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G-Quadruplexes: Targets in Anticancer Drug Design

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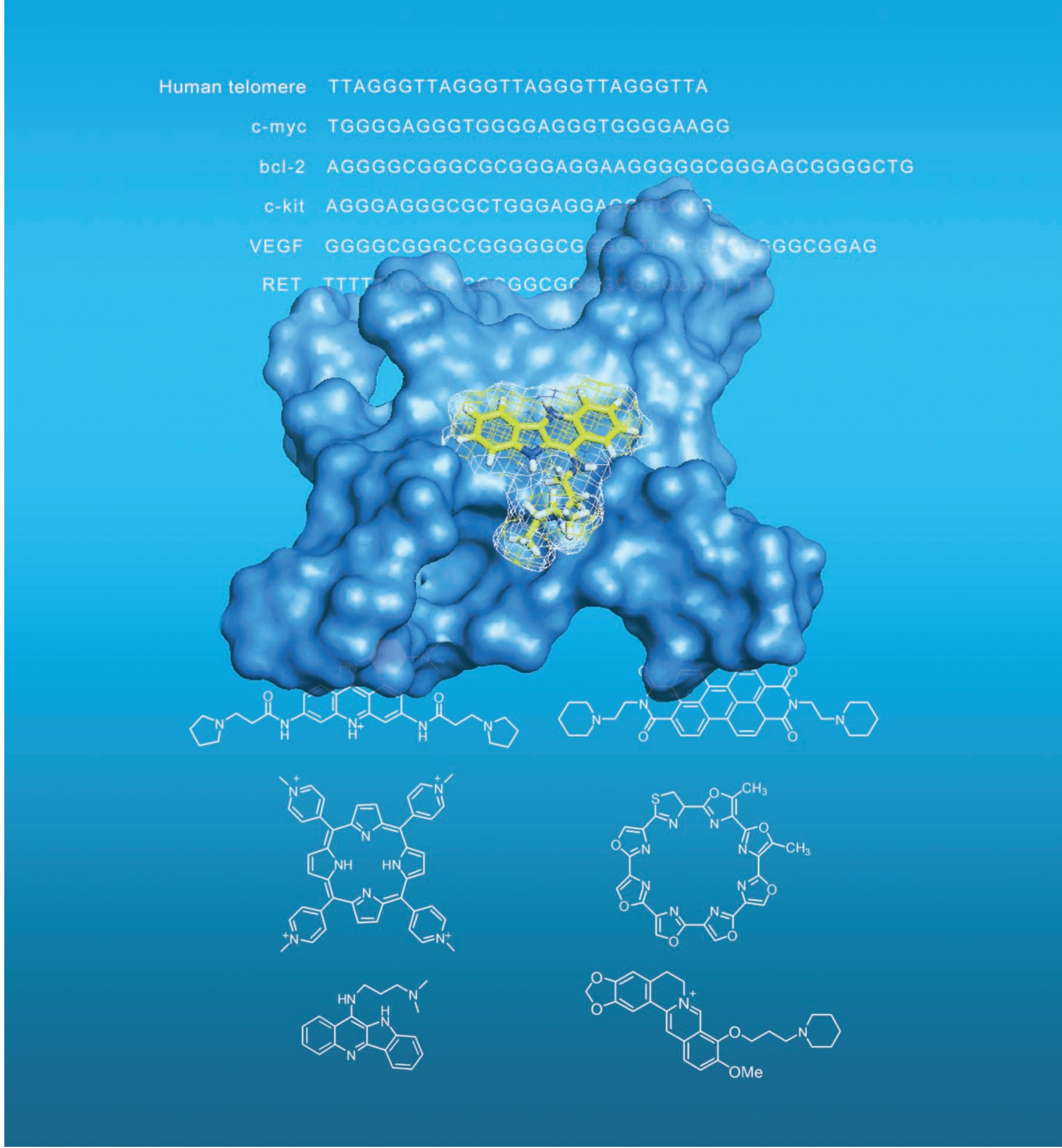

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G-quadruplexes are special secondary structures adopted in some guanine-rich DNA sequences. As guanine-rich sequences are present in important regions of the eukaryotic genome, such as telomeres and the regulatory regions of many genes, such structures may play important roles in the regulation of biological events in the body. G-quadruplexes have become valid targets for new anticancer drugs in the past few decades. Many leading compounds that target these structures have been reported, and a few of them have entered preclinical or clinical trials. Nonetheless, the selectivity of this kind of antitumor compound has yet to be improved in order to suppress the side effects caused by nonselective binding. As drug design targets, the topology and structural characteristics of quadruplexes, their possible biological roles, and the modes and sites of small-ligand binding to these structures should be understood clearly. Herein we provide a summary of published research that has set out to address the above problem to provide useful information on the design of small ligands that target G-quadruplexes. This review also covers research methodologies that have been developed to study the binding of ligands to G-quadruplexes.

1. Introduction

Guanine-rich (G-rich) stretches of DNA have a high propensity to self-associate into planar guanine quartets (G-quartets) to give unusual structures called G-quadruplexes, first reported by Davies and co-workers in 1962.[1] The association of G-quartets can also happen in solution by stacking the planar tetramers on top of each other.

With the advent of X-ray crystallography, nuclear magnetic resonance spectroscopy (NMR) and other powerful technologies, the structures of many G-quadruplexes have been resolved. The common structural characteristic of G-quadruplexes is that the G-rich nucleic acid sequences tend to adopt the G-quadruplex conformation, which is a structure consisting of flat G-quartets of hydrogen bonded guanines. Recent structural studies of various G-quadruplexes have revealed the conformational polymorphism of these structures. For example, human telomeric sequences in physiological ion (K^+ or Na^+) solution have been found to fold into different quadruplex conformations.[2–4]

Quadruplexes are thought to play important roles in some biological events because many guanine-rich regulation regions in the human genome possess the potential to adopt a quadruplex conformation. The formation of quadruplexes in telomeric DNA results in the inhibition of telomerase to extend the telomeres. Moreover, quadruplexes may be present in thousands of gene promoters and hence affect gene expression and subsequent biological processes.

The conformations of G-quadruplexes provide selective recognition sites for small molecules and thus these structures have become important drug-design targets for the treatment of various human disorders (e.g. viral infections and cancer). Since Neidle, Hurley, and co-workers reported the use of 2,6-diamidoanthraquinone derivatives to stabilize the quadruplex structure and inhibit telomerase activity,[5] extensive efforts have been directed toward the development of quadruplex-specific ligands. Although many G-quadruplex ligands have been developed, most of these (such as 2,6-diamidoanthraquinones, telomestatin, and TMPyP4) are not drug-like. Nevertheless, Cylene Pharmaceuticals (San Diego, USA) has developed CX-3543 (quarfloxin), which can disrupt the interaction between the nucleolin protein and a G-quadruplex DNA structure in the ribosomal DNA template, and this compound has now entered phase II clinical trials. It is clear that quadruplex DNA is no longer just a biophysical oddity and must be given serious consideration as an important target for the treatment of various human disorders.

2. Structure, Topology and Biological Roles of G-Quadruplexes

Traditionally, DNA is thought to be an important drug target in anticancer therapies. The design and development of DNA-specific drugs is a challenging task for many scientists. Conventionally, the development of alkylating agents as anticancer agents is highly dependent on the discovery and evolution of the DNA duplex and its associated processes. Unfortunately, many of these drugs are extremely cytotoxic and nonspecific. As such, extensive efforts have been directed toward the discovery of new agents with improved selectivity and less cytotoxicity.[6] Apart from the typical right-handed double helix (B-DNA) proposed by Watson and Crick, DNA can adopt other biologically relevant structures, such as G-quadruplexes. This secondary DNA structure represents a new drug target for DNA-binding compounds. Although much structural information has been presented in other reviews,[7,8] the structural characteristics of G-quadruplexes and the effects of the structural diversity of these structures on the selectivity of drug binding in comparison with duplex DNA is emphasized in this section.

Meanwhile, the unique structural characteristics of G-quadruplexes and their polymorphism may contribute to their varied biological roles. The key regions with the potential to adopt G-quadruplex structures include some oncogene promoter regions,[9] telomeres,[10] ribosomal DNA,[11] mini-satellites,[12] and the immunoglobulin heavy chain switch region,[13] suggesting that G-quadruplexes may have important functions in vivo, which has inspired research focus on further elucidating their biological roles. The major roles of the G-quadruplex may be its ability to “turn on” or “turn off” some physiological

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Zhi-shu Huang received her PhD in organic chemistry in 1999 from Sun Yat-sen University, where she held the position of lecturer (1992–1999), associate professor (2000–2004), and became full professor in 2005. From 2001 to 2003 she was a postdoctoral fellow in medicinal chemistry in the College of Pharmacy at the University of Texas at Austin (USA). In 2003, she returned to Sun Yat-sen University and worked in the Institute of Medicinal Chemistry, School of Pharmaceutical Sciences. Projects in her research group are focused on the research and development of anticancer drugs that target DNA, the interaction of small molecules with biomolecules, and structural modification of natural products and QSAR.

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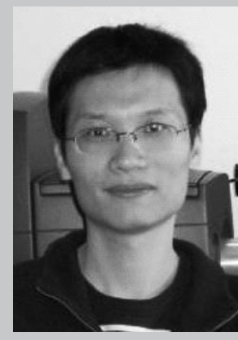
Kwok-yin Wong obtained his BSc (with honors) in chemistry from the University of Hong Kong in 1981 and his PhD degree from the same university in 1986. He was a postdoctoral research fellow at the California Institute of Technology from 1986 to 1987. He worked as a chemist in the Government Laboratory of Hong Kong from 1987 to 1990. He joined the Hong Kong Polytechnic University as a lecturer in 1990, and was promoted to professor in 1995. He was promoted to chair professor in 2005. Currently he also heads the Department of Applied Biology and Chemical Technology of the Hong Kong Polytechnic University. His research interests include electrochemistry, green chemistry, biosensors, and chemical biology.



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events by the transcriptional regulation of genes or telomere length.

For example, many telomeric DNA-binding proteins bind to quadruplexes and/or promote their formation,[14–19] which may also regulate telomerase activity.[20,21] In addition, the *Escherichia coli* RecQ protein can unwind G-quadruplex DNA, and members of the RecQ helicase family are highly conserved and are essential for maintaining genomic stability of many organisms, ranging from *E. coli* to humans.[22–24] These findings further support the cellular role of these highly ordered DNA structures. Furthermore, enrichment of G-quadruplex DNA in the near-upstream region of genes in many organisms allows the genes related to transcription, amino acid biosynthesis, and signal transduction to be predominantly controlled by G-quadruplex DNA.[9] Thus, the important biological functions of potential G-quadruplex-forming regions and the effects of G-quadruplex formation or stabilization make this higher-order structure emerge as a significant target for drug design.

