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Original article

Synthesis of 2,6-diaryl-substituted pyridines and their antitumor activities

Jong-Keun Son [a], Long-Xuan Zhao [b], Arjun Basnet [a], Pritam Thapa [a], Radha Karki [a], Younghwa Na [c], Yurngdong Jahng [a], Tae Cheon Jeong [a], Byeong-Seon Jeong [a], Chong-Soon Lee [d], Eung-Seok Lee [a,*]

[a] College of Pharmacy, Yeungnam University, Kyongsan 712-749, South Korea
[b] Department of Chemistry, Liaoning Normal University, Dalian, Liaoning 116-029, PR China
[c] College of Pharmacy, Catholic University of Daegu, Kyongsan 712-702, South Korea
[d] Department of Biochemistry, Yeungnam University, Kyongsan 712-749, South Korea

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Abstract

For the development of novel antitumor agents, we designed and synthesized 2,6-diaryl-substituted pyridine derivatives bearing three aryl groups, which are the bioisosteres of terpyridine, and evaluated their biological activities. Most of the 18 prepared compounds showed moderate cytotoxicity against several human cancer cell lines. From the structure–activity relationships we may conclude that the number of aryl groups employed would be critical for their biological activities.

Keywords: Terpyridine; Cytotoxicity; Topoisomerase I inhibition; Anticancer agent; Bioisostere

1. Introduction

Terpyridine has been extensively studied as a ligand in a wide range of metal complexes [1,2] and DNA binding agents [3–6]. Metal complexes of terpyridine can efficiently intercalate into nucleic acid [7]. Recently, we reported that terpyridine derivatives showed a strong cytotoxicity against several human cancer cell lines as well as considerable topoisomerase I inhibitory activity [8–12]. Although the cytotoxicity and topoisomerase I inhibitory activity of terpyridine derivatives have been reported [9–12], a systematic study on terpyridine isosteres has not been evaluated yet. From the results of the previous studies, terpyridine isosteres employing four aryl groups showed strong cytotoxicity against several human cancer cell lines or strong topoisomerase I or II inhibitory activity [9,10,12]. However, terpyridine derivatives employing three aryl groups showed relatively weaker cytotoxicity and topoisomerase I or II inhibitory activity [11]. It would be very interesting to design and prepare terpyridine isosteres employing three aryl groups and to evaluate cytotoxicity and topoisomerase I inhibitory activity. In addition, we anticipated that we may obtain valuable information on the correlation between the number of aryl groups employed in terpyridine isosteres and biological activities such as cytotoxicity and topoisomerase I inhibitory activity. In connection with the previous studies, in the present study, we designed and prepared 18 terpyridine bioisosteres employing three aryl groups which have aromatic substituents including phenyl, furyl, thienyl or pyridyl units at 2,6-position on pyridine, and evaluated them for their cytotoxicity against several human cancer cell lines and topoisomerase I inhibitory activity (Fig. 1).

2. Chemistry

Synthetic methods for the preparation *via* the modified Kröhnke synthesis [13,14] of 2,6-diaryl-substituted pyridine

* Corresponding author. Tel.: +82 53 810 2827; fax: +82 53 810 4654.
E-mail address: eslee@yu.ac.kr (E.-S. Lee).

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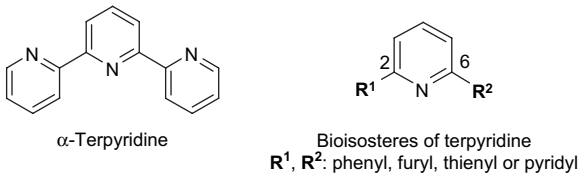


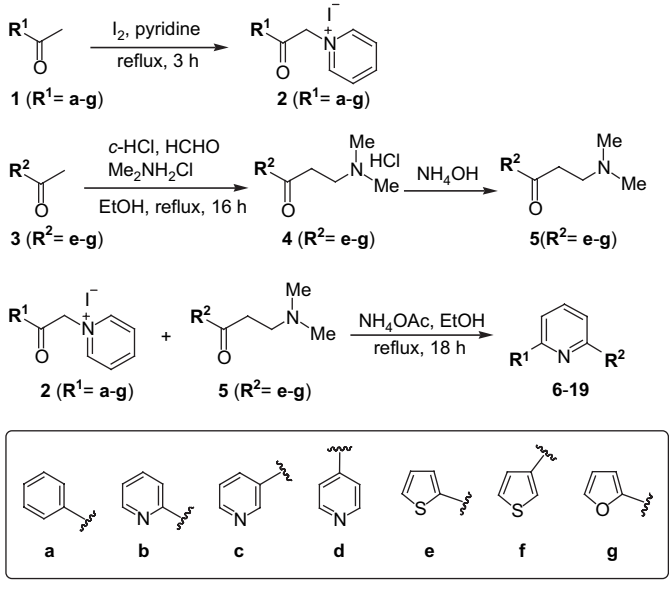
Fig. 1. Structures of α-terpyridine and 2,6-diaromatic substituted pyridine derivatives.

derivatives are summarized in Scheme 1. Treatment of aryl methyl ketone **1** with pyridine-I_2 by refluxing afforded the desired pyridinium salts **2** [15]. The reaction of aryl methyl ketone **3** with Me_2NH_2Cl in the presence of conc. HCl and paraformaldehyde in absolute EtOH generated the Mannich base hydrochlorides **4** [16], which were neutralized by addition of ammonium hydroxide solution to afford the Mannich bases **5**. Subsequent addition reaction with **2** and **5** in the presence of NH_4OAc in hot EtOH afforded compounds **6**–**19** in 20.0–43.3% of overall yields.

