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Human telomere double G-quadruplex recognition by berberine-bisquinolinium imaging conjugates in vitro and cells

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ABSTRACT: Molecular tools of double or multimeric G-quadruplexes have been given higher requirements on detection sensitivity, thermal stabilization and cell imaging to establish functions of these G-quadruplex aggregates and biological mechanisms as anticancer reagents. Here, two smart berberine-bisquinolinium conjugates (**Ber-360A** and **Ber-PDS**) by linking the berberine fluorophore ligand and an established G-quadruplex binder (i.e. bisquinolinium scaffold), have been designed and evaluated their activities and mechanisms for G-quadruplex aggregation. Two conjugates, especially **Ber-PDS**, are two highly selective, sensitive and fluorescent sensors which can distinguish human telomere double G-quadruplexes from other type G-quadruplexes and ds DNA. These two ligands could be the first example to stack two adjacent G-quadruplex units and fluorescently recognize human telomere double G-quadruplexes. Furthermore, conjugate **Ber-PDS** could enter the nucleoli and target G-quadruplex DNA through microscopy experiments, and also display strong telomerase inhibition and antitumor activities.

Keywords

Berberine-bisquinolinium conjugates; G-quadruplex aggregates; optical probes; selective recognition; microscopy; antitumor

1. Introduction

Double and multimeric G-quadruplexes formed by two or more G-quadruplexes, have appeared in the human telomere DNA and RNA regions, especially single-stranded 3'-telomere overhang [1-3], and greatly contributed to telomere maintenance [4-6]. To differentiate double and multimeric G-quadruplexes from a large number of monomeric G-quadruplexes and double-stranded DNA in telomere region, the ligands of these G-quadruplex aggregates have been paid more attention to establish G-quadruplex assembly roles and develop potential antitumor drugs with low side effects [7-17]. Firstly, some monomeric G-quadruplex binders have demonstrated to bind double G-quadruplexes, although with low selectivity [7-10]. Subsequently, improved double or multimeric G-quadruplex binders have been developed, with higher binding affinity and selectivity. For instance, hemin [6] and a chiral cyclic helicene [11] have been reported to specifically intercalate into the cleft formed by two G-quadruplexes, providing an enhanced selectivity towards multimeric G-quadruplexes. In addition, some polymers, including a dinickel-salphen complex, a bisquinolinium (**360A**) dimer and a pyridostatin (**PDS**) dimer reported by our research group, could bind two adjacent G-quadruplex units by π - π stacking and exhibit high selectivity for double or multimeric quadruplexes [12-17]. So the π - π stacking on two adjacent G-quadruplex units and the intercalation into the cleft could be thought as two better binding modes for the design of excellent double or multimeric G-quadruplex ligands.

On the other hand, some fluorescence probes have been reported to display higher binding selectivity towards G-quadruplex aggregates than monomeric G-quadruplexes and ds DNA [17-23]. Such specific probes with high detection sensitivity, thermal stabilization and cell imaging would accurately track G-quadruplex aggregates in cells and better understand the antitumor mechanism. Similarly, monomeric G-quadruplex probes still displayed poor selectivity between monomeric and multimeric G-quadruplexes [18,19]. Subsequently, by the intercalation into the cleft, some fluorescence probes, for example tetrazolylpyrene nucleoside [20] and triaryl-substituted imidazoles linked to naphthalimide derivative [21], displayed high selectivities towards multimeric G-quadruplexes. In addition, by the stacking on the two adjacent G-quadruplex units, two berberine dimers have been reported as highly selective imaging agents for double G-quadruplexes [22,23]. Though these double G-quadruplex probes displayed higher fluorescence responses towards double or multimeric G-quadruplexes, their thermal stabilization and cell imaging were not ideal, which prevents the

accurate tracking in cells and decreases anticancer activities.

With the aim of reaching higher fluorescent detection, selective binding and stabilization towards double G-quadruplexes in vitro and cells, two smart conjugates (Scheme 1) as further development of our recently reported polyether-tethered bisquinolinium dimer [15] and berberine dimers [22,23] have been designed and synthesized. Specifically, the new conjugates retain the berberine fluorophore ligand as high and selective fluorescence detection section towards G-quadruplexes. To increase their thermal stabilization towards G-quadruplexes and cell activities, the suitable polyether linkers tether selected bisquinolinium scaffolds **360A** [24,25] and pyridostatin **PDS** [26-28] with high G-quadruplex thermal stabilization and the potentiality as cancer therapeutic agent. Two conjugates **Ber-PDS** and **Ber-360A** have been evaluated for their binding selectivity, fluorescence response and thermal stabilization towards human telomere double quadruplex DNA (G2T1) in vitro, for the suitability to visualise double G-quadruplex forms in cells through microscopy experiments, as well as for their capability in inhibiting telomerase and producing antitumor effects in a range of cell lines. For comparison, the interaction and fluorescence response of probes **Ber** and **(Ber)₂** towards G2T1 has been discussed.

Scheme 1. Structures of dimer **(Ber)₂** and of berberine-bisquinolinium conjugates **Ber-360A** and **Ber-PDS**.

2. Material and methods

2.1. Oligonucleotides and Compounds

G-quadruplexes (Table 1) from Shanghai Sangon Biological Engineering Technology & Services (Shanghai, China) were dissolved in 10 mM Tris-HCl (100 mM KCl or NaCl, pH

7.0) buffer, annealed by heating to 95 °C for 10 min and then cooled to room temperature overnight. Conjugates **Ber-360A** and **Ber-PDS** were dissolved in DMSO to give 10 mM stock solution and diluted to the appropriate concentration using suitable buffer before use.

