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4-(1*H*-Imidazo[4,5-*f*]-1,10-phenanthrolin-2-yl)phenol-based G-quadruplex DNA binding agents: Telomerase inhibition, cytotoxicity and DNA-binding studies

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ABSTRACT

Four novel 4-(1*H*-imidazo[4,5-*f*]-1,10-phenanthrolin-2-yl)phenol derivatives **1**–**4** have been synthesized, and their G-quadruplex DNA-binding interactions, telomerase inhibition, antiproliferative activity, cell cycle arrest, and apoptotic induction were studied. All compounds show the preferential *h*-telo, *c*-myc, and *c*-kit2 G-quadruplex binding affinity and the G-quadruplex versus duplex selectivity. In the case of the same G-quadruplex target, the compound **1** exhibits better stabilization effect (ΔT_m) than the other three compounds and also gives 80.2% inhibition of telomerase activity at 7.5 μM. All compounds can promote selectively the formation of parallel G-quadruplex structure of both *c*-myc and *c*-kit2 without addition of any cations. Four compounds display the cytotoxicity activities against HeLa and HepG2 cells by MTT assay with IC_{50} values of about 10^{-6} and 10^{-5} M, respectively, and cause a substantial decrease in the G_2/M-phase cell population and a significant increase in the number of apoptotic cells.

1. Introduction

G-quadruplex-forming DNA sequences have been identified in the human telomere[1–3] as well as the promoters of several oncogenes including *c*-myc,[4] kras,[5] *c*-kit,[6,7] and bcl-2.[8] More recently, RNA G-quadruplexes were found in the regions of G-rich telomeric repeat-containing RNA (TERRA),[9,10] the 5′-end of human telomerase RNA (hTR)[11] and 5′-untranslated regions (UTR) of mRNAs.[12–14] The telomeric G-quadruplex DNA-recognizing small molecules can inhibit cancer cell growth via mechanisms that may involve disruption of the telomere and/or the prevention of telomere extension.[15–19] Small molecules that bind and stabilize the *c*-myc and *c*-kit G-quadruplexes also suppress transcription of the proto-oncogenes *c*-myc[20–22] and *c*-kit.[23–25] Consequently, the G-quadruplexes formed by the telomere and the promoters of oncogenes are potential molecular targets for anticancer drugs.[26–28] G-quadruplexes in the 5′ UTR of mRNAs modulate translation and may provide yet another class of targets for small molecule intervention.[12,13,26]

To date, a large number of small molecules have been designed to target G-quadruplex DNAs.[29–33] 1,10-Phenanthroline derivatives can bind to duplex DNA due to their planar structure. Recently, the small organic molecules containing phenanthroline scaffold and their metal complexes have been reported be able to better stabilize the human telomeric G-quadruplex than the duplex DNA.[34–42] For example, platinum(II) phenanthroline complex induces a high degree of quadruplex DNA stabilization and inhibits telomerase activity,[34] another two phenanthroline compounds exhibit better affinity and selectivity for G-quadruplex DNA than duplex,[35] bis-phenanthroline Ni(II) and Cu(II) complexes promote the G-quadruplex folding and inhibit the activity of telomerase,[36] and Pt(II) phenanthroimidazole complexes display a significantly greater binding affinity and selectivity for quadruplex than duplex.[37,38] Also our previous studies have indicated that three phenanthroline-based derivatives induce the formation of antiparallel G-quadruplex structure and inhibit the telomerase activity.[39]

Until now some studies have reported the binding affinity and selectivity of phenanthroline-based compounds for different biologically relevant G-quadruplex-forming DNA sequences, whereas studies on effects of G-quadruplex-binding ligands on cell cycle and apoptosis have been rarely reported.[43–47] Herein, we report the synthesis and characterization of four novel 4-(1*H*-imidazo[4,5-*f*]-1,10-phenanthrolin-2-yl)phenol derivatives (Scheme 1). Their binding interactions with human telomeric (*h*-telo) and promoter (*c*-kit2 and *c*-myc) G-quadruplex DNAs and duplex DNA were studied by FRET melting assay, competitive dialysis, absorption titration, and CD spectroscopy. Furthermore, influences of these compounds on telomerase activity, cell proliferation, cell cycle, and apoptosis were evaluated.

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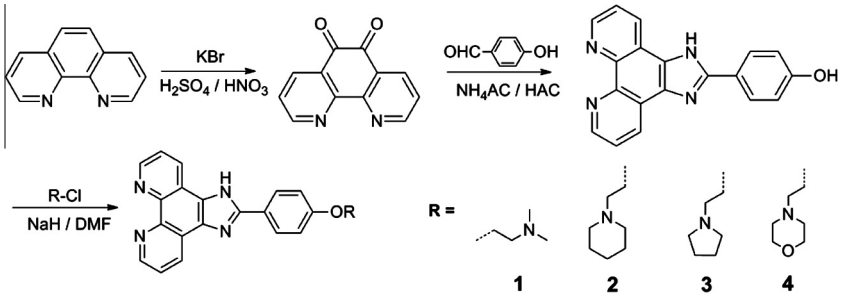

Scheme 1. Synthetic route to compounds **1–4**.

2. Results and discussion

2.1. Synthesis

The general synthetic method for compounds **1–4** is described in Scheme 1. 1,10-Phenanthroline-5,6-dione[48–50] and 4-(1*H*-imidazo[4,5-*f*]-1,10-phenanthrolin-2-yl)phenol (*p*-hpip)[50–52] were prepared following the literature methods. The final products were prepared from ligand *p*-hpip and 2-dimethylaminoethyl chloride hydrochloride (**1**), *N*-(2-chloroethyl)piperidine hydrochloride (**2**), *N*-(2-chloroethyl)pyrrolidine hydrochloride (**3**), or 4-(2-chloroethyl)morpholine hydrochloride (**4**). Structures of all these final products were confirmed by ^{1}H and ^{13}C NMR, IR, elementary analysis, and ESI-MS. The detailed synthetic procedures of *p*-hpip and compounds **1–4** are given in the Experimental.

2.2. Stabilization potential and binding affinity of the compounds to G-quadruplex DNAs

FRET melting analysis was used to determine the G-quadruplex stabilization and selectivity of the series of ligands to particular G-quadruplex target. Here the stabilization potential of four compounds (**1–4**) to *h*-telo, *c*-myc, and *c*-kit2 G-quadruplex DNAs was first studied by FRET melting assay (Figs. S1–S3, Supplementary data). The shifts in melting temperature values (ΔT_m) are given in Figure 1 and Table 1.