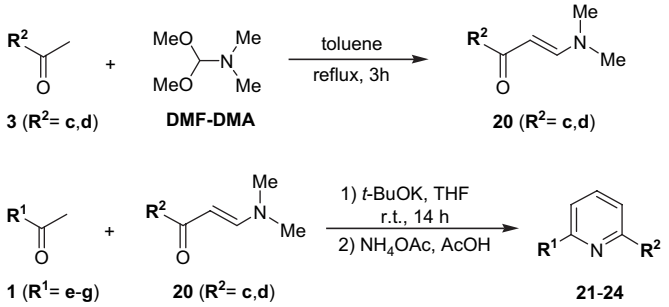
Since some of the products **21**–**24** starting from 3- or 4-acetyl pyridine **3** ($\mathbf{R^2} = \mathbf{c}$, **d**) were not prepared by utilizing pyridinium salt like **2**, a different synthetic method [17] was accomplished as shown in Scheme 2. A toluene solution of **3** ($\mathbf{R^2} = \mathbf{c}$, **d**) in the presence of DMF–DMA was refluxed for 3 h to yield enaminone intermediates **20**. The solution of potassium *tert*-butoxide and **1** ($\mathbf{R^2} = \mathbf{e}$–**g**) in anhydrous THF was stirred at room temperature for 2 h, and each enaminone intermediate **20** was added to the solution and allowed to stir at the same temperature for 14 h. To the mixture were added NH_4OAc and acetic acid to afford products **21**–**24** in 20.5–29.3% of overall yields.

3. Results and discussion

The synthetic method for the preparation of 2,6-diaromatic substituted pyridine derivatives was relatively easy and simple,



Scheme 1. Synthetic pathway for the 2,6-diaryl pyridines **6**–**19**.



Scheme 2. Synthetic pathway for the 2,6-diaryl pyridines **21**–**24**.

and the compounds could be obtained in short steps. The 18 prepared compounds are shown in Fig. 2.

The HPLC results of the prepared compounds displayed that they had high purity in a range of 96.7–100.0%. The data of ^{1}H NMR, ^{13}C NMR, ESI LC/MS, HPLC including retention time, melting point and yield for final products are shown in Table 1.

For the evaluation of antitumor cytotoxicity, four different human cancer cell lines were utilized: A549 (human lung carcinoma), SK-OV-3 (human ovary adenocarcinoma), SK-MEL-2 (human malignant melanoma), and HCT15 (human colon adenocarcinoma). The IC_{50} values of 2,6-diaryl-substituted pyridine derivatives **6**–**19** and **21**–**24** against above human cancer cell lines are shown in Table 2. Most of the prepared compounds showed moderate cytotoxicity, generally 10–80 μM, against human cancer cell lines, although they are less potent than α-terpyridine and doxorubicin used as control references. However, the prepared compounds did not exhibit significant topoisomerase I inhibitory activity. Some of the topoisomerase I inhibitory results are shown in Fig. 3.

In comparison with previous study, terpyridine bioisosteres employing four aryl groups indicated relatively strong cytotoxicity and topoisomerase I or II inhibitory activity [9,10,12]. Some of the prepared compounds showed much stronger cytotoxicity than doxorubicin and α-terpyridine, or much stronger topoisomerase I inhibition than camptothecin. However, terpyridine derivatives employing three aryl groups showed relatively weaker cytotoxicity and topopisomerase I inhibition [11]. Since the prepared compounds in this study were employed three aryl groups, the biological activity would be lower than four aryl groups. From the structure–activity relationships we may conclude that the number of aryl groups employed would be critical for their biological activities.

In conclusion, we have designed and prepared the eighteen 2,6-diaryl-substituted pyridine derivatives employing three aryl groups in connection with previous study, and evaluated their antitumor cytotoxicity and topoisomerase I inhibitory activity. Most of the prepared compounds showed moderate cytotoxicity against several human cancer cell lines although they are less potent than control references. But the prepared compounds did not exhibit significant topoisomerase I inhibitory activity. From the structure–activity relationships we may conclude that the number of aryl groups employed would be critical for their biological activities. This study may provide

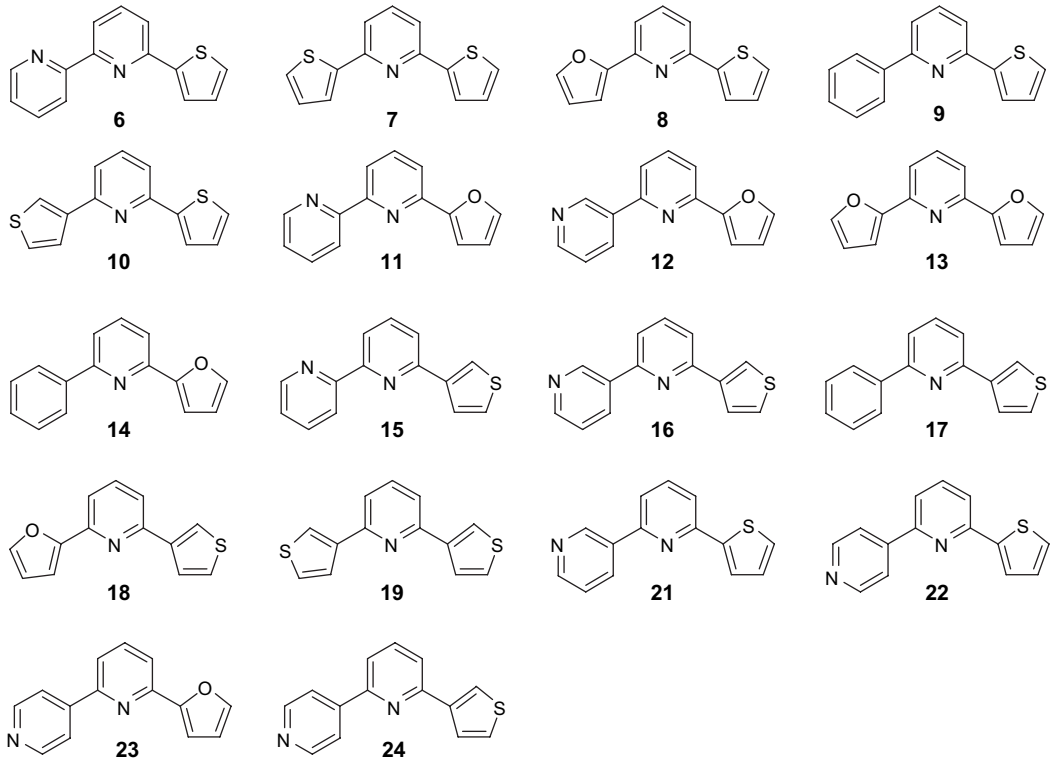


Fig. 2. Structures of the prepared compounds.

valuable information to the researchers working on the development of antitumor agents. Structure–activity relationship study of the 2,6-diaryl-substituted pyridine derivatives indicates that there is not a significant correlation according to substituted aromatic compounds, but in general furan-containing compounds **8**, **11**–**13** and **23** showed relatively weaker cytotoxicity.