Table 1. DNA sequences used in this work.

DNA	Sequence (from 5' to 3')	Structure
ds DNA	CAATCGGATCGAATTCGATCCGATTG +GTTAGCCTAGCTTAAGCTAGGCTAAC	Double stranded
G1	AGGG(TTAGGG) ₃	G4 (monomeric)
c-myc	TGAGGGTGGGGAGGGTGGGGAA	G4 (monomeric)
c-kit1	G ₃ AG ₃ CGCTG ₃ AG ₂ AG ₃	G4 (monomeric)
c-kit2	G ₃ CG ₃ (CG) ₂ (AG ₃) ₂ G	G4 (monomeric)
G2T1	AGGG(TTAGGG) ₇	G4 (double)
G2T2	AGGG(TTAGGG) ₃ TTA(TTAGGG) ₄	G4 (double)
G2T4	AGGG(TTAGGG) ₃ (TTA) ₃ (TTAGGG) ₄	G4 (double)
G2T6	AGGG(TTAGGG) ₃ (TTA) ₅ (TTAGGG) ₄	G4 (double)
Ap7	AGGGTTA ^a GGG(TTAGGG) ₆	G4 (double)
Ap13	AGGGTTAGGGTTA ^a GGG(TTAGGG) ₅	G4 (double)
Ap19	AGGG(TTAGGG) ₂ TTA ^a GGG(TTAGGG) ₄	G4 (double)
Ap25	AGGG(TTAGGG) ₂ TTA ^a GGG(TTAGGG) ₄	G4 (double)
Ap31	AGGG(TTAGGG) ₄ TTA ^a GGG(TTAGGG) ₂	G4 (double)
Ap37	AGGG(TTAGGG) ₅ TTA ^a GGGTTAGGG	G4 (double)
Ap43	AGGG(TTAGGG) ₆ TTA ^a GGG	G4 (double)

^aAp= 2-aminopurine.

2.2. Fluorescence titration studies

Ber-360A and **Ber-PDS** (1.0 μM) were added into concentrate solutions of G-quadruplexes (500 μM) in 10 mM Tris-HCl buffer (100 mM KCl or NaCl, pH 7.0). The fluorescence data were collected at $\lambda_{\text{ex}}/\lambda_{\text{em}}=355/530$ nm and analyzed to give the apparent binding constants (K_a) of **Ber-360A** and **Ber-PDS** towards double and monomeric G-quadruplex DNA by non-linear fitting (seeing supplementary material) [23,29]. The detection limit was calculated according to the equation $3\sigma_{\text{bi}}/m$, wherein σ_{bi} is the standard deviation of blank measurements, and m is the slope of the straight line between the fluorescence intensity of compounds **Ber-360A** and **Ber-PDS** (1 μM) in the absence of human telomere monomeric and double G-quadruplexes (G1 or G2T1) and the concentration of G1 or G2T1 [22,30].

2.3. Determination of quantum yields

The quantum yield values (Φ_x) of conjugate **Ber-360A/Ber-PDS** (0.5 μM) with different DNA (G2T1: 1 μM ; monomeric G1, ds DNA and CT DNA: 2 μM) were measured according to the experimental method from the reported literature (seeing supplementary material) and calculated relative to a 0.1 mol/L H_2SO_4 solution of quinine sulfate ($\Phi_s = 0.54$) [31,32].

2.4. Fluorescence competition studies

Fluorescence competition titrations of two conjugates towards G2T1 in the presence of G1 or ds DNA were performed by maintaining the concentration of each conjugate (1 μM) and G1 (8 μM) or ds DNA (8 μM) with varying the concentration of G2T1 (0~2 μM) in 10 mM Tris-HCl buffer and 100 mM KCl or NaCl (pH 7.0) and measured at $\lambda_{\text{ex}}/\lambda_{\text{em}} = 355/530$ nm. They were also performed by maintaining the concentration of each conjugate (1 μM) and G2T1 (2 μM) with varying the concentration of G1 (0~6 μM) or ds DNA (0~6 μM) in 10 mM Tris-HCl buffer and 100 mM KCl or NaCl (pH 7.0). Fluorescence competition titrations of compound **BRACO 19** and conjugate **Ber-PDS** towards G2T1 or G1 were measured by maintaining the concentration of **Ber-PDS** (1 μM) and mixed-type G2T1 (2 μM) or G1 (4 μM) with varying the concentration of **BRACO 19** (0~10 μM) in 10 mM Tris-HCl buffer and 100 mM KCl (pH 7.0) at $\lambda_{\text{ex}}/\lambda_{\text{em}} = 355/530$ nm.

2.5. 2-Ap titration studies

The annealed double G-quadruplexes modified with 2-aminopurine (Ap) (5 μM) was prepared in 10 mM Tris-HCl buffer and 100 mM NaCl or KCl (pH 7.0). The fluorescence data were collected at $\lambda_{\text{ex}}/\lambda_{\text{em}} = 305/370$ nm by maintaining the concentration of Ap-labelled G-quadruplexes (5 μM) with varying the concentration of **Ber-360A/Ber-PDS** (0~20 μM).

2.6. CD Procedures

CD spectra were measured on annealed G2T1 (5.0 μM) in 10 mM Tris-HCl (100 mM NaCl or KCl, pH 7.0) with **Ber-360A/Ber-PDS**, and nonannealed G2T1 (5.0 μM) in 10 mM Tris-HCl (pH 7.0) with **Ber-360A/Ber-PDS**. CD melting assays of antiparallel or mixed-type G2T1 (5.0 μM) and G1 (10.0 μM) were performed without or with **Ber-360A/Ber-PDS** in 10 mM Tris-HCl (100 mM NaCl or KCl, pH 7.0). The experimental condition is the 100 nm/min

scanning speed, the 2 s response time and the 1 °C/min heating rate. The melting temperature (T_m) was calculated from the melting profiles with the software origin 8.0.

