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9-Substituted berberine derivatives as G-quadruplex stabilizing ligands in telomeric DNA

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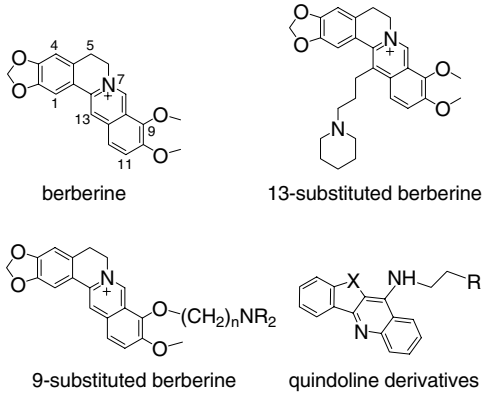
Abstract—The interaction of berberine and its 9-substituted derivatives with human telomeric DNA d[$G_3(T_2AG_3)_3$](telo21) has been investigated via CD spectroscopy, fluorescence spectroscopy, PCR-stop assay, competitive dialysis, and telomerase repeat amplification protocol (TRAP) assay. The results indicated that these semisynthesized compounds could induce and stabilize the formation of anti-parallel G-quadruplex of telomeric DNA in the presence or absence of metal cations. Compared with berberine, the 9-substituted derivatives exhibit stronger binding affinity with G-quadruplex and higher inhibitory activity for telomerase. Introduction of a side chain with proper length of methylene and terminal amino group to the 9-position of berberine would significantly strengthen the binding affinity with G-quadruplex, resulting in increasing inhibitory effects on the amplification of telo21 DNA and on the telomerase activity.

1. Introduction

Human telomeric DNA, located at the very end of chromosomes, consists of repeating sequence of $(TTAGGG/CCCTAA)_n$ which ends as the single strand $(TTAGGG)_n$. In the presence of monovalent cations such as potassium or sodium ions, this single G-rich strand can form unique four-stranded helical conformations called G-quadruplexes.[1–4] Depending on the incubation conditions, the human telomeric sequence telo21 (5′-GGGTTAGGGTTAGGGTTAGGG-3′) is known to form different types of G-quadruplex structure. For example, anti-parallel G-quadruplexes were formed in Na^+ solution, whereas a mixture of parallel and anti-parallel G-quadruplexes was formed in K^+ solution.[5] G-quadruplex formation has been shown in vitro to inhibit the activity of telomerase, an enzyme responsible for the elongation of telomeres in the tumor cells. This unique activity of telomerase makes it an ideal target for anti-cancer drug design.[6,7] A number of small molecules such as ethidium derivatives,[1] disubstituted triazines,[8] telomestatin,[9] acridines,[10] and quindoline derivatives[11] have been shown to induce the formation of G-quadruplexes and inhibit telomerase activity.



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Berberine, an alkaloid isolated from Chinese herbs, was initially used as anti-microbial agent.[12] Berberine and its derivatives were subsequently tested as inhibitors of topoisomerase **I** and **II** which are linked to anti-cancer activity.[13] Tsuruo and coworkers reported the inhibitory activity of berberine on telomerase in 1999, and recently, 13-substituted berberine derivatives were also reported to inhibit telomerase activity by binding to G-quadruplex DNA by Neidle's group.[14,15] With the aim of exploring novel and potent telomerase inhibitors for cancer chemotherapy, we have recently designed and synthesized a new series of 9-substituted berberine derivatives in our laboratories and examined their interaction with G-quadruplex DNA as well as their inhibitory effect on telomerase activity. The corresponding results from these studies are presented here.

2. Results and discussion

2.1. Design of the berberine derivatives

The crystal structures of quadruplex-ligand complexes have revealed that ligands bind onto a G-quartet at the end of a stack, with the planar chromophore moieties participating in π–π stacking interactions with the guanines and substituents lying in the grooves of the structures.[3,16]

In our previous studies, introduction of a side chain with terminal amino group to the outside of the crescent planar chromophore moieties of quindoline led to a significant stabilization effect on G-quadruplex DNA and inhibitory activity on telomerase.[11] Since the ring system of berberine has a similar form with that of quindoline, it is expected that introducing a side chain with terminal amino group on the 9-position of berberine, which also located in the outside of the crescent ring system, may significantly increase interaction between the berberine derivatives and G-quadruplex. On the basis of such assumption, we prepared several berberine derivatives **4a**–**g** (Scheme 1).

2.2. Synthesis of the berberine derivatives

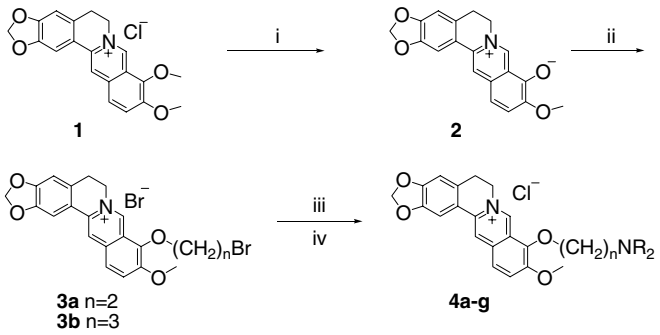
The synthetic pathway for 9-substituted berberine derivatives **4a**–**g** is shown in Scheme 1. Selective demethylation of berberine **1** at 190 °C under vacuum gives berberrubine **2**[17] in 65% yield. Alkylation of **2** with 1,2-dibromoethane or 1,3-dibromopropane at 60 °C in acetonitrile[18] yields **3a** in 65% and **3b** in 70%, respectively. Subsequent ammonolysis and anion exchange afford **4a**–**g** in 20–60% yield.

2.3. CD studies

2.3.1. Inducing anti-parallel G-quadruplex formation by berberine and its derivatives in the absence of metal cations. Human telomeric sequence telo21 was used as G-rich DNA. The formation of telomeric G-quadruplex as induced by berberine or its 9-substituted derivatives and K^+, Na^+ was monitored by CD spectroscopy (Fig. 1). It has been reported that telo21 forms an anti-parallel G-quadruplex in the presence of Na^+, whereas a mixture of parallel and anti-parallel G-quadruplexes is formed in the presence of K^+[11]. We found that the spectral characteristics in the presence of berberine or its derivatives are similar to those in Na^+ solution, with a major positive peak around 295 nm, a negative peak at 262 nm, and a smaller positive peak at 248 nm, which are characteristic of anti-parallel G-quadruplex structure. This finding suggests that telo21 forms an anti-parallel G-quadruplex in the presence of berberine and its derivatives.

