sup>291,378</sup>

The detailed binding mode of QN-302 with G4 has been studied.<sup>383-385</sup> Molecular modelling studies suggest that QN-302 binds at the duplex-G4 junction through the NDI core *via* stacking interactions with the G4 terminal G-quartet. The benzyl-pyrrolidine substituent of QN-302 protrudes significantly into the groove. The phenyl ring stacks efficiently onto the adjacent guanine of the lower G-quartet. The benzyl-pyrrolidine group allows enhanced G4 binding affinity and selectivity, enhanced cellular and *in vivo* potency, and greater selectivity in the pattern of downregulated gene expression. Furthermore, the pharmacokinetic properties of QN-302, such as half-life *in vivo* and bioavailability, are not apparently responsible for its superior activity *in vivo*. These results further demonstrate the importance of the binding affinity and selectivity of G4 ligands for clinical applications.

#### G4 ligands with higher selectivity

As the above examples show, the selectivity of the compound for its target is of utmost importance. High selectivity is critical for improving drug efficacy and reducing side effects, as it allows the highly efficient inhibition of G4-targeted enzymes and prevents off-target enzyme inhibition. High selectivity against the duplex is also important to reduce the genotoxicity of G4 ligands and small compounds targeting other three- and four-stranded nucleic acid structures. High selectivity for G4 ligands requires the involvement of directional interactions in addition to stacking interactions. The energy of electrostatic interactions is highest within non-covalent interactions, but it depends only on the distance between the charges, making it difficult to confer selectivity. This can be particularly detrimental to discriminating between G4 and canonical duplex structures. The energy of hydrogen bonding varies with direction, so many G4 ligands have been modified to introduce hydrogenbonding donors and acceptors to allow hydrogen bonds with the target G4, especially with the loops, grooves, and flanking regions (Fig. 7). The importance of these three regions for specificity is discussed in this subsection.

**Loops.** There are 21 844 possible sequences with three loops in intramolecular G4s with 1–7 nucleotides of which 20 492 are

found at least once in the human genome.<sup>139</sup> The loop region was recently reported to significantly impact the topology and thermal stability of G4, and the kinetics of G4 formation.<sup>123,386,387</sup> This suggests that the binding of G4 ligands to the loop regions can significantly alter the overall G4 structure and thus modulate protein binding and enzymatic activity. In addition, a G4 structure can adapt other nucleic acid structural motifs, such as a hairpin loop. A duplex can be attached to a G4.<sup>167,168</sup> The duplex formed within the loops stabilizes the overall G4 structure compared to an unstructured loop and accelerates G4 folding kinetics.<sup>169,175-177</sup>

**Grooves.** The G4 structure has four grooves: narrow, medium, and wide as defined by the glycosidic bond conformation adopted by any two base-paired 2'-deoxyguanosines of stacked G-quartets.<sup>388</sup> There are eight combinations of groove types.<sup>389</sup> The depth and width of these grooves differ significantly from the major and minor grooves of the duplex structure. Moreover, the Hoogsteen base pairs forming the G-quartet use different hydrogen bond acceptors and donors than the Watson–Crick base pairs in the duplex, so the hydrogen bond acceptors and donors exposed in the grooves are also very different in the G4 and in the duplex structures. The hydration pattern in the G4 grooves is also different from in the duplex grooves.<sup>389</sup> These characteristic grooves are likely important for selective binding to the G4 over the duplex structure.

Flanking: The 5' and 3' flanking regions adjacent to the G4 core region are also targets for hydrogen bonding interactions. The stacking and hydrogen bonding interactions formed between the flanking nucleotide and another flanking nucleotide, the adjacent G-quartet, and the neighboring loop, all contribute to the overall structure and thermodynamics of G4. The flanking nucleotides reportedly influence the binding affinity of the G4 ligand. As discussed above, the importance of detailed structural information on the loop, groove and flanking regions will increase as the design of G4 ligands progresses.

The three-dimensional structure of the loop regions, and the depth and width of the grooves, depend on the nucleotide sequence. The nucleotide sequences for the 5' and 3'-flanking regions determine the target. These differences are important not only for structural selectivity against the canonical duplex, but also for sequence selectivity towards the many G4s present in cells, although only a few examples of G4 ligands have been reported to show sequence selectivity. Selectivity for DNA G4 and RNA G4 may also be important for precise targeting. More three-dimensional G4 structures are needed to predict the overall topology of G4 from the sequence and also the fine structure of the loops, grooves, and flanking regions.

Small molecules that highly selectively target triplex and i-motifs have been reported, as shown for the triplex of MALAT1 discussed above; however, only a few studies show the effects of these molecules on cellular and organoid functions.<sup>120,390</sup> As mentioned above, even small molecules with high selectivity require toxicity testing, improved oral dosing, animal models, and confirmation of compound efficacy and pharmacokinetics. More systematic and quantitative studies of the relationship between structure, selectivity, and function *in vitro* and *in vivo* are required.