2.7. UV-Vis titration studies

UV-Vis spectra were measured by maintaining the concentration of the conjugate (**Ber-360A/Ber-PDS**, 30 μ M) with varying the concentration of G2T1 (0~10 μ M) in 10 mM Tris-HCl buffer and 100 mM NaCl or KCl (pH 7.0) at room temperature.

2.8. Molecular modelling studies

Molecular modelling studies were performed by Schrodinger software package (Schrodinger, LLC, New York, NY, USA). The coordinates of telomeric G-quadruplexes were generated a solved NMR structure (PDB ID: 2MB3) and adjusted with the protein preparation wizard. The compound structures using docking were drawn in Maestro and prepared with LigPrep. The docking simulations were carried out with Glide by setting the active site throughout the G-quadruplexes.

2.9. Cell imaging studies

In live cell staining experiment, HeLa cells were incubated with 10 μ M **Ber-PDS** or **Ber-360A** for 12 h, and subsequently incubated with 5 μ g·mL⁻¹ Hoechst 33342 for 20 min. Lastly, the cells were examined by fluorescence on an Axio Observer microscope.

In fixed cell staining experiment, HeLa cells were fixed with 4% paraformaldehyde for 15 min. Then the cell membrane was permeabilized with 0.3% Triton X-100 for 30 min. Cells were incubated with RNase-free DNase I (100 units·mL⁻¹), or DNase and Protease-free RNase A (100 units·mL⁻¹), or PBS as control, at 37 °C and 5% CO₂ atmosphere for 3 h. Cells were stained with 5 μ M of **Ber-PDS** or **Ber-360A** for 1 h, and subsequently stained with DAPI (5 μ g·mL⁻¹) for 30 min. Lastly, the cells were examined by fluorescence on an Axio Observer microscope.

In the competitive binding experiment of **BRACO 19**, HeLa cells were fixed with 4% paraformaldehyde for 15 min and incubated with 10 μ M **Ber-PDS** for 1 h. Subsequently, the cells were incubated with different concentration of **BRACO 19** (0~80 μ M) for 1 h. Lastly, the cells were examined by fluorescence on an Axio Observer microscope.

2.10. Assay of telomerase inhibition

Telomerase activities with compounds **(Ber)₂**, **Ber-360A** and **Ber-PDS** were measured by the TRAP-LIG assay [33].

2.11. Assay of cytotoxicity

Antitumor activities of compounds **(Ber)₂**, **Ber-360A** and **Ber-PDS** were measured against five cancer cells (HeLa, MCF-7, MDA-MB-231, A549 and HepG2) and a normal liver cell line L02 by MTT assay [15].

3. Results and Discussion

3.1. Synthesis of conjugates **Ber-360A** and **Ber-PDS**

The synthetic route of berberine-bisquinolinium conjugates **Ber-360A** and **Ber-PDS** is shown in Scheme S1. Their structures were fully characterized with NMR (¹H and ¹³C) and MS (LR and HR) (seeing supplementary material, Fig. S1-18).

3.2. Spectroscopic recognition for G2T1

The fluorescence emission of conjugates **Ber-PDS** and **Ber-360A** have been firstly measured in the presence of mixed-type G2T1, antiparallel G2T1, mixed-type G1, antiparallel G1, c-kit 1, c-kit 2, c-myc, ds DNA and CT DNA. Upon the addition of mixed-type and antiparallel G2T1, conjugates **Ber-PDS** and **Ber-360A** resulted in the appearance of a new emission peak at 530 nm and higher fluorescence enhancement than five monomeric G-quadruplexes (mixed-type and antiparallel G1, c-kit 1, c-kit 2 and c-myc), ds DNA and CT DNA, respectively (Fig. S19). Correspondingly, fluorescence colour changed from dark to yellow (Fig. 1A, inset). Furthermore, the photochemical and sensing properties of conjugates **Ber-360A** and **Ber-PDS** have been investigated with the above-mentioned DNA sequences (Fig. 1A). Conjugate **Ber-PDS** exhibited the highest quantum yield ($\Phi_x=0.54$) towards antiparallel G2T1, *ca.* 2.1-fold higher than conjugate **Ber-360A**. The quantum yield of **Ber-PDS** towards antiparallel G2T1 was *ca.* 10.8-fold higher than towards antiparallel G1. Furthermore, **Ber-PDS** towards mixed-type G2T1 showed the high quantum yield ($\Phi_x=0.26$), *ca.* 2.4-fold higher than conjugate **Ber-360A**. The quantum yield of **Ber-PDS** towards mixed-

type G2T1 was *ca.* 6.5~26-fold higher than those for mixed-type G1, c-kit 1, c-kit 2 and c-myc, respectively, and *ca.* 13- and 26-fold higher than those for ds DNA and CT DNA.

In addition, fluorescence competition recognition will be crucial towards G2T1 over a large amount of monomeric G-quadruplexes and double stranded DNA *in vivo*. So fluorescence competition assays of conjugates **Ber-360A** and **Ber-PDS** towards G2T1 over G1 and ds DNA were performed. The results show that the consecutive addition of G2T1 to a solution of conjugate **Ber-PDS** and a high concentration of G1 or ds DNA, resulted in enhanced fluorescence emissions in a similar fashion to the case without G1 (Fig. 1B). In contrast, the presence of G1 (Fig. 1C) and ds DNA (Fig. S20B) did not result in a marked change of the enhanced fluorescence of conjugate **Ber-PDS** with G2T1. These results suggest that the presence of a large number of monomeric G-quadruplexes and ds DNA had little effect on fluorescence recognition of conjugate **Ber-PDS** towards double G-quadruplexes. Moreover, conjugate **Ber-360A** also showed the similar fluorescence competition recognition towards double G-quadruplexes over monomeric G-quadruplexes and ds DNA (Fig. 1C and S20).