2.3.2. Inducing anti-parallel G-quadruplex formation by berberine and its derivatives in the presence of K^+. The conformational characteristics of the human telomeric telo21 G-quadruplex structure induced by 9-substituted berberine derivatives in the presence of K^+ were monitored by CD spectroscopy (Fig. 2). In the presence of 0.1 M K^+, telo21 (5 μM) was induced to form hybrid G-quadruplexes with a shoulder located near 270 nm, a strong positive peak centered at 291 nm, and a small negative peak near 234 nm in the CD spectrum, which is typical of mixed parallel/anti-parallel G-quadruplexes.[5] Upon the addition of compound **4d** (3 molar equivalent to telo21) to a solution of K^+-preformed hybrid G-quadruplex, the CD spectrum dramatically changed, with the disappearance of the shoulder near 270 nm and the negative peak near 234 nm, and the emergence of a distinct negative peak at 261 nm and a small positive peak at 244 nm accompanied by a slight increase of the maximum at 291 nm. This spectrum is similar to that of anti-parallel G-quadruplex induced by Na^+. However, when adding the same amount of berberine (3 molar equivalent to telo21) into the solution of K^+-preformed hybrid G-quadruplex, the resulting CD spectrum is distinctly different from that of adding compound **4d**—although the shoulder near 270 nm also weakened and the maximum of the positive peak at 291 nm increased slightly, there was no negative peak at 261 nm. The other 9-substituted berberine derivatives behave similarly to compound **4d**. The results suggested that 9-substituted berberine derivatives are more effective in initiating the topological change of G-quadruplex compared to unsubstituted berberine.

2.3.3. Thermodynamic stability of the telomeric G-quadruplex by berberine and its derivatives. The stability of G-quadruplex induced by berberine and its derivatives was studied by measuring the thermodynamic stability profile in the absence of monovalent cations. The change in the CD absorption at 295 nm with increasing temperature was monitored, which is a typical index for the conformational change from single strand to G-quadruplex.[11,19] In the presence of **4d**, the CD spectrum of telo21 showed a large positive peak at around 295 nm at room temperature, indicating that the conformation was mainly the G-quadruplex structure. As the temperature increased, a positive peak appeared at around 260 nm in the CD spectrum which is consistent with the destruction of the G-quadruplex to single-stranded DNA (Fig. 3a). Figure 3b shows the normalized CD absorbance of telo21 at 295 nm with 15 μM **4d** in Tris–HCl buffer (pH 7.4) as a function of temperature.



	4a	4b	4c	4d	4e	4f	4g
n	3	3	3	3	3	2	2
$-NR_2$	$-N(CH_3)_2$	$-N(C_2H_5)_2$	$-N\square NH$	$-N\square$	$-N\square O$	$-N(C_2H_5)_2$	$-N[CH(CH_3)_2]_2$

Scheme 1. Reagents and conditions: (i) 190 °C, 20–30 mmHg, 15 min; (ii) $Br(CH_2)_nBr$, CH_3CN, 60 °C, 3 h; (iii) R_2NH, CH_3CN, K_2CO_3, 80 °C, 2–3 h; (iv) Dowex anion exchange resin.

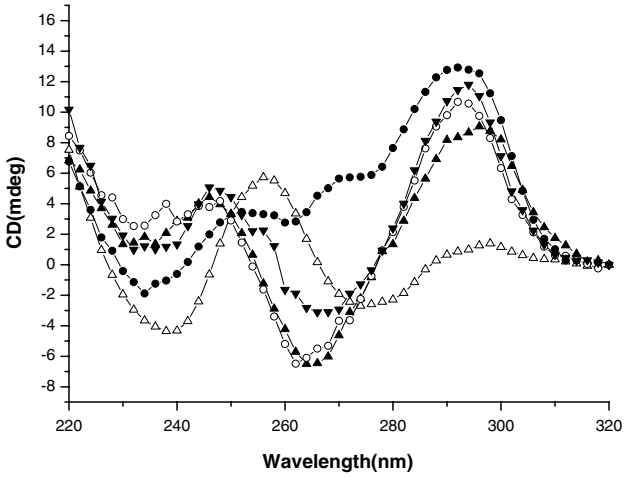


Figure 1. CD spectra of 5 μM telo21 in Tris–HCl buffer, 10 mM, pH 7.4, without cations or drug (△), with 0.1 M Na^+ (▲), 0.1 M K^+ (●), 10 μM **4d** (○), and 10 μM berberine (▼).

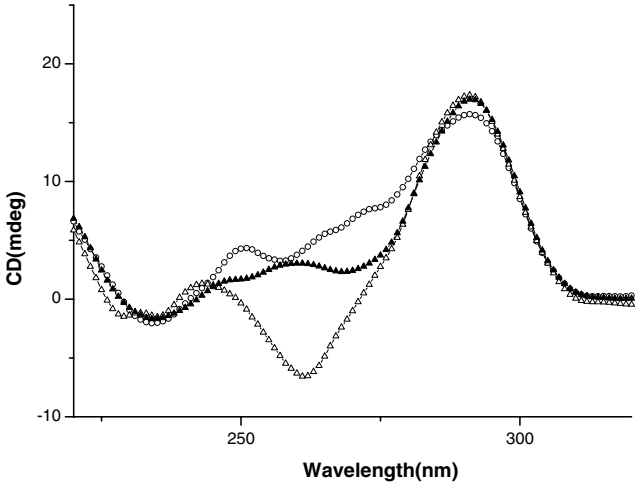


Figure 2. CD spectra of 5 μM telo21 in Tris–HCl buffer, 10 mM, pH 7.4, with 0.1 M K^+ (○), 0.1 M K^+ and 15 μM berberine (▲), 0.1 M K^+ and 15 μM **4d** (△).

