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Ting-Cong Liao a, Tian-Zhu Ma a, Suo-Bin Chen b, Agostino Cilibrizzi c, Meng-Jia Zhang a, Jun-Hui Li a, Chun-Qiong Zhou a

a Guangdong Provincial Key Laboratory of New Drug Screening, School of Pharmaceutical Sciences, Southern Medical University, Guangzhou 510515, P. R. China
b School of Pharmaceutical Sciences, Sun Yat-sen University, Guangzhou 51006, P. R. China
c Institute of Pharmaceutical Science, King’s College London, London SE1 9NH, United Kingdom
† These authors contributed equally.
* Corresponding author. Email: zcqlg@smu.edu.cn (C.-Q. Zhou).

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
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.

<table>
<thead>
<tr>
<th>DNA</th>
<th>Sequence (from 5’ to 3’)</th>
<th>Structure</th>
</tr>
</thead>
<tbody>
<tr>
<td>ds DNA</td>
<td>CAATCGGATCGAATTCGATCCGATTG +GTTAGCCTAGCTAAGCTAGGCTAAC</td>
<td>Double stranded</td>
</tr>
<tr>
<td>G1</td>
<td>AGGG(TTAGGG)₃</td>
<td>G4 (monomeric)</td>
</tr>
<tr>
<td>c-myc</td>
<td>TGAGGGTTGGGGAGGGTGGGGAA</td>
<td>G4 (monomeric)</td>
</tr>
<tr>
<td>c-kit1</td>
<td>G₃AG₃CGCT₃AG₂AG₃</td>
<td>G4 (monomeric)</td>
</tr>
<tr>
<td>c-kit2</td>
<td>G₃CG₃(CG)₂(AG)₃G</td>
<td>G4 (monomeric)</td>
</tr>
<tr>
<td>G2T1</td>
<td>AGGG(TTAGGG)₇</td>
<td>G4 (double)</td>
</tr>
<tr>
<td>G2T2</td>
<td>AGGG(TTAGGG)₄TTA(TTAGGG)₄</td>
<td>G4 (double)</td>
</tr>
<tr>
<td>G2T4</td>
<td>AGGG(TTAGGG)₃(TTA)₃(TTAGGG)₄</td>
<td>G4 (double)</td>
</tr>
<tr>
<td>G2T6</td>
<td>AGGG(TTAGGG)₃(TTA)₃(TTAGGG)₄</td>
<td>G4 (double)</td>
</tr>
<tr>
<td>Ap7</td>
<td>AGGGTTTAp₆GGG(TTAGGG)₅</td>
<td>G4 (double)</td>
</tr>
<tr>
<td>Ap13</td>
<td>AGGGTTAGGGTTAp₉GGG(TTAGGG)₅</td>
<td>G4 (double)</td>
</tr>
<tr>
<td>Ap19</td>
<td>AGGG(TTAGGG)₉TTAPGGG(TTAGGG)₄</td>
<td>G4 (double)</td>
</tr>
<tr>
<td>Ap25</td>
<td>AGGG(TTAGGG)₉TTAPGGG(TTAGGG)₄</td>
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</tr>
<tr>
<td>Ap31</td>
<td>AGGG(TTAGGG)₉TTAPGGG(TTAGGG)₂</td>
<td>G4 (double)</td>
</tr>
<tr>
<td>Ap37</td>
<td>AGGG(TTAGGG)₉TTAPGGG(TTAGGG)₁</td>
<td>G4 (double)</td>
</tr>
<tr>
<td>Ap31</td>
<td>AGGG(TTAGGG)₉TTAPGGG(TTAGGG)₂</td>
<td>G4 (double)</td>
</tr>
</tbody>
</table>

*Ap= 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 λ<sub>ex</sub>/λ<sub>em</sub>=355/530 nm and analyzed to give the apparent binding constants (Kₐ) 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 3σ<sub>bi</sub>/m, wherein σ<sub>bi</sub> 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 ($\Phi_x$) 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$_2$SO$_4$ solution of quinine sulfate ($\Phi_x = 0.54$) [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 $\lambda_{ex}/\lambda_{em} = 355/530$ 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 $\lambda_{ex}/\lambda_{em} = 355/530$ 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 $\lambda_{ex}/\lambda_{em} = 305/370$ 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 \( \text{Ber-360A/Ber-PDS} \), 30 μM with varying the concentration of G2T1 (0~10 μM) 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 μM Ber-PDS or Ber-360A for 12 h, and subsequently incubated with 5 μg·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% CO\(_2\) atmosphere for 3 h. Cells were stained with 5 μM of Ber-PDS or Ber-360A for 1 h, and subsequently stained with DAPI (5 μg·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 μM Ber-PDS for 1 h. Subsequently, the cells were incubated with different concentration of BRACO 19 (0~80 μM) 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 \( (\text{Ber})_2, \text{Ber-360A} \) and \( \text{Ber-PDS} \) were measured by the TRAP-LIG assay [33].