Fig. 1. (A) Fluorescence quantum yields (Φ_x) of two conjugates (0.5 μM) in the absence and presence of a panel of DNA sequences (G2T1: 1 μM ; monomeric G1, ds DNA and CT DNA: 2 μM). Inset: colour changes of two conjugates in the presence of different DNA. (B) Fluorescence intensities of **Ber-PDS** (1 μM) with the addition of increasing amounts of antiparallel G2T1 (0~2 μM) without and with antiparallel G1 (8 μM) and mixed-type G2T1 (0~2 μM) without and with mixed-type G1 (8 μM) and ds DNA (8 μM). (C) Fluorescence intensities of the mixture of conjugate (1 μM) and mixed-type or antiparallel G2T1 (2 μM) with the addition of increasing amounts of mixed-type or antiparallel G1 (0~6 μM).

3.3. Detection limit for G2T1

The detection limit of the optical probes **Ber-360A** and **Ber-PDS** towards G2T1 and G1 through spectrofluorimetric titrations was measured. For comparison, the detection limit of **Ber** and dimer (**Ber**)₂ towards G2T1 and G1 has been also measured and summarized in Table S1 and Fig. S21-23. The detection limit of **Ber-PDS** towards antiparallel G2T1 (0.44 nM) was *ca.* 6 and 13.6-fold lower than the values for **Ber-360A** (2.6 nM) and monomer **Ber** (6.0 nM), respectively, and slightly lower than that of dimer (**Ber**)₂ (0.65 nM) [22]. The detection limit of **Ber-PDS** towards antiparallel G1 was not detected for the lack of stabilization towards antiparallel G1 (Fig. S29D). Like dimer (**Ber**)₂, **Ber-360A** showed lower detection limit towards antiparallel G2T1 over G1 (22 nM). However, monomer **Ber** had comparable detection limit towards antiparallel G2T1 and G1. On the other hand, though the detection limit of **Ber-PDS** for mixed-type G2T1 (2.9 nM) was comparable with the values for (**Ber**)₂ (2.2 nM), this value was *ca.* 1.6- and 3.7-fold lower than the values for **Ber-360A** (4.6 nM) and monomer **Ber** (10.7 nM). In addition, like dimer (**Ber**)₂, probes **Ber-360A** and **Ber-PDS** showed *ca.* 4.9- and 8.8-fold lower detection limit towards mixed-type G2T1 over G1, respectively. Monomer **Ber** still had comparable detection limit towards mixed-type G2T1 and G1. Combined with the results of their fluorescence recognition towards G2T1, two conjugates, especially **Ber-PDS**, were the most efficient optical probes for G2T1.

3.4. Binding affinity towards G2T1

To analyze the interaction of conjugates **Ber-360A** and **Ber-PDS** with different DNA sequences, the DNA-binding affinities (K_a 's) were measured by fluorescence titration assays and summarized in Table 2, Fig. S24 and S26. The results show that **Ber-360A** and **Ber-PDS**

exhibited comparable binding affinities towards antiparallel G2T1 ($19.6 \pm 5.8 \mu\text{M}^{-1}$ for **Ber-360A** and $21.8 \pm 4.0 \mu\text{M}^{-1}$ for **Ber-PDS**, respectively), and showed *ca.*16.3- and 11.5-fold higher binding affinities towards antiparallel G2T1 than G1. Moreover, **Ber-360A** towards mixed-type G2T1 exhibited higher binding affinities than **Ber-PDS** ($16.9 \pm 4.7 \mu\text{M}^{-1}$ for **Ber-360A** and $11.1 \pm 1.0 \mu\text{M}^{-1}$ for **Ber-PDS**, respectively). In addition, **Ber-360A** showed *ca.* 12~16.9 and 33.8-fold higher binding affinity towards mixed-type G2T1 over four monomeric G-quadruplexes (mixed-type G1, c-kit 1, c-kit 2 and c-myc) and ds DNA, respectively. And **Ber-PDS** showed *ca.*10~28 and 10-fold higher binding affinity towards mixed-type G2T1 over four monomeric G-quadruplexes (mixed-type G1, c-kit 1, c-kit 2 and c-myc) and ds DNA, respectively. Thus the two optical probes were suggested to show higher binding activities towards G2T1 *versus* monomeric G-quadruplexes and ds DNA. Their high binding activities and selective visualization towards G2T1 suggest that the future application could focus on constructing a label-free luminescent switch-on G-quadruplex-based assay for in vitro detection of metal ions, protein and enzymes, like the reported literatures [34-37].

Table 2. Binding constants (K_a , μM^{-1}) of conjugates **Ber-PDS** and **Ber-360A** with different DNA sequences by fluorescence titration assays.