4. Experimental

4.1. Materials and methods

Compounds used as starting materials and reagents were purchased from Aldrich Chemical Co., Fluka, Tokyokasei, Junsei and were used without further purification. Thin-layer chromatography (TLC) and column chromatography were performed with Kieselgel 60 F_{254} (Merck) and silica gel Kieselgel 60, (230–400 mesh, Merck), respectively. All compounds containing aromatic ring were visualized on TLC plates with UV light. Nuclear magnetic resonance (NMR) spectra were taken on a Bruker AMX 250 MHz for ^{1}H NMR and 62.5 MHz for ^{13}C NMR, and tetramethylsilane (TMS) was used as an internal standard. Chemical shifts (δ) were recorded in parts per million, and coupling constants (J) in hertz. Melting points were determined in open capillary tubes on electrothermal 1A 9100 digital melting point apparatus and were uncorrected. HPLC analyses were performed in Beckman HP controller and Beckmann UV detector using Beckmann ultraspere HPLC column (4.6 mm × 150 mm, 5 μm Microsorb C-18 column) with a 20 μL injection volume with a gradient elution of 50% B in A to 100% B at a flow rate of 1.0 mL/min at 254 nm UV detection, where mobile phase A was doubly distilled water and mobile phase B was 90% acetonitrile in water. Retention time was given in minutes, and purity of compounds was described as percent (%). ESI LC/MS analyses were performed with a Finnigan LCQ Advantage® LC/MS/MS spectrometry utilizing Xcalibur® program. For ESI LC/MS, LC was performed with a 5 μL injection volume on a GL Science® 5 μm ODS reverse-phase C18 LC/MS column (150 × 1.5 mm, i.d.) with a gradient elution of 10% B in A to 90% B in A for 10 min and retaining 90% B in A for 10 min at a flow rate of 180 μL/min, where mobile phase A was 0.1% formic acid in water and mobile phase B was 0.1% formic acid in acetonitrile. MS ionization conditions were as following: Sheath gas flow rate: 70 arb, Aux gas flow rate: 20 arb, I spray voltage: 4.5 kV, capillary temp.: 215 °C, capillary voltage: 21 V, tube lens offset: 10 V.

*4.1.1. General procedure for the preparation of compounds **2***

A mixture of **1a**–**g** (20 mmol) and I_2 (20 mmol) in pyridine (25 mL) was refluxed for 3 h. After cooling the mixture to 20 °C, the precipitate was filtered and washed with cold pyridine several times, which was used in the next step without further purification.

*4.1.1.1. 1-(2-Oxo-phenylethyl)pyridinium iodide **2a***. Light brown solid; yield 99.9%; mp 175.0–179.0 °C; ^{1}H NMR (250 MHz, $CDCl_3$) δ 9.00 (d, $J = 6.5$ Hz, 2H, pyridinium H-2, H-6), 8.74 (dt, $J = 7.8$, 1.2 Hz, 1H, pyridinium H-4),