Four compounds show moderate stabilization of G-quadruplexes, with ΔT_m values range 6.8–17.8, 4.2–13.1, and 4.0–10.5 °C for *h*-telo, *c*-myc, and *c*-kit2, respectively, in the concentration range 1–3 μM. Because each DNA target gives a different melting temperature (T_m) value, the selectivity among different quadruplex targets cannot be directly compared using ΔT_m. For the same G-quadruplex target, the compound **1** exhibits better stabilization potential than the other compounds, the ΔT_m values were found to be 17.8, 13.1, and 10.5 °C, for *h*-telo, *c*-myc, and *c*-kit2 G-quadruplexes, respectively, at 2 μM concentration, while the compound **4** exhibits the lowest stabilization potential, and the observed ΔT_m is 6.8 °C for *h*-telo, 4.2 °C for *c*-myc, and 4.0 °C for *c*-kit2 at the same concentration. The different stabilization potential of four compounds may be due to different basicity, polarity, and size of amino alkyl side chains.[39,53] All the compounds show preference for stabilizing G-quadruplex DNAs over duplex DNA using competitive FRET melting assay in the presence of 10-fold excess of duplex DNA (ds26, 2 μM). Furthermore, in the presence of 25-fold excess of ds26 (5 μM), the compound **2** also stabilizes the structures of *h*-telo, *c*-myc, and *c*-kit2 G-quadruplexes, with a slight decrease in ΔT_m (2.6, 0.9, and 1.6 °C, respectively) compared to G-quadruplex alone, whereas the compound **1** stabilizes only structures of both *c*-myc and *c*-kit2 G-quadruplexes (with a decrease in ΔT_m of 0.3 and 1.5 °C, respectively). In the case of three G-quadruplex targets, the larger decrease in ΔT_m values (27–76%) was observed for compounds **3** and **4** in the presence of 25-fold excess of ds26, suggesting that both compounds possess moderate binding selectivity for *h*-telo, *c*-myc, and *c*-kit2 G-quadruplexes versus duplex.

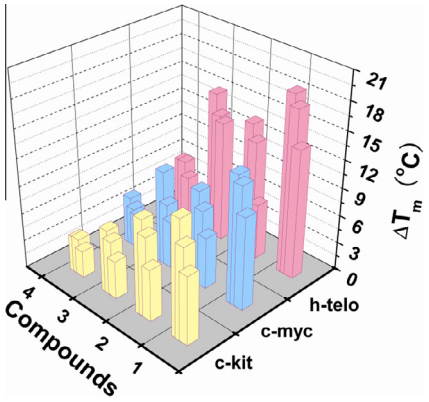

Figure 1. Increase of melting temperature (ΔT_m) of *h*-telo, *c*-myc, and *c*-kit2 G-quadruplex DNAs in the presence of compounds **1**, **2**, **3**, and **4** by FRET melting assay. Concentrations of each compound are 1, 2, and 3 μM from right to left for each G-quadruplex target.

The binding interactions of the compounds with G-quadruplex DNAs were further measured by absorption titration experiment. Isosbestic points, slight blue-shift, and hypochromic effects were observed in the absorption spectra for compound **1** upon titration with G-quadruplexes as depicted in Figure 2. The blue-shift about 1–2 nm may arise from the absorbance of the increasing amount of DNA. The hypochromic phenomenon is attributed to the strong interaction between the compound **1** and G-quadruplex DNA. Using Eq. 1, the binding constants (K_b) of compound **1** with *h*-telo, *c*-myc, and *c*-kit2 G-quadruplexes were calculated to be (1.01 ± 0.09), (1.44 ± 0.12), and $(0.86 \pm 0.03) \times 10^6\ M^{-1}$ at 25.0 °C, respectively (Table 2).

To investigate whether there is a binding selectivity between G-quadruplex and duplex, a parallel absorption titration experiment of compound **1** with ctDNA was performed (Fig. S4, Supplementary data). The K_b value for the ctDNA-compound **1** was determined to be $(6.6 \pm 0.3) \times 10^4\ M^{-1}$, which is approximately 15.3-, 21.8-, and 13.0-fold lower than those for complexes of compound **1** with *h*-telo, *c*-myc, and *c*-kit2 G-quadruplexes, respectively. Similarly, the binding constants of the other compounds for *h*-telo, *c*-myc, and *c*-kit2 G-quadruplexes and for ctDNA were also determined, and the data are summarized in Table 2. These data reveals that the binding affinities of four compounds to G-quadruplex DNAs are preferential over duplex DNA. The correlation between stabilization (ΔT_m) and equilibrium binding (K_b) is not straightforward and thus there is no simple relationship between them.

The relative binding affinities of compounds **1**, **2**, and **3** to different DNA structures were further evaluated by competition dialysis assay, and the results obtained in PBS buffer containing

Table 1
G-quadruplex stabilization potential (ΔT_m) by FRET melting assay

Compounds	ΔT_m (°C) at 2 μM compound concentration								
	h-telo[a]	*h*-telo+ds26[a]		*c*-myc[b]	*c*-myc+ds26[b]		*c*-kit2[a]	*c*-kit2+ds26[a]	
		2 μM[c]	5 μM[d]		2 μM[c]	5 μM[d]		2 μM[c]	5 μM[d]
1	17.8	14.5	12.0	13.1	12.8	12.8	10.5	10.2	9.0
2	12.6	10.8	10.0	9.1	8.3	8.2	8.8	9.3	7.2
3	13.5	13.1	7.4	6.8	7.2	4.7	6.0	4.7	4.0
4	6.8	6.6	2.4	4.2	4.0	1.0	4.0	3.1	2.1

[a] With 100 mM KCl.
[b] With 40 mM KCl.
[c] With 2 μM duplex DNA (ds26).
[d] With 5 μM duplex DNA (ds26).