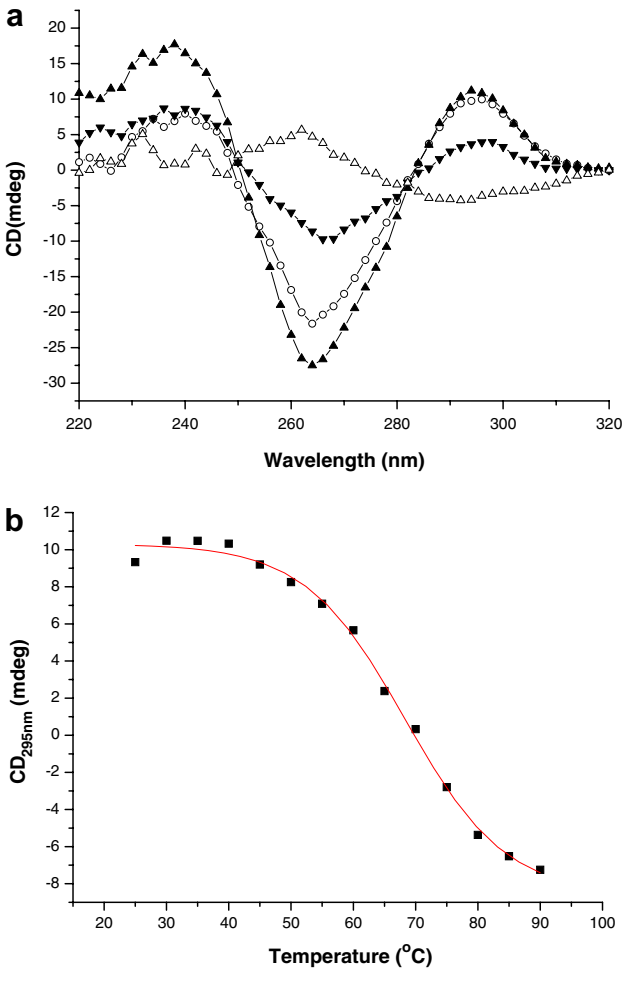


Figure 3. (a) CD spectra change of telo21 in the presence of 25 μM **4d**, at 25 °C (○), 50 °C (▲), 70 °C (▼), and 90 °C (△). (b) CD melting profiles of telo21 with 25 μM **4d**. All spectra were collected in a strand concentration of 5 μM in Tris–HCl buffer, 10 mM, pH 7.4, without metal ions.

The T_m values for G-quadruplex structure of telo21 induced by berberine and its derivatives **4a**–**g** were calculated from the CD melting curves at 295 nm using a

non-linear least-squares fitting. The T_m and ΔT_m values are shown in Table 1. Compared with the T_m value of 44.0 °C for G-quadruplex structure of telo21 induced by berberine, it is clear that compounds **4a**–**g** can increase the melting temperature of the G-quadruplex, indicating a higher stabilizing ability of these compounds to telo21 than berberine. Furthermore, the thermodynamic stability data indicated that the berberine derivatives with aminopropyl side chain at the 9-position interact more strongly with G-quadruplex than those with aminoethyl side chain.

2.4. Binding affinities of berberine and its derivatives to G-quadruplex

The binding affinities of berberine and its derivatives to telo21 were also investigated by means of fluorescence titration. In this experiment, hybrid G-quadruplexes in the presence of K^+ were added to a fixed amount of berberine or its derivatives (1 μM) until no further change in fluorescence was observed. Figure 4a shows the fluorescence spectra of compound **4d** in the presence of various concentrations of telo21. The fluorescence intensity of **4d** increased rapidly with the concentration of DNA until the binding had reached saturation. Table 2 summarizes the DNA binding constants K_i that were obtained by Scatchard analysis (Fig. 4b) of the fluorescence-binding data.[1,20] The relative binding affinities as indicated by the binding constant K_i are in the order **4d** > **4a** ≈ **4b** ≈ **4c** > **4e** > **4f** > **4g** > berberine. All 9-substituted amino derivatives have higher DNA-binding affinities comparing to berberine. This indicates that introduction of an amino side chain onto the 9-position of berberine can increase the binding affinity to the G-quadruplex. Among them, the 9-aminopropyl derivatives were found to have higher affinities than those with aminoethyl side chain.

2.5. Inhibition of amplification in telo21 by berberine and its derivatives

The induction to form G-quadruplex structures in telo21 by berberine and its derivatives was investigated by PCR stop assay. The specific binding of ligands with intramolecular G-quadruplex of telo21 could inhibit the hybridization process catalyzed by DNA polymerase.[21] In the presence of berberine derivatives, the single strand telo21 was induced into a G-quadruplex structure that blocked hybridization with a complementary strand. Under such circumstances, 5′–3′ extension with Taq polymerase was inhibited and the final double-stranded DNA PCR product could not be detected.

In the PCR-stop assay, 0.4 μM telo21 and ligands (berberine and **4a**–**g**) at 1.5, 2.1, and 2.8 μM were used with

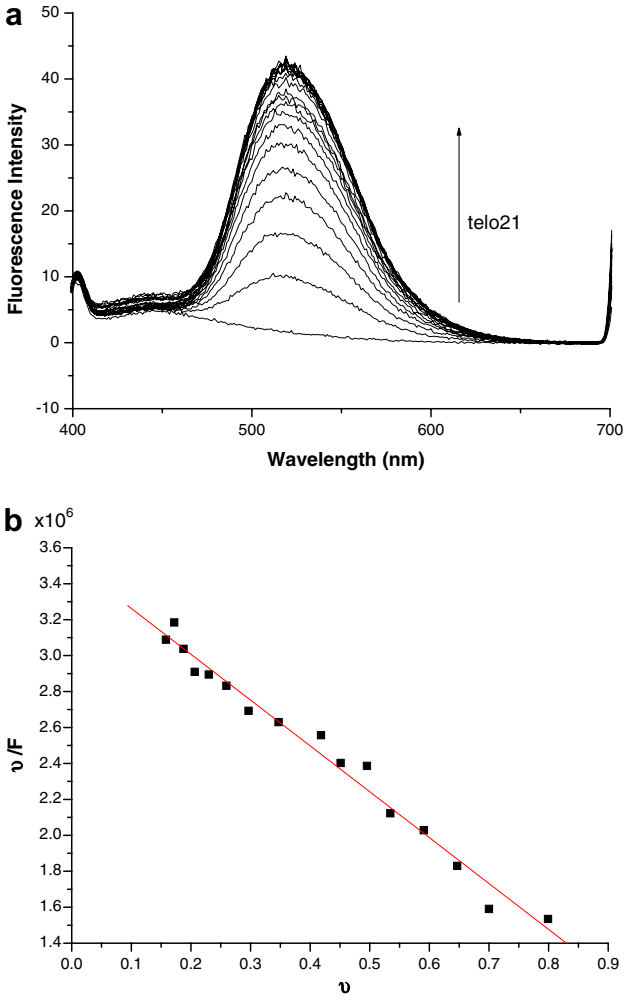


Figure 4. (a) Fluorescence properties of **4d** in the presence of a quadruplex-formed telo21 at room temperature. Excitation was set at 355 nm. (b) Scatchard analysis of the fluorescence-binding data.

