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PAPER

Promoting the formation and stabilization of human telomeric G-quadruplex DNA, inhibition of telomerase and cytotoxicity by phenanthroline derivatives†

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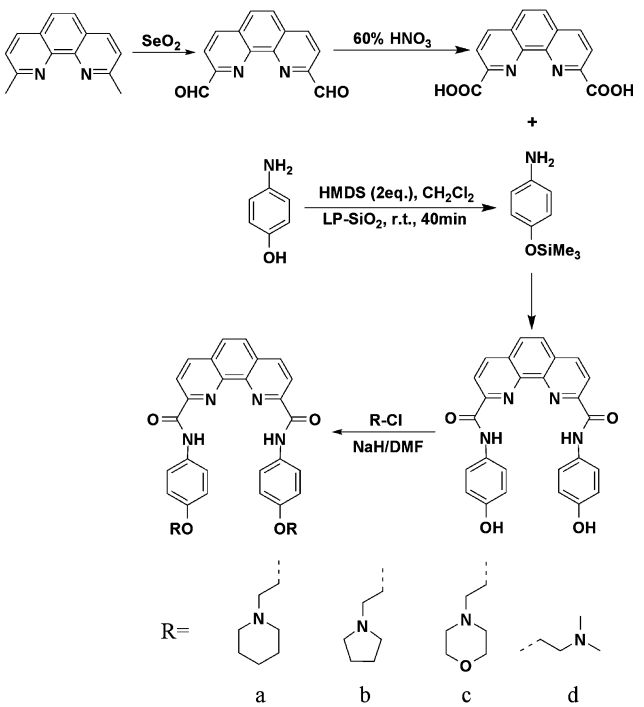
Four new di-substituted phenanthroline-based compounds **a–d** have been designed and prepared, and they have been shown to induce the formation of anti-parallel structure of human telomeric G-quadruplex DNA by CD spectra. FRET assay indicates that the melting temperature increases (ΔT_m values) of G-quadruplex in buffer (pH 7.4) containing 100 mM NaCl are 31.6, 34.6, 17.8 and 32.6 °C for the compounds (1.0 μM) **a**, **b**, **c** and **d**, respectively. Competitive FRET assay shows that the four compounds exhibit a high G-quadruplex DNA selectivity over duplex DNA. Three of the compounds are the potent telomerase inhibitors and HeLa cell proliferation inhibitors.

Introduction

G-quadruplex DNAs and RNAs exist in pivotal genomic regions such as telomeres, promoters of oncogenes, most growth control genes, 5′ and 3′ untranslated regions and introns.[1] Targeting G-quadruplex DNAs with small molecules is emerging as a rational and promising strategy for anti-cancer drug design, since they might inhibit telomerase activity and block transcription or translation of a particular gene by stabilizing G-quadruplex DNAs.[2] Significant progress has been made during the past few years in the design of small molecules that target G-quadruplex DNA, and a number of classes of ligands have been reported.[3] As a strategy for G-quadruplex ligand design, high selectivity between duplex DNA and G-quadruplex DNA is the crucial step, because ligand interaction with duplex DNA leads to acute toxic and intolerable side effects on normal tissues. A number of small molecules have been identified to selectively bind and stabilize the telomeric G-quadruplex DNA *in vitro*.[4]

Phenanthroline derivatives attracted our attention not only because of their pharmacological effects in many drugs, but also because of their functional role in DNA ligands. Recently, Neidle and co-workers reported that a platinum-phenanthroline square complex can induce a high degree of quadruplex DNA stabilization and inhibit telomerase activity.[5a] Teulade-Fichou and colleagues suggested that another two phenanthroline compounds exhibit exceptional affinity and selectivity for G-quadruplex DNA.[5b] These exciting results prompted us to develop the phenanthroline family of compounds to act as G-quadruplex binding ligands.

Herein we synthesized four new phenanthroline derivatives **a–d** by attaching cationic amino side chains to the aromatic chromophore (Scheme 1). Surprisingly, compounds **a–d** can efficiently induce the formation and stabilization of anti-parallel G-quadruplex structure of human telomeric sequence ($AG_3(T_2AG_3)_3$, HTG22), and even lead to the conversion



Scheme 1 Synthetic route of compounds **a**, **b**, **c** and **d**.

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of hybrid G-quadruplex to anti-parallel G-quadruplex. The four compounds can also selectively bind and stabilize G-quadruplex in the presence of excessive duplex DNA. Importantly, the compounds **a**, **b** and **d** can significantly inhibit telomerase activity and HeLa cell proliferation.