2.11. Assay of cytotoxicity

Antitumor activities of compounds \( (\text{Ber})_2, \text{Ber-360A} \) and \( \text{Ber-PDS} \) were measured against five cancer cells (HeLa、MCF-7、MDA-MB-231、A549 and HepG2) an a normal liver cell line L02 by MTT assay [15].

3. Results and Discussion

3.1. Synthesis of conjugates \( \text{Ber-360A} \) and \( \text{Ber-PDS} \)

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

3.2. Spectroscopic recognition for G2T1

The fluorescence emission of conjugates \( \text{Ber-PDS} \) and \( \text{Ber-360A} \) 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 \( \text{Ber-PDS} \) and \( \text{Ber-360A} \) 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 \( \text{Ber-360A} \) and \( \text{Ber-PDS} \) have been investigated with the above-mentioned DNA sequences (Fig. 1A). Conjugate \( \text{Ber-PDS} \) exhibited the highest quantum yield \( (\Phi_x=0.54) \) towards antiparallel G2T1, ca. 2.1-fold higher than conjugate \( \text{Ber-360A} \). The quantum yield of \( \text{Ber-PDS} \) towards antiparallel G2T1 was ca. 10.8-fold higher than towards antiparallel G1. Furthermore, \( \text{Ber-PDS} \) towards mixed-type G2T1 showed the high quantum yield \( (\Phi_x=0.26) \), ca. 2.4-fold higher than conjugate \( \text{Ber-360A} \). The quantum yield of \( \text{Ber-PDS} \) 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 ($\Phi_x$) of two conjugates (0.5 μM) in the absence and presence of a panel of DNA sequences (G2T1: 1μM; 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 ± 5.8 μM⁻¹ for Ber-360A and 21.8 ± 4.0 μM⁻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 ± 4.7 μM⁻¹ for Ber-360A and 11.1 ± 1.0 μM⁻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 (Kₐ, μM⁻¹) of conjugates Ber-PDS and Ber-360A with different DNA sequences by fluorescence titration assays.

<table>
<thead>
<tr>
<th>DNA</th>
<th>Ber-PDS</th>
<th>Ber-360A</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anti G2T1</td>
<td>21.8 ± 4.0</td>
<td>19.5 ± 5.8</td>
</tr>
<tr>
<td>Anti G2T2</td>
<td>5.1 ± 0.6</td>
<td>6.3 ± 0.8</td>
</tr>
<tr>
<td>Anti G2T4</td>
<td>3.5 ± 0.6</td>
<td>3.2 ± 0.7</td>
</tr>
<tr>
<td>Anti G2T6</td>
<td>1.4 ± 0.5</td>
<td>1.1 ± 0.2</td>
</tr>
<tr>
<td>Mixed G2T1</td>
<td>11.1 ± 1.0</td>
<td>16.9 ± 4.7</td>
</tr>
<tr>
<td>Mixed G2T2</td>
<td>6.3 ± 0.7</td>
<td>6.1 ± 0.5</td>
</tr>
<tr>
<td>Mixed G2T4</td>
<td>3.1 ± 0.8</td>
<td>2.4 ± 0.7</td>
</tr>
<tr>
<td>Mixed G2T6</td>
<td>1.0 ± 0.6</td>
<td>1.1 ± 0.5</td>
</tr>
<tr>
<td>Anti G1</td>
<td>1.9 ± 0.6</td>
<td>1.1 ± 0.4</td>
</tr>
<tr>
<td>Mixed G1</td>
<td>1.0 ± 0.1</td>
<td>1.0 ± 0.3</td>
</tr>
<tr>
<td>c-kit 1</td>
<td>1.1 ± 0.3</td>
<td>1.1 ± 0.3</td>
</tr>
<tr>
<td>c-kit 2</td>
<td>0.4 ± 0.1</td>
<td>0.7 ± 0.3</td>
</tr>
<tr>
<td>c-myc</td>
<td>1.0 ± 0.3</td>
<td>1.2 ± 0.3</td>
</tr>
<tr>
<td>ds DNA</td>
<td>1.1 ± 0.4</td>
<td>0.6 ± 0.1</td>
</tr>
</tbody>
</table>
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.