Table 1
Spectral data of the prepared compounds

Compd. no.	Purity by HPLC: % (retention time: min)	^{1}H NMR (200 MHz), δ (ppm), J (Hz)/^{13}C NMR (62.5 MHz), δ (ppm)/ESI LC/MS ($[MH]^+$)/melting point (°C)/total yield (%)
6	100.0 (13.5 min)	8.68 (ddd, $J = 4.8$, 1.8, 0.9 Hz, 1H, pyridine H-6′), 8.58 (ddd, $J = 7.8$, 1.2, 0.9 Hz, 1H, pyridine H-3′), 8.29 (dd, $J = 7.8$, 0.9 Hz, 1H, pyridine H-3), 7.85 (dt, $J = 7.8$, 1.8 Hz, 1H, pyridine H-4′), 7.82 (t, $J = 7.8$ Hz, 1H, pyridine H-4), 7.67 (dd, $J = 7.8$, 0.9 Hz, 1H, pyridine H-5), 7.66 (dd, $J = 3.6$, 1.1 Hz, 1H, thiophene H-5), 7.42 (dd, $J = 5.1$, 1.1 Hz, 1H, thiophene H-3), 7.32 (ddd, $J = 7.8$, 4.8, 1.2 Hz, 1H, pyridine H-5′), 7.14 (dd, $J = 5.1$, 3.7 Hz, 1H, thiophene H-4)/155.81, 155.52, 151.79, 148.99, 145.24, 137.61, 136.91, 127.99, 127.56, 124.50, 123.81, 121.31, 118.99, 118.56/239/76.2 °C/27.5%
7	97.3 (14.4 min)	7.63 (dd, $J = 8.5$, 7.2 Hz, 1H, pyridine H-4), 7.62 (dd, $J = 3.7$, 1.1 Hz, 2H, thiophene H-5, H-5′), 7.47 (d, $J = 7.8$ Hz, 2H, pyridine H-3, H-5), 7.40 (dd, $J = 5.0$, 1.1 Hz, 2H, thiophene H-3, H-3′), 7.11 (dd, $J = 5.0$, 3.7 Hz, 2H, thiophene H-4, H-4′)/152.09, 144.83, 137.28, 127.91, 127.73, 124.68, 116.66/244/75.1° C/24.0%
8	98.8 (14.8 min)	7.69 (t, $J = 7.8$ Hz, 1H, pyridine H-4), 7.61 (dd, $J = 3.7$, 1.1 Hz, 1H, thiophene H-5), 7.53 (dd, $J = 7.8$, 0.9 Hz, 1H, pyridine H-3), 7.52 (dd, $J = 1.8$, 0.8 Hz, 1H, furan H-5), 7.49 (dd, $J = 7.8$, 0.9 Hz, 1H, pyridine H-5), 7.39 (dd, $J = 5.1$, 1.1 Hz, 1H, thiophene H-3), 7.17 (dd, $J = 3.4$, 0.7 Hz, 1H, furan H-3), 7.10 (dd, $J = 5.1$, 3.7 Hz, 1H, thiophene H-4), 6.53 (dd, $J = 3.4$, 1.8 Hz, 1H, furan H-4)/153.64, 152.20, 148.98, 144.92, 143.22, 137.22, 127.90, 127.60, 124.65, 116.85, 116.40, 112.02, 108.98/228/84.3 °C/38.7%
9	99.0 (16.6 min)	8.12 (dd, $J = 7.8$, 1.1 Hz, 2H, pyridine H-3, H-5), 7.74 (t, $J = 7.8$ Hz, 1H, pyridine H-4), 7.65 (dd, $J = 3.6$, 1.0 Hz, 1H, thiophene H-5), 7.62–7.57 (m, 2H, phenyl H-2, H-6), 7.53–7.44 (m, 3H, phenyl H-3, H-4, H-5), 7.25 (dd, $J = 5.0$, 1.0 Hz, 1H, thiophene H-3), 7.12 (dd, $J = 5.0$, 3.7 Hz, 1H, thiophene H-4)/157.52, 153.09, 146.33, 139.78, 138.25, 129.96, 129.55, 128.79, 128.46, 127.76, 125.35, 119.14, 117.82/238/74.8 °C/25.0%
10	100.0 (16.5 min)	8.00 (dd, $J = 3.0$, 1.0 Hz, 1H, thiophene H-5), 7.74 (dd, $J = 5.0$, 1.0 Hz, 1H, thiophene H-3), 7.70 (t, $J = 7.8$ Hz, 1H, pyridine H-4), 7.62 (d, $J = 3.7$ Hz, 1H, thiophene H-2′), 7.50 (dd, $J = 13.7$, 7.8 Hz, 2H, pyridine H-3, H-5), 7.40 (dd, $J = 5.0$, 0.7 Hz, 1H, thiophene H-5′), 7.40 (dd, $J = 5.0$, 2.1 Hz, 1H, thiophene H-4′), 7.08 (dd, $J = 5.0$, 3.7 Hz, 1H, thiophene H-4)/152.91, 152.18, 145.32, 141.99, 137.34, 127.91, 127.60, 126.28, 126.15, 124.46, 123.81, 118.12, 116.62/244/104.1 °C/39.0%
11	95.9 (11.5 min)	8.68 (ddd, $J = 4.8$, 1.7, 0.9 Hz, 1H, pyridine H-6′), 8.54 (ddd, $J = 7.7$, 1.2, 0.9 Hz, 1H, pyridine H-3′), 8.29 (dd, $J = 7.8$, 1.0 Hz, 1H, pyridine H-3), 7.85 (t, $J = 7.8$ Hz, 1H, pyridine H-4), 7.84 (dt, $J = 7.7$, 1.7 Hz, 1H, pyridine H-4′), 7.71 (dd, $J = 7.8$, 1.0 Hz, 1H, pyridine H-5), 7.56 (dd, $J = 1.8$ Hz, 0.7 Hz, 1H, furan H-5), 7.32 (ddd, $J = 7.7$, 4.8, 1.2 Hz, 1H, pyridine H-5′), 7.19 (dd, $J = 3.4$, 0.7 Hz, 1H, furan H-3), 6.56 (dd, $J = 3.4$, 1.8 Hz, 1H, furan H-4)/156.00, 155.70, 153.93, 149.04, 148.76, 143.22, 137.55, 136.84, 123.77, 121.24, 119.15, 118.39, 112.00, 108.62/223/52.5 °C/21.3%