Results and discussion

Synthesis of phenanthroline-based compounds

The general synthetic route of compounds **a–d** is shown in Scheme 1. 1,10-phenanthroline-2,9-dicarbaldehyde and 1,10-phenanthroline-2,9-dicarboxylic acid were prepared using previously reported procedures.[5a,6a–6c] Trimethylsilyl-protected 4-aminophenol was synthesized according to the literature.[6d] The detailed description of synthesis and characterization of ligand and the target compounds **a–d** is given in the Experimental section.

Promoting the formation and stabilization of G-quadruplex by compounds a–d

G-quadruplex formed from human telomere sequence (HTG22) in the presence of compounds **a–d** was monitored by CD spectroscopy that is very useful for distinguishing DNA secondary structures.[7a] In the absence of salt, the CD spectrum of randomized HTG22 oligonucleotide was found to have a negative band centered at 232 nm, a major positive band at 252 nm, a minor negative band at 275 nm, and a positive band at near 295 nm (Fig. 1A, black line). Upon addition of 2 mol equiv. of compounds **a–d**, the dramatic changes in the CD spectra were observed. The bands at 252, 275 and 295 nm are shifted to 245, 265 and 290 nm, respectively; also, the bands centered at 265 and 290 nm significantly increased, which is a typical anti-parallel G-quadruplex structure.[7b] These results indicate that compounds **a–d** could induce the human telomeric guanine-rich DNA to form the anti-parallel G-quadruplex conformation under cations deficient conditions.

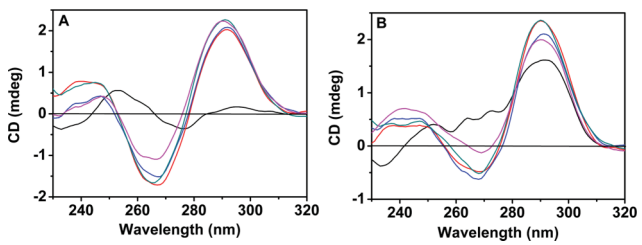

Fig. 1 CD spectra of 5 μM HTG22 in 10 mM Tris-HCl buffer without (A) and with 100 mM K^+ (B) (pH 7.4): without compound (black); with compounds **a** (blue), **b** (green), **c** (pink) and **d** (red) at 10 μM.

CD studies with human telomeric DNA show the existence of the anti-parallel and hybrid G-quadruplex conformations in the presence of Na^+ and K^+, respectively.[7b,7c] To study whether compounds **a–d** show selectivity for either the parallel or the anti-parallel conformations, CD experiments were performed in the presence of 100 mM K^+ (Fig. 1B). The CD spectrum of HTG22 in Tris-HCl/KCl buffer shows two positive maxima at about 265 and 295 nm and a negative band at 235 nm, which is indicative of a hybrid of anti-parallel and parallel conformations.[7c] After addition of 2 mol equiv. of compounds **a–d** into this DNA solution, the band at 290 nm increases while a positive band at 265 nm becomes a negative band. This result clearly suggests that compounds **a–d** strongly stabilize the anti-parallel conformation of HTG22 quadruplex DNA. These results that the compounds **a–d** can induce the formation and stabilization of G-quadruplex were also observed for other G-quadruplex ligands.[8]

As shown in Fig. 2, in the presence of Na^+ the CD spectrum of HTG22 shows a strong positive band at 290 nm and a negative band at 265 nm, which indicates the formation of an anti-parallel G-quadruplex structure.[7b] After addition of the compounds **a–d** into this DNA solution, the intensities of both positive and negative bands are increased gradually with the increasing concentration of compounds from 1 to 3 mol equiv., which suggests that an anti-parallel structure is stabilized by compounds **a–d**.

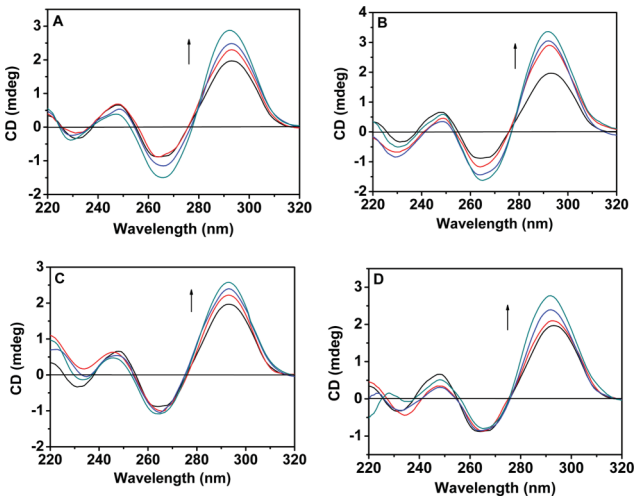

Fig. 2 CD titration spectra of HTG22 (5 μM) at increasing concentrations (arrows: 0–3 mol equiv.) of compounds **a** (A), **b** (B), **c** (C) and **d** (D) in 10 mM Tris-HCl and 100 mM NaCl buffer at pH 7.4.