2.1. General features of G-quadruplexes

2.1.1. The building blocks of G-quadruplexes

The building block of G-quadruplexes is the G-quartet (also known as a G-tetrad), which was first reported by Gellert et al. over 40 years ago.[1] The substructure arises from the association of four guanines into a cyclic Hoogsteen hydrogen bonding motif in which each guanine base forms two hydrogen bonds with its neighbors (Figure 1). The crystal structure of a G-quadruplex shows that the G-quartet can be considered as a square aromatic surface, the dimensions of which are much bigger than the Watson–Crick base pairs (Figure 1), and this

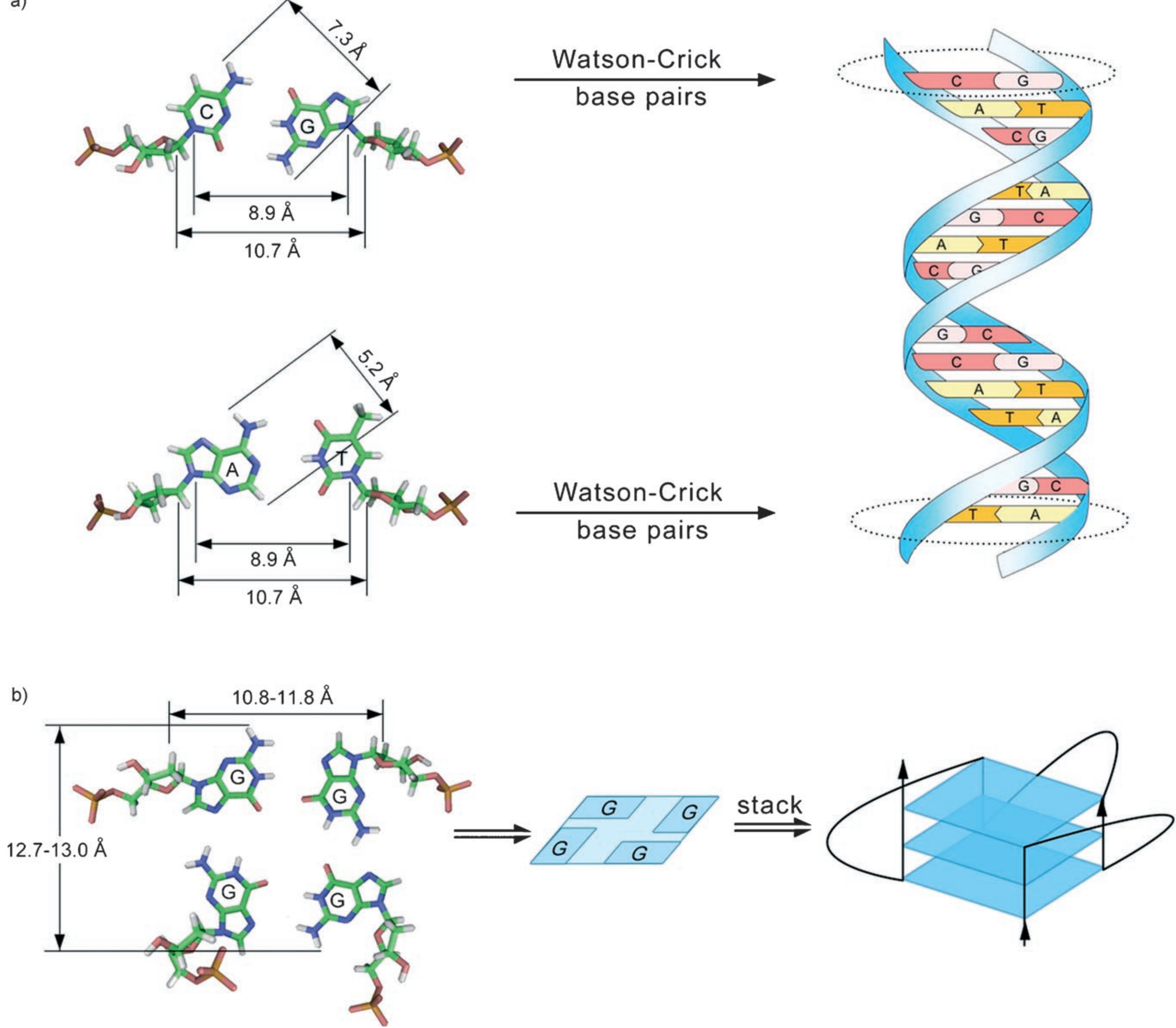

Figure 1. Comparison of the dimensions of duplex and G-quadruplex DNA structures. a) The double helix and its base pair surface. b) The quadruplex structure and the G-quartet surface.

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difference constitutes the basis for designing G-quadruplex-specific ligands.[25,26]

2.1.2. The basic topology and structure of G-quadruplexes

G-quartets can stack on top of each other to form four-stranded G-quadruplexes (Figure 1). These structures exhibit extensive structural diversity and polymorphism relative to duplex DNA. In general, structural polymorphism arises mostly from the nature of the loop, such as variations of strand stoichiometry, strand polarity, glycosidic torsion angle, and the location of the loops that link the guanine strand(s). Meanwhile, the solution environment, such as the presence of metal ions, ligands, or molecular crowding conditions, may also influence the topology of quadruplex. G-quadruplexes can be folded from a single G-rich sequence intramolecularly or by the intermolecular association of two (dimeric) or four (tetrameric) separate strands. Potential G-quadruplex-forming sequences have been summarized by Burge et al. (see Table 1).[7] The relative arrangement of strand polarity can also give rise to structural polymorphism. For example, the polarities of the four strands in a G-quadruplex can be parallel, three parallel and one antiparallel, adjacent parallel, or alternating parallel, resulting in different conformations named as parallel, propeller, and antiparallel quadruplexes.

Variations in strand polarity also affect the location of the linkers, or loops (X_n, X_o, and X_p) between G-rich segments (G_m; Table 1). As shown in Figure 2, parallel G-strands require a connecting loop to link the bottom G-tetrad with the top one, leading to propeller-type (double-chain-reversal) loops. Antiparallel G-strands can be linked either by diagonal or lateral (edgewise) loops, depending on whether the strands are adjacent or diagonally opposed. In general, the sequence and size of the loops play an important role in determining the topology of quadruplexes. In addition, loop residues can themselves form stacking and hydrogen bonding interactions, further stabilizing (or destabilizing) G-quadruplex folds.[27–31] A recent molecular dynamics simulation has revealed that the sequences of the connecting loops in quadruplexes are the major contributors to quadruplex flexibility and are the potential sites for drug binding.[32]

The guanine glycosidic torsion angles are another important parameter in characterizing G-quadruplexes. All parallel quadruplexes have guanine glycosidic torsion angles that are characteristic of an *anti* conformation, whereas guanine tetrads in

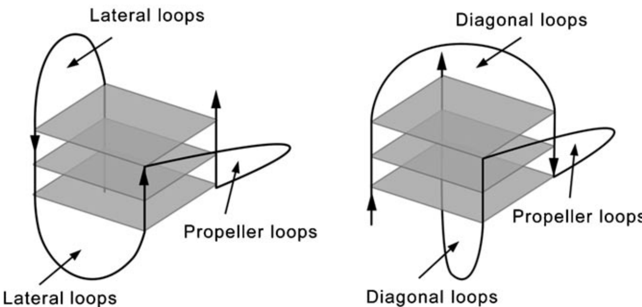

Figure 2. Loop regions in G-quadruplex structures.

antiparallel quadruplexes are found to adopt both *syn* and *anti* conformations characteristic of a given topology and a set of strand orientations.

Many G-quadruplexes can coordinate cations such as K^+ and Na^+. The hole between G-tetrads is well suited to coordinating cations of this size because the two planes of tetrads are lined by eight carbonyl O6 atoms (with strong negative electrostatic potential) that create a central negatively charged channel inside the G-tetrad stack (Figure 3).

In solution, many quadruplex-forming sequences adopt multiple folding topologies depending on the length of individual G-quartets, loop composition and size, and the type of cation. It has been reported that human genes contain as many as 376 000 quadruplex-forming sequences.[33] Databases used in screening and identifying G-quadruplex structures are very common.[34–36] With the increasing number of quadruplexes reported in genomic studies, drug design must be directed not only to differentiating between duplex and quadruplex DNA species, but also to recognizing different quadruplex species.

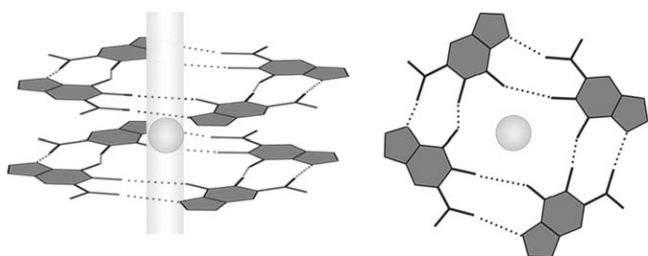

Figure 3. $(G)_8 \cdot K^+$ in a G-quadruplex ion channel.

Table 1. G-rich sequences with the potential to form G-quadruplexes.

Name	Sequence	Description
Intramolecular G4	$G_m X_n G_m X_o G_m X_p G_m$	*m* represents the number of G residues in each short G-tract; X_n, X_o, and X_p can be any combination of residues, including G, forming the loops.
Bimolecular G4	Two $X_n G_m X_o G_m X_p$	X_n and X_p are any non-guanine nucleotide of length *n* and *p*, G_m is any number of guanines involved in tetrad formation of length *m*, and X_o is any nucleotide of length *o* involved in loop formation.
Tetramolecular G4	Four $X_n G_m X_o$	X_n and X_o are any nucleotide of length *n* and *o*, and G_m is any number of guanines involved in tetrad formation of length *m*.

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The area of G-quartets, loop regions, groove dimensions, and the negative electrostatic potential of the anionic backbone and the central channel of G-quadruplexes are critical elements that need to be considered in order to improve the binding selectivity of drug candidates.

2.2. Important G-quadruplexes in the human genome

A number of important regions in the human genome have been reported to adopt G-quadruplex structures. These include telomeric ends, immunoglobulin switch regions, mutational hot spots, and regulatory elements with oncogene promoters.[37] Some of these are recognized as promising targets for the design of antitumor drugs. The sequences of some cancer-related genes are shown in Table 2. Because of the polymorphism of G-quadruplexes, the importance of the various topologies and conformations of these structures for drug–DNA recognition events are discussed.

2.2.1. Telomeres

Telomeric DNA in human somatic cells of tandem repeats of the sequence d(TTAGGG) is typically 5–8 kilobases (kb) long, with a single-stranded 3′ overhang of 100–200 bases. Two intramolecular G-quadruplex structures have been identified from the human telomeric sequence as the basket-type and propeller-type, depending on the incubation conditions (Table 2, Entry A). In Na^+ solution, NMR studies have revealed an antiparallel basket-type G-quadruplex conformation that contains both diagonal and lateral TTA loops.[38] In the presence of K^+, a solid-state, intramolecular propeller-type G-quadruplex conformation has been reported. Propeller-type G-quadruplexes have no loop regions available for end-stacking of G-quadruplex ligands, and their guanine columns are also in a homogeneous parallel arrangement.[10] Because the structure in K^+ solution is considered to be biologically more relevant, a number of structural studies of the telomeric G-quadruplex in K^+ solution have been performed, and several structures were found that are inconsistent with the crystal structure (Table 2). The equilibrium of G-quadruplex species in K^+ solution can be altered by several additional factors. For example, platinum-based cross-linking studies have shown that the basket-type structure coexists with other quadruplexes in both Na^+ and K^+ solutions.[39] A subsequent ^{125}I-radioprobing study has revealed that a chair-type conformation is the major species in K^+ solution.[40] Recently, sedimentation and fluorescence studies have revealed that the crystal structure of telomeric DNA (referred to as H22 in the report) is unlikely to be the major species in K^+ solution, and various forms are energetically similar.[41] A mixture of chair-type and parallel/antiparallel hybrid structures may coexist for H22 in K^+ solution.[2] In the latter study, antiparallel/parallel strands with one propeller and two lateral loops in K^+ solution (mixed-type) has also been observed in two NMR studies.[3,4] However, the NMR structures all have flanking sequences that confer additional stabilization, so they cannot be directly compared with the crystal structure. Recently, it was reported that human telomeric DNA forms parallel-stranded intramolecular G-quadruplexes in K^+ solution under molecular crowding conditions.[42] Moreover, others have suggested a compact stacking structure for multimers of hybrid-type and parallel-type G-quadruplexes in human telomeric DNA.

