/ Review

i-Motif-Binding Ligands and Their Effects on the Structure and Biological Functions of i-Motif

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The i-motif is a high-order DNA structure formed by hemiprotonated cytosine–cytosine base pairs under acidic conditions. It is less well studied than other high-order DNA structures, such as G-quadruplexes. However, increasing numbers of i-motif-binding ligands are being reported, and they are being used as tools to investigate the i-motif's structure and biological functions. It has become clear that the i-motif has a functional role in the regulation of gene expression. In this review, we provide an overview of i-motif ligands so far reported, and we summarize their effects on the structure and their use as tools to investigate its biological functions.

Key words i-motif; ligand; interacting agent; DNA high-order structure; DNA application

1. Introduction

There is increasing interest in various non-canonical (non-B) DNA structures, and it is becoming clear that they have important biological functions. Small molecules that target these DNA structures are therefore useful as research tools to probe their functions, and are also widely considered to have great therapeutic potential.^{1–5)} For example, G-quadruplexes (G4s), which are among the most extensively investigated non-B DNA structures, have roles in telomerase inhibition, transcriptional regulation, and replication. These structures occur in guanine-rich sequences of telomeric DNA and gene promoter regions, where they are formed by stacking of guanine tetrads (G-quartets) (Fig. 1). The stabilization of G4 with small molecules has potential for cancer therapeutics,^{6–9)} and our group has developed a series of macrocyclic compounds, 7 Oxazole Telomestatin Derivatives (OTD) and 6OTD derivatives, that interact with the G-quartet plane of G4 through π – π interaction and stabilize the structure.^{10–16)} Many G4 ligands have been used in research aimed at understanding the roles of this structure.^{5,17–19)} On the other hand, hemiprotonated cytosine–cytosine base pairs ($C-C^+$) can form a structure called the i-motif in cytosine-rich sequences under acidic conditions. This structure is less well studied than other DNA structures, in part due to its limited stability. However, interest has greatly increased in recent years, because the i-motif is known to be present in oncogene promoters, and can be regarded as complementary to G4.^{20–23)} In addition, the increasing number of i-motif-interacting agents has provided new tools for research on its biological roles and its recognition in the nuclei of human cell.^{18,24–30)} The most representative strategy for development of i-motif ligands is utilizing the already known G4-interacting agents and their analogues, and also hits from chemical library screening.

Here, we first briefly describe the structure of the i-motif, and then we review recently developed i-motif-interacting agents, together with their effects on the i-motif structure and its functions.

2. The i-Motif Structure

The i-motif, which was recognized in 1993, is a tetraplex structure formed by hemiprotonated cytosine–cytosine ($C-C^+$) base pairs under acidic conditions in DNA sequences enriched in cytosine.³¹⁾ This structure has two wide grooves and two narrow grooves, and its $C-C^+$ plane is smaller than that of the G-quartet in G4. Cytosine-rich sequences can fold into intra- and intermolecular i-motifs *in vitro* (Table 1). Sequences with one tract of cytosines have the potential to fold into tetramolecular structures (intermolecular i-motifs) such as the first reported i-motif based on 5'-TCCCCC-3' (Fig. 2c). Sequences with two tracts of cytosines can form bimolecular i-motif structures (Fig. 2b), which are favored by longer linkers. Most natural long cytosine-rich sequences fold into intramolecular i-motifs (Fig. 2a), which are more stable than the intermolecular structures.^{31–36)}

The formation and stability of i-motifs depend upon several factors. As an important principle, cytosines in i-motif-forming sequences require protonation of their nitrogen atom at position 3 (N3) to form the $C-C^+$ base pairs. The pK_a of the N3 in cytosine is close to 4.8 and the optimum pH for i-motif formation is close to the pK_a . Under strongly acidic conditions, all the cytosines in a sequence will be protonated and folding cannot occur, while under neutral or basic conditions the formation of an i-motif is difficult.³⁷⁾ However, there is evidence for the formation of i-motifs at neutral pHs under supercoiling conditions,³⁸⁾ molecular crowding conditions,^{39,40)} and in the presence of interacting agents^{41,42)} and cations such

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Fig. 1. Non-canonical Structures of DNA: G-Quadruplex (G4) and i-Motif
(Color figure can be accessed in the online version.)