The ability of these compounds to stabilize telomeric G-quadruplex (*FAM*-$G_3[T_2AG_3]_3$-*TAMRA*, F21T) was further evaluated by a fluorescence resonance energy transfer (FRET) melting assay.[9] As shown in Fig. 3 (also see Fig S1, ESI†), compounds **a**, **b** and **d** appear as remarkably strong quadruplex stabilizers with an increase in the melting temperature (ΔT_m) of 31.6, 34.6 and 32.6 °C

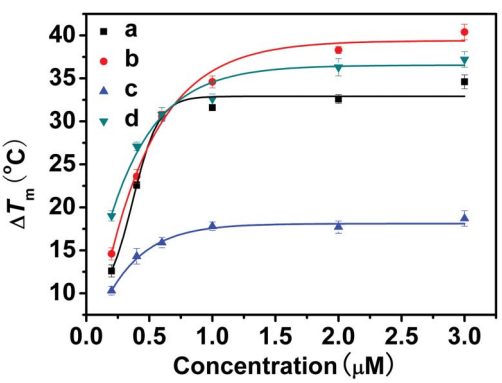

Fig. 3 FRET stabilization curves for compounds **a**, **b**, **c** and **d** with various concentrations upon binding to F21T DNA. The values are the mean ± SE of three independent experiments.

Table 1 Stabilization of G-quadruplex DNA by 0.4 and 1.0 μM compounds **a–d** in the presence of or absence of ds26 using FRET melting assay in buffer containing either 100 mM NaCl or 100 mM KCl. The values are the mean ± SE of three independent experiments

	ΔT_m at 1.0 μM compound concentration (°C)				ΔT_m at 0.4 μM compound concentration (°C)	
Compounds	F21T[a]	F21T + ds26[a,c]	F21T + ds26[a,d]	F21T[b]	F21T[a]	F21T[b]
a	31.6 ± 0.3	31.6 ± 0.4	28.3 ± 0.4	26.7 ± 0.7	22.6 ± 0.4	22.7 ± 0.3
b	34.6 ± 0.7	34.2 ± 0.6	33.8 ± 0.5	27.9 ± 0.6	23.6 ± 0.8	24.4 ± 0.7
c	17.8 ± 0.5	17.3 ± 0.4	17.0 ± 0.4	20.9 ± 0.4	14.3 ± 0.7	15.1 ± 0.5
d	32.6 ± 0.6	32.3 ± 0.4	31.4 ± 0.5	28.5 ± 0.6	27.0 ± 0.8	26.1 ± 0.6

[a] 100 mM Na^+. [b] 100 mM K^+. [c] Concentration of ds26 is 2.0 μM. [d] Concentration of ds26 is 5.0 μM.

at 1.0 μM in buffer containing 100 mM NaCl, respectively, while a more modest effect is obtained for **c** with a ΔT_m of 17.8 °C at 1.0 μM (Table 1). The compound **c** has two morpholine rings in the side chains, and the oxygen atom on the morpholine ring leads to a greater polarity of compound **c** than the other three compounds, and the basicity of side chain amines is also different,[10] which should give rise to the difference of the ΔT_m values. Similar results have also been observed for tri-substituted isoalloxazine and triarylpyridine derivatives,[11] where the ΔT_m values are lower for derivatives with a morpholine ring in the side chain than those with other amino alkyl side chains.