DNA	Ber-PDS		Ber-360A	
	K_a	RA ^c	K_a	RA
Anti ^a G2T1	21.8 ± 4.0	21.8	19.5 ± 5.8	19.5
Anti G2T2	5.1 ± 0.6	5.1	6.3 ± 0.8	6.3
Anti G2T4	3.5 ± 0.6	3.5	3.2 ± 0.7	3.2
Anti G2T6	1.4 ± 0.5	1.4	1.1 ± 0.2	1.1
Mixed ^b G2T1	11.1 ± 1.0	11.1	16.9 ± 4.7	16.9
Mixed G2T2	6.3 ± 0.7	6.3	6.1 ± 0.5	6.1
Mixed G2T4	3.1 ± 0.8	3.1	2.4 ± 0.7	2.4
Mixed G2T6	1.0 ± 0.6	1.0	1.1 ± 0.5	1.1
Anti G1	1.9 ± 0.6	1.9	1.1 ± 0.4	1.1
Mixed G1	1.0 ± 0.1	1.0	1.0 ± 0.3	1.0
c-kit 1	1.1 ± 0.3	1.1	1.1 ± 0.3	1.1
c-kit 2	0.4 ± 0.1	0.4	0.7 ± 0.3	0.7
c-myc	1.0 ± 0.3	1.0	1.2 ± 0.3	1.2
ds DNA	1.1 ± 0.4	1.1	0.6 ± 0.1	0.6

^a Anti = antiparallel; ^b Mixed = mixed-type; ^c RA = relative affinity of DNA sequences and mixed-type G1.

3.5. Induction and stabilization towards G2T1

The effects of two conjugates on the conformation of G-quadruplexes were studied by CD experiments. The addition of **Ber-PDS** and **Ber-360A** caused no significant structural changes towards antiparallel G2T1 (Fig. S29A and S29B). However, upon addition of **Ber-PDS** into mixed-type G2T1, a significant increase of the positive peak at *ca.* 265 nm and a decrease of the positive peak at *ca.* 295 nm were observed (Fig. 2A). Different from the result of **Ber-PDS**, addition of **Ber-360A** into mixed-type G2T1 exhibited a marked increase of the positive peak at *ca.* 295 nm and the negative peak at *ca.* 260 nm, as well as a decrease of the shoulder peak at *ca.* 270 nm (Fig. S29C). These findings demonstrate that **Ber-PDS** and **Ber-360A** did not change the hybrid conformation though the conformation of mixed-type G2T1 was disturbed [38]. Furthermore, without potassium, sodium or any compounds, addition of each conjugate into non-annealed G2T1 exhibited a distinct positive peak at *ca.* 295 nm, a shoulder peak at 275 nm and a negative peak at *ca.* 260 nm (Fig. 2B and S29E), which demonstrates that two conjugates could induce the formation of mixed-type G-quadruplexes.

Fig. 2. CD spectra of (A) mixed-type G2T1 (5 μ M) in 10 mM Tris-HCl and 100 mM KCl and (B) non-annealed G2T1 (5 μ M) in 10 mM Tris-HCl with varying equivalents of conjugate **Ber-PDS**: (1) 0 equiv, (2) 2 equiv, (3) 4 equiv, (4) 6 equiv and (5) 8 equiv, respectively. (C) Plot of ΔT_m of antiparallel G2T1, antiparallel G1 and ds DNA in the presence of compounds (**Ber**)₂, **Ber-360A** and **Ber-PDS** in 10 mM Tris-HCl and 100 mM NaCl (pH 7.0). (D) Plot of ΔT_m of mixed-type G2T1, mixed-type G1, c-kit 1, c-kit 2 and c-myc in the presence of compounds (**Ber**)₂, **Ber-360A** and **Ber-PDS** in 10 mM Tris-HCl and 100 mM KCl (pH 7.0). ([compound]/[G2T1] = 3:1, [compound]/[G1] = 1.5:1, [compound]/[ds DNA] = 3:1).

In addition, the melting of G2T1 was carried out in the presence of **Ber-360A** and **Ber-PDS**. The results suggest that conjugate **Ber-360A** had higher ΔT_m values (26.2 °C) towards antiparallel G2T1 than **Ber-PDS** and (**Ber**)₂ (Fig. 2C and S31), which was related to the high thermal stabilization of monomer **360A** towards antiparallel G-quadruplexes [15]. These data demonstrate that the two conjugates possessed higher or comparable thermal stabilization than those reported antiparallel G2T1 ligands [12-15,22,39]. And two conjugates exhibited higher thermal stabilization towards antiparallel G2T1 than antiparallel G1 and ds DNA. On the other hand, conjugate **Ber-PDS** had higher ΔT_m values (15.8 °C) towards mixed-type G2T1 than **Ber-360A** and (**Ber**)₂ (Fig. 2D and S31), which was also related to the high thermal stabilization of monomer **PDS** ($\Delta T_m > 34.5^\circ\text{C}$) towards mixed-type G-quadruplexes [25-26]. Moreover, **Ber-PDS** showed higher thermal stabilization towards mixed-type G2T1 than mixed-type G1, c-kit1, c-kit2 and c-myc, respectively. On base of the findings of CD spectra and CD-melting, two conjugates, especially **Ber-PDS**, could induce the formation of mixed-type G-quadruplexes and display higher thermal stabilization towards double G-quadruplexes than monomeric G-quadruplexes and ds DNA.

3.6. Binding modes and fluorescence turn-on mechanism towards G2T1

The binding modes were further analyzed by spectrometric titrations and CD-melting studies. Upon addition of antiparallel G2T1, **Ber-360A** caused two obvious hypochromicities (25%) for the peaks at *ca.* 348 nm (with a marked red shift of 8 nm), and *ca.* 433 nm (Fig. 3A). Similarly, the addition of mixed-type G2T1 to **Ber-360A** and antiparallel and mixed-type G2T1 to **Ber-PDS** also led to a visible hypochromicity for the peak (at *ca.* 346 nm for **Ber-360A** and at *ca.* 327 nm for **Ber-PDS**) with a red shift (Fig. S32). The noticeable redshift at above 300 nm suggested a π - π stacking effect of the two conjugates on G2T1 [22,23,40].