12	97.4 (8.6 min)	9.27 (dd, $J = 2.2$, 0.7 Hz, 1H, pyridine H-2′), 8.66 (dd, $J = 4.8$, 1.6 Hz, 1H, pyridine H-4′), 8.40 (ddd, $J = 8.0$, 2.1, 1.8 Hz, 1H, pyridine H-5′), 7.82 (t, $J = 7.8$ Hz, 1H, pyridine H-4), 7.69 (dd, $J = 7.8$, 0.9 Hz, 1H, pyridine H-3), 7.62 (dd, $J = 7.7$, 0.9 Hz, 1H, pyridine H-5), 7.56 (dd, $J = 1.7$, 0.7 Hz, 1H, furan H-5), 7.43 (ddd, $J = 8.0$, 4.8, 0.8 Hz, 1H, pyridine H-6′), 7.20 (dd, $J = 3.4$, 0.7 Hz, 1H, furan H-3), 6.54 (dd, $J = 3.4$, 1.8 Hz, 1H, furan H-4)/154.34, 153.69, 149.93, 149.59, 148.33, 143.43, 137.61, 134.68, 134.39. 123.53, 118.51, 117.45, 112.11, 109.09/223/82.8 °C/20.0%
13	100.0 (10.8 min)	7.73 (dd, $J = 8.5$, 7.2 Hz, 1H, pyridine H-4), 7.55 (d, $J = 7.8$ Hz, 2H, pyridine H-3, H-5), 7.54 (d, $J = 0.8$ Hz, 2H, furan H-5, H-5′), 7.14 (dd, $J = 3.4$, 0.7 Hz, 2H, furan H-3, H-3′), 6.53 (dd, $J = 3.4$, 1.8 Hz, 2H, furan H-4, H-4′)/153.67, 149.13, 143.27, 137.19, 116.68, 111.98, 108.87/212/92.3 °C/36.6%
14	100.0 (15.6 min)	8.08 (dd, $J = 8.1$, 1.5 Hz, 2H, pyridine H-3, H-5), 7.76 (t, $J = 7.8$ Hz, 1H, pyridine H-4), 7.64–7.58 (m, 2H, phenyl H-2, H-6), 7.54 (dd, $J = 1.8$, 0.8 Hz, 1H, furan H-5), 7.52–7.41 (m, 3H, phenyl H-3, H-4, H-5), 7.19 (dd, $J = 3.4$, 0.8 Hz, 1H, furan H-3), 6.54 (dd, $J = 3.4$, 1.8 Hz, 1H, furan H-4)/156.93, 154.07, 149.19, 143.16, 139.20, 137.31, 129.50, 129.00, 128.65, 128.28, 126.95, 118.54, 116.74, 112.00, 108.72/222/oil/26.2%
15	96.7 (11.1 min)	8.69 (ddd, $J = 4.8$, 1.5, 0.8 Hz, 1H, pyridine H-6′), 8.59 (d, $J = 8.0$ Hz, 1H, pyridine H-3′), 8.31 (dd, $J = 7.8$, 0.7 Hz, 1H, pyridine H-3), 8.02 (dd, $J = 3.0$, 1.2 Hz, 1H, thiophene H-2), 7.85 (t, $J = 7.8$ Hz, 1H, pyridine H-4), 7.81 (dd, $J = 5.1$, 1.2 Hz, 1H, pyridine H-4′), 7.80 (td, $J = 7.8$, 1.3 Hz, 1H, thiophene H-4), 7.65 (dd, $J = 7.8$, 0.8 Hz, 1H, pyridine H-5), 7.43 (dd, $J = 5.0$, 3.0 Hz, 1H, thiophene H-5), 7.32 (ddd, $J = 7.5$, 4.8, 1.1 Hz, 1H, pyridine H-5′)/156.21, 155.65, 152.67, 149.04, 142.34, 137.67, 136.84, 126.33, 126.22, 123.74, 123.55, 121.22, 120.12, 118.99/239/81.4 °C/24.8%
16	98.0 (8.5 min)	9.30 (d, $J = 2.0$ Hz, 1H, pyridine H-2′), 8.66 (dd, $J = 4.8$, 1.6 Hz, 1H, pyridine H-6′), 8.43 (dt, $J = 8.0$, 1.9 Hz, 1H, pyridine H-4′), 8.03 (dd, $J = 3.0$, 2.0 Hz, 1H, thiophene H-2), 7.82 (t, $J = 7.8$ Hz, 1H, pyridine H-4), 7.78 (dd, $J = 5.0$, 1.8 Hz, 1H, thiophene H-4), 7.65 (t, $J = 7.7$ Hz, 2H, pyridine H-3, H-5), 7.43 (dd, $J = 7.8$, 4.8 Hz, 1H, pyridine H-5′), 7.42 (dd, $J = 5.0$, 3.0 Hz, 1H, thiophene H-5)/154.21, 153.47, 149.88, 148.34, 142.05, 137.69, 134.78, 134.31, 126.33, 126.24, 123.90, 123.51, 119.15, 118.32/239/80.6 °C/27.0%
17	99.6 (15.3 min)	7.97 (dd, $J = 3.0$, 1.2 Hz, 1H, thiophene H-2), 7.73 (t, $J = 7.8$ Hz, 1H, pyridine H-4), 7.71 (dd, $J = 5.1$, 3.8 Hz, 1H, thiophene H-4), 7.57 (dd, $J = 7.8$, 0.9 Hz, 1H, pyridine H-3), 7.53 (dd, $J = 1.7$, 0.8 Hz, 1H, furan H-5), 7.47 (dd, $J = 7.8$, 0.9 Hz, 1H, pyridine H-5), 7.39 (dd, $J = 5.1$, 3.0 Hz, 1H, thiophene H-5), 7.16 (dd, $J = 3.4$, 0.7 Hz, 1H, furan H-3), 6.54 (dd, $J = 3.4$, 1.8 Hz, 1H, furan H-4)/156.80, 153.08, 142.50, 139.37, 137.42, 128.96, 128.65, 126.93, 126.38, 126.10, 123.61, 118.45, 118.33/238/73.4 °C/43.3%
18	100.0 (15.8 min)	8.14–8.10 (m, 2H, phenyl H-2, H-6), 8.02 (dd, $J = 3.0$, 1.2 Hz, 1H, thiophene H-2), 7.79 (dd, $J = 5.1$, 1.3 Hz, 1H, thiophene H-4), 7.77 (t, $J = 7.8$ Hz, 1H, pyridine H-4), 7.63 (dd, $J = 7.8$, 0.9 Hz, 1H, pyridine H-5), 7.56 (dd, $J = 7.7$, 0.9 Hz, 1H, pyridine H-3), 7.53–7.45 (m, 3H, phenyl H-3, H-4, H-5), 7.41 (dd, $J = 5.0$, 3.0 Hz, 1H, thiophene H-5)/153.96, 153.11, 149.13, 143.17, 142.17, 137.31, 126.31, 126.16, 123.74, 118.38, 116.46, 112.00, 108.68/228/105.7 °C/28.8%