Telomeres are non-coding DNAs located at the termini of linear chromosomes and can form protective structures at these regions, preventing them from being recognized as DNA double-strand breaks and consequently degraded or fused by DNA repair mechanisms.[43] Stringent control of telomere length is important for cell cycle control, cellular immortalization, and tumorigenesis.[44] Briefly, telomere shortening is thought to lead to the loss of structural integrity of the telomere nucleoprotein, thus resulting in the activation of p53 and Rb tumor suppressor pathways and cellular senescence, which is an important tumor suppressor mechanism.[45] Telomere-induced senescence is as effective as apoptosis in decreasing cancer incidence,[46,47] particularly in preventing oncogene-expressing cells from progressing to malignancy.[48] Normal human somatic cells exhibit weak telomerase activity, which is insufficient to maintain a constant telomere length, whereas more than 90% of human tumor cell populations have high telomerase activity and show various telomere lengths.[49,50]

The folding and stabilization of a variety of G-quadruplexes in G-rich sequences at the end of telomeres may affect telomere length and hence the normal regulation of telomeres in the cell cycle or other events (Figure 4). The effects of stabilization of these structures by small ligands have been extensively studied over the past decade. The interactions of G-quadruplexes with ligands give rise to various effects on telomere functions. For example, many small ligands capable of stabilizing G-quadruplexes are effective in telomerase inhibition, which is the most common parameter for evaluating the telomeric G-quadruplex ligands.[51–54] Other reported effects of ligands binding to G-quadruplexes include shortening of telomere length,[55] induction of senescence, and inhibition of cell growth.[56–58] Moreover, end-to-end fusions of chromosomes have been observed with telomeres in the presence of G-quadruplex ligands.[59] Recently, some G-quadruplex ligands have been found to interfere with the conformation and length of the telomeric G-overhang;[60] other studies have also shown that some G-quadruplex ligands may function by dissociation of the telomere binding proteins POT1 (protection of telomeres 1) and TRF2 (telomeric repeat binding factor 2) and uncapping telomeres to make them available for extension.[61–64]

Additionally, the biological functions of telomeres are likely to be dependent on the nature of their various structural states. For example, hybrid quadruplexes can occur within a 3′-end overhang, and the subsequent packaging enables the formation of a so-called t-loop at the end of the chromosome, which is likely to provide flexibility for responding to such environmental changes as protein binding.[2]

2.2.2. c-myc

The nuclear hypersensitivity element III_1 (NHE III_1) upstream of the P1 promoter of *c-myc* is a G-rich strand containing a 27-

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Table 2. Promoter sequences and G-quadruplex structures in disease-related genes.

Gene	Entry		Promoter Sequence	Structure
Human telomere	A		**T**TAG**G**GTTA**G**GGTT**A**GGGT**T**AGGG**T**TA 1 5 10 15 20 25	A
c-myc	B	Pu27	**T**GGG**G**AGGG**T**GGGG**A**GGGT**G**GGGA**A**GG 1 5 10 15 20 25	B
	C	Myc-2345	**T**GAG**G**GTGG**G**GAGG**G**TGGG**G**AA 1 5 10 15 20	C
	D	Myc-1245	**T**GGG**G**AGGG**T**TTTT**A**GGGT**G**GGGA 1 5 10 15 20	D
	E	Pu24I[a]	T**G**AGGG**T**GGIG**A**GGGT**G**GGGA**A**GG 5 10 15 20 25	E
	F	Myc22-G14T/G23T	T**G**AGGG**T**GGGT**A**GGGT**G**GGTA**A** 5 10 15 20 25	F

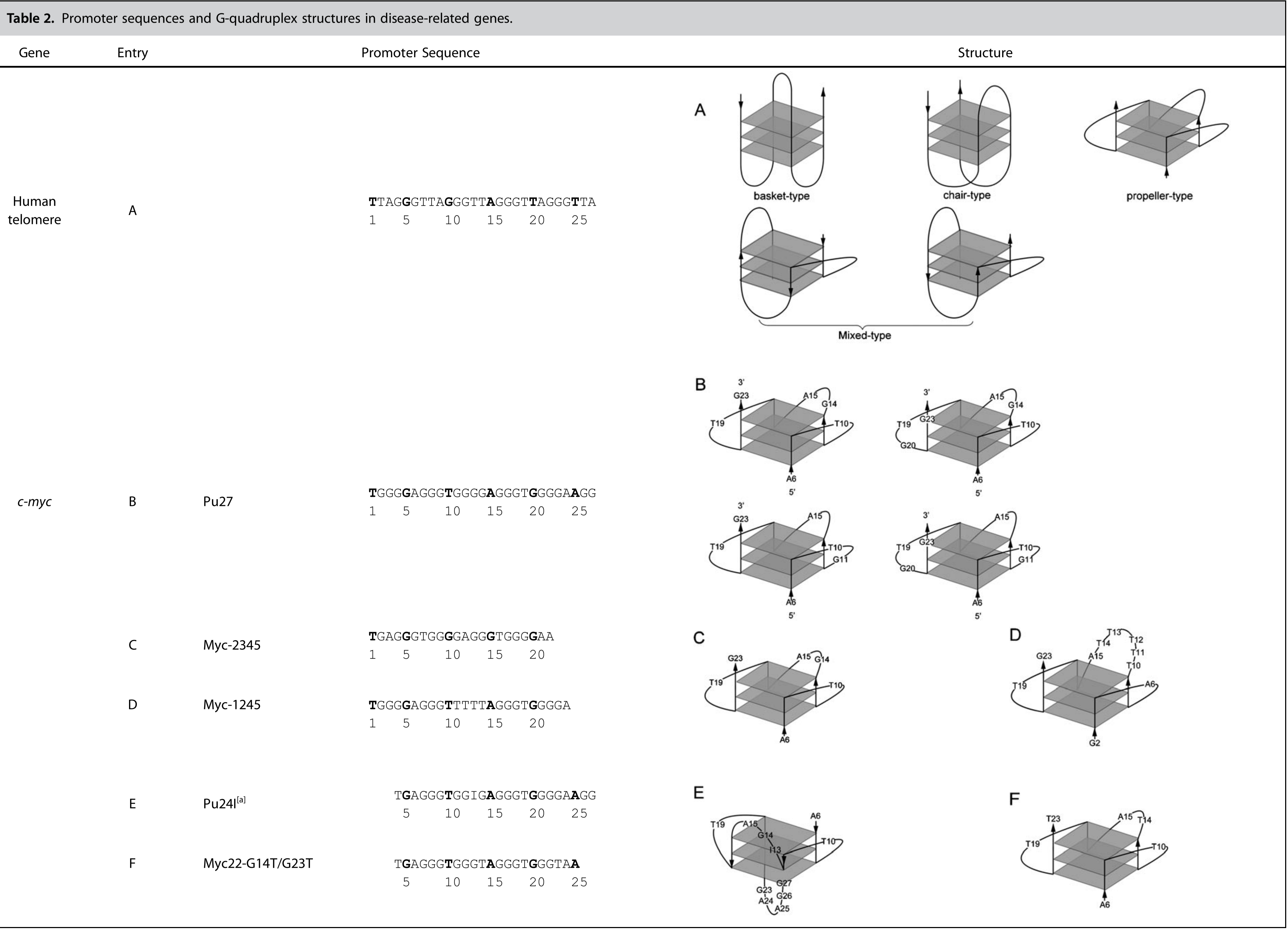

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Table 2. (Continued)

Gene	Entry		Promoter Sequence	Structure
c-kit	G	c-kit native	**A**GGG**A**GGGC**G**CTGG**G**AGGA**G**GGGC**T**G 1 5 10 15 20 25	
	H	c-kit87up	**A**GGG**A**GGGC**G**CTGG**G**AGGA**G**GG 1 5 10 15 20	H
	I	c-kit21	**C**GGG**C**GGGC**G**CGAG**G**GAGG**G**AGG**G**C 1 5 10 15 20 25	
bcl-2	J	Pu39	**A**GGG**G**CGGG**C**GCGG**G**AGGA**A**GGGG**G**CGGG**A**GCGG**G**GCTG 1 5 10 15 20 25 30 35	
	K	5′G4	**A**GGG**G**CGGG**C**GCGG**G**AGGA**A**GGGG**G**C 1 5 10 15 20 25	
	L	3′G4	**C**GCGG**G**AGGA**A**GGGG**G**CGGG**A**GCGG**G**GCTG 10 15 20 25 30 35	
	M	MidG4	CGGG**C**GCGG**G**AGGA**A**GGGG**G**CGGG**A**GC 10 15 20 25 30 35	
	N	MidG4-G15T/G16T	CGGG**C**GCGG**G**AGGA**A**TTGG**G**CGGG**A**GC 10 15 20 25 30	N
VEGF	O		**G**GGG**C**GGGC**C**GGGG**G**CGGG**G**TCCC**G**GCGG**G**GCGG**A**G 1 5 10 15 20 25 30 35	
RET	P		**T**TTT**T**AGGG**G**CGGG**G**CGGG**G**CGGG**G**GTTT**T**T 1 5 10 15 20 25 30	
HIF-1α	Q		**G**CGA**G**GGCG**G**GGGA**G**AGGG**G**AGGG**G**CGCG 1 5 10 15 20 25	
k-ras	R		**G**GGA**G**GGAG**G**GAAG**G**AGGG**A**GGGA**G**GGA 1 5 10 15 20 25	
Rb	S		**C**GGG**G**GGTT**T**TGGG**C**GGC 1 5 10 15	

[a] I = inosine base in the sequence.

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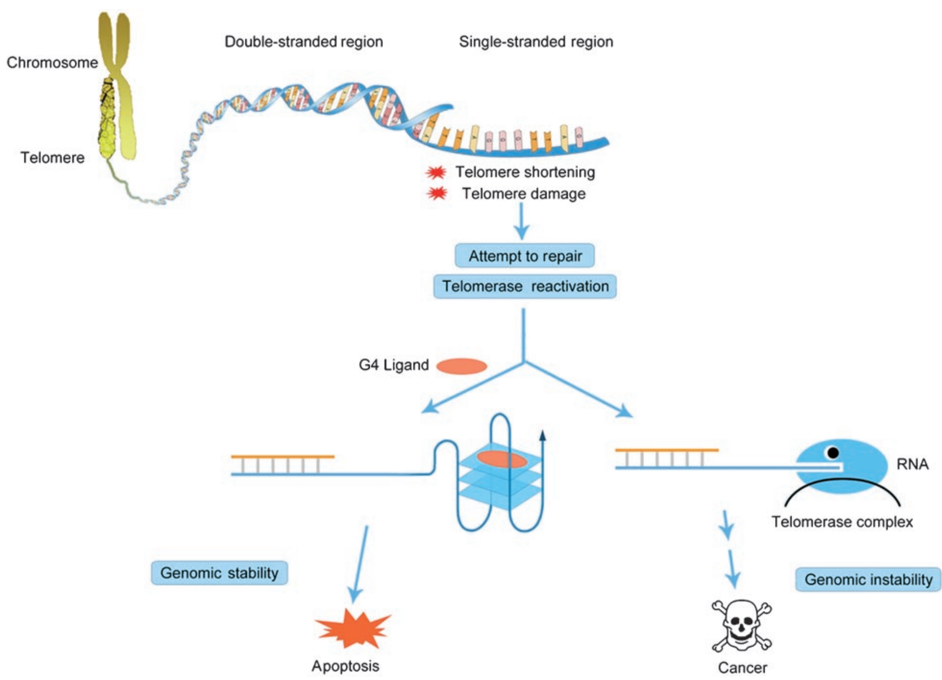