In addition, two conjugates, especially **Ber-PDS**, displayed no obvious isobestic points towards antiparallel and mixed-type G2T1 (Fig. 3A and S32), suggesting the complex binding modes. Furthermore, the binding affinities and fluorescence response of **Ber-360A** and **Ber-PDS** were performed towards G1 and double quadruplexes G2Tn (n=1, 2, 4 and 6, number of TTA subunits in the linkers) (Table 1, Fig. S25 and S27). Conjugate **Ber-PDS** showed *ca.* 11.1, 6.3, 3.1 and 1.0-fold higher binding affinities towards mixed-type G2T1, G2T2, G2T4 and G2T6 over G1, respectively. And conjugate **Ber-360A** exhibited *ca.* 16.9, 6.1, 2.4 and 1.1-fold higher binding affinities towards mixed-type G2T1, G2T2, G2T4 and G2T6 over G1, respectively. Similarly, two conjugates displayed higher binding affinities towards antiparallel G2T1 than antiparallel G1 and other double G-quadruplexes with longer TTA linkers. In addition, with the gradual extension of TTA linkers in double G-quadruplexes, the fluorescence response of two conjugates towards double G-quadruplexes decreased gradually (Fig. 3B). These results demonstrate that the two conjugates possibly bound the two adjacent G-quartets in one G2T1.

Furthermore, the possible binding sites were discussed by fluorescence titrations with Ap-labelled G-quadruplexes [12,14,15,21,23], which do not perturb the quadruplex topology (Fig. S30). As shown in Fig. 3C and S33A, addition of two probes into mixed-type G2T1 caused more significant decrease on the fluorescence intensities of Ap19 and Ap31 at two G-tetrads of the 5' and 3' ends, and Ap13, Ap25 and Ap37 in the quadruplex grooves than Ap7 and Ap43 on two external propeller loops. These results suggest that two probes could bind four tetrads by π - π stacking modes and have low binding affinities towards two external propeller loops. By comparison, the addition of **Ber-PDS** into antiparallel G2T1 determined a more marked effects on the fluorescence intensities of Ap13, Ap19, Ap25, Ap31 and Ap37 than those of Ap7 and Ap43 (Fig. S33B), whereas the addition of **Ber-360A** led to a more pronounced decrease of the fluorescence intensities towards Ap7, Ap13, Ap25, Ap37 and Ap43 compared to Ap19 and Ap31 (Fig. S33C). These results indicate that **Ber-PDS** showed weaker binding affinity for Ap7 and Ap43 bases of the TTA loops in antiparallel G2T1, while **Ber-360A** displayed weaker binding affinity for Ap19 and Ap31 bases of the TTA loops in antiparallel G2T1. Nonetheless, the data clearly suggest that the two optical probes interacted with four tetrads and the TTA loop linking the two G-quadruplex units. In addition, a 2:1 binding ratio between conjugate and G2T1 was got according to Fig. 3D and S28. Thus all the results indicate that two molecules of **Ber-PDS** or **Ber-360A** possibly stacked the adjacent two G-quartets in one G2T1.

Fig. 3. (A) UV-Vis titration spectra of **Ber-360A** (30 μM) upon increasing concentration (0~10 μM) of antiparallel G2T1. (B) Fluorescence intensity of antiparallel and mixed-type G1 (10 μM) and G2Tn (n=1, 2, 4 and 6, 5 μM) in the presence of **Ber-PDS** and **Ber-360A** ([conjugate]:[G2T1] = 3:1, [conjugate]:[G1] = 1.5:1), respectively. Inset: colour changes of two conjugates in the presence of different DNA. (C) Plot of normalized fluorescence intensity F/F_0 at 370 nm of 2-Ap individually labelled mixed-type G2T1 (Ap7, Ap13, Ap19, Ap25, Ap31, Ap37 and Ap43, respectively) *versus* binding ratio of $[\text{Ber-PDS}]/[\text{G2T1}]$, respectively. (D) Plot of fluorescence intensity at 530 nm of conjugate **Ber-PDS** *vs* binding ratio of $[\text{Anti-G2T1}]/[\text{Ber-PDS}]$. Inset: plot of fluorescence intensity at 530 nm of conjugate **Ber-PDS** *vs* binding ratio of $[\text{Mixed-G2T1}]/[\text{Ber-PDS}]$, $\lambda_{\text{ex}} = 355 \text{ nm}$.

Furthermore, molecular modelling studies have been used to discuss the binding modes and luminescent mechanism of conjugates **Ber-PDS** and **Ber-360A** with G2T1 as shown in Fig. 4. The molecular modelling indicates that two molecules of **Ber-PDS** or **Ber-360A** could bind with the adjacent two G-quartets. Take the interaction between **Ber-PDS** and antiparallel G2T1 for example (Fig. 4A, left), the **Ber** moiety of one **Ber-PDS** stacked on one G-quartet, and the **PDS** moiety stacked on the adjacent G-quartet and the TTA loop for the