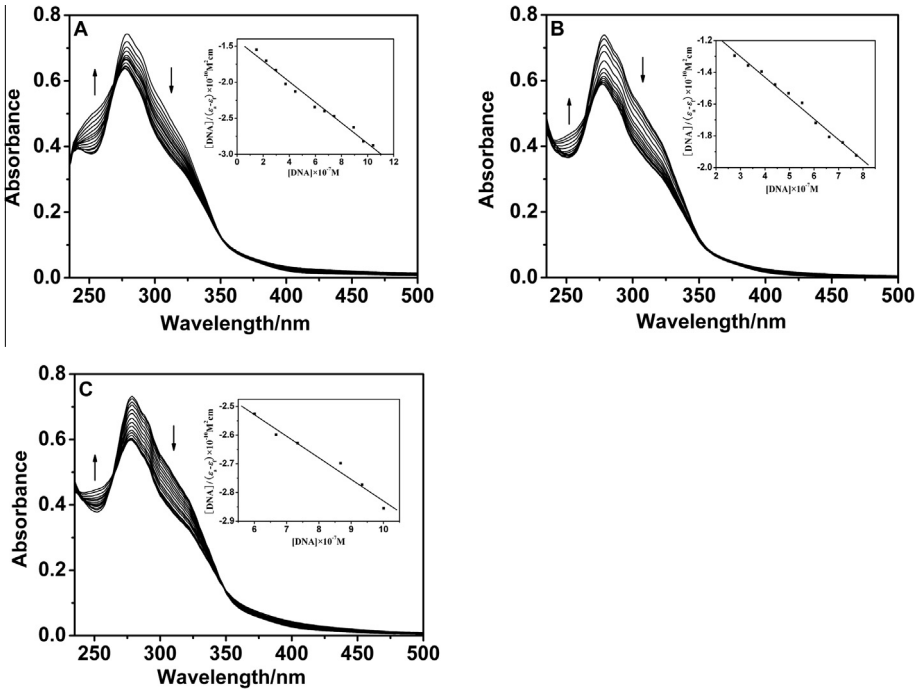

Figure 2. Absorption titration spectra of compound **1** (30 μM) with increasing amounts of *h*-telo (A), *c*-myc (B), and *c*-kit2 (C) G-quadruplex DNAs in 10 mM tris–HCl buffer containing 100 mM KCl (pH 7.4). The arrow indicates the absorbance changes upon increasing DNA concentration. Insert is the curve fit by Eq. 1. Absorbance was monitored at 315 nm.

Table 2
G-quadruplex and ctDNA binding constants (K_b) measured by Eq. 1

Compounds	K_b[a] ($\times 10^6$ M^{-1})			
	h-telo	*c*-myc	*c*-kit2	ctDNA
1	1.01 ± 0.09	1.44 ± 0.12	0.86 ± 0.03	0.066 ± 0.003
2	1.17 ± 0.12	1.35 ± 0.11	0.77 ± 0.06	0.081 ± 0.002
3	0.93 ± 0.06	1.26 ± 0.13	0.61 ± 0.15	0.11 ± 0.02
4	0.36 ± 0.04	0.64 ± 0.08	0.39 ± 0.03	0.14 ± 0.06

[a] Values are means ± S.E.

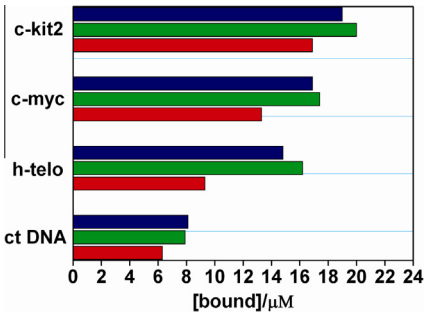

Figure 3. Relative affinities of compounds **1–3** to *h*-telo, *c*-myc, and *c*-kit G-quadruplexes and ctDNA determined by competitive dialysis. The amount of bound compound was plotted against each DNA structure as a bar. **1** (red), **2** (green), and **3** (blue).

100 mM KCl are depicted in Figure 3. All the compounds bind preferentially to *h*-telo, *c*-myc, and *c*-kit2 G-quadruplex DNAs, demonstrating that these have the selectivity for quadruplex over duplex. However, in the case of particular G-quadruplex target, the binding selectivity of each compound is not remarkable, especially for compounds **2** and **3**. In addition, for all the compounds the bound amount for *c*-kit2 G-quadruplex is the highest, which fact is inconsistent with results obtained by absorption titration. The difference may result from significant non-specific binding of the compounds to *c*-kit2 G-quadruplex. The number of experimental points in Scatchard plot shown in the inset in Figure 2C is lower than that of spectra in the titration, because the initial data points from the absorption titration deviate from the linear plot and were

deleted from Scatchard plot-these data may arise from non-specific binding. However, the similar phenomenon is not observed for both *h*-telo and *c*-myc G-quadruplexes. Because the bound compound concentration obtained in the equilibration dialysis includes a sum of specific and non-specific binding, this results in the larger amount of the bound compound for *c*-kit2 than for *h*-telo and *c*-myc.

2.3. Promoting the formation of parallel G-quadruplex DNAs

G-quadruplexes formed from *h*-telo, *c*-kit2, and *c*-myc sequences in the presence of compounds **1**–**4** were monitored by CD spectroscopy (Fig. 4). The CD spectrum of *c*-kit2 sequence in the absence of any added salts shows a major positive peak at 262 nm, a minor peak at 294 nm, and a negative band at 240 nm. Upon titration of the compounds **1–4**, a concentration-dependent increase of intensity at 262 nm is not accompanied by associated increase of the peak intensity at 294 nm, and these features are indicative of selective induction of a parallel G-quadruplex structure.[7,54] In addition, the CD spectrum of *c*-myc sequence in the absence of any added salt was found to have a positive peak at 260 nm and a negative band at 240 nm. Upon addition of the compound (2 mol equiv), the increase of intensity at 262 nm was observed suggesting that compounds **1–4** induce the formation of a parallel G-quadruplex structure (Fig. S5A, Supplementary data).[55] In contrast, for *h*-telo a compound-dependent induction of G-quadruplex structure change was not observed even in the presence of 2 mol equiv of the compound (Fig. S5B, Supplementary data). These results show that four compounds are able to promote selectively the formation of parallel G-quadruplex structures of both *c*-kit2 and *c*-myc and therefore stabilize their structures under cation-deficient conditions.