Table 1 (*continued*)

Compd. no.	Purity by HPLC: % (retention time: min)	^{1}H NMR (200 MHz), δ (ppm), J (Hz)/^{13}C NMR (62.5 MHz), δ (ppm)/ESI LC/MS ([MH]$^+$)/melting point (°C)/total yield (%)
19	98.9 (14.2 min)	7.99 (dd, $J = 3.0$, 1.2 Hz, 2H, thiophene H-2, H-2′), 7.76 (dd, $J = 5.1$, 1.2 Hz, 2H, thiophene H-4, H-4′), 7.73 (dd, $J = 8.3$, 7.3 Hz, 1H, pyridine H-4), 7.51 (d, $J = 7.6$ Hz, 2H, pyridine H-3, H-5), 7.41 (dd, $J = 5.1$, 3.0 Hz, 2H, thiophene H-5, H-5′)/153.50, 142.84, 137.84, 126.77, 126.55, 124.02, 118.62/244/158.3 °C/30.3%
21	98.0 (8.9 min)	9.29 (d, $J = 1.6$ Hz, 1H, pyridine H-2′), 8.65 (dd, $J = 4.8$, 1.6 Hz, 1H, pyridine H-6′), 8.42 (dt, $J = 8.1$, 2.0 Hz, 1H, pyridine H-4′), 7.76 (t, $J = 7.8$ Hz, 1H, pyridine H-4), 7.63 (dd, $J = 3.7$, 1.1 Hz, 1H, thiophene H-5), 7.60 (dd, $J = 8.1$, 0.8 Hz, 1H, pyridine H-5′), 7.59 (dd, $J = 7.8$, 0.8 Hz, 1H, pyridine H-3), 7.42 (dd, $J = 5.1$ Hz, 1.1 Hz, 1H, thiophene H-3), 7.40 (dd, $J = 7.8$, 0.8 Hz, 1H, pyridine H-5), 7.12 (dd, $J = 5.1$, 3.7 Hz, 1H, thiophene H-4)/153.93, 152.53, 149.89, 148.18, 144.82, 137.56, 134.24, 134.22, 127.95, 127.87, 124.73, 123.47, 118.14, 117.57/239/78.9 °C/27.2%
22	100.0 (9.2 min)	8.74 (dd, $J = 4.5$, 1.6 Hz, 2H, pyridine H-2′, H-6′), 8.01 (dd, $J = 4.5$, 1.6 Hz, 2H, pyridine H-3′, H-5′), 7.81 (t, $J = 7.8$ Hz, 1H, pyridine H-4), 7.68 (dd, $J = 7.1$, 2.6 Hz, 2H, pyridine H-3, H-5), 7.66 (dd, $J = 3.7$, 1.1 Hz, 1H, thiophene H-5), 7.44 (dd, $J = 5.1$, 1.1 Hz, 1H, thiophene H-3), 7.14 (dd, $J = 5.1$, 3.7 Hz, 1H, thiophene H-4)/153.88, 152.75, 150.42, 145.89, 144.74, 137.73, 128.09, 128.07, 124.96, 120.97, 118.65, 118.58/239/88.2 °C/23.8%
23	99.7 (8.9 min)	8.74 (dd, $J = 4.5$, 1.6 Hz, 2H, pyridine H-2′, H-6′), 7.98 (dd, $J = 4.6$, 1.6 Hz, 2H, pyridine H-3′, H-5′), 7.85 (t, $J = 7.8$ Hz, 1H, pyridine H-4), 7.74 (dd, $J = 7.8$, 0.9 Hz, 1H, pyridine H-3), 7.67 (dd, $J = 7.8$, 0.9 Hz, 1H, pyridine H-5), 7.57 (dd, $J = 1.6$, 0.7 Hz, 1H, furan H-5), 7.21 (dd, $J = 3.4$, 0.5 Hz, 1H, furan H-3), 6.57 (dd, $J = 3.4$, 1.8 Hz, 1H, furan H-4)/154.56, 153.97, 150.82, 150.65, 150.07, 146.64, 143.98, 138.15, 121.62, 121.52, 119.22, 118.85, 112.59, 109.68/223/45.5 °C/29.3%
24	97.8 (12.3 min)	8.74 (dd, $J = 4.5$, 1.6 Hz, 2H, pyridine H-2′, H-6′), 8.03 (dd, $J = 3.0$, 1.1 Hz, 1H, thiophene H-2), 8.00 (dd, $J = 4.5$, 1.6 Hz, 2H, pyridine H-3′, H-5′), 7.80 (t, $J = 7.8$ Hz, 1H, pyridine H-4), 7.78 (dd, $J = 5.1$, 1.1 Hz, 1H, thiophene H-4), 7.68 (t, $J = 7.8$ Hz, 2H, pyridine H-3, H-5), 7.42 (dd, $J = 5.0$, 3.0 Hz, 1H, thiophene H-5)/153.98, 153.53, 150.38, 146.33, 141.92, 137.77, 126.41, 126.24, 124.04, 121.03, 120.13, 118.63/239/152.6 °C/20.5%

8.28 (t, $J = 6.8$ Hz, 2H, pyridinium H-3, H-5), 8.08–8.05 (m, 2H, phenyl H-2, H-6), 7.83–7.76 (m, 1H, phenyl H-4), 7.69–7.63 (m, 2H, phenyl H-3, H-5), 6.49 (s, 2H, $-CO-CH_2-$).

4.1.1.2. 1-[2-Oxo-2-(pyridine-2-yl)ethyl]pyridinium iodide ***2b***. Grey-black solid; yield 78.4%; mp > 300 °C; ^{1}H NMR (250 MHz, $CDCl_3$) δ 9.01 (d, $J = 6.4$ Hz, 2H, pyridinium H-2, H-6), 8.87 (dd, $J = 4.8$, 0.9 Hz, 1H, pyridine H-6), 8.73 (dt, $J = 7.8$, 1.2 Hz, 1H, pyridinium H-4), 8.28 (t, $J = 6.6$ Hz, 2H, pyridinium H-3, H-5), 8.13–8.02 (m, 2H, pyridine H-3, H-4), 7.83 (ddd, $J = 7.2$, 4.8, 1.5 Hz, 1H, pyridine H-5), 6.51 (s, 2H, $-CO-CH_2-$).

4.1.1.3. 1-[2-Oxo-2-(pyridine-3-yl)ethyl]pyridinium iodide ***2c***. Brown solid; yield 72.0%; mp 180.0–183.0 °C; ^{1}H NMR (250 MHz, $CDCl_3$) δ 9.23 (br, 1H, pyridine H-2), 8.99

Table 2
Cytotoxicity of the prepared compounds against several human cancer cell lines

Compd. no.	IC_{50}[a] (μM)					
	R^2	R^1	A549	SK-OV-3	SK-MEL-2	HCT15
6	2-Thienyl (**e**)	2-Pyridyl (**b**)	49.85	68.17	76.52	53.49
7	2-Thienyl (**e**)	2-Thienyl (**e**)	38.13	43.37	34.86	37.60
8	2-Thienyl (**e**)	2-Furyl (**g**)	52.83	80.11	55.70	69.03
9	2-Thienyl (**e**)	Phenyl (**a**)	33.18	44.16	47.83	31.61
10	2-Thienyl (**e**)	3-Thienyl (**e**)	36.71	40.34	33.58	23.51
11	2-Furyl (**g**)	2-Pyridyl (**b**)	53.85	>100	>100	84.02
12	2-Furyl (**g**)	3-Pyridyl (**c**)	>100	>100	>100	98.96
13	2-Furyl (**g**)	2-Furyl (**g**)	>100	>100	>100	>100
14	2-Furyl (**g**)	Phenyl (**a**)	78.04	>100	87.29	42.53
15	3-Thienyl (**f**)	2-Pyridyl (**b**)	26.84	58.26	77.24	42.15
16	3-Thienyl (**f**)	3-Pyridyl (**c**)	53.14	52.06	79.04	37.28
17	3-Thienyl (**f**)	Phenyl (**a**)	45.26	89.29	87.40	51.44
18	3-Thienyl (**f**)	2-Furyl (**g**)	50.43	52.71	57.28	47.06
19	3-Thienyl (**f**)	3-Thienyl (**f**)	>100	>100	>100	>100
21	2-Thienyl (**e**)	3-Pyridyl (**c**)	51.74	42.83	26.69	27.11
22	2-Thienyl (**e**)	4-Pyridyl (**d**)	26.31	28.45	21.09	14.18
23	2-Furyl (**g**)	4-Pyridyl (**d**)	>100	>100	>100	90.63
24	3-Thienyl (**f**)	4-Pyridyl (**d**)	43.25	37.40	49.73	33.29
α-Terpyridine			0.25	0.08	0.18	0.23
Doxorubicin			0.12	0.23	0.16	0.27
Camptothecin			0.004	0.014	0.165	0.083

[a] Each value represents mean IC_{50} of three determinations. The error range was within 5% of mean IC_{50}.