Figure 4. Structure and biological roles of telomeres. Telomeres are noncoding regions of DNA located at the termini of chromosomes and end with a single-stranded region. Repair mechanisms or telomerase reactivation is be triggered by telomere shortening or damage. While repair or reactivation in excess would induce or trigger tumorigenesis, G4 ligands could induce or stabilize the G-quadruplex structure in this region and thus block telomerase from binding to the terminal single-stranded end of telomeres.

base-pair sequence (Pu27), which has the propensity to adopt a G-quadruplex structure. The presence of a quadruplex within this promoter region was initially proposed based on chemical probe studies, gel mobility measurements, and fluorescence resonance energy transfer (FRET) spectroscopy.[65,66] In later studies, the topological structures of several *c-myc* quadruplex sequences were determined by circular dichroism (CD), NMR, and mutational experiments.[67,68] The G-rich region of *c-myc* contains more than four consecutive G-strands, resulting in the formation of a dynamic mixture of four parallel G-quadruplex loop isomers in the native Pu27 region (Table 2, Entry B). Furthermore, two different sequences derived from the Pu27 region have been analyzed by NMR,[69] which revealed that both Myc-2345 and Myc-1245 (Table 2, Entries C and D) form intramolecular propeller-type G-quadruplexes in K^+ solution. In this case, the core of three G-tetrads is formed by four G-stretches oriented in the same direction, with all anti- guanines and three loops adopting double-chain-reversal structures similar to the crystal telomeric G-quadruplex structure in K^+ solution. Similar structures have also be found in the Pu24I (Table 2, Entry E) and Myc22-G14T/G23T sequences (Table 2, Entry F).[70,71] A remarkable structural feature of Pu24I was revealed by NMR studies: a guanine base (G24) at the 3′ end plugs back into the G-tetrad core by participating in G-tetrad formation and displacing another guanine (G10) of a continuous guanine tract in a loop. This configuration is maintained by a stable diagonal loop, which contains a G·(A-G) triad stacking on and capping the G-tetrad core. These new folding features result from the presence of five guanine tracts in the sequence that are different from the four guanine tracts in the *c-myc* sequences studied previously. TMPyP4 has been found to stack on the other terminal G-tetrad, being sandwiched against one of the base-pair platforms with little perturbation from the ligand-free Pu24I quadruplex structure.

c-myc belongs to the Myc gene family and was one of the first oncogenes identified. It was subsequently linked to a wide range of human cancers. *c-myc* functions as a gene-specific transcription factor through its protein product, c-Myc, which is thought to regulate 10–15% of all cellular genes and involved in cell cycle regulation, apoptosis, metabolism, cellular differentiation, and cell adhesion (Figure 5).[72] As a result, the aberrant overexpression of *c-myc* is associated with a variety of malignant tumors including those of breast, colon, cervix, small-cell lung, osteosarcomas, glioblastomas, and myeloid leukemia.[73] In particular, c-Myc has been identified as one of the main activating factors for the human telomerase reverse transcriptase (hTERT) catalytic domain of the telomerase enzyme.[74]

The NHE III_1 region of *c-myc* controls 85–90% of transcriptional activation. The specific G-quadruplex structure formed in this region functions as a transcriptional repressor element (Figure 5).[67] Stabilization of G-quadruplexes in this region by small molecular ligands can suppress *c-myc* transcriptional activation,[67,68,75] down-regulate *c-myc* expression,[76,77] inhibit cell proliferation, and induce delayed apoptosis of leukemia cells.[78]

2.2.3. c-kit

Recently, two G-rich sequences (c-kit native and c-kit21) in the promoter region of the human *c-kit* gene have been identified, and biophysical studies have shown that these sequences can form G-quadruplexes.[79–81] In the c-kit native sequence, 87 base pairs upstream of the transcription start site of the human *c-kit* gene, a single G-quadruplex structure forms in K^+ solution. An NMR study has shown that the c-kit87up sequence forms a new intramolecular G-quadruplex (Table 2, Entry H).[82] Most strikingly, an isolated guanine (G10) is involved in G-tetrad core formation, despite the presence of four three-guanine tracts. There are four distinctive loops, including two single-residue and double-chain-reversal loops (A5, C9), a two-residue loop (C11, T12), and a five-residue stem-loop (A16,

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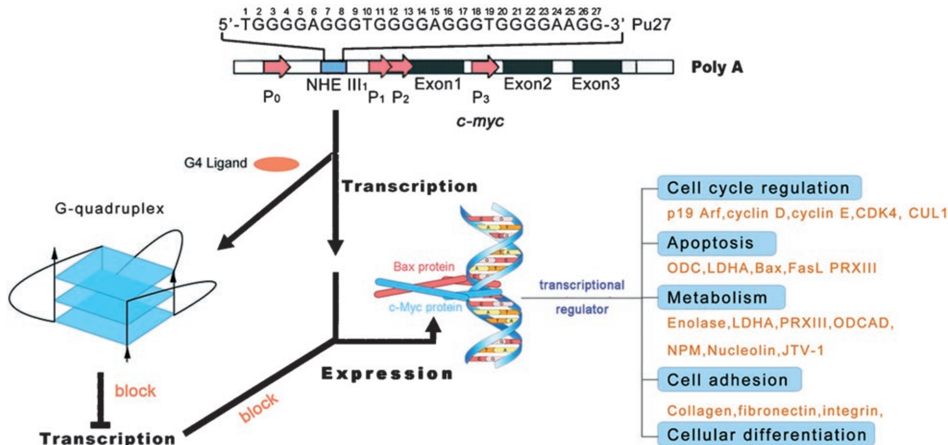

Figure 5. Structure and biological roles of *c-myc*. The G-rich region NHE III_1 is located upstream of the P1 promoter of *c-myc*, which could form a parallel G-quadruplex structure in the presence of G4 ligand. The overexpression of *c-myc* has been found in a variety of tumors, as its product, the c-Myc protein, is a transcriptional regulator, the target genes of which are involved in cell cycle regulation, apoptosis, metabolism, cell adhesion, and cellular differentiation (genes highlighted in orange). The G4 ligand would induce or stabilize the G-quadruplex structure in this region, and thus play a role to "turn on" or "turn off" the transcription of *c-myc*.

G17, G18, A19, G20). In view of the importance of predicting G-quadruplex topologies from sequence information, these new folds in which G residues in non-G-tract regions can participate in structural core formation are particularly worthy of attention. In the case of the c-kit21 sequence, a variety of quadruplex conformations have been identified. This sequence needs to be mutated in order to form a single quadruplex species, probably with a parallel fold.

The proto-oncogene *c-kit*, which encodes a membrane-bound glycoprotein of the family of growth factor receptors with tyrosine kinase activity, constitutes a cell signaling system that can stimulate cell proliferation, differentiation, migration, and survival.[83] Because it is closely linked with the regulation of cell division, its activity plays a very crucial role in human neoplasm. *c-kit* expression has been reported in a variety of human solid tumors such as mast cell tumors, germ cell tumors, ovarian carcinomas, malignant melanomas, gastrointestinal stromal tumors, small-cell lung cancer, neuroblastoma, and breast carcinoma.[84]

The G-rich strand upstream the *c-kit* transcription initiation site is essential for promoter activity.[79,85] In particular, the quadruplex-forming sequence shows a high level of sequence conservation across human, mouse, rat, and chimpanzee, indicating its common biophysical characteristics of this sequence.[80]

2.2.4. bcl-2

The human *bcl-2* gene contains a GC-rich region upstream of the P1 promoter, which is critical for the regulation of *bcl-2* gene expression. It can form a mixture of three distinct intramolecular G-quadruplexes (5′G4, MidG4, and 3′G4) resulting from the six runs of guanines, including one run of five guanines, two runs of four guanines each, and three runs of three guanines each (Table 2, Entries J–M). With more than four consecutive G-tracts in the sequence, the G-quadruplex in *bcl-2* has the ability to form either three or six different loop isomers.[86] The central G-quadruplex (MidG4), which is the most stable of the major species formed in the *bcl-2* promoter region, is likely to form a mixed parallel/antiparallel structure consisting of three tetrads connected by loops and to give rise to three possible loop isomers. An NMR study of the shorter and mutated *bcl-2* quadruplex bcl2 MidG4Pu23-G15T/G16T has showed that one of the topologies for this mixed parallel/antiparallel intramolecular quadruplex has two lateral loops and one propeller loop, similar to one of the 22-mer telomeric quadruplex topologies (Table 2, Entry N).[87,88]

bcl-2 is a proto-oncogene, and its oncogenic property arises from decreasing the rate of cell death.[89] Its protein product, Bcl-2, is a mitochondrial membrane protein, which is present in delicate balance with other related proteins and is involved in the control of programmed cell death, functioning as an apoptosis inhibitor.[90] Overexpression of *bcl-2* has been found in a wide range of human cancers, including B-cell and T-cell lymphomas, breast, cervical, non-small-cell lung, prostate, and colorectal. In addition, it also functions in chemotherapy-induced apoptosis,[89] which indicates its potential role in drug resistance.

The G-rich strand located in the *bcl-2* P1 promoter plays a significant role in the regulation of *bcl-2* transcription.[91] Although the effects of G-quadruplex ligands on *bcl-2* expression remain to be unearthed, some studies have shown that some G-quadruplex ligands can induce apoptosis.[57,58,92–94] In particular, the ligand 12459 has been found to induce apoptosis characterized by dysfunction of Bcl-2.[95] These findings suggest the possible role of G-quadruplex formation in the *bcl-2* promoter during apoptosis.

2.2.5. VEGF and Rb

The formation of G-quadruplexes in the G-rich sequences of the human vascular endothelial growth factor (VEGF) and the *neuroblastoma* (*Rb*) oncogene promoter has been investigated by chemical footprinting and CD studies.[96,97] These quadruplexes, which are induced to form from the duplex sequences with the ligands telomestatin or TMPyP4, are likely to adopt the parallel conformation.

VEGF is a pluripotent cytokine and angiogenic growth factor.[98] When it binds with its cognate receptor, the complex can stimulate the proliferation, migration, survival, and permeability of endothelial cells, thus resulting in the formation of

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new blood vessels.[99] The formation of new blood vessels adjacent to primary tumors promotes the growth of cancer cells by providing oxygen and nutrients. Because VEGF can initiate angiogenesis to aid in tumor cell survival, its expression is usually elevated in many types of cancer.[100] The transition from duplex to quadruplex conformation in the promoter region of VEGF can be driven by G-quadruplex ligands such as TMPyP4 (Figure 13, below) and telomestatin (Figure 18, below), and the transition may affect the enzymes that bind to this region,[97] indicating that the formation of G-quadruplexes may play a role in VEGF expression.

The *Rb* gene encodes a nuclear phosphoprotein that acts as a tumor suppressor by affecting the cell cycle. The Rb protein regulates G_1/S-phase cell cycle progression and is a critical mediator of antiproliferative signaling. Moreover, Rb is functionally inactivated in the majority of human cancers and is aberrant in one-third of all breast cancer cases.[101] Given the important roles of the Rb/E2F pathway in controlling cell growth and a series of biological events leading to DNA replication and S phase, oncogenic mutations of the *Rb* gene are thought to disrupt the normal function of the pathway.[102] The formation of G-quadruplexes at the 5′ terminus of the *Rb* gene impedes the activity of DNA polymerase, indicating a possible biological role for the quadruplex structure in the *Rb* gene.[96]

2.2.6. Other G-quadruplexes

Putative quadruplexes corresponding to the sequences of the hypoxia-inducible factor 1α (HIF-1α) promoter region, *k-ras* and *RET* oncogenes, are shown in Table 2.[103–105] Other types of G-quadruplexes have also been found in the RNA region,[106] peptide nucleic acids (PNA)[107] and locked nucleic acids (LNA).[108] All these sequences constitute the G-quadruplex family.