To understand whether G-quadruplex DNA undergoes conformational changes during compound–DNA interaction, CD spectra of *h*-telo, *c*-kit2, and *c*-myc G-quadruplexes in the absence and presence of compounds **1–4** at [compound]/[DNA] ratios of 2 were determined (Fig. S6, Supplementary data). The CD spectrum of free *h*-telo in buffer containing KCl has a negative band at 235 nm and a positive band at 290 nm with a shoulder near 265 nm, which is characteristic of G-quadruplex DNA in the hybrid form.[56] With the exception of the compound **4**, a slight intensity increase of the positive peak at 290 nm was observed upon addition of the other compounds. The CD spectra of both *c*-myc and *c*-kit2 G-quadruplexes show a parallel conformation in the presence of 100 mM K^+, with a positive peak at 262 nm and a negative peak at 241 nm. Upon addition of 2 mol equiv of the compounds **1–4** to either *c*-myc or *c*-kit2 G-quadruplex, the compound-induced CD spectral changes were less prominent. In summary, the binding of four compounds fails to affect significantly the conformation of *h*-telo, *c*-myc, and *c*-kit2 G-quadruplexes in buffer containing 100 mM KCl.

2.4. Telomerase inhibition of compounds in cell-free system

Human telomeric G-quadruplex-binding ligands are known to inhibit telomerase activity. The above results obtained by FRET melting assay and the moderate stabilization potential of the compounds for *h*-telo G-quadruplex prompted us to investigate if these compounds would also show telomerase inhibitory ability using a modified telomerase repeat amplification protocol assay (TRAP-LIG assay).[39,57] As shown in Figure 5, except the compound **4**, the other compounds were able to inhibit telomerase activity, but the effects were different. Formation of G-quadruplex structure in TS primer sequence hinders its elongation by telomerase and thus the intensity of the ladder decreases. Based on this principle, the compound **1** is a strong inhibitor that inhibits 80.2% of telomerase activity at 7.5 μM, whereas 21.5% and 14.4% inhibitions are obtained for compounds **2** (15 μM) and **3** (10 μM), respectively.

The current results indicate that the potency of in vitro telomerase inhibition is in accordance with the stabilization potential. In comparison with compounds **2** and **3**, the compound **1** stabilizes the structure of the *h*-telo G-quadruplex more efficiently and thus exhibits a stronger inhibitory effect on the telomerase activity (80.2% at 7.5 μM). The compound **4** presents a poor inhibitory activity (3.4% at 15 μM) owing to its lower stabilization potential and/or weak binding affinity to *h*-telo G-quadruplex DNA.

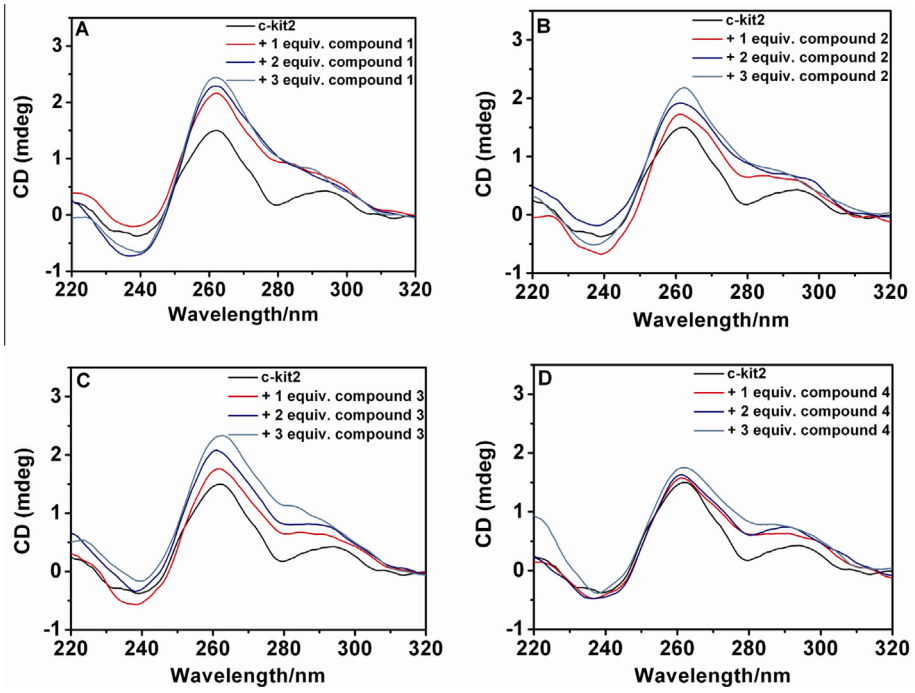

Figure 4. CD spectra of 5 μM *c*-kit2 DNA in the presence of different concentrations of compounds **1** (A), **2** (B), **3** (C), and **4** (D) in 10 mM tris–HCl buffer without any added salts.

2.5. In vitro cytotoxicity

2.5.1. Antiproliferative activity

The in vitro antiproliferative activity of compounds **1–4** was evaluated against two human cancer cell lines HeLa and HepG2 by treating cells with various concentrations for 72 h. The number of viable cells, determined by MTT assay, decreases with increasing concentrations of compounds **1–4** in both tested lines (Fig. 6), which shows that compounds **1–4** display significant antitumor activity with the IC_{50} values of 1.0, 0.60, 0.80, and 0.69×10^{-6} M for HeLa cells and 9.1, 8.7, 11, and 16×10^{-6} M for HepG2 cells, respectively. All compounds inhibit the growth of HeLa cells better than that of HepG2 cells. The compound **4** is also a potent antiproliferative agent, especially for HeLa cells, due to other reasons such as differences in cell uptake, alternative binding position, or cell modification .