Table 1. Folding of Cytosine-Rich Sequences into Different Conformations

Oligonucleotides	Conformation	Ref.
CCCTAACCCT	Intermolecular (bimolecular)	35
TCCTTTTCCA	Intermolecular (bimolecular)	36
CCCCCTTTCCCC	Intermolecular (bimolecular)	34
CCCCCTCCCC	Intermolecular (tetramolecular)	34
TCCCC	Intermolecular (tetramolecular)	31
CCTTTCCTTACCTTCC	Intramolecular	32
CCCTAACCCTAACCTAACCT	Intramolecular	32

as Ag^+ .⁴³⁾ Due to the negatively charged phosphate backbone, DNA is an anionic structure at neutral pH. This negative charge can be affected by ionic strength, resulting in destabilization and conformational change. It has been reported that increasing the concentration of NaCl to 100 mM destabilizes i-motifs, which are more stable at low ion concentration due to the effect of cations on the pK_a of N3 of cytosine.³⁷⁾

The stability of DNA structure is known to be influenced by molecular crowding. For example, co-solvents and crowding agents can increase the stability of DNA secondary structures based on Hoogsteen hydrogen bonding by dehydration. The effects of 40% (w/v) of co-solvents such as 1,2-dimethoxyethane, 2-methoxyethanol, ethylene glycol, 1,3-propanediol and glycerol on i-motif formation by 5'-CCCCCT-3' have been studied, but these low-molecular-weight co-solvents did not affect its stability. On the other hand, the crowding agent polyethylene glycol (PEG)-300 stabilizes the i-motif at near-physiological pH by increasing its pK_a . The concentration and molecular weight of PEG are important for i-motif induction, and excessive crowding can lead to nonspecific interaction of DNA with PEG, so that the formation of i-motif structure is not promoted.^{39,40)} Based on the limited stability of i-motifs, they were initially employed as pH sensors in nanotechnol-

ogy.^{44,45)} Their biological functions remained unknown until the development of i-motif-interacting ligands.

3. The i-Motif Ligands

3.1. i-Motif Interacting Agents Inspired by G4 Ligands

3.1.1. Tetra-(*N*-methyl-4-pyridyl)porphyrin (TMPyP4)

The first study of a small molecule binding to i-motif was reported in 2000, when Hurley and colleagues investigated the interaction of TMPyP4 (**1**) with tetramolecular i-motif structure from a human telomeric sequence⁴⁶⁾ (Fig. 3). This compound interacts with G4, inhibits telomerase, and down-regulates *c-myc* expression.^{47,48)} The interaction of TMPyP4 with i-motif was confirmed by electrophoretic mobility shift assay (EMSA), though the melting temperature of the DNA was not altered. The results of NMR experiments indicated that two molecules of TMPyP4 (**1**) interact with i-motif, at the top and bottom of the structure. The absence of marked changes of the imino proton signals in ^1H -NMR titration experiments indicated that TMPyP4 (**1**) does not interact with the C-C⁺ base pairs, and the mechanism of the interaction is proposed to be groove binding, not intercalation.⁴⁶⁾ Gargallo and colleagues confirmed the non-intercalative binding of this compound with other i-motif telomeric sequences⁴⁹⁾ and fur-

Fig. 2. Structures of a) Intramolecular i-Motif b) Bimolecular i-Motif c) Tetramolecular i-Motif

Fig. 3. The Structure of TMPyP4 (1) and Its Interaction Modes with i-Motif

ther investigated the interaction of TMPyP4 (1) with *bcl-2* i-motif-forming sequence. In the case of the *bcl-2* sequence, the structure is disrupted at pH 6.1, which might indicate preferential binding of TMPyP4 (1) to partially unfolded regions of DNA.⁵⁰⁾ Qin *et al.* further showed that the potassium ion concentration in buffers can affect the binding mode of TMPyP4 (1) to i-motifs, and suggested two possible interaction modes: intercalation and groove binding. The results of UV-vis absorption spectroscopy and induced circular dichroism (ICD) spectroscopy suggested that high ion concentrations promote the groove binding mode.⁵¹⁾ It seems clear that many factors, in addition to sequence, length of loops and DNA topology, can affect the interaction of ligands with i-motifs.