3. G-quadruplexes as Targets for Anticancer Drug Design

3.1. Interaction modes between ligands and G-quadruplexes

The particular geometry of the G-quadruplex structure is thought to allow specific recognition by small ligands through various binding modes (Figure 6) in a manner analogous to that of double-helical DNA intercalators. Several groups of compounds, including cationic porphyrin (TMPyP4),[109] acridine (BSU6039),[110] polycyclic acridine (RHPS4),[111] and *N*,*N*′-bis-[2-(1-piperidino)ethyl]-3,4,9,10-perylenetetracarboxylic diimide (PIPER),[112] have been identified as interacting specifically with G-quadruplexes.

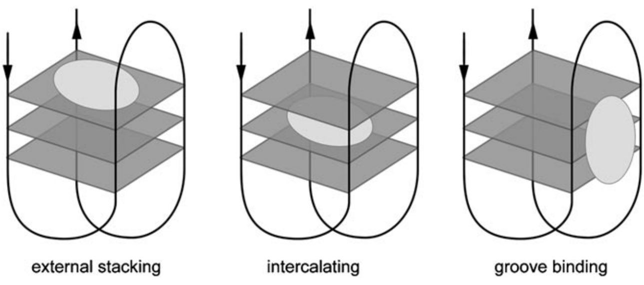

Figure 6. A representation of a ligand–G4 complex with a) external stacking mode on the surface of the terminal quartet, b) intercalating mode between the stacks of G-tetrads, and c) groove binding mode.

Despite the emergence of such compounds, the interactions of these compounds with G-quadruplexes in vitro are far more complicated than expected. For example, NMR studies have shown that in the presence of RHPS4, the structure of the core quadruplex $d(TTAGGGT)_4$ remained unperturbed because the compound does not intercalate within the G-quadruplex, but instead stacks on the surface of the terminal quartet (Figure 7 a).[111] The crystal structure of a complex of BSU6039 (Figure 7 b) with the *Oxytricha* dimeric intermolecular quadruplex formed from two strands of d(GGGGTTTTGGGG) has been reported, and the results show that the acridine moiety binds at one end of the G-quartets within one of the thymine loops.[110] Duplex intercalators such as ethidium or proflavin, which are usually poor quadruplex binders,[113] also show a similar phenomenon. Several peptide–hemicyanine conjugates have been proposed to be specific for binding to quadruplex grooves and loops without significant π stacking with DNA bases.[114]

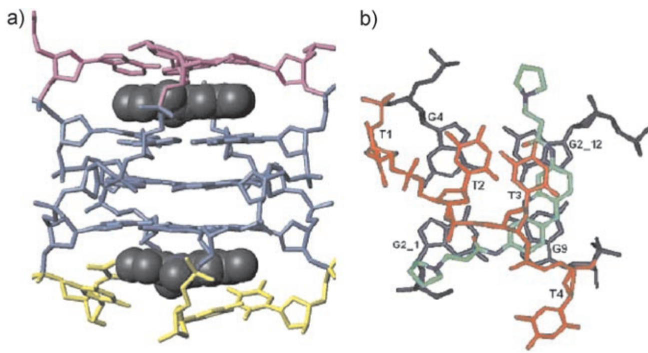

Figure 7. a) Energy-minimized structure of the 2:1 RHPS4–$d(TTAGGGT)_4$ complex showing the AGGGT core with RHPS4 intercalated at the A3–G4 and G6–T7 steps. (Figure reprinted with permission from reference [111]; copyright 2003, Elsevier.) b) A view onto the plane of BSU6039 (cyan), showing its stacking onto the adjacent G-quartet (blue). (Figure reprinted with permission from reference [110]; copyright 2003, Elsevier.)

Intercalator binding between G-tetrads inside the quadruplex is thought to be difficult, not only because the G-quadruplex is an extremely stable and rigid structure, but also because distortion of quadruplex integrity requires a very high energy cost. Thus, stacking of the drug on the outer planes of G-tetrads appears to be a more energetically favorable and probable mode for ligands that have the potential to intercalate with G-quadruplex DNA.

3.2. Common methods for characterizing G-quadruplex–ligand interactions

To gain a better understanding of receptor–ligand interactions, it is necessary to answer the questions posed by Scatchard[115]

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more than 50 years ago: "How many? How tight? Where? Why? What is it?". The first two questions can be answered by equilibrium binding studies, whereas the answers for the remaining three questions can be found from the results of structural and functional studies.

Quadruplex–ligand interactions can be investigated by the techniques used to study duplex DNA–ligand interactions. Titration experiments analyzed by spectroscopic methods such as ultraviolet (UV), fluorescence, and CD can provide useful information on equilibrium ligand–quadruplex binding, including the location of the binding sites and the nature of the binding interactions. NMR spectroscopy and X-ray crystallography can also be used to obtain detailed structural information on quadruplex–ligand complexes.

Calorimetric techniques such as differential scanning calorimetry (DSC) and isothermal titration calorimetry (ITC) can provide quantitative information on the thermodynamics of ligand–quadruplex binding. Melting experiments monitored by UV, FRET, and CD measurements can also be used to assess ligand–quadruplex interactions.

Electrospray ionization mass spectrometry (ESIMS) can be used to probe the noncovalent interactions of quadruplexes with ligands. Surface plasmon resonance (SPR) spectroscopy is an alternative choice for studying quadruplex–ligand interactions. SPR can monitor molecular binding interactions in a real-time manner and provide useful information (such as K_d values) on the binding system.

Gel band-shift assays have been widely used to examine DNA–ligand interactions, including electrophoresis mobility shift assays (EMSA) and dimethyl sulfate (DMS) methylation protection assays. Molecular modeling and molecular dynamic studies are also important approaches for estimating the interaction energies and predicting the possible structures of quadruplex–ligand complexes. Polymerase chain reaction (PCR) stop assays have been used to evaluate the effects of ligand interactions on the formation of G-quadruplexes. Some of these approaches are discussed below, and examples of the methods and applications are presented.

3.2.1. UV/Vis spectroscopy

A conventional method for identifying quadruplex-interacting ligands involves the use of UV/Vis spectroscopy. G-quadruplexes often produce a remarkable bathochromic shift and a hypochromic effect on the Soret band of the bound chromophore. This hypochromic phenomenon is thought to arise from the strong interaction between the electronic states of the chromophore and DNA bases. This approach has been used to study the interaction of cationic porphyrin (TMPyP4) with quadruplex sequences in *c-myc*.[116] Pronounced changes in the UV/Vis spectrum of the compound were observed, including a red shift in the absorbance maximum (λ_{max}), and a remarkable attenuation in absorbance (~35%). These observations indicated an intercalative interaction between the TMPyP4 molecule and the quadruplex structure.

3.2.2. Circular dichroism

Polarized light spectroscopy offers the possibility to characterize nucleic acid conformations and their complexes with proteins or other ligands. For example, CD can provide structure information on G-quadruplex and quadruplex–ligand complexes.[3,14,117] In most cases, CD can be used to distinguish between parallel and antiparallel folded quadruplexes[118,119] because of the distinctive separation of the marker bands for these structures; parallel quadruplexes usually show a strong positive CD band at 270 nm and a negative band at 240 nm, whereas antiparallel quadruplexes exhibit a positive band at 295 nm and a negative band at 260 nm.

In general, nonchiral molecules are CD-inactive. Upon binding to quadruplexes, however, nonchiral molecules often give characteristic CD signals due to induced CD (ICD) resulting from the chiral environment of the molecule in the bound state.[120,121] An ICD signal corresponding to the absorption by a nonchiral ligand represents direct evidence of interaction with DNA, and usually provides some information on the ligand–DNA interaction. However, the use of ICD for characterizing different types of quadruplex–ligand interactions, such as the orientation and location of the ligand within its binding site, appears to be challenging.

3.2.3. NMR spectroscopy

Most in vitro structural studies of quadruplex-forming oligonucleotides have been carried out with NMR spectroscopy. This technique can provide rich structural information in which spectral parameters are usually related to quadruplex conformation and dynamics. The first step in the NMR analysis of quadruplex-forming oligonucleotides is the assignment of resonance frequencies to individual proton nuclei of the oligonucleotide (in the case of ^{13}C- and ^{15}N-labeled oligonucleotides, ^{13}C and ^{15}N resonances can also be assigned). The nuclear Overhauser effect (NOE)-based strategy developed for resonance assignment of linear duplex DNA is only partially feasible for quadruplexes because of their poor chemical shift dispersion, the possible presence of nucleotides in the *syn* conformation, or the folding of the phosphate backbone.[122]

The quality of the spectrum of the exchangeable proton resonances is a good indication of how amenable the quadruplex spectra will be for structural analysis. The ^{1}H spectrum in 90% H_2O/D_2O can provide a wealth of structural information on the quadruplex. The number of slowly exchanging imino protons gives information about strand stoichiometry, the number of G-quartets, and the symmetry of the quadruplex structure.[123,124] The range of resonance frequencies indicates the presence of Watson–Crick base pairs, Hoogsteen-bonded imino protons, hydrogen-bonded amino protons, and exchange-protected imino protons from unpaired residues. In general, imino proton chemical shifts <12.5 ppm are indicative of Watson–Crick base pairs (NH···H hydrogen bonds). Imino proton chemical shifts in the range of 10.5–12 ppm are indicative of guanine NH···O hydrogen bonds that appear in Hoogsteen alignments of the G-quartets.

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3.2.4. X-ray crystallography

X-ray crystallographic techniques have played a significant role in probing the structures of G-quadruplexes in DNA and RNA. The single-crystal analysis of short-length oligonucleotides can provide precise and detailed structural information on G-quadruplexes and G-quadruplex–ligand complexes. There are more than 20 structures of quadruplex DNA or RNA that have been solved by X-ray crystallography available in the RCSB protein data bank (PDB). The crystals are commonly produced by vapor diffusion with sitting or hanging drops in a solution that contains the cacodylate salt of a monovalent cation, 3–6 mM spermine, and 5–15% (*v*/*v*) of a polymer [methylpentanediol or poly(ethylene glycol)] at pH 6.0–7.0. The presence of a divalent cation has been reported to increase the order within the crystals.[125]

X-ray crystal structures are unambiguous, and provide a definitive view of the location of the cations within the structure, the hydration within the grooves, and the binding mode of the ligand with the G-quadruplex. However, it is possible that the crystal structure is not representative of the primary species in solution; the crystal packing forces and the crystallization conditions may influence the structure. A more thorough discussion of X-ray crystallographic techniques applied to the study of G-quadruplex structures can be found in a review by Campbell and Parkinson.[126]

3.2.5. FRET assays

Fluorescence resonance energy transfer can give valuable information on the structure of nucleic acids[127–129] because this photophysical phenomenon is distance- and orientation-dependent. FRET has been successfully used to probe the secondary structure of oligonucleotides containing G-rich sequences in which a fluorescence donor and acceptor are attached to opposite ends of the oligonucleotide. The formation of a quadruplex in the G-rich sequence is often accompanied by a decrease in the distance between the donor and acceptor, thus leading to a more efficient energy transfer between the two chromophores. This approach is very useful for assessing the effects of ligand binding on the structural stability of G-quadruplexes. With ligand bound, the fluorescent quadruplex is often stabilized, thus showing an increased melting temperature.[130] FRET has been applied to real-time PCR experiments and allows the rapid screening of a large pool of different ligands.[131]

3.2.6. ITC and DSC

Calorimetric techniques such as isothermal titration calorimetry and differential scanning calorimetry provide direct quantitative information on the thermodynamics of quadruplex–ligand binding and are reliable methods for obtaining such information. These techniques, however, usually require substantially more material than most spectrophotometric methods, and may not be suitable for determining large binding constants.