\[ \text{Anti} = \text{antiparallel; Mixed} = \text{mixed-type; RA} = \text{relative affinity of DNA sequences and mixed-type G1.} \]

\[ \Delta T_m (\circ C) \]
Fig. 2. CD spectra of (A) mixed-type G2T1 (5 μM) in 10 mM Tris-HCl and 100 mM KCl and (B) non-annealed G2T1 (5 μM) 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 (ΔT_m > 34.5°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 [Ber-PDS]/[G2T1], respectively. (D) Plot of fluorescence intensity at 530 nm of conjugate Ber-PDS vs binding ratio of [Anti-G2T1]/[Ber-PDS]. Inset: plot of fluorescence intensity at 530 nm of conjugate Ber-PDS vs binding ratio of [Mixed-G2T1]/[Ber-PDS], $\lambda_{ex} = 355$ 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-quarter, and the PDS moiety stacked on the adjacent G-quarter 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 kcal·mol⁻¹ for Ber-PDS and -25.6 kcal·mol⁻¹ for Ber-360A) over mixed-type G2T1 (-22.9 kcal·mol⁻¹ for Ber-PDS and -23.3 kcal·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 ($\lambda_{ex} = 355$ 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.

<table>
<thead>
<tr>
<th>A</th>
<th>Bright field</th>
<th>Ber-360A</th>
<th>DAPI</th>
<th>Merged</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td><img src="image1" alt="Control Bright field" /></td>
<td><img src="image2" alt="Control Ber-360A" /></td>
<td><img src="image3" alt="Control DAPI" /></td>
<td><img src="image4" alt="Control Merged" /></td>
</tr>
<tr>
<td>DNase I</td>
<td><img src="image1" alt="DNase I Bright field" /></td>
<td><img src="image2" alt="DNase I Ber-360A" /></td>
<td><img src="image3" alt="DNase I DAPI" /></td>
<td><img src="image4" alt="DNase I Merged" /></td>
</tr>
<tr>
<td>RNase A</td>
<td><img src="image1" alt="RNase A Bright field" /></td>
<td><img src="image2" alt="RNase A Ber-360A" /></td>
<td><img src="image3" alt="RNase A DAPI" /></td>
<td><img src="image4" alt="RNase A Merged" /></td>
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Fig. 5. (A) and (B) Microscopy images of fixed HeLa cells stained with 5 μM 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 μM Ber-PDS and 2 μM mixed-type G2T1 or 4 μM 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 μM Ber-PDS and different concentrations of BRACO 19 (0–80 μM). \( \lambda_{ex}/\lambda_{em} = 365/445 \) nm for DAPI and \( \lambda_{ex}/\lambda_{em} = 470/525 \) 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 (IC₅₀ = 0.8 ± 0.2 μM and 1.2 ± 0.2 μM, respectively, Fig. 6) than dimer (Ber)₂ (IC₅₀ = 12.0 ± 2.6 μM, Fig. S38A), 360A (IC₅₀ = 3.2 ± 0.6 μM, Fig. S38B) and Ber (IC₅₀ = 75 μM) [48]. And two conjugates showed comparable telomerase inhibition with PDS (IC₅₀ = 0.4 ± 0.1 μM, 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. IC\textsubscript{50} values (μM) of compounds (Ber)\textsubscript{2}, Ber-360A and Ber-PDS against a panel of cell lines.

<table>
<thead>
<tr>
<th>Compound</th>
<th>IC\textsubscript{50} (μM)</th>
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<tbody>
<tr>
<td></td>
<td>HeLa</td>
</tr>
<tr>
<td>(Ber)\textsubscript{2}</td>
<td>36.0 ± 2.3</td>
</tr>
<tr>
<td>Ber-360A</td>
<td>10.9 ± 1.2</td>
</tr>
<tr>
<td>Ber-PDS</td>
<td>9.2 ± 0.8</td>
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*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)\textsubscript{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)\textsubscript{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 $\pi$-$\pi$ 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.

**Acknowledgement**

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**References**


Graphical Abstract

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

Ting-Cong Liao †, Tian-Zhu Ma †, Suo-Bin Chen, Agostino Cilibrizzi, Meng-Jia Zhang, Jun-Hui Li, Chun-Qiong Zhou*

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.