3.1.2. Phenanthroline and Acridine Derivatives

Many G-quadruplex ligands have characteristic cores, such as phenanthroline, naphthalene diimide, anthraquinone and acridine. These cores have been modified and various analogues have been investigated as G4 binders.⁵²⁾ Phenanthroline

derivatives are known to interact with G4 and inhibit telomerase activity.^{53,54)} Wei and colleagues investigated phenanthroline derivatives (2–7) with different functional groups (Fig. 4); UV melting analysis indicated that these compounds increase the T_m of the i-motif, although the CD spectra suggested perturbation of the i-motif structure. UV-Vis absorption titration experiments suggested that phenanthroline derivatives (2–7) might stack on the C–C⁺ base pair of i-motif structure. The binding constants of these compounds for i-motif are lower than those for G-quadruplex.^{55,56)} As for acridine, monomeric acridine with two propylamine side chains (MonoA (8)) and acridine dimer linked by diethylenetriamine (BisA (9)) (Fig. 4) were studied by fluorescence resonance energy transfer (FRET) melting assay to evaluate their interaction with the i-motif. The melting temperatures (T_m) of both i-motif and G4 were increased by BisA (9), but MonoA (8) had no effect.⁵⁷⁾ Subsequent studies on modified acridine compounds have mostly focused on their role as G4 ligands.^{58,59)}

a) Phenanthroline derivatives

b) Acridine derivatives

Fig. 4. Structures of a) Phenanthrolines (2–7) and b) Acridine Derivatives MonoA (8) and BisA (9)

Fig. 5. Structures of Telomestatin (10) and OTD Derivatives: 7OTD (11), 6OTD (12), L2H2-5OTD (13), and L2H2-4OTD (14)

3.1.3. Macrocyclic Tetraoxazoles, L2H2-4OTD

A series of macrocyclic poly-oxazole compounds has been developed by our group as G4 ligands,^{10–16} inspired by the natural product telomestatin (10),⁶⁰ a potent telomerase inhibitor. Telomestatin has a planar cyclic structure including seven oxazoles and a thiazoline, which resemble the G-quartet plane of G4 and interact with G4 in a π - π stacking mode.⁶¹ We synthesized both macrocyclic hepta (7OTD (11)) and hexa-oxazoles (6OTD (12)) derivatives, which are much easier to access than telomestatin; many derivatives can easily be synthesized by installing various functional groups. These compounds interact with G-quartet in the same binding mode as telomestatin.^{10–16} Thus, we decided to see if these compounds

could be modified to target the i-motif. The planar structure of i-motif is smaller than that of G4, so we synthesized penta- (L2H2-5OTD (13)) and tetra-oxazoles (L2H2-4OTD (14)) bearing amine functional groups in the side chains⁶² (Fig. 5).

Decreasing the size of the macrocycle reduces its stabilizing ability, and L2H2-4OTD (14) did not show potent stabilizing ability for G4-forming sequences.⁶² On the other hand, it interacted with i-motif forming sequences. In CD titration experiments, incremental addition of L2H2-4OTD (14) decreased the ellipticity of the characteristic spectral signal of telomeric i-motif at 288 nm and the peak was slightly shifted towards lower wavelength. Nuclear Overhauser effect spectroscopy (NOESY) and chemical shift perturbation (CSP)

analyses indicated that the preferred binding site of L2H2-4OTD (**14**) is Loop 1 and Loop 3 of telomeric i-motif and their neighboring region (Fig. 6). This interaction causes slight distortion of the C3–C15 base pair, which is consistent with the decrease of the ellipticity in the CD experiments, though the structure of the i-motif is retained. When a second molecule of L2H2-4OTD (**14**) binds to the next most preferred binding site, the C2–C14 and C8–C20 base pairs are affected as well. Electrospray mass spectroscopy confirmed that two molecules of **14** are involved in this interaction, and these molecules bind cooperatively to the i-motif structure.⁶³⁾

3.2. Ligands Other than G4-Inspired Interacting Agents

3.2.1. Mitoxantrone, Tilorone and Tobramycin

Mitoxantrone (**15**) is an antitumor agent which has been studied by Waller and colleagues by screening the MicroSource library for compounds interacting with i-motif

Fig. 6. Interaction of L2H2-4OTD (**14**) with Telomeric i-Motif

The binding site is close to loops 1 and 3. (Color figure can be accessed in the online version.)

Fig. 7. Structures of Mitoxantrone (**15**), Tilorone (**16**) and Tobramycin (**17**)

Fig. 8. Structures of SWCNTs (**18**) and GQDs (**19**) and Their Effects on i-Motif Formation at Physiological pH

DNA (Fig. 7). FRET melting curves indicate that mitoxantrone stabilizes the i-motif. In addition, the i-motif was formed under neutral conditions (pH 7.4) upon incremental addition of mitoxantrone (**15**). It also stabilized G4, but less effectively than i-motif. SPR analysis indicates that its binding affinity for double-stranded DNA is weaker than its affinity for the i-motif. Nevertheless, assuming that the decrease in ellipticity of the characteristic CD signal of the i-motif at 288nm reflects interaction and not destabilization, this can be one of the ligands that stabilizes the i-motif under neutral conditions.⁶⁴⁾ The same group has discovered other i-motif binding ligands, such as tilorone (**16**) and tobramycin (**17**) (Fig. 7), by screening of compound libraries using thiazole orange fluorescence intensity displacement (FID) assay.⁶⁵⁾