influence of two loops steric hindrance (Fig. S34A). And both **Ber** and **PDS** moieties of a second **Ber-PDS** could pile up on two G-quartets (Fig. S34B). Similar results of binding mode have been gotten between **Ber-PDS** and mixed-type G2T1 (Fig. 4B, left). Different from the binding modes between **Ber-PDS** and G2T1, the **360A** moiety of one **Ber-360A** stacked on one G-quartet, but the **Ber** moiety stacked on the adjacent G-quartet and the TTA loop though both **Ber** and **360A** moieties of a second **Ber-360A** could pile up on two G-quartets (Fig. 4A, 4B and S35). According to the binding modes, due to the **Ber** plane π - π stacking the G-quartet in G2T1, the **Ber**-plane rotation was probably prevented, which induced to the conformational changes in the excited state of the **Ber** plane, and the fluorescence light-up of conjugate by G2T1. Due to both **Ber** planes of two **Ber-PDS** molecules π - π stacking on the G-quartets, **Ber-PDS** showed stronger fluorescence response towards G2T1 than **Ber-360A**. Furthermore, with the rise of the content of glycerol in the glycerol-water solution, **Ber-PDS** displayed more significant increase on the fluorescence intensity at *ca.* 530 nm than **Ber-360A** (Fig. 4C and 4D), which further verifies this assumption of luminescent mechanism [22,41,42]. In addition, two conjugates exhibited higher binding affinities towards antiparallel G2T1 ($-26.1 \text{ kcal}\cdot\text{mol}^{-1}$ for **Ber-PDS** and $-25.6 \text{ kcal}\cdot\text{mol}^{-1}$ for **Ber-360A**) over mixed-type G2T1 ($-22.9 \text{ kcal}\cdot\text{mol}^{-1}$ for **Ber-PDS** and $-23.3 \text{ kcal}\cdot\text{mol}^{-1}$ for **Ber-360A**), which is consistent with the K_a values.

Fig. 4. (A) and (B) Docking models of two conjugates towards antiparallel and mixed-type G2T1, respectively. (C) and (D) Fluorescence spectra of **Ber-PDS** and **Ber-360A** in aqueous glycerol solution containing different concentrations of glycerol ($\lambda_{\text{ex}} = 355$ nm), respectively.

3.7. Cell imaging

Encouraged by the results of fluorescence response towards double G-quadruplexes, the intracellular application of conjugates **Ber-360A** and **Ber-PDS** in live and fixed cells was further investigated with an inverted fluorescence microscope [43,44]. Live HeLa cells stained with the optical probe **Ber-360A** displayed distinct fluorescent foci. However, these foci were mainly localized in the cytoplasm (Fig. S36A). After the luminescence of DAPI had been verified not to interfere with the probe fluorescence (Fig. S37A), the fixed cell staining assays had been further carried out. When fixed HeLa cells were stained with **Ber-360A**, the fluorescent signal completely disappeared upon treatment with ribonuclease (RNase A), whereas the fluorescent signal did not obviously change upon treatment with deoxyribonuclease (DNase I) (Fig. 5A and S37B). Therefore, we conclude that **Ber-360A** could not enter into the nucleoli and probably bind RNA in the cytoplasm. Interestingly, a

strong emission response was found in the nucleoli of live cells stained with **Ber-PDS** (Fig. S36B). Furthermore, when fixed HeLa cells were stained with **Ber-PDS**, the fluorescence signals in the nucleoli clearly disappeared upon DNase I treatment, but not upon RNase A treatment (Fig. 5B and S37C). These results suggest that the optical probe **Ber-PDS** probably bound DNA in nucleoli.

For probe **Ber-PDS** exhibited a strong fluorescence response towards G-quadruplexes, especially double G-quadruplexes, such significant emissions in nucleoli have been proved by the competitive binding experiment of a classical G-quadruplex DNA binder **BRACO 19** [21,45] and these two probes towards G-quadruplex DNA in vitro and cells. Firstly, in vitro, the consecutive addition of binder **BRACO 19** resulted in significant reduction on fluorescence emissions of the mixture of **Ber-PDS** and G-quadruplexes, especially G2T1 (Fig. 5C). Then binder **BRACO 19** has further been used to competitively bind G-quadruplexes with probe **Ber-PDS** in cells. As shown in Fig. 5D, addition of **BRACO 19** caused the significant decrease on the number of corresponding fluorescence foci, which further verifies the specificity of conjugate **Ber-PDS** towards G-quadruplex DNA, probably G2T1, in the nucleoli.

Fig. 5. (A) and (B) Microscopy images of fixed HeLa cells stained with 5 μ M **Ber-360A** and **Ber-PDS** after DNase I or RNase A treatment, respectively, which are the enlarged images in the white boxes in Fig. S37B and S37C. (C) Plot of normalized fluorescence intensity at 530 nm of the mixture of 1 μ M **Ber-PDS** and 2 μ M mixed-type G2T1 or 4 μ M mixed-type G1 *versus* molar ratio of [BRACO 19]/[Ber-PDS] in 10 mM Tris-HCl and 100 mM KCl (pH 7.0). (D) Microscopy images of fixed HeLa cells stained with 10 μ M **Ber-PDS** and different concentrations of **BRACO 19** (0~80 μ M). $\lambda_{\text{ex}}/\lambda_{\text{em}} = 365/445$ nm for DAPI and $\lambda_{\text{ex}}/\lambda_{\text{em}} = 470/525$ nm for **Ber-360A** and **Ber-PDS**, respectively.

3.8. Telomerase inhibition and cytotoxicity

As a specific target for anticancer reagents, formation and stabilization of G-quadruplexes in the telomere region has been shown to inhibit the telomerase activity [46,47]. The above

results confirm that conjugates **Ber-360A** and **Ber-PDS** displayed strong stabilization towards mixed-type G2T1 in K^+ buffer and induced the formation of mixed-type G-quadruplexes. So their telomerase inhibition activities have been studied by TRAP-LIG assay. Conjugates **Ber-PDS** and **Ber-360A** displayed a concentration-dependent inhibition and higher inhibition of telomerase activities ($IC_{50} = 0.8 \pm 0.2 \mu M$ and $1.2 \pm 0.2 \mu M$, respectively, Fig. 6) than dimer **(Ber)₂** ($IC_{50} = 12.0 \pm 2.6 \mu M$, Fig. S38A), **360A** ($IC_{50} = 3.2 \pm 0.6 \mu M$, Fig. S38B) and **Ber** ($IC_{50} = 75 \mu M$) [48]. And two conjugates showed comparable telomerase inhibition with **PDS** ($IC_{50} = 0.4 \pm 0.1 \mu M$, Fig. S38C). The strong telomerase inhibition of two conjugates is probably related with the high binding affinity and stabilization towards G2T1 and the strong telomerase inhibition of **360A** and **PDS**.