In buffer containing 100 mM KCl the ΔT_m values are 26.7, 27.9, 20.9 and 28.5 °C for compounds **a**, **b**, **c** and **d** at 1.0 μM, respectively (Table 1 and Fig. S2, ESI†). Compared with ΔT_m in NaCl buffer, the lower values in KCl buffer are mainly due to the difficulty in accurate determination of a too high T_m, because both ΔT_m values in buffer containing either 100 mM NaCl or 100 mM KCl are similar for the compounds **a**, **b**, **c** and d at 0.4 μM (Table 1, Fig. S2, ESI†). Altogether, these data reflect a very high level of quadruplex stabilization for compounds **a**, **b** and **d** and represent a significant improvement as compared to another modified phenanthroline platinum(II) complex (20 °C at 1.0 μM in buffer containing 50 mM K^+)[5a] and are competitive with another two bisquinolinium compounds (29.7 and 28.5 °C at 1.0 μM in buffer containing 100 Na^+, respectively).[5b]

A DNA competition FRET-melting assay was used to verify G-quadruplex selectivity over duplex in buffer containing 100 mM NaCl, which shows that the ΔT_m values for compounds **a–d** at 1.0 μM are not significantly decreased even at levels of 10- and 25-fold excess of duplex DNA(ds26) (Table 1, Fig. S3, ESI†). These results indicate that compounds **a–d** exhibit an exquisite quadruplex selectivity over duplex.

Inhibition of telomerase activity in cell-free system

The above encouraging results obtained by FRET and CD for compounds **a–d** prompted us to investigate if these compounds would also show telomerase inhibition using a modified TRAP assay (TRAP-LIG assay).[12] On the basis of TRAP-LIG assay, ligands that stabilize G-quadruplex structures show telomerase inhibitory activity. In this experiment, solutions of phenanthroline derivatives at certain concentrations were added to the telomerase reaction mixture containing extract from lysed HeLa cell lines. As shown in Fig. 4, with increasing concentrations of the compounds **a**, **b** and **d**, a decrease in the intensity of the ladder was observed, whereas this decrease was not observed for compound **c**. The ladder in Fig. 4 was the elongation product of the TS primer, in which each band corresponded to a product that the hexanucleotide TTAGGG telomeric DNA repeat are added to TS primer by telomerase (see Experimental section). Formation of G-quadruplex structures within the TS sequence will hinder its elongation by telomerase and thus the intensity of the ladder will decrease. These results show that compounds **a**, **b** and **d** are potential *in vitro* inhibitors of telomerase.

The compound **c** presented a poor inhibitory activity, probably due to the different thermodynamic stabilization of the complexes between these compounds and G-quadruplex DNA. This speculation was consistent with the current thinking that a stronger

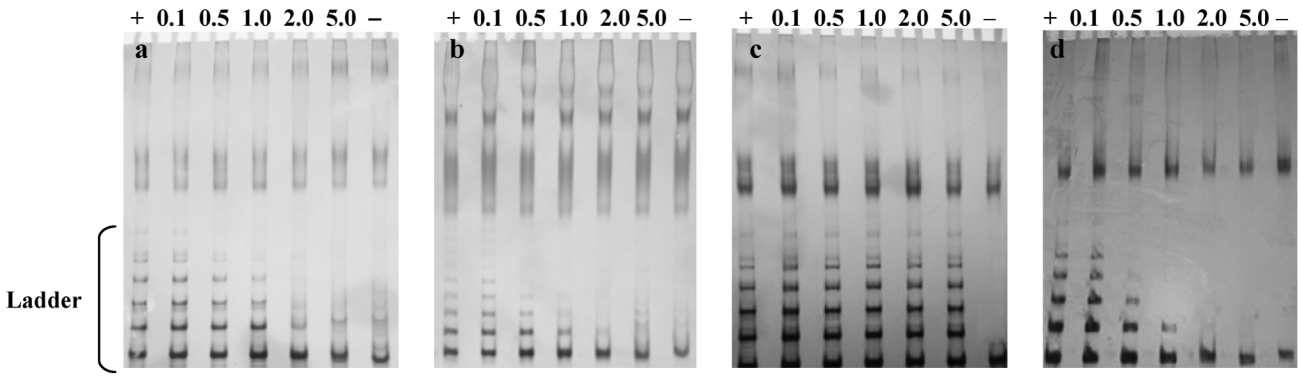

Fig. 4 Inhibition of telomerase activity by compounds **a–d** at concentrations of 0.1, 0.5, 1.0, 2.0 and 5.0 μM. A positive control was run with telomerase, but with no ligand. A negative control was run without either telomerase or ligand. Positive and negative control lanes are indicated by + and – labels, respectively. Ladder shows the product of telomerase elongation.

binding ligand of the G-quadruplex was also a good inhibitor of telomerase activity.[5b,9c,13] Investigations into the thermodynamics properties of the interactions are currently underway in our laboratory.