3.2.2. Carboxylic Acid-Modified Single-Walled Carbon Nanotubes (SWCNTs) and Graphene Quantum Dots (GQDs)

In 2006, SWCNTs with carboxylic acid modification (Fig. 8) were introduced as a selective i-motif binders, as well as inducers of i-motif formation under physiological conditions. Addition of SWCNTs (**18**) had almost no effect on the T_m of G4, while it significantly increased the T_m of telomeric i-motif, and slightly decreased the intensity of the characteristic CD signal of the i-motif. The carboxylic acid modification increases the solubility of SWCNTs.⁴¹⁾ From water activity measurements under conditions of molecular crowding, it has been found that SWCNTs (**18**) cause water molecules to be released from DNA structure and stabilize the i-motif.⁶⁶⁾ In 2012, SWCNTs were reported to inhibit telomerase activity by stabilization of i-motif structure, based on TRAP assay *in vitro* and *in vivo*.⁶⁷⁾ However, it is not clear whether the i-motif directly inhibits telomerase activity or whether it induces the complementary G4, which plays the major role in this inhibition. Graphene quantum dots (GQDs (**19**)) (Fig. 8) modified with carboxylic acid were shown to intercalate with DNA, and (**19**) were used to study i-motif-forming sequences of human telomeres and *c-myc*. Under conditions where the i-motif is not folded (pH 8), addition of GQDs promotes the formation

of this structure, based on CD spectral measurements. This result has been confirmed by NMR spectroscopy, where the characteristic i-motif proton signals appear upon addition of GQD. 2-Ap fluorescence assay showed a decrease of fluorescence intensity in three loops, indicating the interaction of GQDs with the loop regions in an end-stacking mode. It has suggested that the interaction reduced the solvent-accessible area, and stabilized the i-motif structure.⁴²⁾

3.3. Ligands Used to Study the Biological Functions of i-Motif Formation of i-motif structure in cytosine-rich sequences of the promoter regions of various genes,^{68–75)} such as RET,⁶⁸⁾ Rb,⁶⁹⁾ *bcl-2*,⁷⁰⁾ *c-myc*,⁷¹⁾ and telomeric DNA,⁷⁶⁾ is associated with important biological functions. These promoters form i-motifs with different topologies, loop lengths and transitional pHs, which are thus potential targets of small-molecular ligands for studies of their biological activities.

Through the screening of a chemical library (NCI Diversity Set I) by FRET high-throughput assay for *bcl-2* i-motif-forming sequences, two small molecules with opposite effects on the fluorescence intensity were selected, *i.e.*, IMC-48 (**20**) and IMC-76 (**21**) (Fig. 9). The *bcl-2* cytosine-rich sequence is in

equilibrium between i-motif and hairpin structures. NMR titration revealed that IMC-48 (**20**) stabilized the i-motif, while IMC-76 (**21**) promoted the formation of hairpin structure. This conclusion was supported by CD analysis, bromine footprinting, and naphthodeoxyuridine (NdU) fluorescence assay. The biological effects of these compounds were studied in lymphoma cell lines. IMC-48 (**20**), the i-motif stabilizer, caused transcriptional activation and increased gene expression, whereas IMC-76 (**21**), which promotes the formation of hairpin structure, caused transcriptional repression and decreased the gene expression. This study by Hurley and colleagues provided the first definitive evidence for regulation of gene expression by i-motif structure.²⁴⁾

After establishing that the *k-ras* mid-region sequence is in equilibrium between i-motif, a hybrid i-motif/hairpin, and a hairpin structure, the same group identified nitidine (**22**) (Fig. 9) by screening of a chemical library (NCI/NIH diversity Set III and Mechanistic) for compounds with activity to modulate the dynamic equilibrium of *k-ras* i-motif/hairpin structures.²⁵⁾ Nitidine was one of a series of alkaloids previously studied for interaction with DNA structures, especially G4.²⁶⁾ It (**22**)

Fig. 9. Structures of IMC-48 (**20**), IMC-76 (**21**), Nitidine (**22**), NSC309874 (**23**) and Acridone Derivative (**24**)