Taq DNA polymerase as the catalyst. The PCR results are given in Figure 5. Derivatives **4c** and **4d** showed inhibitory effect on the hybridization of telo21 when the concentration was 1.5 μM (Fig. 5a). Derivatives **4b** and **4e** also showed inhibitory effect on the hybridization when the concentration was increased to 2.1 μM (Fig. 5b). Other derivatives including **4a**, **4f**, **4g**, and berberine exhibited inhibitory effect on the hybridization process only at a higher concentration of 2.8 μM (Fig. 5c). To clarify, the concentrations that inhibited hybridization by 50% (IC_{50}) are listed in Table 3. A correlation can be drawn between Table 1 and Figure 5—berberine derivatives that are more effective stabilizers of the G-quadruplex structure as revealed by their higher ΔT_m values are better inhibitors of the gene expression. Compounds **4c** and **4d**, with six-membered cyclic

Table 1. T_m and ΔT_m of the G-quadruplex induced by berberine and **4a**–**g**[a]

Compound	G-Quadruplex alone	Berberine	**4a**	**4b**	**4c**	**4d**	**4e**	**4f**	**4g**
T_m (°C)	40.1	44.0	65.2	60.1	63.1	68.3	56.8	57.0	51.7
ΔT_m (°C)	—	3.9	25.1	20.0	23.0	28.2	16.7	16.9	11.6

[a] Measured by CD spectra in 10 mM Tris–HCl buffer (*p*H 7.4) without metal ions.

Table 2. Binding constants (K_i, M^{-1}) of berberine and its derivatives (**4a–g**) to G-quadruplex

Compound	Berberine	4a	4b	4c	4d	4e	4f	4g
K_i ($\times 10^{-6}$ M^{-1})	1.79	3.06	3.03	3.01	3.52	2.44	2.16	2.05

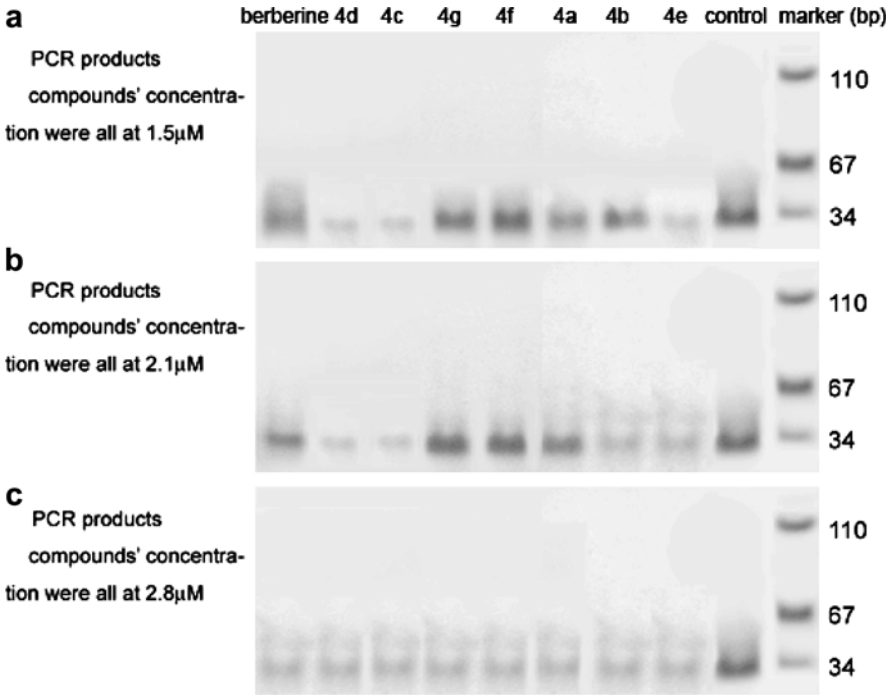


Figure 5. Effect of increasing concentrations of berberine and **4a–g** on the PCR-stop assay with telo21.

Table 3. The IC_{50} values of berberine and its derivatives (**4a–g**) in the PCR stop assay

Compound	Berberine	4a	4b	4c	4d	4e	4f	4g
IC_{50} (μmol/L)	2.1	1.5	1.4	0.7	0.7	1.3	2.3	2.3

amino group in the side chain terminal, are most effective in these aspects.

2.6. Competition dialysis

To evaluate the selectivities of berberine and its derivatives for G-quadruplex and other DNA structures, we performed a competitive dialysis experiment using different types of DNA including telo21, HTC21, $d(A)_{21}$:$d(T)_{21}$, $d(A)_{21}$:2 $d(T)_{21}$, and $d(A)_{21}$. Amongst these, telo21 forms the G-quadruplex structure, HTC21 forms the i-motif structure, $d(A)_{21}$:$d(T)_{21}$ forms the duplex structure, $d(A)_{21}$:2 $d(T)_{21}$ is associated to a triplex structure, whereas $d(A)_{21}$ is a single strand structure. Higher binding affinity was reflected by the higher concentration of ligands accumulated in the dialysis unit containing the specific DNA.[22]

In this study, the five different types of DNA were dialyzed simultaneously against a certain free ligand solution. The amount of bound ligand was directly proportional to the binding constant for each conformational form of DNA. The data are shown in Figure 6 in which the amount of bound ligand was plotted against the five types of DNA. It is clear that derivative **4d** interacts preferentially with G-quadruplex (telo21) with a much weaker affinity for other types of DNA structures.