Overall, the compounds **a**, **b** and **d** could remarkably stabilize the structure of the G-quadruplex and thus exhibited a strong inhibitory effect on telomerase activity.

Antiproliferative activity of compounds a–d

By means of MTT assay the short-term *in vitro* cytotoxicity of the compounds **a–d** was evaluated by using HeLa cell. Fig. 5 depicts the percent cell viability (relative to control) *versus* the concentration of the compounds **a–d** for an incubation period of 72 h. It was found that compounds **a**, **b** and **d** displayed much more potent antitumour activities than compound **c**. From the cytotoxicity profiles, the IC_{50} values of compounds **a**, **b** and **d** were determined to be 2.0, 2.1 and 2.3 μM, respectively. These results are consistent with above results from the TRAP assay and FRET-melting assay; the compound **c** has less antitumour activity than the other compounds because of its lower G-quadruplex stability and telomerase inhibitory activity, and in contrast the high quadruplex affinities and significant telomerase inhibitory activities for compounds **a**, **b** and **d** result in the enhanced *in vitro* biological activities.

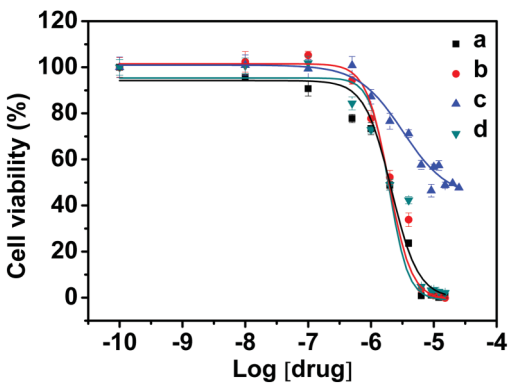

Fig. 5 Effect of compounds **a–d** on HeLa cell viability. HeLa cells were treated with varying concentrations of compounds (0–15 μM) for 72 h. The values are the mean ± SE of three independent experiments.

Conclusion

In summary, four new phenanthroline-based compounds have been designed and synthesized. The interactions between these compounds and G-quadruplex DNA have been studied by FRET-melting assay and CD spectroscopy. From these studies we have identified that the compounds **a–d** induce high stabilization of telomeric G-quadruplex DNA and high selectivity for quadruplex *vs.* duplex DNA. In order to examine the abilities to bind strongly to telomeric quadruplex DNA and to inhibit cancer proliferation, TRAP-LIG assays and the short-term *in vitro* cytotoxicities of the four compounds have been carried out. These studies indicate that compounds **a**, **b** and **d** are potent telomerase inhibitors and HeLa cell proliferation inhibitors.

Experimental

Materials, methods and instrumentation

All biochemical reagents and chemical solvents were purchased from commercial sources. All solvents were distilled over standard drying agents and degassed with nitrogen prior to use. IR spectra were recorded on the Shimadzu FTIR 8400S spectrometer. ^{1}H and ^{13}C NMR spectra were recorded on a Bruker DRX300 spectrometer at room temperature. ESI-MS spectra were obtained using Waters Q-TOF MicroTM instrument.

All oligomers/primers used in this study were purchased from Sangon, China.

Compounds **a–d** were dissolved in DMSO to yield a 10 mM stock solution, which was then diluted using corresponding buffer to the appropriate concentrations to be tested.

Synthesis

N,*N*′-Bis(4-aminophenol)-1,10-phenanthroline-2,9-carboxamide (L)[5a,5b,14]

EDCI (1.5 g, 7.8 mmol) and HOAt (0.1 g, 0.74 mmol) were successively added to a solution of 1,10-phenanthroline-2,9-dicarboxylic acid (1.0 g, 3.7 mmol) and trimethylsilyl-protected 4-aminophenol (1.4 g, 7.8 mmol) in DMF (10 mL). The reaction mixture was then stirred at room temperature for 2 h, and the formed precipitate was collected and washed repeatedly with a solution of $NaHCO_3$ (1%) and diethyl ether. The product was obtained as a yellow solid (1.3 g, 78%). M.p. > 270 °C. IR (KBr, cm^{-1}): 3377, 3338, 1676 (C═O), 1618, 1550, 1517, 1440, 1274, 1242, 1137, 1101, 1008, 865, 833, 819, 703. ^{1}H-NMR (300 MHz, DMSO-d_6): δ (ppm) 11.25 (s, 2H), 9.40 (s, 2H), 8.81 (d, J = 8.4 Hz, 2H), 8.55 (d, J = 8.4 Hz, 2H), 8.23 (s, 2H), 7.91 (d, J = 8.4 Hz, 4H), 6.85(d, J = 8.4 Hz, 4H); ^{13}C-NMR (75 MHz, DMSO-d_6): δ (ppm) 163.3, 155.5, 151.5, 145.1, 140.0, 131.8, 129.6, 123.2, 122.8, 116.7. ^{1}H and ^{13}C NMR spectra were shown in Fig. S4 and S5 (ESI†).