Fig. 10. Structures of PBP1 (**25**) and PBP2 (**26**), Their Interaction and Effect on *bcl-2* DNA and Gene Expression

was reported to disrupt the hairpin in hybrid i-motif/hairpin structures and downregulate *k-ras* gene expression. This compound did not bind to *bcl-2* and *c-myc* i-motifs, and showed selectivity for the hairpin species on the *k-ras* i-motif structure.²⁵⁾ Hurley and colleagues also reported a benzothiophene-carboxamide compound, NSC309874 (**23**) (Fig. 9), which selectively binds to the platelet-derived growth factor receptor (PDGFR)- β i-motif-forming sequence and downregulates its promoter activity.²⁷⁾ Subsequently, an acridone derivative **24** (Fig. 9) was reported to selectively interact with i-motif over G4 and single-stranded DNA, and this interaction caused the down-regulation of *c-myc* gene. Shu *et al.* have studied analogues of acridone based on the initial finding of stabilizing ability for the *c-myc* i-motif. FRET melting assay, macroscale thermophoresis (MST) data, and SPR analysis confirmed the selectivity and high affinity of the acridone derivative for *c-myc* i-motif. The results of CD spectroscopy and electropho-

retic mobility shift assay indicated that the acridone derivative induces i-motif formation at pH 6.8. This observation was further supported by NMR titration experiments; the acridone derivative increased the characteristic i-motif signal at 15–16 ppm, which are related to imino protons shared between cytosine–cytosine base pairs. Electrospray mass spectroscopy indicated a 1:1 ratio of DNA:ligand in the complex; interestingly, at pH 7.0, where the i-motif is not formed, a complex peak was not observed; this suggests that the acridone derivative does not bind to the single-stranded i-motif-forming sequence.²⁸⁾

Dash and colleagues recently reported that PBP1 (**25**) and PBP2 (**26**) (Fig. 10), which are peptidomimetic ligands bearing bis-triazole, interact selectively with G4 and i-motif.²⁹⁾ Peptidomimetic ligand containing triazole, pyridine and prolinamide is known to interact well with four-stranded G4 due to its conformational flexibility.⁷⁷⁾ Through FRET melting assay,

Table 2. The i-Motif Ligands, and Their Effects on i-Motif and Cells

Compound	i-motif DNA	Effect on i-motif	Biological function	Ref.
 IMC-48 (20)	 <i>bcl-2</i> i-motif	- stabilize the <i>bcl-2</i> i-motif structure	Upregulate the <i>bcl-2</i> gene expression	24
 IMC-76 (21)	 <i>bcl-2</i> hairpin	- stabilize the <i>bcl-2</i> hairpin structure	Downregulate the <i>bcl-2</i> gene expression	24
 Nitidine (22)	 <i>k-ras</i> i-motif/hairpin	- destabilize the hairpin on hybrid i-motif/hairpin structure - stabilize <i>k-ras</i> G4	Downregulate the <i>k-ras</i> gene expression	25
 NSC309874 (23)	 PDGFR- β i-motif	- stabilize the PDGFR-β i-motif structure - no significant interaction with complementary G4	Downregulate the PDGFR- β gene expression	27
 acridone derivative (24)	 <i>c-myc</i> i-motif	- stabilize the <i>c-myc</i> i-motif structure - no significant interaction with complementary G4	Downregulate the <i>c-myc</i> gene expression	28
 PBP1 (25)	 <i>bcl-2</i> i-motif	- stabilize the <i>bcl-2</i> i-motif structure and promote its formation in neutral pHs	Upregulate the <i>bcl-2</i> gene expression	29

Fig. 11. The Steric Hindrance between Two Structures of G4 and i-Motif Might Be the Reason for Mutual Exclusivity

(Color figure can be accessed in the online version.)

thiazole orange (TO) displacement assay and fluorescence binding titration, it was established that PBP1 (**25**) shows selectivity for i-motif and PBP2 (**26**) interacts with G4. Single molecule FRET (SmFRET) and fluorescence lifetime analysis under conditions where DNAs are unfolded revealed that addition of PBP1 (**25**) and PBP2 (**26**) induced the formation of i-motif and G4 from their respective sequences. Cellular studies indicated that PBP1 (**25**) upregulates *bcl-2* gene expression and PBP2 (**26**) downregulates *bcl-2* gene expression in cancer cells. These observations demonstrate that small structural differences in the para and meta isomers of **25** and **26** can markedly alter the interactions of the ligands and their biological activity.²⁹⁾

The above ligands, their properties, and their biological functions are summarized in Table 2. The variety of binding sites for these ligands and the differences in their effects on the i-motif structure make it difficult at this stage to know whether stabilization or destabilization of i-motif structures promotes or downregulates gene expression.