### Conclusion and perspective

DNA and RNA sequences encode not only genetic information but also higher order information, such as three-dimensional structures and stabilizing factors, and they respond to environmental factors, all of which are critical for gene regulation. Moreover, it has been found that DNA and RNA undergo LLPS in living cells to regulate the central dogma at many steps involving the replication of genomic DNA and viral RNA; the transcription of mRNA, rRNA, and non-coding RNA; the processing and localization of RNAs; and translation from RNA to protein.<sup>391-395</sup> One fundamental property of LLPS is responsiveness to cellular stresses, such as high temperatures, nutrient starvation, toxins, and reactive oxygen species. The structure and stability of nucleic acids depend on the molecular environment as we described in this review. Interestingly, recent studies suggest that LLPS of nucleic acids is dependent of their secondary structures.<sup>239,285</sup> Thus, DNA and RNA sequences may further encode phase behavior. Despite our knowledge of the biology, chemistry, and physics of noncanonical nucleic acid structures, many aspects are still opening for further investigation. In this regard, researches on the non-canonical structures of DNA and RNA utilizing small ligands in vitro and in vivo are essential for elucidating their roles in different biological processes. In spite of recent findings of ligands that target non-canonical structures, the number of structure-specific ligands is very limited. Moreover, noncanonical structural ligands that can target a specific sequence are further needed. To develop structure and sequence-selective ligands, at least the following are required.

(1) Three-dimensional structures of nucleic acid-ligand complexes: Structural information is essential for the rational design of ligands. Even though biochemical and biophysical information for G4 ligand complexes is relatively abundant, there are only a limited number of RNA and DNA G4 ligand complexes with structures that have been solved. Moreover, the structure of an i-motif and ligand complex has not been reported yet.

(2) Dynamic properties of non-canonical structures: Noncanonical structures are not static. Their formation and thermal stability largely depend on molecular environmental factors and protein binding. In the case of DNA, non-canonical structures always compete against the canonical duplex. In the case of RNA, structural switches among the most stable and metastable structures may be important for their biological roles as in the case of riboswitches. Thus, the dynamic and kinetic properties of non-canonical structures should be studied under cell-mimicking conditions and in living cells.

(3) High-throughput screening systems: since not only high affinity but also high selectivity of ligands are required for cellular applications, screening systems that can evaluate biorthogonality among canonical and non-canonical structures are needed. For example, many i-motif and G4 ligands also bind G4s and i-motifs, respectively. Moreover, it is difficult to eliminate non-specific binding of ligands to the duplex. In addition to structure-specific ligands, sequence-specific ligands are required, because as shown for triplexes and quadruplexes, there are many putative sequences that form certain non-canonical structures.

A wide variety of G4 ligand screening systems, such as fluorescent intercalator displacement (FID), are used to identify ligands,<sup>396–398</sup> but these methods are low-throughput. Obtaining structure- or sequence-selective ligands using such methods requires multiple steps. Our group established new G4 ligand screening systems that are optimized to provide structure- or sequence-selective G4 ligands,<sup>399,400</sup> although a molecular probe exhibiting target selectivity is required. Furthermore, cell-based FID assays can provide ligands that bind intracellular G4s efficiently.<sup>401</sup> These reports demonstrate that G4 ligands exhibiting structure selectivity can efficiently target G4s within cells. Further structural information on target-selective ligands can enrich target-selective ligand databases such as G4 LDB<sup>290</sup> and NALDB.<sup>402</sup> A systematic quantitative structure-activity relationship (OSAR) workflow in 2022 was reported to broadly discover RNA ligands using HIV-1 transactivation response RNA as a model system.<sup>403</sup> This method can evaluate binding parameters, including affinity and binding kinetics, bypassing the need for structural information. Therefore, the discovery of target-selective ligands not only aids understanding of the scaffolds, but also the rational design of ligands for targetselective binding.

(4) Widely useable databases: bioinformatic and omics studies have provided a massive amount of data sets to researchers, but it is almost impossible to make effective use of them by examining individual studies. Thus, to unify and utilize a large amount of data, databases and machine learning systems for wide and open use are desired.

In conclusion, structure- and sequence-selective ligands could be the key to decoding hidden information in nucleic acids and to develop small molecular drugs targeting nucleic acids. Nucleic acids -DNA, RNA, as well as artificial ones- are now coming of age as drug targets and targeting biomolecules.

# Data availability

No primary research results, software or code have been included, and no new data were generated or analyzed as part of this review.

# Conflicts of interest

There are no conflicts to declare.

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