Fig. 6. Telomerase inhibition in the presence of conjugates **Ber-360A** and **Ber-PDS** by TRAP-LIG assay. Lanes 1~7: 0, 0.3, 0.5, 1.6, 3.1, 5.0 and 10 μM , respectively.

Having confirmed telomerase inhibition properties of the two conjugates, their cytotoxicity towards five cancerous cell lines (i.e. HeLa, A549, HepG2, MDA-MB-231 and MCF-7) and a normal cell line (i.e. L02) have been evaluated in Table 3 with the MTT assay. Dimer **(Ber)₂** had been tested as reference compound. Overall, **Ber-360A** and **Ber-PDS** exhibited higher inhibitory activities against HeLa and HepG2 cells in comparison to A549, MDA-MB-231 and MCF-7 cells. And **Ber-PDS** and **Ber-360A** exhibited higher inhibitory activities against HeLa and HepG2 cells than dimer **(Ber)₂**, which is parallel exactly to their G-quadruplex stabilization and telomerase inhibition properties. In addition, **Ber-PDS** and **Ber-360A** exhibited higher inhibitory activities against liver cancer cells HepG2 than normal liver cells L02. However, **(Ber)₂** is a more potent inhibitor for normal liver cells L02 than liver cancer cells HepG2, and also displays the stronger cytotoxicity in normal liver cells L02 than **Ber-360A** and **Ber-PDS**, which will be further studied in the future.

Table 3. IC₅₀ values (μM) of compounds **(Ber)₂**, **Ber-360A** and **Ber-PDS** against a panel of cell lines.

Compound	IC ₅₀ (μM)					
	HeLa	MCF-7	MDA-MB-231	A549	HepG2	L02 ^a
(Ber)₂	36.0 ± 2.3	41.7 ± 2.9	48.9 ± 1.7	28.7 ± 1.1	97.8 ± 2.2	19.9 ± 1.6
Ber-360A	10.9 ± 1.2	24.1 ± 2.1	27.3 ± 2.8	36.3 ± 2.7	12.9 ± 2.2	117.8 ± 5.2
Ber-PDS	9.2 ± 0.8	26.8 ± 2.3	20.9 ± 1.0	37.3 ± 2.1	10.1 ± 0.3	22.9 ± 1.2

^a L02 is a normal liver cell line.

4. Conclusions

In summary, two smart berberine-bisquinolinium optical probes were designed to image double G-quadruplexes in vitro and cells. Their fluorescence response, binding selectivity and thermal stabilization towards human telomere double G-quadruplexes have been discussed by CD spectroscopy, CD-melting and spectrometric titrations. They retained the advantage of dimer **(Ber)₂** on high fluorescence response and detection sensitivities towards human telomere double G-quadruplex DNA. Furthermore, they also made up the lack of dimer **(Ber)₂** on thermal stabilization and showed higher binding abilities and stabilization towards G2T1 than G1. On the base of spectroscopic and molecular modelling studies, two molecules of **Ber-360A** or **Ber-PDS** have been hypothesized to stack with the adjacent two G-quartets in one G2T1. The **Ber** plane of conjugate π - π stacking the G-quartet in G2T1, probably prevented the **Ber**-plane rotation, induced to the conformational changes in the excited state for the **Ber** plane, and resulted in the “light-up” of conjugate by G2T1. Then cell microscopy studies indicate that the optical probe **Ber-PDS** entered into the nucleoli and allowed fluorescence detection of G-quadruplex DNA including double G-quadruplexes, which contributes to the accurate tracking of G-quadruplexes in cells.

Overall, these results suggest that the combination of an efficient optical tag and a well-known G-quadruplex binder is a relevant strategy to identify new imaging agents for double G-quadruplex structures, combining high selectivity, thermal stabilization and fluorescence recognition properties towards double G-quadruplexes. In addition, the two conjugates developed in this study also possessed strong telomerase inhibition and significant anticancer activities toward tumour cell lines. This study provides a new way to design specific recognition binders towards double G-quadruplexes to accurately track G-quadruplexes in

cells, establish G-quadruplex assembly roles and develop potential antitumor drugs with low side effects.

Declaration of competing interest

The authors declare that they have no conflict of interest.

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Graphical Abstract

Human telomere double G-quadruplex recognition by berberine-bisquinolinium imaging conjugates in vitro and cells

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Two smart berberine-bisquinolinium conjugates, especially **Ber-PDS**, are two highly selective, sensitive and fluorescent sensors which can distinguish human telomere double G-quadruplexes from other type G-quadruplexes and ds DNA. Conjugate **Ber-PDS** could enter the nucleoli and target G-quadruplex DNA through microscopy experiments, and also display strong telomerase inhibition and antitumor activities.

Highlights

- Two highly selective probes for double G-quadruplexes (G2T1) were developed.
- Probes specially targeted two adjacent G-quadruplex units in one G2T1.
- Probe **Ber-PDS** achieved the imaging of G-quadruplexes in cells by microscopy.
- Probes displayed strong telomerase inhibition and anticancer activities.