ITC is a powerful technique that allows the direct monitoring of many bimolecular binding interactions in a label-free manner.[132–135] ITC gives a binding isotherm that is used to determine the binding enthalpy (ΔH°), the equilibrium binding constant (K_b), and binding stoichiometry (n) for an equilibrium binding system. The thermodynamic parameters, Gibbs free energy change (ΔG°) and entropy change (ΔS°), can be calculated by using equations (1) and (2):

$$\Delta G^\circ = -\Delta RT \ln K_b \quad (1)$$

$$\Delta S^\circ = (\Delta H^\circ - \Delta G^\circ)/T \quad (2)$$

This method has been successfully used to study the interaction of cationic porphyrin and anthraquinone ligands with G-quadruplexes.

DSC allows the continuous measurement of the apparent specific heat of a system as a function of temperature.[136–138] Thus, DSC can be used to examine physicochemical processes initiated by temperature, such as phase transitions or conformational changes. A single DSC experiment can provide a wealth of thermodynamic information, much of which cannot obtained by other techniques. The area under the experimental heat capacity curve can be used to determine the calorimetric transition enthalpy (ΔH). This calorimetric transition enthalpy is model-independent and is therefore unrelated to the nature of the transition. The temperature at which excess heat capacity occurs at a maximum defines the transition temperature (T_m). Differences in the initial and final baselines provide a measure of the heat capacity change that accompanies the transition. The DSC methodology offers a convenient and powerful means to characterize the thermodynamics of quadruplex stability. This technique has been widely used to study the effects of ligand binding on G-quadruplexes.

3.2.7. ESIMS

Many noncovalent interactions between nucleic acids and small ligands are biologically important processes, and ESIMS is recognized as a useful tool in the characterization of such binding.[139–142] The stoichiometry and selectivity of the binding of small molecules to oligonucleotides can usually be obtained with minimal sample consumption. For G-quadruplexes, mass spectra are usually obtained with an LCQ ion trap mass spectrometer equipped with a heated capillary electrospray source, or a Q-TOF mass spectrometer equipped with a Z-spray source. Both instruments can be operated in the negative ion mode. The use of ESIMS to investigate the selectivity, binding stoichiometry, and binding mode of the small ligands *N*,*N*′-bis-(2-morpholinylpropyl)-3,4,9,10-perylenetetracarboxylic acid diimide (Tel01), distamycin A, and diethylthiocarbocyanine iodide (DTC) to the parallel-stranded G4-quadruplex $[d(T_2G_5T)]_4$ has been reported.[142] Moreover, the ability of ESIMS to distinguish the binding selectivity between quadruplex and duplex DNA and to differentiate between various binding modes is of interest.

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3.2.8. SPR biosensor assays

Surface plasmon resonance is a sensitive technique for characterizing quadruplex–ligand interactions.[129,143–146] Upon interaction of a small ligand with a quadruplex, a change in refractive index at the sensor surface occurs. The change in SPR response is directly related to the changes in the refractive index at the sensor surface. The refractive index at the surface is directly related to the number of molecules bound at the sensor surface. A change in refractive index at the sensor surface causes a shift in the reflection angle. In general, a quadruplex that interacts with ligands is immobilized onto the sensor chip, and the binding of the ligand to quadruplex results in a change in refractive index. SPR is a rapid and powerful tool for screening small-molecule libraries, and allows kinetic and thermodynamic characterization of the interactions of active ligands with macromolecules.

3.2.9. EMSA

Electrophoresis mobility shift assays are a convenient way to determine conformational changes in DNA, including the formation or destabilization of a quadruplex, and the binding between ligands and quadruplexes.[118,145,147] The oligonucleotide in question is incubated with the test compound and is then subject to electrophoresis in native polyacrylamide gel conditions followed by phosphorescent image analysis (phosphorimaging). Stabilization of the quadruplex structure by ligand binding usually leads to a proportional mobility shift (decrease in migration rate) of the oligonucleotide relative to that of the quickly migrating quadruplex alone.

3.2.10. DMS methylation protection assays

DMS methylation protection, which is one of the techniques of chemical footprinting assays, is very useful for studying the interaction of small ligands with quadruplexes and mapping the binding site with high accuracy.[68,145,148] In this assay, the band from an EMSA representing the shifted quadruplex species is subjected to DMS treatment to methylate guanine bases. The sample is then subjected to piperidine cleavage at the sites of methylated guanine residues and is analyzed with a native polyacrylamide gel assay. The protection of the guanine residues from methylation by ligand–quadruplex interactions can be assessed by this method.

3.2.11. Competition dialysis assays

Competition dialysis assays can be used to probe the binding selectivity of ligands to various nucleic acids structures[25,139,149] based on the principle of equilibrium dialysis. A macromolecule solution is usually placed in a semipermeable dialysis membrane that allows small ligands to pass through, whereas large molecules are retained. At equilibrium, the chemical potential of the free ligand is virtually equal inside and outside the dialysis membrane, and any excess ligand on the macromolecule side of the membrane can be attributed to binding to the macromolecule in question.

3.2.12. PCR stop assays

The induction of biologically relevant G-quadruplex formation in specific sequences by G-quadruplex ligands can be investigated using a PCR stop assay. The specific binding of ligands with G-quadruplexes in the promoter regions of genes or telomeric DNA can block the binding of RNA polymerases to the sequences and thus inhibit polymerase activity. There are two types of PCR stop assay in current use: one employs a ^{32}P-labeled long G-rich template and a short primer complementary with the 5′ end of the template; products representing full-length, major arrest site, and primer can be evaluated by electrophoresis.[67] The other type employs a pair of nonlabeled primers that are partially complementary; double-stranded products can be evaluated by electrophoresis and staining (SYBR, silver, or ethidium bromide).[150] Both of these PCR stop assay techniques have been used widely, and are chosen based on the given laboratory resources available.

3.3. Small-molecule binding to G-quadruplex structures

Since Zahler and co-workers demonstrated in 1991 that K^+-stabilized G-quadruplex structures are able to inhibit telomerase activity,[151] G-quadruplexes have emerged as a significant target for telomerase inhibitors. Several research groups have searched for small organic molecules that can bind to G-quadruplexes and hence block telomerase activity or inhibit G-quadruplex-related gene expression. Although there is still a long way to go in the development of potent drugs that target G-quadruplexes, some promising lead compounds have been achieved by structure-based design and organic synthesis.

3.3.1. Anthraquinones and related inhibitors

The first quadruplex-interactive ligand as a telomerase inhibitor is a symmetric molecule, the 2,6-disubstituted aminoalkylamido anthraquinone BSU-1051 (Figure 8) with a telomerase inhibitory value ($^{tel}IC_{50}$) of 23 μм.[5] Systematic structure–activity studies were subsequently reported[132,133] for a wide range of substituted amido anthraquinones (AQ, Figure 8), such as 1,4-, 1,5-, 1,8-, 2,6-, and 2,7-regioisomers with substituents at various positions on the chromophore. Some structure–activity relationships of these compounds, such as the significant factors of side chain length and the size and nature of the side chain end groups, are summarized.

Computer modeling was used to establish the DNA quadruplex–AQ complexes based on a "threading" intercalation binding mode. This molecular model indicates that tetrad–ligand π–π overlap is feasible, and that any flexible pendant side chains can be accommodated in the wider quadruplex grooves.

A series of 2,7-fluorenone (2,7-FO, Figure 8) analogues have been designed to decrease cytotoxicity by prevention of redox

Figure 8. Structures of G-quadruplex ligands: anthraquinone regioisomers and 2,7-fluorenones.

cycling through removal of one of the quinone carbonyl moieties.[152] The most potent compounds in this series have $^{tel}IC_{50}$ values of 8–12 μM, with a 2–10-fold decrease in conventional cytotoxicity against a panel of human-tumor-derived cell lines relative to their equivalent AQ analogues. In general, the substituent effects on telomerase inhibition observed with the FO analogues is similar to the those of the AQ series.

Comparison of 2,7-AQ and 2,7-FO analogous demonstrates that the latter are slightly less active telomerase inhibitors possibly because of the curved arrangement and decreased electron deficiency of the 2,7-FO chromophore, which disfavor π-stacking interactions with the putative G-quartet binding site.

3.3.2. Acridine analogues

The importance of introducing a positive charge in the central ring of the chromophore has been the impetus for extensive studies of the use of a series of 3,6-bisamidoacridines as telomerase inhibitors (Figure 9)[134,153] because the positively charged ring is likely to be complementary to the channel of negative electrostatic potential of G-quadruplexes. In comparison with the analogous 2,7-bisamidoanthraquinones, these compounds do not have clearly improved inhibitory activity toward telomerase. This can be ascribed to the weak basicity of the ring nitrogen atom for the poor electron-donating effect of the 3,6-bisamido groups. Thus, the electronic properties of the acridine chromophore appear to be similar to those of the amidoanthraquinone moiety.

Figure 9. Structures of G-quadruplex ligands: 3,6-bisamidoacridines.

The selectivity of ligands for G-quadruplex over duplex DNA has been studied by computer modeling methods.[143,154] As mentioned above, G-quadruplexes usually have distinct structural character with duplex DNA, notably the presence of four quasi-equivalent grooves in the structure. A number of 3,6,9-trisubstituted acridines (Figure 10) were rationally designed and synthesized.[154] Each of the two substituent amido chains lies in the two widest grooves, the anilino group at the 9-position of the acridine fits into a third groove in this model,[134] and the 9-substituent enhances the basicity of the acridine central ring nitrogen atom. The enhanced telomerase inhibitory activity of these compounds was evaluated with that of the 3,6-disubstituted compounds, correlating with increased quadruplex binding affinity measured by SPR and binding energy calculated by computer modeling. These 3,6,9-trisubstituted acridines interact selectively with G-quadruplex over duplex DNA. The 3,6-disubstituted acridines have approximately the same binding constant for duplex and quadruplex DNA, whereas the 3,6,9-trisubstituted acridines bind to quadruplex DNA with 30–40-fold greater affinity than to duplex. Recently, members of the new 3,6,9-trisubstituted acridine family[155] have been found to have telomerase inhibitory activity in the range of 10–20 nM. Some of these have entered the preclinical development stage.

Figure 10. Structures of G-quadruplex ligands: 3,6,9-trisamidoacridines and the pentacyclic acridine RHPS4.