***N*,*N*′-Bis(phenyl-4-ethoxypiperidine)-1,10-phenanthroline-2,9-carboxamide (a).** Ligand **L** (0.1 g, 0.235 mmol) was suspended in dry and degassed DMF (10 mL). NaH (60% dispersion in mineral oil) (0.07 g, 2.3 mmol) was added to this suspension under a nitrogen atmosphere and heated to 110 °C. 1-(2-Chloroethyl)piperidine hydrochloride (0.175 g, 0.95 mmol) was dissolved in DMF (3.3 mL) and the resulting solution was added dropwise to the above reaction mixture. After the addition was complete the mixture was reacted at 110 °C for one day under nitrogen, and then stirred at room temperature for 3 days. The reaction mixture was filtered to give a solution which was concentrated under reduced pressure to obtain a yellow solid. This solid was dissolved in methanol, and adjusted to pH 7.0 with HCl (aq) and NEt_3. The resulting solution was concentrated under vacuum and purified by column chromatography on silica gel using a gradient of ethanol (0–10%) in ethyl acetate with 0.8% NEt_3 to give compound **a** as a yellow solid (0.5 g, 57%). M.p. 156–158 °C. IR (KBr, cm^{-1}): 3431, 3338, 2933, 1677 (C═O), 1596, 1542, 1512, 1496, 1247, 1135, 1108, 1037, 950, 867, 827, 705. ^{1}H-NMR (300 MHz, DMSO-d_6): δ (ppm) 11.28 (s, 2H), 8.83 (d, J = 8.4 Hz, 2H), 8.58 (d, J = 8.1 Hz, 2H), 8.25 (s, 2H), 8.02 (d, J = 7.8 Hz, 4H), 7.08 (d, J = 8.1 Hz, 4H), 4.12 (m, 4H), 2.68 (m, 4H),

2.46 (m, 8H), 1.52 (m, 8H), 1.40 (m, 4H); ^{13}C-NMR (75 MHz, DMSO-d_6): δ (ppm) 160.3 153.4, 148.2, 141.9, 136.8, 130.0, 128.7, 126.4, 119.7, 112.9, 64.1, 55.8, 52.7, 23.93, 22.2. ESI(+)-MS (*m*/*z*): 249.1 (100%), [**a** + 2H + Na + H_2O + CH_3OH]$^{3+}$; 437.1 (90%), [**a** + H + Na + H_2O + 5CH_3OH]$^{2+}$. ^{1}H and ^{13}C NMR spectra were shown in Fig. S6 and S7 (ESI†).

***N*,*N*′-Bis(phenyl-4-ethoxypyrrole)-1,10-phenanthroline-2,9-carboxamide (b).** Compound **b** was prepared from ligand **L** and *N*-(2-chloroethyl)pyrrolidine hydrochloride according to the procedure described for compound **a** above. Yellow solid (0.53 g, 64%); m.p. 158–160 °C. IR (KBr, cm^{-1}): 3446, 3321, 2956, 2925, 2775, 1678 (C═O), 1596, 1542, 1512, 1475, 1245, 1132, 1107, 1033, 867, 827, 707, 667. ^{1}H-NMR (300 MHz, DMSO-d_6): δ (ppm) 11.29 (s, 2H), 8.81 (d, *J* = 8.4 Hz, 2H), 8.56 (d, *J* = 8.1 Hz, 2H), 8.25 (s, 2H), 8.06 (d, *J* = 8.7 Hz, 4H), 7.09 (d, *J* = 9.0 Hz, 4H), 4.14 (t, *J* = 5.7 Hz, 4H), 2.85 (t, *J* = 5.7 Hz, 4H), 2.58 (m, 8H), 1.72 (m, 8H); ^{13}C-NMR (75 MHz, DMSO-d_6): δ (ppm) 162.6, 155.6, 150.4, 144.1, 139.1, 132.3, 130.9, 128.6, 122.0, 115.1, 67.4, 54.9, 54.6, 23.7; ESI(+)-MS(*m*/*z*): 235.1 (100%), [**b** + 2H + Na + 2H_2O]$^{3+}$; 485.2 (90%), [**b** – $(CH_2)_4N(CH_2)_2OC_6H_4NH$ – H + 2Na]$^+$. ^{1}H and ^{13}C NMR spectra were shown in Fig. S8 and S9 (ESI†).