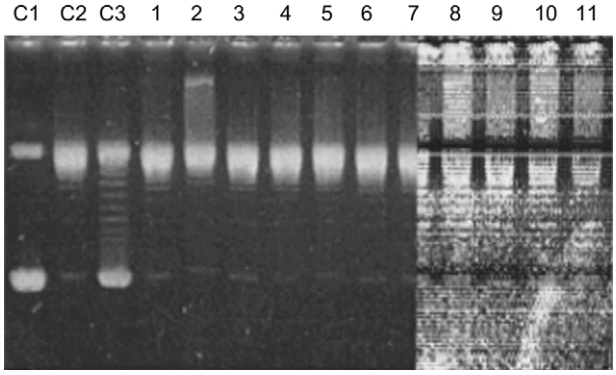


Fig. 3. Topoisomerase I inhibitory activity of compounds. Lane C1. pBR322 DNA (supercoiled form). Lane C2. pBR322 DNA + Topoisomerase I (relaxed form). Lane C3. pBR322 DNA + Topoisomerase I + camptothecin (final conc.: 20 μg/mL). Lanes 1–11. pBR322 DNA + Topoisomerase I + *Prepared compounds* (final conc.: 50 μg/mL) (1, **6**; 2, **21**; 3, **22**; 4, **7**; 5, **8**; 6, **9**; 7, **10**; 8, **11**; 9, **12**; 10, **23**; 11, **13**).

(d, $J = 6.5$ Hz, 2H, pyridinium H-2, H-6), 8.92 (dd, $J = 4.8$, 1.5 Hz, 1H, pyridine H-6), 8.76 (dt, $J = 7.8$, 1.2 Hz, 1H, pyridinium H-4), 8.42 (ddd, $J = 8.0$, 1.5, 0.6 Hz, 1H, pyridine H-4), 8.30 (t, $J = 6.6$ Hz, 2H, pyridinium H-3, H-5), 7.72 (dd, $J = 7.7$, 4.8 Hz, 1H, pyridine H-5), 6.52 (s, 2H, $-CO-CH_2-$).

4.1.1.4. 1-[2-Oxo-2-(pyridine-4-yl)ethyl]pyridinium iodide ***2d***. Light brown solid; yield 66.7%; mp 129.0–132.0 °C; ^{1}H NMR (250 MHz, $CDCl_3$) δ 8.98 (d, $J = 6.4$ Hz, 2H, pyridinium H-2, H-6), 8.95 (ddd, $J = 4.8$, 1.4, 0.9 Hz, 2H, pyridine H-2, H-6), 8.76 (dt, $J = 7.8$, 1.1 Hz, 1H, pyridinium H-4), 8.30 (t, $J = 6.7$ Hz, 2H, pyridinium H-3, H-5), 7.95 (ddd, $J = 4.8$, 1.4, 0.9 Hz, 2H, pyridine H-3, H-5), 6.49 (s, 2H, $-COCH_2-$).

4.1.1.5. 1-[2-Oxo-2-(thiophen-2-yl)ethyl]pyridinium iodide ***2e***. Yellow solid; yield 97.6%; mp 170.5–171.5 °C; ^{1}H NMR (250 MHz, $CDCl_3$) δ 9.02 (d, $J = 6.5$ Hz, 2H, pyridinium H-2, H-6), 8.73 (dt, $J = 7.8$, 1.2 Hz, 1H, pyridinium H-4), 8.27 (t, $J = 6.8$ Hz, 2H, pyridinium H-3, H-5), 8.23 (d, $J = 5.0$ Hz, 1H, thiophene H-3), 7.71 (d, $J = 3.8$ Hz, 1H, thiophene H-5), 7.41 (dd, $J = 5.0$, 3.8 Hz, 1H, furan H-4), 6.42 (s, 2H, $-CO-CH_2-$).

4.1.1.6. 1-[2-Oxo-2-(thiophen-3-yl)ethyl]pyridinium iodide ***2f***. Light brown solid; yield 89.4%; mp 160.0 °C; ^{1}H NMR (250 MHz, $CDCl_3$) δ 8.98 (d, $J = 6.5$ Hz, 2H, pyridinium H-2, H-6), 8.76 (dd, $J = 2.7$, 1.3 Hz, 1H, thiophene H-2), 8.71 (dt, $J = 7.8$, 1.2 Hz, 1H, pyridinium H-4), 8.26 (t, $J = 6.8$ Hz, 2H, pyridinium H-3, H-5), 7.79 (dd, $J = 5.1$, 2.8 Hz, 1H, thiophene H-4), 7.61 (dd, $J = 5.1$, 1.3 Hz, 1H, thiophene H-5), 6.39 (s, 2H, $-CO-CH_2-$).

4.1.1.7. 1-[2-(Furan-2-yl)-2-oxoethyl]pyridinium iodide ***2g***. Brown solid; yield 99.9%; mp 162.0–163.4 °C; ^{1}H NMR (250 MHz, $CDCl_3$) δ 8.99 (d, $J = 6.5$ Hz, 2H, pyridinium H-2, H-6), 8.73 (dt, $J = 7.8$, 1.1 Hz, 1H, pyridinium H-4), 8.27 (t, $J = 6.9$ Hz, 2H, pyridinium H-3, H-5), 8.21 (d, $J = 1.5$ Hz, 1H, furan H-5), 7.71 (d, $J = 3.7$ Hz, 1H, furan H-3), 6.88 (dd, $J = 3.7$, 1.5 Hz, 1H, furan H-4), 6.26 (s, 2H, $-CO-CH_2-$).