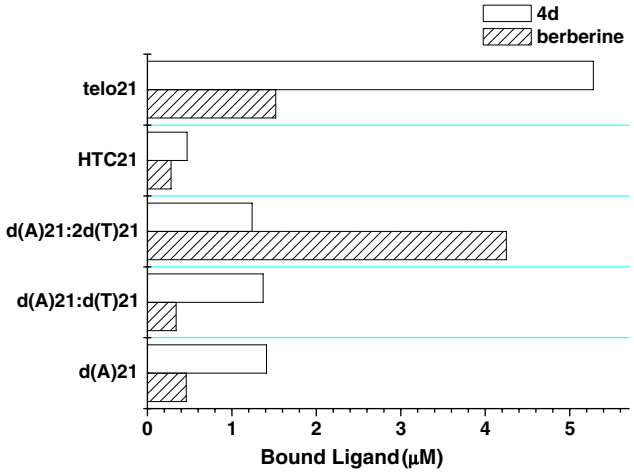


Figure 6. Competition dialysis assay. All measurements were performed in a pH 7.4 Tris–HCl buffer containing 100 mM NaCl.

2.7. In vitro inhibition of telomerase activity by berberine and its derivatives

The effects of berberine and its derivatives on telomerase activity were examined in cell-free system using a telomerase repeat amplification protocol (TRAP) assay. In this experiment, solutions of berberine and its derivatives were added to a telomerase reaction mixture containing extract from MCF-7 cells, which express high levels of telomerase activity. The in vitro inhibitory effect of berberine and its derivatives **4a**–**g** toward the process of telomerase was studied in a dose-dependent manner.

As shown in Figure 7 and Table 4, the inhibitory activities of the berberine derivatives can be revealed from the telomere ladder. Derivatives **4c** and **4d** inhibited the telomerase activity remarkably, with an IC_{50}^{tel} (concentration that inhibited telomerase activity by 50%) of 7.5 μM (**4c**) and 12.5 μM (**4d**). Derivatives **4a**, **4b**, and **4e** inhibited telomerase activity with an IC_{50}^{tel} of 17.5 μM. Derivatives **4f** and **4g** showed a relatively weaker inhibitory activity with an IC_{50}^{tel} of 25 μM. Nevertheless, berberine showed a much weaker inhibitory effect on telomerase activity, with an IC_{50}^{tel} of about 75 μM. As a control, inhibition of ITAS (Taq polymerase activity) was not observed at the concentrations for telomerase activity.

The results revealed clearly that the telomerase inhibitory properties of these berberine derivatives were significantly improved upon introduction of electron-donor groups into the 9-position, which is in accord with the experimental data from the thermodynamics stability study and the PCR stop assay.

3. Conclusions

Berberine and semisynthetic 9-substituted berberine derivatives were investigated for their interaction with telomeric G-quadruplex. Our results indicated that berberine and its 9-substituted derivatives could induce and stabilize the anti-parallel G-quadruplex structure formation in the presence or absence of metal cations. Introduction of an alkyl amino group, such as a six-membered cyclic amino group, into the 9-position of berberine improves the selective binding with G-quadruplex and increases the inhibitory effect on the hybridization, resulting in blocking of the gene expression. 9-Substituted berberine derivatives also showed higher inhibitory effect against telomerase activity comparing with berberine. This study suggested that the berberine derivatives might be potential lead compounds for the development of new telomerase inhibitors.

4. Experimental

^{1}H NMR spectra were recorded at 300 MHz on a Mercury-Plus spectrometer using TMS as an internal standard in DMSO-d_6 or $CD_3OD/CDCl_3$ (v/v = 1:1);

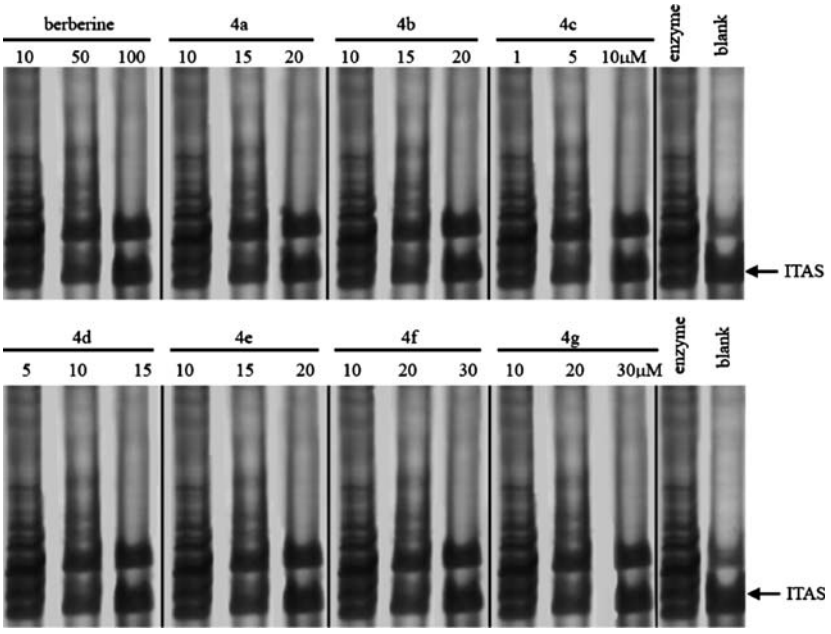


Figure 7. Telomerase inhibition by berberine and **4a**–**g** in a TRAP assay. Increasing concentrations of compound were added in the TRAP mixture in the presence of an internal control (ITAS).

Table 4. The IC_{50}^{tel} values of berberine and its derivatives (**4a**–**g**) in the TRAP assay

Compound	Berberine	4a	4b	4c	4d	4e	4f	4g
IC_{50}^{tel} (μmol/L)	75	17.5	17.5	7.5	12.5	17.5	25	25

FAB-MS spectra were obtained using a VG ZAB-HS spectrometer; Elemental analysis was carried out on an Elementar Vario EL CHNS Elemental Analyzer. The CD spectra were recorded on a Jasco J-810 spectropolarimeter, and in the melting studies, the temperature of the sample was maintained by a Jasco Pelter temperature controller. Fluorometric measurements were run on a Shimadzu RF-5301PC spectrofluorophotometer. PCR-stop assay was carried out on the Eppendorf thermocycler for DNA amplification (Mastercycler personal, 5332).

All oligonucleotides were purchased from Invitrogen China. Telomerase extract was prepared from MCF-7 cells as described before.[23] Berberine chloride was isolated from Chinese herbal medicine ('Huang-Lian') and recrystallized from hot water. Compounds **2**, **3a**, and **3b** were synthesized according to Refs. 17 and 18.