Heterogeneous nuclear ribonucleoprotein LL (hnRNP LL) was the first protein reported to bind to i-motif structure. EMSA and SPR experiments indicated that this protein has a high affinity for the *bcl-2* i-motif-forming sequence, but the CD spectra suggested that the i-motif was unfolded. Bromine footprinting experiments confirmed the unfolding of the i-motif and the formation of stable single-stranded structure after interaction with hnRNP LL. This interaction caused transcriptional activation and increased gene expression.⁷⁸⁾ hnRNP LL has four RNA recognition motifs (RRMs), and it appeared that RRM1 and RRM2 cause the unfolding of the i-motif structure, based on CD experiments. These two motifs were found to be necessary for increasing the gene expression, in contrast to RRM3 and RRM4, which interact weakly with the i-motif.⁷⁹⁾ Similarly, hnRNP K has reported to unfold the *k-ras* i-motif and upregulate gene expression.²⁵⁾

Based on the above results, the i-motif is thought to have regulatory functions in the genome. Recent NMR studies of DNA in living human cells have supported the stability of the i-motif in the promoter regions of *DAP*, *HIF-1 α* , *PDGF-A* and *JAZF1* genes in nuclei of human cells.⁸⁰⁾ These sequences can form i-motifs under near-physiological conditions.^{81,82)} NMR observation of the imino proton signals of C–C⁺

base pairs indicated the stability of i-motif in nuclei of living HeLa cells.⁸⁰⁾ Further, an antibody fragment (iMab) has been reported to recognize the i-motif with high affinity and selectivity in nuclei of human cells. In that work, Zeraati *et al.* screened a fragment library to select an i-motif-specific antibody fragment. Enzyme-linked immunosorbent assay (ELISA) and bio-layer interferometry (BLI) were used to characterize the interacting antibody. The results indicated that iMab does not interact with other DNAs, such as dsDNA, hairpin, G4 and single-stranded i-motif-forming sequences. Indirect immunofluorescence staining of iMab indicated the presence of i-motif in the nuclei of human cell lines. Furthermore, the level of i-motif formation was high in the late G1 phase of the cell cycle, which involves transcription. This observation supports a regulatory role of i-motif in promoters. On the other hand, the formation of G4 during the cell cycle was different from that of i-motif, and it was speculated that these two structures might be mutually exclusive and play opposite roles in the regulation of gene expression.³⁰⁾ The mutual exclusivity of i-motif and G4 has been supported by Mao's group, using a laser tweezer method under conditions that promote the formation of both G4 and i-motif structures in dsDNA. The results indicated that G4 and i-motif cannot form at the same time, possibly due to steric hindrance between the two structures (Fig. 11). It is not clear whether this mutual exclusivity also holds in the genome but there are indications that G4 and i-motif might each control specific biological activities in cells.^{83,84)} i-Motif-interacting agents will be important tools for investigating this structure and its functions in the genome in cells.

3.4. Ligands Used as Fluorescent Probes The i-motif has been employed to develop nanomachines, biosensors and logic gates,^{44,85–88)} which utilize the reactivity of i-motif structure under acidic conditions, as well as its formation and disruption due to pH changes, to switch the fluorescence of DNA-interacting compounds. Thiazole orange (**27**),⁸⁹⁾ 2,2'-diethyl-9-methylselenocarbocyanine bromide (DMSB) (**28**),⁹⁰⁾ crystal violet (**29**),⁹¹⁾ berberine (**30**),⁹²⁾ neutral red (**31**),⁹³⁾ thioflavin T (**32**),⁹⁴⁾ perylene tetracarboxylic acid diimide derivative (PTCDI) (**33**),⁹⁵⁾ (Fig. 12) and various complexes (**34–37**) have been used as fluorescent probes for i-motif structure, although most of them had been previously investigated as probes for G4.

Thiazole orange (TO (**27**)) is a non-fluorescent compound in aqueous solutions, and it shows fluorescence upon interacting with nucleic acids. TO has high affinity for G4 DNA compared to dsDNA and ssDNA. This selectivity is proposed to be due to twisting of the structure of TO during its interaction with tetraplex DNAs.^{96–98)} This compound also interacts with i-motif and exhibit fluorescence upon interaction (Fig. 13a). The binding site of TO in i-motif has not been confirmed yet, but the interaction of this compound with nucleic acids involves intercalation between base pairs and interaction with the negatively charged phosphate backbone of DNA and minor grooves.⁸⁹⁾ TO has also been used in fluorescent intercalator displacement (FID) assay to screen compounds interacting with DNA structures.⁹⁷⁾ This screening method has been used for exploring compounds interacting with i-motif, and has yielded new i-motif ligands.⁶⁴⁾ Since the i-motif is known to form under acidic conditions and in the presence of Ag⁺ via formation of C–Ag⁺–C at neutral pH,⁴³⁾ i-motif-based label-