***N*,*N*′-Bis(phenyl-4-ethoxymorpholine)-1,10-phenanthroline-2,9-carboxamide (c).** Compound **c** was prepared from ligand **L** and 4-(2-chloroethyl)morpholine hydrochloride according to the procedure described for compound **a** above. Yellow solid (0.61 g, 68%); m.p. 192–194 °C. IR (KBr, cm^{-1}): 3433, 3334, 3296, 2945, 1681 (C═O), 1596, 1539, 1514, 1471, 1253, 1112, 1132, 1006, 873, 829, 709. ^{1}H-NMR (300 MHz, DMSO-d_6): δ (ppm) 11.27 (s, 2H), 8.81 (s, *J* = 8.4 Hz, 2H), 8.56 (d, *J* = 8.4 Hz, 2H), 8.23 (s, *J* = 10.1 Hz, 2H), 8.05 (d, *J* = 8.7 Hz, 4H), 7.08 (d, *J* = 8.7 Hz, 4H), 4.16 (t, *J* = 5.7 Hz, 4H), 3.60 (m, 8H), 2.73 (m, 4H), 2.49 (m, 8H); ^{13}C-NMR (75 MHz, DMSO-d_6): δ (ppm) 162.5, 155.4, 150.3, 144.0, 138.9, 132.2, 130.8, 128.5, 121.8, 115.1, 66.5, 65.9, 57.5, 54.0. ESI(+)-MS(*m*/*z*): 672.2 (100%), [**c** + H]$^+$; 472.2 (90%), [**c** – $(C_6H_4O(CH_2)_2C_4H_6ON)$ + H]$^+$. ^{1}H and ^{13}C NMR spectra were shown in Fig. S10 and S11 (ESI†).

***N*,*N*′-Bis(phenyl-4-ethoxydimethylamino)-1,10-phenanthroline-2,9-carboxamide (d).** Compound **d** was prepared from ligand **L** and 2-dimethylaminoethyl chloride hydrochloride according to the procedure described for compound **a** above. Yellow solid (0.46 g, 59%); m.p. 176–178 °C. IR (KBr, cm^{-1}): 3454, 3323, 2956, 2927, 1666 (C═O), 1595, 1542, 1514, 1467, 1384, 1250, 1172, 1134, 993, 867, 825, 810, 707, 667. ^{1}H-NMR (300 MHz, methanol-d_4): δ (ppm) 8.69 (d, *J* = 8.4 Hz, 2H), 8.56 (d, *J* = 8.4 Hz, 2H), 8.10 (s, 2H), 9.96 (d, *J* = 9.0 Hz, 4H), 7.18 (d, *J* = 9.0 Hz, 4H), 4.45 (m, 4H), 3.64 (m, 4H), 3.03 (m, 12H); ^{13}C-NMR (75 MHz, DMSO-d_6): δ (ppm) 164.0, 156.1, 151.7, 145.5, 140.5, 134.3, 132.4, 130.1, 123.3, 116.8, 65.1, 57.7, 45.2; ESI(+)-MS(*m*/*z*): 323.1 (100%), [**d** + Na + CH_3OH]$^{2+}$; 681.2 (90%), [**d** – 3H + 4Na]$^+$. ^{1}H and ^{13}C NMR spectra were shown in Fig. S12 and S13 (ESI†).

CD spectroscopy

CD spectra were recorded on a dualbeam DSM 1000 CD spectrophotometer (Olis, Bogart, GA). Each measurement was the average of three repeated scans recorded from 220 to 320 nm with a 0.1 mm quartz cell at 25 °C. The scanning rate (22 nm min^{-1}) was automatically selected by the Olis software as a function of the signal intensity to optimize data collection. Prior to use the oligomer HTG22 d[AGGG(TTAGGG)$_3$] dissolved in corresponding buffer was heated to 95 °C for 5 min, then gradually cooled to room temperature and incubated at 4 °C overnight. HTG22 at a final concentration of 5 μM was resuspended in 10 mM Tris-HCl buffer (pH 7.4) with or without the specific cations to be tested. CD titration was performed at a fixed HTG22 concentration (5 μM) with various concentrations of the compounds in the corresponding buffer. After each addition of compound, the reaction was stirred and allowed to equilibrate for at least 15 min (until no elliptic changes were observed) and a CD spectrum was collected. A background CD spectrum of corresponding buffer solution with or without compounds was subtracted from the average scan for each sample. Final analysis of the data was carried out using Origin 7.5 (OriginLab Corp.)