4.1. General procedure for the preparation of derivatives 4a–g

The substituent amine (2 mmol) was added to a magnetically stirred solution of **3a** or **3b** (2 mmol) and anhydrous K_2CO_3 in dry acetonitrile (15 mL). The reaction mixture was heated at 80 °C for 2–3 h and the reaction was monitored by TLC. The resulting solid was filtered at room temperature and subjected to anion exchange into chloride form. The crude product was chromatographed on an Al_2O_3 column, eluted with $CHCl_3$/MeOH (9:1, v/v) to give the proposed compound.

4.1.1. 9-*O*-3-(Dimethylamino)propylberberine (4a). 9-*O*-3-Bromopropylberberine (**3b**) was treated with dimethylamine according to general procedure to give the desired product **4a** as a light yellow solid, yield 52%; ^{1}H NMR (DMSO-d_6, 300 MHz): δ 9.70 (s, 1H), 8.88 (s,1H), 8.16 (d, 1H, J = 9.2), 8.01 (d, 1H, J = 9.2), 7.75 (s, 1H), 7.06 (s, 1H), 6.13 (s, 2H), 4.90 (t, 2H, J = 5.5), 4.31 (t, 2H, J = 5.7), 4.04 (s, 3H), 3.36 (t, 2H, J = 7.8), 3.20 (t, 2H, J = 5.7), 2.85 (s, 6H), 2.22 (m, 2H); FAB-MS m/z: 407 $[M-Cl]^+$; Anal. Calcd for $C_{24}H_{27}ClN_2O_4$: C, 65.08; H, 6.14; N, 6.32. Found: C, 65.27; H, 6.42; N, 6.05.

4.1.2. 9-*O*-3-(Diethylamino)propylberberine (4b). 9-*O*-3-Bromopropylberberine (**3b**) was treated with diethylamine according to general procedure to give the desired product **4b** as a light yellow solid, yield 58%; ^{1}H NMR (DMSO-d_6, 300 MHz): δ 9.73 (s, 1H), 8.90 (s,1H), 8.20 (d, 1H, J = 9.1), 8.04 (d, 1H, J = 9.1), 7.78 (s, 1H), 7.09 (s, 1H), 6.16 (s, 2H), 4.92 (t, 2H, J = 5.6), 4.36 (t, 2H, J = 5.8), 4.07 (s, 3H), 3.38 (t, 2H, J = 7.5), 3.22 (m, 6H), 2.24 (m, 2H), 1.26 (t, 6H, J = 7.2); FAB-MS m/z: 435 $[M-Cl]^+$; Anal. Calcd for $C_{26}H_{31}ClN_2O_4$: C, 66.30; H, 6.63; N, 5.95. Found: C, 66.61; H, 6.37; N, 6.21.

4.1.3. 9-*O*-3-(1-Piperazinyl)propylberberine (4c). 9-*O*-3-Bromopropylberberine **3b** was treated with piperazine according to general procedure to give the desired product **4c** as a yellow solid, yield 29%; ^{1}H NMR (CD_3OD/$CDCl_3$, 300 MHz): δ 9.93 (s, 1H), 8.50 (s,1H), 7.94 (d, 1H, J = 9.2), 7.88 (d, 1H, J = 9.1), 7.47 (s, 1H), 6.82 (s, 1H), 6.02 (s, 2H), 5.01 (t, 2H, J = 5.7), 4.46 (t, 2H, J = 5.5), 4.02 (s, 3H), 3.49 (t, 2H, J = 5.5), 3.24-3.16 (m, 8H), 2.94 (t, 2H, J = 7.6), 2.38 (m, 2H); FAB-MS m/z: 448 $[M-Cl]^+$; Anal. Calcd for $C_{26}H_{30}ClN_3O_4$: C, 64.52; H, 6.25; N, 8.68. Found: C, 64.77; H, 6.06; N, 8.74.

4.1.4. 9-*O*-3-(1-Piperidine)propylberberine (4d). 9-*O*-3-Bromopropylberberine (**3b**) was treated with piperidine according to general procedure to give the desired product **4d** as a light yellow solid, yield 48%; ^{1}H NMR (CD_3OD:$CDCl_3$, 300 MHz): δ 9.69 (s, 1H), 8.55(s,1H), 7.97 (d, 1H, J = 9.3), 7.94 (d, 1H, J = 9.3), 7.52 (s, 1H), 6.88 (s, 1H), 6.09 (s, 2H), 4.93 (t, 2H, J = 6.2), 4.42 (t, 2H, J = 6.3), 4.08 (s, 3H), 3.24 (t, 2H, J = 6.3), 2.62 (t, 2H, J = 7.7), 2.51 (br s, 4H), 2.12 (m, 2H); 1.64 (m, 4H), 1.51 (m, 2H); FAB-MS m/z: 447 $[M-Cl]^+$; Anal. Calcd for $C_{27}H_{31}ClN_2O_4$: C, 67.14; H, 6.47; N, 5.80. Found: C, 67.25; H, 6.70; N, 5.53.

4.1.5. 9-*O*-3(4-Morphiline)propylberberine (4e). 9-*O*-3-Bromopropylberberine **3b** was treated with morphiline according to general procedure to give the desired product **4e** as a light yellow solid, yield 60%; ^{1}H NMR (CD_3OD/$CDCl_3$, 300 MHz): δ 9.65 (s, 1H), 8.48 (s,1H), 7.92 (d, 1H, J = 9.1), 7.88 (d, 1H, J = 9.1), 7.46 (s, 1H), 6.80 (s, 1H), 6.01 (s, 2H), 4.90 (t, 2H, J = 6.3), 4.39 (t, 2H, J = 6.4), 4.01 (s, 3H), 3.67 (t, 4H, J = 4.4), 3.19 (t, 2H, J = 6.4), 2.62 (t, 2H, J = 7.4), 2.51 (t, 4H, J = 4.4); 2.09 (m, 2H); FAB-MS m/z: 449 $[M-Cl]^+$; Anal. Calcd for $C_{26}H_{29}ClN_2O_5$: C, 64.39; H, 6.03; N, 5.78. Found: C, 64.66; H, 5.89; N, 6.02.