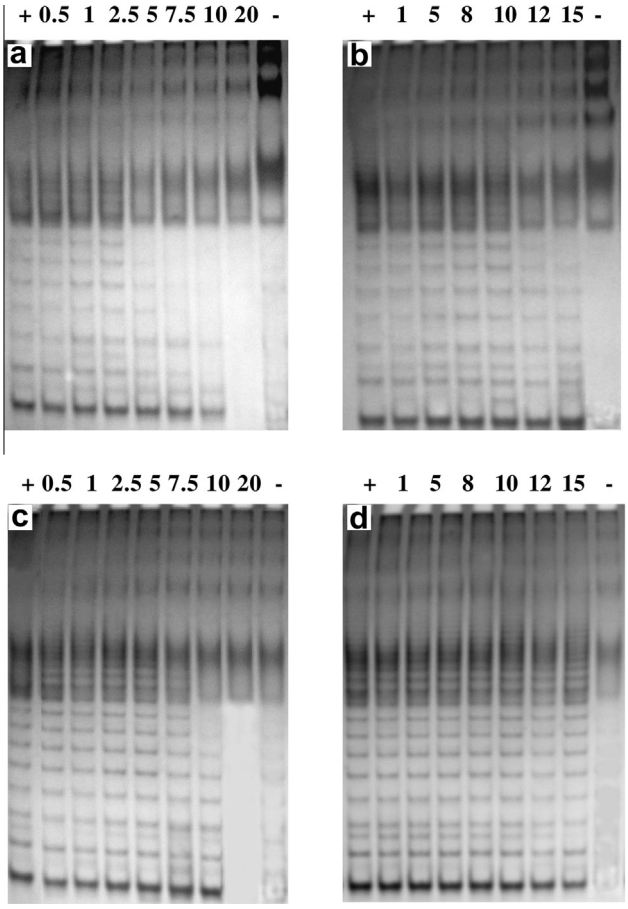

Figure 5. TRAP assay performed in the presence of increasing concentrations of compounds **1** (a), **2** (b), **3** (c) and **4** (d). The positive control was run with telomerase without ligand. The negative control was run without either telomerase or ligand. Positive and negative control lanes are indicated by + and − labels, respectively.

2.5.2. Cell cycle analysis and detection of apoptosis

All of the target compounds were further evaluated for their effects on the cell cycle and for the apoptotic induction in both HeLa and HepG2 cells by flow cytometric assay that enables quantification of the total cellular population in the different phases of the cell cycle (G_0/G_1, S, and G_2/M), as well as the sub-G_1 apoptotic population.

Four compounds delay the cell cycle progression of both HeLa and HepG2 cells upon treatment for 48 and 30 h, respectively, at their corresponding IC_{50} values and arrest cells in G_0/G_1 and/or S phases. This causes a substantial decrease in the G_2/M phase cell population that was zero for HepG2 cells and near to zero for HeLa cells. The treatment also leads to a significant increase in the number of apoptotic cells, as shown by an increase in the subG_0/G_1 content from 12.8% to 53.5% for HeLa cells and from 2.64% to 55.4% for HepG2 cells (Table 3, Figs. S7 and S8, Supplementary data).

It was reported that apoptosis of AML cells induced by telomestatin might be related to the reduction of telomere length,[43] whereas RHPS4 is a potent in vitro telomerase inhibitor and blocks the cells in the G_2/M phase of the cell cycle, but it fails to reduce telomere length in tumor cells.[44,45] 2,6-Pyridine-dicarboxamide derivatives displaying a strong selectivity for G4 structures and strong inhibition of telomerase inhibit cell proliferation and then induce apoptosis, but these effects are not associated with telomere shortening.[46] HXDV, a G-quadruplex stabilizer, exhibits

Table 3
Flow cytometric analysis of HeLa and HepG2 cell cycles upon the 48 and 30 h treatment, respectively, with compounds **1–4** at their corresponding IC_{50} concentration[a]

Compounds	G_0/G_1 (%)	S (%)	G_2/M (%)	subG_0/G_1 (%)
HeLa				
Control	56.8 ± 2.1	26.9 ± 0.9	16.3 ± 0.8	12.8 ± 0.2
1	69.2 ± 1.9	22.9 ± 1.1	7.9 ± 0.2	31.0 ± 0.5
2	62.7 ± 1.9	33.9 ± 1.1	3.4 ± 0.2	53.5 ± 0.5
3	50.9 ± 1.0	48.4 ± 1.2	0.7 ± 0.08	25.1 ± 1.2
4	59.8 ± 1.3	40.2 ± 1.2	0	19.6 ± 0.7
HepG2				
Control	65.3 ± 2.1	24.0 ± 0.9	10.7 ± 0.4	2.64 ± 0.2
1	75.2 ± 1.9	24.8 ± 1.1	0	30.5 ± 0.62
2	49.5 ± 2.3	50.5 ± 1.4	0	49.9 ± 1.4
3	66.5 ± 1.0	33.5 ± 1.2	0	52.2 ± 1.2
4	76.4 ± 1.4	25.4 ± 1.2	0	55.4 ± 0.7

[a] Values are means ± S.E. of three independent experiments.

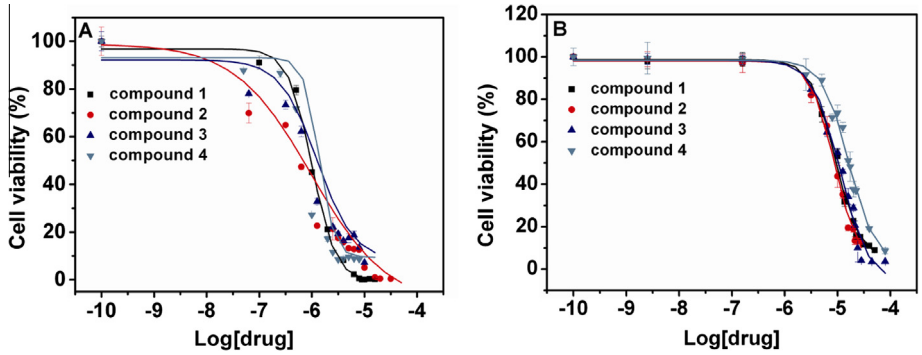

Figure 6. Effect of compounds **1–4** on HeLa (A) and HepG2 (B) cell viability. Both cells were treated with various concentrations of compounds for 72 h. The values are means ± S.E. of three independent experiments.

antiproliferative activity by a mode of action independent on telomerase and also inhibits the cell cycle progression causing a M phase cell cycle arrest.[47] In our present work, the compound **1** is a potent telomerase inhibitor compared to compounds **2**, **3**, and **4**, but all the compounds arrest the cell cycle and induce the cell apoptosis. In the case of our current results, the relationship between telomerase inhibition and the cell cycle and apoptosis is unclear. In addition, the moderate stabilization potential and high binding affinities of compounds for both *c*-kit and *c*-myc G-quadruplexes may interfere with the cell function including arrest of cell cycle and induction of apoptosis due to suppression of gene expression. Meanwhile, the possible interactions of the compound with RNA G-quadruplexes also inhibit gene expression and further affect cell function. Thus the in-depth investigation is deserved to be performed in future work.