Fig. 12. Structures of Thiazole Orange (27), DMSB (28), Crystal Violet (29), Berberine (30), Neutral Red (31), Thioflavin T (32), PTCDI (33), Iridium Complex (34), Ruthenium Complexes (35, 36) and Terbium Complex (37)

Fig. 13. Types of Fluorescence Change Mechanism in Interacting Ligands

a) Type A: i-Motif is formed in the presence of H^+ or Ag^+ , then the ligand interacts with i-motif and exhibits fluorescence, b) Type B: The ligand exhibits fluorescence by interaction with the cavity of hairpin structure, and formation of i-motif causes release of the ligand with loss of fluorescence, and c) Type C: Aggregation of the ligand causes fluorescence quenching, but i-motif induces disaggregation with an increase in fluorescence. (Color figure can be accessed in the online version.)

free fluorescence sensing systems have been developed as logic gates. For example, the INHIBIT logic gate (Fig. 14a) can respond to H^+ and Ag^+ as inputs, where the presence and absence of inputs are defined as 1 and 0, respectively. Ag^+ promotes the formation of i-motif at neutral pH and induces TO fluorescence as the output ($\text{FI}=1$), while cysteine (Cys) disrupts the $\text{C}-\text{Ag}^+-\text{C}$ bond and switches off the fluorescence of TO ($\text{FI}=0$). The reversibility of this system was demonstrated by addition of Ag^+ and Cys alternately.⁸⁹⁾ Another ex-

ample of this kind of ligand is DMSB (28), which binds to the groove of G4, resulting in enhanced fluorescence. DMSB (28) also exhibits fluorescence upon interaction with i-motif, while its fluorescence is reduced in the presence of ssDNA. DMSB was used in an OR logic gate (Fig. 14b), where an acidic condition (H^+) or the presence of Ag^+ are used as inputs to generate an increase in fluorescence intensity as the output.⁹⁰⁾

Crystal violet (CV, 29), a triphenylmethane, is a selective fluorescent probe for i-motif and G4 over ssDNA and dsDNA.

Fig. 14. i-Motif-Based Logic Gates

a) INHIBIT logic gate, b) OR logic gate, and c) AND logic gate.

CV (**29**) interacts weakly with single-stranded nucleic acids and the fluorescence is reduced, while its interaction with G4 and i-motif causes an increase in its fluorescence, based on studies with G-rich and C-rich sequences exposed to K⁺ and H⁺, respectively. Formation of G4 and i-motif enhances the CV (**29**) fluorescence, and the structural transition of ssDNA to the corresponding tetraplex DNAs can be monitored. CV (**29**) has been used in an OR logic gate (Fig. 14b) with K⁺ and H⁺ as inputs.⁹¹ The interaction of berberine (**30**) with nucleic acids, especially G4, has also been widely studied, and may be related to the biological activities.^{99,100} This ligand exhibits fluorescence in response to the formation of i-motif under acidic conditions, and this phenomenon was utilized in INHIBIT, OR and AND logic gates. In the presence of Ag⁺ the interaction of berberine (**30**) with i-motif is weak, and therefore in the AND logic gate (Fig. 14c), even under acidic conditions, the fluorescence intensity is low (FI=0) when the Ag⁺ is present in the system, but after addition of cysteine, which disrupts C–Ag⁺–C, the fluorescence intensity is increased (FI=1) due to formation of i-motif under acidic conditions in the presence of berberine (**30**).⁹²

Among the ligands used as fluorescence probes, neutral red (**31**) selectively interacts with i-motif, exhibiting a significant increase in fluorescence. This compound exists in neutral and protonated forms. The fluorescence intensity of neutral red (**31**) did not change significantly in the presence of G4, ssDNA or dsDNA, showing that this compound specifically recognizes the i-motif structure.⁹³

Thioflavin T (ThT, **32**) containing a benzothiazole moiety has been used for recognition of ds-DNA¹⁰¹ and G-quadruplexes.^{102,103} This compound is not fluorescent in the twisted state, but when it interacts with nucleic acids, the twisting is released and it exhibits fluorescence. Formation of i-motifs of RET and Rb has been monitored in terms of the change of fluorescence of ThT (**32**). Interestingly, the behavior of **32** fluorescence was totally different with RET and Rb i-motifs. The RET sequence forms a hairpin at neutral pH, and this has a cavity for interaction with ThT (**32**), causing an increase in the fluorescence of ThT (**32**) (Fig. 13b). On the other hand, the Rb i-motif contains a long adenine loop, providing a cavity

for interaction of **32** and enhancing its fluorescence. Although ThT (**32**) has been used to probe the formation of i-motifs in RET and Rb sequences, the interaction mechanism of ThT (**32**) has not been confirmed, and **32** might not selectively recognize the i-motif structure.⁹⁴