A pentacyclic acridine 3,11-difluoro-6,8,13-trimethyl(8*H*)-quino[4,3,2-*kl*]acridinium methylsulfate (RHPS4, Figure 10) has also been identified as a potent telomerase inhibitor, with an $^{tel}IC_{50}$ value of 0.33 μM. This compound was found to inhibit cell proliferation within 2–3 weeks at non-cytotoxic concentrations.[156,157]

3.3.3. Quindoline analogues

Molecular modeling and structure–activity studies of amidoanthraquinones and acridines have revealed that a tricyclic chro-

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mophore is possibly insufficient for ligand stacking on the surface of the terminal quartet. Two classes of tetracyclic chromophore ligands with four aromatic rings have been studied, one of which has a five-membered ring in the middle and is fused in a linear arrangement to produce crescent-shaped molecules. One derivative of benzo[*b*]naphtho[2,3-*d*]furan (Figure 11 a) with a single substituent has an $^{tel}IC_{50}$ value of 7.0 μM,[158] and the disubstituted derivatives of the natural product quindoline have modest activity, with $^{tel}IC_{50}$ values in the range of 6–16 μM (Figure 11 b,c).[159,160]

Figure 11. Structures of G-quadruplex ligands: a) benzo[*b*]naphtho[2,3-*d*]furan, b,c) disubstituted quindolines, and d) 11-substituted quindolines.

It is interesting that 11-subsitituted quindolines (Figure 11 d) designed and synthesized by our research group show stronger telomerase inhibitory activity than the disubstituted quindolines, with $^{tel}IC_{50}$ values of 0.44–12.3 μM.[55,161] This can be attributed to the nitrogen atom in the pyridine ring of quindoline. Electron-donating groups such as substituted amino groups at the 11-position can enhance the basicity of the nitrogen atom in the pyridine ring, which is protonated at physiological pH, resulting in an increase in the electrostatic interaction between the quindoline derivatives and the negative electrostatic center of the G-quadruplex. The results indicate that this 11-substituted quindoline not only stabilizes the G-quadruplex structure but also induces the G-rich telomeric repeat sequence to fold into a quadruplex structure. Our recent studies have shown that these derivatives have the ability to induce and stabilize the G-quadruplexes in *c-myc*, which lead to down-regulation of *c-myc* in the HepG2 cell line.[76] We also found that derivatives with terminal amino groups in their side chains can selectively bind to the *c-myc* G-quadruplex isomers with the double nucleotide loops in the absence of K^+.

3.3.4. Quinacridine analogues

Pentacyclic crescent-shaped dibenzophenanthroline (quinacridine) derivatives have the ability to stabilize G-quadruplexes, as shown by FRET assays. The chromophores of these molecules have five aromatic rings, which are fused in a linear arrangement to produce crescent-shaped molecules. These ligands bind to the human intramolecular quadruplex, as evidenced by changes in G-quadruplex DNA melting temperature (ΔT_m), which correlates well with the extent of inhibitory activity against telomerase. The two most active compounds (Figure 12 a,b) have ΔT_m values of 19.7 and 12.5 °C, and $^{tel}IC_{50}$ values of 0.028 and 0.5 μM, respectively.[127]

Figure 12. Structures of G-quadruplex ligands: a,b) disubstituted quinacridines, c) cyclo-bis-quinacridine BOQ_1, and d) the platinum–quinacridine hybrid Pt-MPQ.

Cyclic bis-intercalating ligands have the ability to preferentially bind nucleic acid motifs containing exposed bases such as hairpin loops. This selective behavior can be attributed to their cyclic framework, which disfavors their intercalation into a DNA double helix. This observation has recently been obtained with a cyclic macrocycle containing two quinacridine moieties bridged by polyammonium linkers.[129] The compound BOQ_1 (Figure 12 c) appears to be a very promising potential drug candidate for G-quadruplexes because the combination of large subunits that are able to stack on a G-quartet and the macrocyclic scaffold makes it likely to exhibit favorable groove/loop interactions. BOQ_1 seems to have a higher propensity to bind to quadruplex ($\Delta T_m = +28$ °C) than duplex DNA.

More recently, a novel platinum–quinacridine hybrid (Pt-MPQ, Figure 12 d),[162] comprising a monofunctional Pt moiety and mono-*para*-quinacridine, was found to interact with quadruplex DNA by a dual noncovalent/covalent binding mode. Pt-MPQ is the first prototype hybrid molecule that is able to inter-

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act with quadruplex DNA simultaneously through stacking interactions and platination of bases.

3.3.5. Cationic porphyrins and related analogues

3.3.5.1. Cationic porphyrins

Porphyrins are well-established binding agents for duplex DNA, and there is evidence that they may have unusual intercalation interactions with certain double-stranded DNA sequences. It is generally believed that the planar arrangement of the aromatic rings in porphyrin analogues can bind to G-quadruplexes by stacking with the G-tetrads. Therefore, porphyrins are nonselective ligands that bind to all nucleic acids. By using CD and NMR, the porphyrin analogue TMPyP4 (tetra-(*N*-methyl-4-pyridyl)porphyrin, Figure 13 a) was found to bind

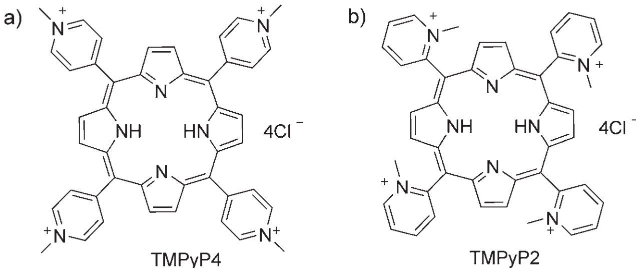

Figure 13. Structure of a) TMPyP4 and b) its isomer TMPyP2.

to and stabilize both parallel and antiparallel G-quadruplex structures, but with only twofold greater affinity for quadruplex over duplex DNA.[109,163] Furthermore, TMPyP4 was found to inhibit telomerase by interacting with G-quadruplexes, whereas its isomer, TMPyP2 (tetra-(*N*-methyl-2-pyridyl)porphyrin, Figure 13 b), with the *N*-methyl groups in the sterically hindered 2-position, showed very weak activity.[164,165] Based on the results of photocleavage assays, a model for the end stacking of TMPyP4 on the terminal G-tetrads of the G-quadruplex was proposed.[164] NMR chemical shift changes and line broadening have been observed upon addition of TMPyP4 to quadruplex DNA samples. Furthermore, UV titration experiments on intramolecular quadruplex showed sharp isosbestic points, hypochromicity, and a remarkable red-shift. Both the UV and NMR data strongly suggest that TMPyP4 binds to quadruplex DNA by stacking externally on the G-tetrads rather than by intercalating between these structures.[109]

More recently, the crystal structure of a bimolecular human telomeric quadruplex with sequence d(TAGGGTTAGGG) in complex with TMPyP4 was reported with a resolution of 2.09 Å[166] (Figure 14). The porphyrin molecule was found to stack on the TTA nucleotides, either at part of the external loop structure or at the 5′ region of the stacked quadruplex. The stacking interactions of TMPyP4 with the quadruplex involve the hydrogen bonded base pairs that are not involved in the formation of G-tetrads, thus precluding direct ligand interactions with the G-tetrads. This is consistent with the lower selectivity of TMPyP4 for quadruplex DNAs relative to duplex DNA.

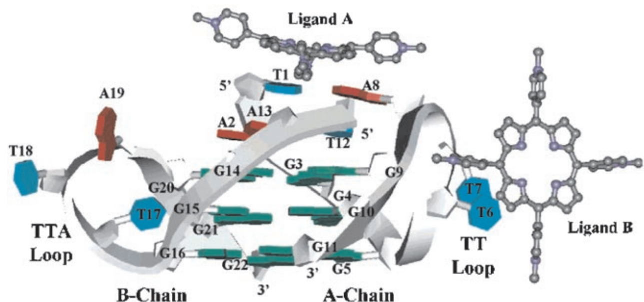

Figure 14. Schematic view of the bimolecular quadruplex–TMPyP4 structure, showing the folding topology, the numbering of nucleotides, the extended TTA loop geometry, and the two TMPyP4 molecules bound per asymmetric unit. Guanine bases are colored green, adenines red, and thymines cyan. (Figure reprinted with permission from reference [166]; copyright 2007, American Chemical Society.)

Microcalorimetry (such as ITC and DSC), electronic spectroscopy (UV/Vis and CD) and molecular modeling have been used to examine the interactions of TMPyP4 with the *c-myc* Pu27 quadruplex.[116] The stoichiometry of TMPyP4/Pu27 G-quadruplex at saturation was found to be 4:1. The four independent TMPyP4 binding sites fall into one of two modes. The two binding modes are different from affinity, enthalpy change, and entropy change for formation of the 1:1 and 2:1, or 3:1 and 4:1 complexes. Because of the specific structure of TMPyP4, it has become the standard molecular model for probing the interactions between small ligands and novel G-quadruplexes.

3.3.5.2. Pentacationic manganese(III) porphyrin

A pentacationic manganese(III) porphyrin (Figure 15) containing a central aromatic core and four flexible cationic arms was recently found to be able to differentiate between quadruplex and duplex DNA; the selectivity differs by about 10000-fold.[167] The bulky cationic substituents surrounding the manganese(III) porphyrin, which preclude a close interaction with the double-stranded DNA structures, are likely to decrease the affinity for duplex DNA. Currently, this porphyrin derivative is the most ef-

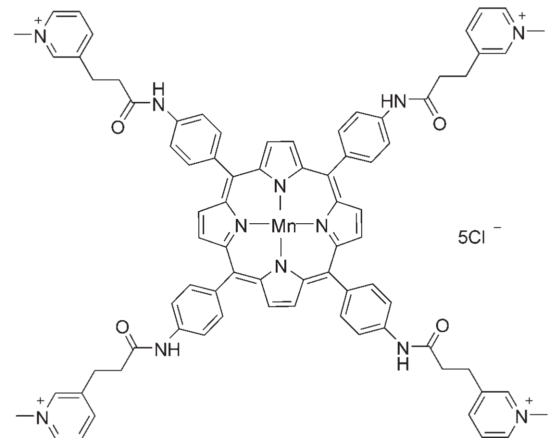

Figure 15. Structure of pentacationic manganese(III) porphyrin.

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ficient agent that can distinguish between quadruplex and duplex DNA.

3.3.5.3. Expanded porphyrin

Due to the structural polymorphism in G-quadruplexes, different G-quadruplex topologies may lead to different signaling pathways and biological functions. To achieve higher therapeutic selectivity for G-quadruplexes, it is necessary to design drugs that can discriminate different types of G-quadruplexes. To this end, a core-modified expanded porphyrin analogue, 5,10,15,20-[tetra(*N*-methyl-3-pyridyl)]-26,28-diselenasapphyrin chloride (Se2SAP, Figure 16), was designed and synthesized.[145]

Figure 16. Structure of Se2SAP.

In this study, Se2SAP was found to selectively bind the *c-myc* G-quadruplex in the presence of duplex DNA and other G-quadruplexes, and to convert the parallel *c-myc* G-quadruplex into a mixed parallel/antiparallel G-quadruplex. More importantly, Se2SAP was found to be less photoactive and cytotoxic than TMPyP4. CD studies have shown that Se2SAP is likely to affect the conformation of the telomeric G-quadruplex and convert the preformed basket G-quadruplex into the hybrid structure that has strong parallel and antiparallel characteristics.[144] A recent study has also shown that Se2SAP can further stabilize the G-quadruplex structures found in the promoter region of the *bcl-2* oncogene.[86]

3.3.5.4. Porphyrazines

Tetrapyridinoporphyrazines are nonsymmetrical phthalocyanine azo analogues in which the four pyridine moieties substitute the four benzene groups in the macrocycle periphery. They differ from porphyrins by having nitrogen atoms in the *meso* positions linked with the individual pyrrole units. The pyridyl groups of the 3,4-tetrapyridinoporphyrazines (3,4-TPyPz, Figure 17 a) compounds can be methylated to give 3,4-tetramethylpyridinium porphyrazines (3,4-TMPyPz, Figure 17 b). Both 3,4-TMPyPz and 3,4-TMPyPz zinc(II) (Figure 17 c) were found to bind strongly and selectively to human telomeric G-quadruplex DNA, inducing the formation of antiparallel quadruplexes in a manner similar to that of molecular chaperones.[168]

Figure 17. Structures of a) 3,4-TPyPz, b) 3,4-TMPyPz, c) 3,4-TMPyPz zinc(II), and d) ZnPc.