3. Conclusions

In this paper, four novel compounds have been synthesized, which show a moderate stabilization potential for G-quadruplexes. For a particular G-quadruplex target, the compound **1** appears to be a potent G-quadruplex DNA stabilization agent with ΔT_m of about 17.8, 13.1, and 10.5 °C for *h*-telo, *c*-myc, and *c*-kit2, respectively, at 2 μM concentration, which also exhibits 80.2% telomerase inhibitory activity at 7.5 μM. All compounds also exhibit selectivity for *h*-telo, *c*-kit2, and *c*-myc G-quadruplexes over duplex DNA, moreover the compound **2** can recognize selectively and stabilize *h*-telo, *c*-kit2, and *c*-myc G-quadruplexes even in the presence of 25-fold excess of ds26, although the compound **1** only stabilizes both *c*-kit2 and *c*-myc G-quadruplexes under the same condition. Four compounds can also induce the formation of a parallel G-quadruplex structure for both *c*-myc and *c*-kit2 under cation-deficient conditions. The antiproliferative effect of all compounds was observed on HeLa and HepG2 cell lines in a concentration-dependent response pattern, with IC_{50} values of 10^{-6} and 10^{-5} M, respectively. These compounds cause the cell cycle arrest at S and/or G_0/G_1 phases and induce cell apoptosis at their corresponding IC_{50} concentration.

4. Materials and methods

4.1. Materials

All biochemical reagents and chemical solvents were purchased from commercial sources. DMF was distilled over standard drying agents and degassed with nitrogen prior to use. IR spectra were recorded on a Shimadzu FTIR 8400S spectrometer. ^{1}H and ^{13}C NMR spectra were taken on a Bruker DRX300 spectrometer at room temperature. ESI-MS spectra were obtained using a LCQ instrument (Finnigan, USA). Elemental analysis was made on an Elementar Vario MICRO instrument (Germany). For all experiments, the compound was dissolved in DMSO as 10 mM stock solution and further dilutions were made in the corresponding buffer.

Calf thymus (ct) DNA was purchased from Sigma, the quadruplex-forming oligonucleotides $dG_3(T_2AG_3)_3$ (*h*-telo), $d(CG_3CG_3CGCGAG_3AG_4)$ (*c*-kit2) and $d(TGAG_3TG_4AG_3TG_4A_2)$ (*c*-myc) were purchased from SBS Genetech Co., Ltd (Beijing, China). The HeLa (human cervix epitheloid carcinoma) cells and the HepG2 (human hepatocellular carcinoma) cells were bought from Boster Bio-Engineering Limited Company (China).

4.2. Synthesis

4-(1*H*-Imidazo[4,5-*f*]-1,10-phenanthrolin-2-yl)phenol (*p*-hpip) the 1,10-phenanthroline-5,6-dione was prepared by oxidation of 1,10-phenanthroline following the literature methods,[48–50] and the product was recrystallized from methanol. For *p*-hpip,[50–52] a mixture of 4-hydroxybenzaldehyde (3.5 mmol, 0.43 g), 1,10-phenanthroline-5,6-dione (2.5 mmol, 0.53 g), ammonium acetate (50 mmol, 3.9 g) and glacial acetic acid (7 mL) were refluxed for 2 h. The cooled deep red solution was diluted with 25 mL water, and neutralized with ammonium hydroxide to obtain the precipitates. This mixture was then filtered and the precipitates were washed with water, dried and recrystallized from ethanol and acetonitrile to get *p*-hpip as a pale yellow solid (0.65 g, 84%). ^{1}H NMR (300 MHz, DMSO-d_6): δ (ppm) 13.52 (s, 1H), 9.98 (s, 1H), 9.03 (d, 2H), 8.94 (d, J = 7.8 Hz, 2H), 8.13 (d, J = 8.4 Hz, 2H), 7.84 (m, 2H), 6.99 (d, J = 8.4 Hz, 2H). ^{13}C NMR (75 MHz, DMSO-d_6): δ (ppm) 167.1, 159.3, 155.6, 151.4, 137.7, 136.1, 131.3, 129.2, 123.9.

4.2.1. 2-[4-(1*H*-Imidazo[4,5-*f*]-1,10-phenanthrolin-2-yl)phenoxy]-*N*,*N*-dimethyl-ethanamine (1)

NaH (60% dispersion in mineral oil) (3.43 mmol, 0.13 g) was added to the solution of *p*-hpip (0.7 mmol, 0.21 g) in dry and degassed DMF (20 mL), and the resulting mixture was heated with stirring for 30 min at 110 °C under N_2 atmosphere. A solution of 2-dimethylaminoethyl chloride hydrochloride (0.7 mmol, 0.20 g) in DMF (40 mL) was added dropwise to the resulting mixture over a period of 1 h, during which time the reaction solution turned from deep red to transparent brown-orange. This solution was further refluxed for 72 h under nitrogen. After the reaction mixture was cooled, the solid was filtered off and the filtrate was concentrated on a rotary evaporator to yield a brown-yellow solid. This solid was dissolved in methanol, to which either HCl (aq) or NEt_3 was added until the pH value of solution reached 7, the resulting solution was evaporated under reduced pressure to obtain a solid. This solid was purified by chromatographic column on silica gel using MeOH–$CHCl_3$–glacial acetic acid (1:2:0.02, v/v/v) as an eluent to give the compound **1** as a yellow solid (38%, 0.1 g). ^{1}H NMR (300 MHz, DMSO-d_6): δ (ppm) 9.01 (m, 4H), 8.29 (d, J = 8.4 Hz, 2H), 7.82 (m, 2H), 7.14 (d, J = 8.4 Hz, 2H), 4.16 (m, 2H), 2.67 (m, 2H), 2.25 (m, 6H). ^{13}C NMR (75 MHz, DMSO-d_6): δ (ppm) 160.0, 151.9, 148.0, 143.5, 130.6, 128.5, 123.8, 122.4, 115.2, 66.4, 58.1, 46.0. IR (KBr, cm^{-1}): 3411, 2939, 2769, 1577, 1523, 1483, 1448, 1352, 1249, 1180, 1031, 837, 804, 740, 649. ESI(+)-MS (*m*/*z*): 384.58 (100%), $[M+H]^+$; 789.33 (100%), $[2M+Na]^+$. Anal. Calcd for $C_{23}H_{21}ON_5$: C, 72.05; H, 5.52; N, 18.26. Found: C, 72.10; H, 5.38; N, 18.38.