4.1.6. 9-*O*-2-(Diethylamino)ethylberberine (4f). 9-*O*-2-Bromoethylberberine **3a** was treated with diethylamine according to general procedure to give the desired product **4f** as a light yellow solid, yield 42%; ^{1}H NMR (DMSO-d_6, 300 MHz): δ 9.81 (s, 1H), 9.00 (s, 1H), 8.19 (d, 1H, J = 9.2), 8.06 (d, 1H, J = 9.2), 7.76 (s, 1H), 7.07 (s, 1H), 6.14 (s, 2H), 4.89 (t, 2H, J = 5.5), 4.51 (t, 2H, J = 4.0), 4.07 (s, 3H), 3.68 (t, 2H, J = 4.0), 3.34 (m, 4H), 3.22 (t, 2H, J = 5.5), 1.29 (t, 6H, J = 7.0); FAB-MS m/z: 421 $[M-Cl]^+$; Anal. Calcd for $C_{25}H_{29}ClN_2O_4$: C, 65.71; H, 6.40; N, 6.13. Found: C, 65.45; H, 6.65; N, 6.28.

4.1.7. 9-*O*-2-(Diisopropylamino)ethylberberine (4g). 9-*O*-2-Bromoethylberberine **3a** was treated with diisopropylamine according to general procedure to give the desired product **4g** as a light yellow solid, yield 20%; ^{1}H NMR (CD_3OD/$CDCl_3$, 300 MHz): δ 9.86 (s, 1H), 8.58 (s,1H), 8.02 (d, 1H, J = 9.0), 7.97 (d, 1H, J = 9.0), 7.56 (s, 1H), 6.90 (s, 1H), 6.11 (s, 2H), 4.96 (t, 2H, J = 5.7), 4.39 (t, 2H, J = 6.7), 4.11 (s, 3H), 3.30 (m, 2H), 3.13 (t, 2H, J = 6.7), 3.02 (t, 2H, J = 5.7), 1.11 (d, 12H, J = 6.3); FAB-MS m/z: 449 $[M-Cl]^+$; Anal. Calcd for $C_{27}H_{33}ClN_2O_4$: C, 66.86; H, 6.86; N, 5.78. Found: C, 66.71; H, 6.92; N, 5.57.

4.2. CD measurements

CD spectra were recorded using a quartz cell of 1-mm optical path length and an instrument scanning speed

of 100 nm/min with a response time of 1 s. CD spectra were obtained by taking the average of at least two scans made from 200 to 400 nm. All DNA samples at a final concentration of 5 μm were dissolved in Tris buffer (10 mM, pH 7.4) and heated to 90 °C for 5 min, gently cooled at the rate of 3 °C min^{-1}, and incubated at 4 °C for several hours. The compounds were dissolved with double-distilled water and titrated into the DNA samples at 0.5 mol equiv up to 5 mol equiv. Where appropriate, the sample also contained 0.1 M KCl or NaCl. Melting curves of the G-quadruplexes were measured with CD intensity at 295 nm. Before the CD spectroscopy, all the samples were thermally treated as described above. The heating rate was 1.0 °C min^{-1}.

4.3. Fluorescence titration experiment

Fluorescence titration was performed at a fixed concentration of drugs (1 μM) in Tris buffer (pH 7.4) at room temperature. Small aliquots of telo21 G-quadruplexes induced in the presence of K^+ were added into the solution at final concentrations from 0 to 7 μM and stirred for 5 min after each addition. Fluorescence intensity was measured at E_x 355 nm and E_x/E_m 5/5 nm. Binding constants were derived from the modified Scatchard equation, $r/C_f = K_i(1 - N_r)[(1 - N_r)/[1 - (N - 1)_r]]^{n-1}$, where r is the molar ratio of bound ligand to DNA, C_f the free ligand concentration, K_i the binding constant, and n the binding size in base pairs.

4.4. PCR stop assay

The PCR stop assay was performed with a modified protocol of the reference 24. Telo21 and the corresponding complementary sequence d[ATCGCTTCTCGTCCC TAACC] were used here. The reaction was performed in 1× PCR buffer, containing 10 pmol of each pair of oligomers, 0.16 mM dNTP, 2.5 U Taq polymerase, and the 1.5, 2.1, 2.8 μM berberine or its derivatives. Reaction mixtures were incubated in a thermocycler with the following cycling conditions: 94 °C for 2 min, followed by 30 cycles of 94 °C for 30 s, 58 °C for 30 s, and 72 °C for 30 s. Amplified products were resolved on 15% non-denaturing polyacrylamide gels in 1× TBE and silver stained. IC_{50} values were calculated using optical density read from LadWorks software.

4.5. Competition dialysis

One hundred and fifty milliliter Tris–HCl buffer (pH 7.4) containing 100 mM NaCl and 1 μM **4d** was placed into a beaker. A volume of 500 μL at 50 μM monomeric unit (single strands, double strands, i-motif, triplet or quartet) of each of the nucleic acid samples was pipeted into a separate Dialyzer unit (Pierce). All 5 dialysis units were then placed in the beaker containing dialysate solution. The beaker was covered with Parafilm and wrapped in foil, and its contents were allowed to equilibrate with continuous stirring for 24 h at room temperature. At the end of the equilibration period, DNA samples were carefully removed to microfuge tubes and treated with 1% SDS. The ligand concentration in each sample was determined by UV absorbance.

4.6. TRAP assay

Telomerase extract was prepared from MCF-7 cells. TRAP assay was performed by using a modification of the TRAP assay.[25–27] PCR was performed in a final 50 μL reaction volume composed of a 45 μL reaction mix containing 20 mM Tris–HCl (pH 8.0), 50 μM deoxynucleotide triphosphates, 1.5 mM $MgCl_2$, 63 mM KCl, 1 mM EGTA, 0.005% Tween 20, 20 μg/mL BSA, 3.5 pmol of primer Telo21 (5′-GGGATTGGGATTGG GATTGGGTT-3′), 18 pmol of primer TS (5′-AA TCCGTCGAGCAGAGTT-3′), 22.5 pmol of primer CXext (5′-GTGCCCTTACCCTTACCCTTACCCT AA-3′), 7.5 pmol of primer NT (5′-ATCGCTTCTCGG CCTTTT-3′), 0.01 amol of TSNT internal control (5′-ATTCCGTCGAGCAGAGTTAAAAGGCCGAGAA GCGAT-3′), 2.5 U of Taq DNA polymerase, and 100 ng of telomerase. Compounds or distilled water was added under a volume of 5 μL. PCR was performed in an Eppendorf Mastercycler equipped with a hot lid and incubated for 30 min at 30 °C, followed by 92 °C 30 s, 52 °C 30 s, and 72 °C 30 s for 30 cycles. After amplification, 8 μl of loading buffer (containing 5× Tris–Borate–EDTA buffer (TBE buffer), 0.2% bromophenol blue, and 0.2% xylene cyanol) was added to the reaction. A 15 μL aliquot was loaded onto a 16% non-denaturing acrylamide gel (19:1) in 1× TBE buffer and electrophoresed at 200 V for 1 h. Gels were fixed and then stained with $AgNO_3$. IC_{50}^{tel} values were calculated using optical density read from LadWorks software.