FRET assay

FRET assay was carried out on a Varian Cary Eclipse Fluorescence spectrometer equipped with a Peltier temperature control accessory with excitation at 483 nm and detection at 500–700 nm. Both excitation and emission slit are 5 nm. The fluorescent labeled oligonucleotide F21T [5′-FAM-d(GGG[TTAGGG]$_3$)-TAMRA-3′], (donor fluorophore FAM: 6-carboxyfluorescein, and acceptor fluorophore TAMRA: 6-carboxytetramethylrhodamine) used as the FRET probes was diluted from stock to the correct concentration (400 nM) in either 10 mM sodium cacodylate buffer (pH 7.4) containing 100 mM NaCl or 10 mM K_2HPO_4/KH_2PO_4 buffer (pH 7.4) containing 100 mM KCl and then annealed by heating to 90 °C for 5 min, followed by cooling to room temperature. Samples were prepared by aliquoting 1 mL of the annealed F21T (at 2× concentration, 400 nM) into cell, followed by 1 mL of buffer or the compound solutions (at 2× concentration) and further incubated for 1 h. The melting of G-quadruplex was monitored alone or in the presence of various concentrations of compounds and/or of either 2.0 or 5.0 μM double stranded competitor ds26 (5-CAATCGGATCGAATTCGATCCGATTG-3′). Fluorescence readings at 533 nm were taken at intervals of 2–3 °C over the range of 5–95 °C, with a constant temperature being maintained for 5 min prior to each reading to ensure a stable value. Final analysis of the data was carried out using Origin 7.5 (OriginLab Corp.).

TRAP assay

The ability of ligands to inhibit telomerase in a cell-free system was assessed with the TRAP-LIG assay following previously published procedures.[12] Protein extracts from exponentially growing HeLa cells were used. Briefly, 0.1 μg of TS forward primer (5′-AATCCGTCGAGCAGAGTT-3′) was elongated by telomerase (500 ng protein extract) in TRAP buffer (20 mM Tris-HCl [pH 8.3], 68 mM KCl, 1.5 mM $MgCl_2$, 1 mM EGTA, and 0.05% Tween 20) containing 125 μM dNTPs and 0.05 μg BSA. The mix was added to tubes containing freshly prepared ligand at various concentrations and to a negative control containing no ligand. The initial elongation step was carried out for 20 min at 30 °C, followed by 94 °C for 5 min and a final maintenance of the mixture at 20 °C. To purify the elongated product and to remove the bound ligands, the QIA quick nucleotide purification kit (Qiagen) was used according to the manufacturer's instructions. The purified

extended samples were then subject to PCR amplification. For this, a second PCR master mix was prepared consisting of 1 μM ACX reverse primer (5′-GCGCGG[CTTACC]$_3$CTAACC-3′), 0.1 μg TS forward primer (5′-AATCCGTCGAGCAGAGTT-3′), TRAP buffer, 5 μg BSA, 0.5 mM dNTPs, and 2 units of *Taq* polymerase. A 10 μL aliquot of the master mix was added to the purified telomerase extended samples and amplified for 35 cycles of 94 °C for 30 s, of 61 °C for 1 min, and of 72 °C for 1 min. Samples were separated on a 10% PAGE in 0.5× TBE buffer and visualized with silver-stained.

Cytotoxic assay

HeLa cell were cultivated in DulbeccoGs modified EagleGs medium (DMEM) containing 10% fetal bovine serum (FBS). Cells in the logarithmic growth-phase were seeded on 96-well culture plates (2.5×10^3/well) in 200 μL volumes. Ligands with various concentrations were added inside the 96-well plates. Five parallel wells were set for each of the treated or control groups. After 72 h of treatment at 37 °C in a humidified atmosphere of 5% CO_2, 20 μL of 5 mg mL^{-1} methyl thiazolyl tetrazolium (MTT) solution was added to each well and further incubated for 4 h. The cells in each well were then treated with dimethyl sulfoxide (DMSO) (150 μL for each well) and the optical density (OD) was recorded at 490 nm. The IC_{50} values were derived from the mean OD values of the five tests *versus* drug concentration curves.

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