4.2.2. 2-[4-[2-(1-Piperidyl)ethoxy]phenyl]-1*H*-imidazo[4,5-*f*]-1,10-phenanthroline (2)

Compound **2** was prepared from ligand *p*-hpip and *N*-(2-chloroethyl)piperidine hydrochloride as described for compound **1** above. The product was purified by chromatographic column on silica gel using MeOH–$CHCl_3$–glacial acetic acid (1:2:0.02, v/v/v) as an eluent. Yellow solid (0.08 g, 29%). ^{1}H NMR (300 MHz, DMSO-d_6): δ (ppm) 8.99–8.92 (m, 4H), 8.31 (d, J = 8.1 Hz 2H), 7.75 (m, 2H), 7.07 (d, J = 8.1 Hz, 2H), 4.13 (m, 2H), 2.67 (m, 6H), 1.49 (m, 4H), 1.37 (m, 2H). ^{13}C NMR (75 MHz, DMSO-d_6): δ (ppm) 159.2, 151.6, 147.3, 142.5, 130.4, 127.9, 123.2, 121.7, 114.4, 65.4, 57.1, 54.2, 25.3, 23.7. IR (KBr, cm^{-1}): 3411, 2933, 2852, 1577, 1526, 1483, 1443, 1413, 1359, 1251, 1180, 1043, 1012, 837, 740, 650. ESI(+)-MS (*m*/*z*): 424.50 (58%), $[M+H]^+$; 446.42 (64%), $[M+Na]^+$; 869.33 (100%), $[2M+Na]^+$. Anal. Calcd for $C_{26}H_{25}ON_5$: C, 73.73; H, 5.95; N, 16.54. Found: C, 73.57; H, 5.83; N, 16.73.

4.2.3. 2-[4-(2-Pyrrolidin-1-ylethoxy)phenyl]-1*H*-imidazo[4,5-*f*]-1,10-phenanthroline (3)

Compound **3** was prepared from ligand *p*-hpip and *N*-(2-chloroethyl)pyrrolidine hydrochloride as described for compound **1** above. The product was purified by chromatographic column on silica gel using MeOH–$CHCl_3$–glacial acetic acid (1:4:0.02, v/v/v)

as an eluent. Yellow solid (0.07 g, 25%). ^{1}H NMR (300 MHz, DMSO-d_6): δ (ppm) 9.05 (m, 2H), 9.00 (m, 2H), 8.36 (d, J = 8.4 Hz, 2H), 7.84 (m, 2H), 7.13 (d, J = 8.4 Hz, 2H), 4.17 (t, J = 5.7 Hz, 2H), 2.83 (t, J = 5.7 Hz, 2H), 2.55 (m, 4H), 1.70 (m, 4H).^{13}C NMR (75 MHz, DMSO-d_6): δ (ppm) 161.9, 153.4, 149.5, 142.5, 130.6, 126.4, 124.6, 121.7, 117.2, 68.9, 56.4, 31.6, 25.5. IR (KBr, cm^{-1}): 3411, 2925, 2873, 1579, 1524, 1481, 1452, 1402, 1359, 1249, 1178, 1074, 1045, 835, 740, 659. ESI(+)-MS (m/z): 410.42 (100%), [M+H]$^+$. Anal. Calcd for $C_{25}H_{23}ON_5$: C, 73.34; H, 5.66; N, 17.10. Found: C, 73.18; H, 5.57; N, 17.31.

4.2.4. 4-[2-[4-(1*H*-Imidazo[4,5-*f*]-1,10-phenanthrolin-2-yl)phenoxy]ethyl]morpholine (4)

Compound **4** was prepared from ligand *p*-hpip and 4-(2-chloroethyl)morpholine hydrochloride as described for compound **1** above. The product was purified by chromatographic column on silica gel using C_2H_5OH–glacial acetic acid (1:0.02, v/v) and MeOH–$CHCl_3$–glacial acetic acid (1:4:0.02, v/v/v) as an eluent. Yellow solid (0.09 g, 31%). ^{1}H NMR (300 MHz, DMSO-d_6): δ (ppm) 9.00 (m, 4H), 8.32 (m, 2H), 7.78 (m, 2H), 7.09 (m, 2H), 4.18 (m, 2H), 3.61 (m, 4H), 2.70 (m, 2H), 2.28 (m, 4H). ^{13}C NMR (75 MHz, DMSO-d_6): δ (ppm) 161.8, 153.1, 148.6, 143.1, 133.6, 129.9, 123.1, 120.0, 116.6, 68.1, 67.4, 58.9, 57.9. IR (KBr, cm^{-1}): 3421, 2931, 2854, 1580, 1524, 1446, 1427, 1344, 1249, 1178, 1116, 1024, 948, 835, 742, 663. ESI(+)-MS (m/z): 245.42 (100%), [M+2Na+H+H_2O]$^{3+}$; 973.17 (58%), [2M+5H_2O+CH_3OH]. Anal. Calcd for $C_{25}H_{23}O_2N_5$: C, 70.57; H, 5.45; N, 16.46. Found: C, 70.33; H, 5.32; N, 16.69.

4.3. FRET melting assay

FRET melting 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 slits were 5 nm. The G-quadruplex-forming sequences comprise the human telomeric DNA (*h*-telo, 5′-FAM-d($G_3[T_2AG_3]_3$)-TAMRA-3′) and two promoter DNAs (*c*-kit2, 5′-FAM-d($CG_3CG_3CGCGAG_3AG_4$)-TAMRA-3′, and *c*-myc, 5′-FAM-d($TGAG_3TG_4AG_3TG_4A_2$)-TAMRA-3′. Donor fluorophore FAM: 6-carboxyfluorescein, and acceptor fluorophore TAMRA: 6-carboxytetramethylrhodamine), which was diluted from stock to the correct concentration (400 nM) in 10 mM K_2HPO_4/KH_2PO_4 buffer (PBS) (pH 7.4) containing either 100 mM KCl (for *h*-telo and *c*-kit2) or 40 mM KCl (for *c*-myc) and then annealed by heating to 90 °C for 5 min, followed by cooling to room temperature. Because the T_m value of *c*-myc G-quadruplex alone is above 80 °C in buffer containing 100 mM KCl, it is difficult to measure accurately their T_m values in the presence of compounds, thus the buffer solution containing 40 mM KCl was used for *c*-myc FRET experiment. Samples were prepared by adding aliquoting 1 mL of the annealed G-quadruplex (at 2× concentration, 400 nM) into cells, followed by addition of buffer or the compound solutions (at 2× concentration) 1 mL, and further incubated for 1 h, thus the final concentration of G-quadruplex is 0.2 μM. The melting of G-quadruplex was monitored in the presence of various concentrations of compounds (0, 1, 2, and 3 μM) without and with 2.0 and 5.0 μM double stranded DNA (ds26, 5′-CAATCGGATCGAATTCGATCCGATTG-3′) competitor. Fluorescence readings at 533 nm were recorded at intervals of 2–3 °C over the range 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.).