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References and notes

1. Koeppel, F.; Riou, J.-F.; Laoui, A.; Mailliet, P.; Arimondo, P.-B.; Labit, D.; Petitgenet, O.; Hélène, C.; Mergny, J.-L. *Nucleic Acids Res.* **2001**, *29*, 1087.
2. Prtraccone, L.; Erra, E.; Esposito, V.; Randazzo, A.; Mayol, L.; Nasti, L.; Barone, G.; Giancola, C. *Biochemistry* **2004**, *43*, 4877.
3. Haider, S. M.; Parkinson, G.-N.; Neidle, S. *J. Mol. Biol.* **2003**, *326*, 117.
4. Wlodarczyk, A.; Grzybowski, P.; Patkowski, A.; Dobek, A. *J. Phys. Chem. B* **2005**, *109*, 3594.
5. Rezler, E. M.; Seenisamy, J.; Bashyam, S.; Kim, M.-Y.; White, E.; Wilson, W.-D.; Hurley, L.-H. *J. Am. Chem. Soc.* **2005**, *127*, 9439.
6. Zahler, A.-M.; Williamson, J.-R.; Cech, T.-R.; Prescott, D.-M. *Nature* **1991**, *350*, 718.
7. Neidle, S.; Parkinson, G. *Nat. Rev. Drug Disc.* **2002**, *1*, 383.
8. Riou, J. F.; Guittat, L.; Mailliet, P.; Laoui, A.; Renou, E.; Petitgenet, O.; Megnin-Chanet, F.; Helene,

C.; Mergny, J. L. *Proc. Natl. Acad. Sci. U.S.A.* **2002**, 2672–2677.
9. Seenisamy, J.; Bashyam, S.; Gokhale, V.; Vankayalapati, H.; Sun, D.; Siddiqui-Jain, A.; Streiner, N.; Shin-ya, K.; White, E.; Wilson, W.-D.; Hurley, L.-H. *J. Am. Chem. Soc.* **2005**, *127*, 2944.
10. Read, M. A.; Wood, A.-A.; Harrison, J.-R.; Gowan, S.-M.; Kelland, L.-R.; Dosanjh, H.-S.; Neidle, S. *J. Med. Chem.* **1999**, *42*, 4538.
11. Zhou, J.-L.; Lu, Y.-J.; Ou, T.-M.; Zhou, J.-M.; Huang, Z.-S.; Zhu, X.-F.; Du, C.-J.; Bu, X.-Z.; M, L.; Gu, L.-Q.; Li, Y.-M.; Chan, A.-S.-Z. *J. Med. Chem.* **2005**, *48*, 7315.
12. Ghosh, A. K.; Bhattacharyya, F. K.; Ghosh, D. K. *Exp. Parasitol.* **1985**, *60*, 404.
13. Sanders, M.-M.; Liu, A.-A.; Li, T.-K.; Wu, H.-Y.; Desai, S.-D.; Mao, Y.; Rubin, E.-H.; LaVoie, E.-J.; Makhey, D.; Liu, L.-F. *Biochem. Pharmacol.* **1998**, *56*, 1157.
14. Naasani, I.; Seimiya, H.; Yamori, T.; Tsuruo, T. *Cancer Res.* **1999**, *59*, 4004.
15. Franceschin, M.; Rossetti, L.; D'Ambrosio, A.; Schirripa, S.; Bianco, A.; Ortaggi, G.; Savino, M.; Schultes, C.; Neidle, S. *Bioorg. Med. Chem. Lett.* **2006**, *16*, 1707.
16. Clark, G. R.; Pytel, P. D.; Squire, C. J.; Neidle, S. *J. Am. Chem. Soc.* **2003**, *125*, 4066.
17. Iwasa, K.; Kamigauchi, M.; Ueki, M.; Taniguchi, M. *Eur. J. Med. Chem.* **1996**, *31*, 469.
18. Long, Y.-H.; Bai, L.-P.; Qin, Y.; Pang, J.-Y.; Chen, W.-H.; Cai, Z.-W.; Xu, Z.-L.; Jiang, Z.-H. *Bioorg. Med. Chem.* **2006**, *14*, 4670.
19. Balagurumoorthy, P.; Brahmachari, S.-K. *J. Biol. Chem.* **1994**, *269*, 21858.
20. Zhong, W.-Y.; Yu, J.-S.; Huang, W.-L.; Ni, K.-Y.; Liang, Y.-Q. *Biopolymers* **2001**, *62*, 315.
21. Kim, M.-Y.; Gleason-Guzman, M.; Izbicka, E.; Nishioka, D.; Hurley, L.-H. *Cancer Res.* **2003**, *63*, 3247.
22. Granotier, C.; Pennarun, G.; Riou, L.; Hoffschir, F.; Gauthier, L.-R.; Cian, A.-D.; Gomez, D.; Mandine, E.; Riou, J.-F.; Mergny, J.-L.; Mailliet, P.; Dutrillaux, B.; Boussin, F.-D. *Nucleic Acids Res.* **2005**, *33*, 4182.
23. Fu, W.; Begley, J.-G.; Killen, M.-W.; Mattson, M.-P. *J. Biol. Chem.* **1999**, *274*, 7264.
24. Gomez, D.; Lemarteleur, T.; Lacroix, L.; Mailliet, P.; Mergny, J.-L.; Riou, J.-F. *Nucleic Acids Res.* **2004**, *32*, 371.
25. Kim, N. W.; Wu, F. *Nucleic Acids Res.* **1997**, *25*, 2595.
26. Krupp, G.; Kuhne, K.; Tamm, S.; Klapper, W.; Heidorn, K.; Rott, A.; Parwaresch, R. *Nucleic Acids Res.* **1997**, *25*, 919.
27. Kim, N. W.; Piatyszek, M. A.; Prowse, K. R.; Harley, C. B.; West, M. D.; Ho, P. L.; Coviello, G. M.; Wright, W. E.; Weinrich, S. L.; Shay, J. W. *Science (Washington, DC)* **1994**, *266*, 2011.