Another porphyrazine analogue,[169] octacationic quaternary ammonium zinc phthalocyanine (ZnPc, Figure 17 d), is also a potent G-quadruplex ligand and displays strong inhibitory activity against telomerase with an $^{tel}IC_{50}$ value of 0.23 μM. Interestingly, ZnPc not only induces G-quadruplex structure transition from the antiparallel to parallel structure, but also triggers parallel structure formation under cation-deficient conditions.

3.3.6. Telomestatin

Telomestatin (SOT-095, Figure 18), a macrocyclic natural product consisting of seven oxazole rings and one thiazoline ring, isolated from the actinomycete *Streptomyces anulatus* 3533-SV4, is currently the most efficient in vitro telomerase inhibitor, with an $^{tel}IC_{50}$ value of 5 nM.[170,171] Telomestatin appears to interact preferentially with intramolecular G-quadruplexes rather

Figure 18. Structures of a) telomestatin (SOT-095), b) HXDV, and c) bistrioxazole acetate.

than intermolecular quadruplexes. This compound has stronger selectivity for intramolecular G-quadruplexes than duplex DNA; the selectivity increases by about 70-fold.[172] As mentioned below, the effects of telomestatin on telomere/telomerase biological functions have been further studied and reported.

More recently, some synthetic derivatives of telomestatin such as macrocyclic hexaoxazole HXDV and bistrioxazole acetate (Figure 18) have been reported.[173,174] HXDV is one quadruplex-binding ligand that binds exclusively to quadruplex DNA and not to duplex or triplex DNA. Experimental results show that HXDV binds to G-quadruplex DNA with concomitant de-stacking of adenine residues from the terminal G-tetrads without altering the length of the quadruplex. This indicates an entropically driven non-intercalative "terminal capping" binding mode of interaction between HXDV and quadruplex DNA. Bistrioxazole acetates, which have a macrocyclic bisamide structure, were designed on the basis of the telomestatin structure. One of these compounds (Figure 18c) showed telomerase inhibitory activity with an $^{tel}IC_{50}$ value of 2 μM.

3.3.7. Perylene derivatives

The polycyclic compound PIPER (Figure 19) is based on the perylene skeleton and is an effective telomerase inhibitor, the $^{tel}IC_{50}$ value of which lies in the low-μM range. This compound

Figure 19. Structure of PIPER.

was designed by the DOCK program and used to screen for potential G-quadruplex DNA binding compounds with the solution structure of the human $d[AG_3(T_2AG_3)_3]$ DNA.[112] An NMR model of quadruplex binding indicates that PIPER binds to a variety of G4-DNA structures with ligand/G-quadruplex stoichiometries of 1:2, 1:1, and 2:1, depending on the sequence of the G4-DNA. PIPER appears to be able to perform chaperone-like functions, capable of accelerating the assembly of G-quadruplex structures in a cell-free system.[175] PIPER can induce the transition from duplex to G-quadruplex in the Pu27 sequence from the *c-myc* promoter region.[176]

3.3.8. Berberine derivatives

Berberine (Figure 20a), an alkaloid isolated from Chinese herbs, has long been used as an antimicrobial agent. The inhibitory activity of berberine on telomerase was first reported in 1999,[177] and a number of 13-substituted berberine derivatives (Figure 20b) have been found in recent years to be able to inhibit telomerase activity by binding to G-quadruplex DNA.[178]

More recently, our research group has studied the interactions of human telomeric G-quadruplex DNA with berberine and its 9-substituted derivatives (Figure 20c), and the effects

Figure 20. Structures of a) berberine, b) 13-substituted berberine, and c) 9-substituted berberine.

of these compounds on telomerase activity.[179] Our results indicate that these semisynthetic berberine derivatives can induce antiparallel G-quadruplex formation and stabilize this structure in the presence or absence of metal cations. In contrast to berberine, the 9-substituted derivatives exhibit stronger binding affinity for G-quadruplexes and higher inhibitory activity against telomerase, presumably due to the introduction of a side chain of proper length in methylene units and a terminal amino group at the 9-position.

3.3.9. Quinoanthroxazine derivatives

The polycyclic fluoroquinoanthroxazines (FQAs, Figure 21), derived from the extended aromatic skeleton of fluoroquinolone, possess various degrees of poisoning ability against topoisomerase II and binding affinities for G-quadruplex DNA.[148,180]

Figure 21. Structures of QQ58, FQA-CS/FQA-CR, and FQA-TS/FQA-TR.

The fluoroquinolones (such as norfloxacin) are well-known antimicrobial agents that can inhibit bacterial DNA gyrase, but some tetracyclic quinolone analogues also have strong anticancer activity mediated by interaction with topoisomerase II. The extended phenoxazine ring of FQAs was found to enhance their stacking interactions with G-quadruplexes, thus improving telomerase inhibition while maintaining the poisoning effect on topoisomerase II.

3.3.10. Bistriazole ligands

A new class of highly selective G-quadruplex-stabilizing ligands, the bistriazole derivatives, has been reported (Figure 22). These compounds were prepared by the "click

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Figure 22. Structures of bistriazole derivatives.

chemistry" approach.[181] The ability of these derivatives to stabilize G-quadruplex DNA and the selectivity of these compounds for G-quadruplex DNA over duplex DNA have been investigated by using a high-throughput FRET assay. The results show these compounds have excellent affinity and selectivity for G-quadruplexes.

3.3.11. Bisquinolinium compounds

A series of triazine compounds have been found by FRET experiments to induce the folding of a human telomeric DNA oligonucleotide into a G-quadruplex. Bisquinoline-substituted triazines (Figure 23) such as 115405 and 12459, can serve as inhibitors against human telomerase at nanomolar concentrations.[56] The ligand-induced stabilization of the quadruplex was found to be related to the inhibition of telomerase activity, telomere shortening, and the induction of apoptosis in human telomerase-positive cells.[182,183]

Figure 23. Structures of bisquinoline derivatives 12459, 115405, 307A, and 360A.

A new series of bisquinoline derivatives (Figure 23) such as 307A and 360A were recently synthesized. The two quinolinium moieties were connected through the 2,6-pyridodicarboxamide unit. These new compounds have higher binding selectivity for telomeric G-quadruplex than for double-stranded DNA, and have relatively strong inhibitory activity against telomerase, with $^{tel}IC_{50}$ values of 0.22–0.45 μM. these compounds also have a high selectivity ratio (33–150) for telomeric G-quadruplexes with respect to *Taq* polymerase, as revealed by the telomere repeat amplification protocol (TRAP) assay.[57] Ligands 307A and 360A from the 2,6-pyridodicarboxamide series are about 10-fold more selective than the previously described bisquinoline-substituted triazine derivative 115405. It is well known that the central pyridodicarboxamide unit, adopting an internally organized H-bonded *syn–syn* conformation, can lock the ligand in a crescent-shaped conformation, which is highly favorable for G-quartet overlap. Based on this property, expanding the aromaticity of the central core, without disrupting the hydrogen bond network, through replacement of the pyridine core by bipyridine and phenanthroline units, has been reported (Figure 24).[26] Phenanthroline bisquinolinium derivatives are recognized as strong quadruplex stabilizers with high selectivity for quadruplex DNA over duplex DNA, and behave similarly to telomestatin under similar conditions.

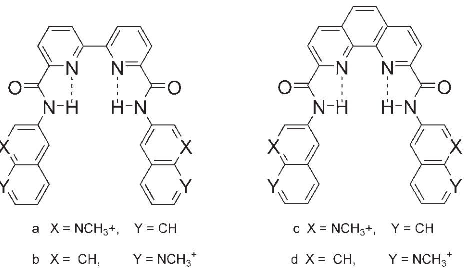

Figure 24. Structures of bipyridine-connected bisquinolines (a and b) and phenanthroline-connected bisquinolines (c and d).

3.3.12. Other G-quadruplex-interactive ligands

The DOCK program can be used to identify ligands that can bind to G-quadruplex DNA grooves. A carbocyanine dye, 3,3′-diethyloxyadicarbocyanine (DODC, Figure 25), is able to specifically probe hairpin G-quadruplexes.[184] In addition, quercetin,[185] a derivative of flavonoid, and its glycoside rutin (Figure 25)[186] were found to be able to interact with monomeric and dimeric G-quadruplexes through different binding modes.

Figure 25. Structures of DODC, quercetin, and rutin.

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3.4. Design of small molecules specific for binding with G-quadruplex structures

Prerequisites for the rational design of small molecules that bind G-quadruplexes include investigation of the structure of the G-quadruplex in question and the ligand–quadruplex binding mode. In principle, inner intercalation between tetrads, outside or end stacking, groove binding, or a combination of two or more of these binding modes can give rise to various degrees of binding specificity. Electrostatic interactions between cationic ligands and the anionic backbone or the negative electrostatic center of the G-quadruplex are very important for improving their binding affinity.

The binding interactions of most ligands to G-quadruplexes usually follow the following principles:

1. The extended planar chromophores that are similar in size and shape to that of a G-quartet stack on the surface of this structure by π–π interactions. For example, the cationic porphyrins TMPyP4, the expanded porphyrin Se2SAP, and telomestatin have a macrocycle chromophore with a size similar to that of the G-tetrad. Moreover, the crescent-shaped polycyclic chromophores of quinacridine derivatives, quindoline derivatives, and berberine derivatives are also similar to the shape of a G-tetrad.
2. The side chains of cationic substituents usually interact with the anionic phosphate backbone on one side of the G-quadruplex or two sides or more by electrostatic interaction.
3. The central cationic aromatic core of G-quadruplex-binding ligands usually interacts with the negative electrostatic center of the G-quadruplex by electrostatic interaction. Examples include pentacationic manganese(III) porphyrin, 3,4-TMPyPz zinc(II), 3,6,9-trisamidoacridines, 11-substituted quindolines, and berberine derivatives.

4. Summary and Outlook

Over the past decade, DNA quadruplex research has rapidly moved from fundamental to clinical studies. The biological significance of G-quadruplexes has been recognized by numerous research efforts. With the advent of X-ray crystallography and NMR studies in this field, the structure and topology of G-quadruplexes have become clearer than ever before. This structural information is particularly important for the design of drug-like molecules specific for G-quadruplexes. The G-quadruplex is recognized as a significant drug target for cancer and other diseases, and extensive efforts have been directed toward the discovery of promising lead compounds capable of stabilizing G-quadruplexes. In this regard, it is worth noting that some candidates have entered preclinical or clinical trials.

Unlike the clear progress in structural studies, there are fewer reports of progress in medicinal chemistry studies, especially in the discovery of real drug-like G-quadruplex ligands. There are likely two primary reasons for this: 1) too many research efforts are focused on some model molecules that do not have a realistic drug-like structure, and fewer ligands with higher selectivity and decreased side effects have been found; 2) the real biological functions of G-quadruplexes and the genes containing G-quadruplex structures in vivo require further illumination.

Nevertheless, given the clinical significance of the G-quadruplex, research activities on telomeric and genomic quadruplexes will continue to grow. With a better understanding of the biological functions and structural properties of G-quadruplexes, it is expected that a wealth of new drugs that are less cytotoxic and that have higher selectivity will emerge in the near future.

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Keywords: antitumor agents · drug design · G-quadruplexes · nucleic acids

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