As another example, aggregation-induced quenching of PTCDI (**33**) was used to design a pH sensor system in combination with i-motif.⁹⁵ PTCDI is in equilibrium between aggregated form and the monomeric form in aqueous solution,¹⁰⁴ and single-stranded oligonucleotide can promote the aggregation of PTCDI because of the strong electrostatic interaction between the monomer and the anionic phosphate backbone. Aggregation causes fluorescence quenching. Under neutral conditions, when the i-motif-forming sequence is in a single-stranded state, PTCDI aggregates and this causes fluorescence quenching. By changing the pH to acidic to promote i-motif structure formation, probe aggregation is reduced and the fluorescence signals of released PTCDI can be detected (Fig. 13c). To investigate the application of this fluorescence system in extracellular and intracellular environments of HeLa cells, cell suspension and lysates were extracted and the fluorescence intensity of PTCDI was monitored in response to formation of i-motif. This method was effective when the cell density was controlled.⁹⁵ The above ligands, are summarized in Table 3.

In addition to the described ligands, there are complexes which are studied in this field (Fig. 12). Ruthenium complexes are used as light switches based on their luminescence increase in the presence of DNA structures.^{105–107} Recently, these complexes have been introduced as anticancer agents.¹⁰⁸ [Ru(bpy)₂(dppz)]²⁺ (**35**) and [Ru(phen)₂(dppz)]²⁺ (**36**) (Fig. 12) bind to G4 and induce G4 formation in the absence of ions. As for i-motif DNA, addition of these complexes caused a slight change in the characteristic CD signal of i-motif under acidic conditions, suggesting that an interaction with i-motif occurs. However, the induction of this structure was not observed in pH 7.0 upon addition of **35** and **36**. By comparing the results of luminescence experiments and UV melting studies, it appears that these complexes preferentially bind to G4 over i-motif, and the interaction with i-motif might be nonspecific

Table 3. Ligands Used as Fluorescent Probes, Their Types of Fluorescence Change Mechanism and the Utilized Logic Gates

Compound	Mechanisms of fluorescence change (See Fig. 13)	Logic gate (See Fig. 14)	Ref.
 Thiazole orange (TO, 27)	Type A	INHIBIT	89
 DMSB(28)	Type A	OR	90
 Crystal violet (CV, 28)	Type A	OR	91
 Berberine(29)	Type A	INHIBIT OR AND	92
 Neutral red (30)	Type A	—	93
 Thioflavin T (ThT, 31)	Type A and Type B (depending on i-motif sequence)	—	94
 PTCDI (32)	Type C	—	95

or might involve binding to the negatively charged phosphate backbone of DNA.^{109,110} Terbium-amino acid complexes (37) have also been investigated. The lanthanide complexes have been used in luminescence resonance energy transfer (LRET). They decrease the T_m , but the fluorescence enhancement was higher for G4 than for i-motif.¹¹¹ The iridium(III) complex (34) has also used for monitoring these DNA structures. This complex showed an increase in luminescence in response to the formation of i-motif (selectively over ssDNA and dsDNA), but it also interacts with G4 with a similar luminescence enhancement to that in the presence of i-motif. This switch-on probe has used to monitor terminal deoxynucleotidyl transfer (TdT) activity in diluted cell extracts.¹¹²

4. Conclusion

Various i-motif-interacting compounds have been developed as tools for probing the i-motif structure and biological functions. Here, we have briefly reviewed them in four groups: (1) i-motif interacting agents inspired by G4 ligands, (2) ligands other than G4-inspired interacting agents, (3) ligands used to study the biological functions of i-motif, and (4) ligands used

as fluorescent probes. Some of these ligands have been shown to modulate gene expression. In other applications, these ligands have been used as fluorescent pH sensors, based the recognition that the i-motif responds to pH changes. Nevertheless, it is not yet clear whether the i-motif directly regulates biological activities, or whether the stabilization and destabilization of this structure is a functional phenomenon in cells. Therefore, new and selective i-motif interacting agents are still needed as tools to investigate these issues.

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Conflict of Interest The authors declare no conflict of interest.

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