4.4. Absorption spectroscopy

Absorption spectra were measured on a Cary 50 spectrophotometer with a 1 cm path length quartz cell. Absorption titrations were carried out by the stepwise addition of either ctDNA or G-quadruplex DNA solution to a cell containing 30, 30, 20, and 25 μM compounds **1**, **2**, **3,** and **4**, respectively, and the spectrum was collected after equilibration for 10 min at 25 °C. Prior to use, the oligomers *h*-telo (d[$AG_3(T_2AG_3)_3$]), *c*-kit2 ([$CG_3CG_3CGCGAG_3AG_4$]), and *c*-myc (d[$TGAG_3TG_4AG_3TG_4A_2$]) dissolved in PBS buffer containing 100 mM KCl were heated to 95 °C for 5 min, then gradually cooled to room temperature and incubated at 4 °C overnight. The formation of G-quadruplex was confirmed by CD spectra. CtDNA was dissolved in buffer and kept at 4 °C for 48 h, a solution of ctDNA in the buffer gave a ratio of UV absorbances of about 1.9 at 260 and 280 nm, indicating that the DNA is sufficiently free of protein. The titration was terminated when the wavelength and intensity of the absorption band for compounds did not change any more upon three successive additions of DNA. The binding constant, K_b, was determined from a [DNA]/($\varepsilon_a-\varepsilon_f$) versus 1/($\varepsilon_b-\varepsilon_f$) plot according to

$$[DNA]/(\varepsilon_a - \varepsilon_f) = [DNA]/(\varepsilon_b - \varepsilon_f) + 1/[(\varepsilon_b - \varepsilon_f)K_b], \quad (1)$$

where [DNA] is the concentration of DNA in base pairs for ctDNA and quartets for G-quadruplex DNA, ε_a is the extinction coefficient observed for the spectral band at a given DNA concentration, ε_f is the extinction coefficient of the compound free in solution, ε_b is the extinction coefficient of the compound when fully bound to DNA.

4.5. Competition dialysis analysis

A PBS buffer containing 100 mM KCl (pH 7.4) was used for all experiments. For each competition dialysis assay, 400 mL of dialysis solution containing 1 μM compounds **1**, **2,** or **3** was placed into a beaker. A volume of 0.5 mL at 40 μM monomeric unit of each of the DNA samples was pipetted into a separate 0.5 mL dialysis bag. The entire dialysis bag was then placed in the beaker containing the dialysis solution. The contents were allowed to equilibrate with continuous stirring for 24 h at 22–23 °C. After equilibration, DNA samples were carefully removed to microfuge tubes and were treated with a final concentration of 1% (w/v) triton x-100. The total concentration of compound within each dialysis bag was then determined by absorption spectroscopy using extinction coefficients at 350 nm of 5005, 2490, and 3302 M^{-1} cm^{-1} for compounds **1**, **2,** and **3**, respectively. An appropriate correction was made to account for volume changes due to the addition of triton x-100 solution. The amount of bound drug was determined by difference ($C_b = C_t - C_f$, where C_f is the concentration of bulk drug solution, C_t is the total drug concentration that enters the dialysis bag, and C_b is the concentration of drug bound to the nucleic acid). The data were plotted as a bar graph using Origin 7.5. Competitive dialysis for every sample was repeated at least three times.

4.6. CD spectroscopy

CD spectra were recorded on a dual-beam 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 cm 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. G-quadruplex or oligomer at a final concentration of 5 μM in the presence of various concentrations of the compounds was tested. After each addition of the compound, the reaction was stirred and allowed to equilibrate for at least 10 min (until no elliptic changes were observed) and the CD spectrum was collected. A background CD spectrum of corresponding buffer solution was subtracted from the average scan for each sample. Final analysis of the data was carried out using Origin 7.5.

4.7. TRAP assay

The ability of compounds to inhibit telomerase in a cell-free system was assessed with the TRAP-LIG assay following previously published procedures.[39,57] 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 in various concentrations. 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 subjected 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-staining.

4.8. MTT assay

HeLa and HepG2 cells were cultivated in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS) (v/v). Cells in the logarithmic growth-phase was seeded on 96-well culture plates (2.5×10^3/well) in 200 μL volumes. Compounds with various concentrations were inserted into the 96-well plates. Five parallel wells were set for each of the treated or control groups. After 72 h treatment at 37 °C in a humidified atmosphere of 5% CO_2, 20 μL of 5 mg/mL 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.

4.9. Flow cytometric assay

HeLa or HepG2 cells were seeded on 60 mm culture dish at 2.5×10^5 cells per dish in DMEM containing 10% (v/v) FBS and incubated for 20 h in a humidified atmosphere with 5% CO_2. Then HeLa and HepG2 cells were treated with compounds at their corresponding IC_{50} concentrations for 48 and 30 h in DMEM supplemented with 10% FBS, respectively. Untreated cells were also included in this experiment for comparison. After treatments, the cells were harvested and washed twice with 4 °C PBS and then fixed in 4 °C PBS (500 μL) and ice-cold 75% ethanol (2 mL) at −20 °C overnight. Before measurement the fixed cells were centrifuged and washed twice with PBS, followed by resuspension in PBS containing 7.5 μM propidium iodide (PI) and 100 μg/mL RNase A at 37 °C for 30 min in the dark. DNA content and subG_0/G_1 peak were analyzed with flow cytometer (BeckMan Coulter CYTOMICS FC-500). At least 10,000 cells were measured for each analysis.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bmc.2012.11.059.

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