4.1.2. General procedure for the preparation of compounds ***5***

A mixture of **3** ($\mathbf{R^2 = e–g}$) (100 mmol), Me_2NH_2Cl (120 mmol), paraformaldehyde (120 mmol) and conc. HCl (0.5 mL) in EtOH (15 mL) was refluxed for 16 h. The mixture was cooled to 0 °C, and the precipitated brown solid **4** was filtered and washed with cold EtOH (5 mL). After addition of conc. NH_4OH solution and ethyl ether, the ethyl ether layer was extracted and washed with water, dried with $MgSO_4$, and the solvent was evaporated to afford the Mannich bases **5**, which was used directly in subsequent reaction without further purification due to its hygroscopic property.

4.1.2.1. 3-(Dimethylamino)-1-(thiophene-2-yl)propan-1-one HCl ***4e***. Mp 178.0–180.0 °C; ^{1}H NMR (250 MHz, DMSO-d_6) δ 8.06 (dd, $J = 4.8$, 1.8 Hz, 1H, thiophene H-3), 8.05 (dd, $J = 1.9$, 0.8 Hz, 1H, thiophene H-5), 7.28 (dd, $J = 4.8$, 4.0 Hz, 1H, thiophene H-4), 3.57 (t, $J = 7.1$ Hz, 2H, $-CH_2-N=$), 3.38 (t, $J = 7.5$ Hz, 2H, $-CO-CH_2-$), 2.77 (s, 6H, $-N(CH_3)_2$).

4.1.2.2. 3-(Dimethylamino)-1-(thiophene-3-yl)propan-1-one HCl ***4f***. Mp 153.0–157.0 °C; ^{1}H NMR (250 MHz, DMSO-d_6) δ 8.59 (dd, $J = 2.8$, 1.2 Hz, 1H, thiophene H-2), 7.67 (dd, $J = 5.1$, 2.8 Hz, 1H, thiophene H-5), 7.52 (dd, $J = 5.1$, 1.2 Hz, 1H, thiophene H-4), 3.52 (t, $J = 7.1$ Hz, 2H, $-CH_2-N=$), 3.35 (t, $J = 5.8$ Hz, 2H, $-CO-CH_2-$), 2.77 (s, 6H, $-N(CH_3)_2$).

4.1.2.3. 3-(Dimethylamino)-1-(furan-2-yl)propan-1-one HCl ***4g***. Mp 168.5–170.5 °C; ^{1}H NMR (250 MHz, DMSO-d_6) δ 8.04 (dd, $J = 1.5$, 0.6 Hz, 1H, furan H-5), 7.54 (dd, $J = 3.6$, 0.6 Hz, 1H, furan H-3), 6.75 (dd, $J = 3.6$, 1.7 Hz, 1H, furan H-4), 3.38 (dd, $J = 11.0$, 5.8 Hz, 4H, $-CH_2-CH_2-$), 2.76 (s, 6H, $-N(CH_3)_2$).

4.1.3. Preparation of 2-phenyl-6-(thiophen-3-yl)pyridine ***17***

A mixture of **5e** ($\mathbf{R^2 = e}$) (4.6 mmol), **2a** (400 mg, 5.5 mmol) and NH_4OAc (45.5 mmol) in EtOH (20 mL) was refluxed for 18 h. After cooling to 5 °C, the precipitated solid was filtered and purified by silica gel chromatography with a gradient elution of EtOAc:*n*-hexane (1:15, v:v) to afford a white solid **17** (yield; 43.3%). TLC (EtOAc:*n*-hexane = 1:10, v:v), $R_f = 0.3$.

With the same method described above, products **6–19** were obtained.

4.1.4. General procedure for the preparation of compounds ***20***

A solution of **3** ($\mathbf{R^2 = c}$, **d**) (165 mmol) and *N*,*N*-dimethylformamide dimethyl acetal (201 mmol) in toluene (100 mL) was heated to reflux for 3 h. Methanol was gradually removed by fractional distillation. The toluene was removed on a rotary

evaporator and the product was crystallized by addition of cyclohexane. Filtration of crystal afforded yellow crystalline enaminone **20**.

*4.1.4.1. (E)-3-(Dimethylamino)-1-(pyridine-3-yl)prop-2-en-1-one **20c***. Yield; 96.0%; mp > 300 °C; ^{1}H NMR (250 MHz, $CDCl_3$) δ 9.08 (d, $J = 2.1$ Hz, 1H, pyridine H-2), 8.67 (dd, $J = 4.8$, 1.7 Hz, 1H, pyridine H-6), 8.19 (dt, $J = 7.9$, 1.9 Hz, 1H, pyridine H-4), 7.85 (d, $J = 12.3$ Hz, 1H, =CH−N=), 7.36 (ddd, $J = 7.9$, 4.8, 0.7 Hz, 1H, pyridine H-5), 5.69 (d, $J = 12.3$ Hz, 1H, −CO−CH=), 3.07 (d, 6H, $-N(CH_3)_2$).

*4.1.4.2. (E)-3-(Dimethylamino)-1-(pyridine-4-yl)prop-2-en-1-one **20d***. Yield; 93.2%; mp 115.0–117.0 °C; ^{1}H NMR (250 MHz, $CDCl_3$) δ 9.08 (d, $J = 2.1$ Hz, 1H, pyridine H-2), 8.67 (dd, $J = 4.8$, 1.7 Hz, 1H, pyridine H-6), 8.19 (dt, $J = 7.9$, 1.9 Hz, 1H, pyridine H-4), 7.85 (d, $J = 12.3$ Hz, 1H,=CH−N=), 7.36 (ddd, $J = 7.9$, 4.8, 0.7 Hz, 1H, pyridine H-5), 5.69 (d, $J = 12.3$ Hz, 1H, −CO−CH=), 3.07 (d, 6H, $-N(CH_3)_2$).

*4.1.5. Preparation of 6-(thiophen-2-yl)-2,4′-bipyridine **22***

A solution of potassium *tert*-butoxide (30 mmol) and **1e** (15 mmol) in anhydrous THF (60 mL) was stirred for 2 h at room temperature, and enaminone **20d** (15 mmol) was added to the mixture in a single portion. The solution, which gradually turned to deep red, was allowed to stir for 14 h. The mixture was treated with NH_4OAc (150 mmol) and AcOH (40 mL). THF was removed by slow distillation for 2 h. The remaining AcOH was removed by evaporation under reduced pressure. After the residue was formed, ethyl acetate was added, and the organic layer was successively washed with water and 10% Na_2CO_3 solution three times. The organic layer was extracted and dried with $MgSO_4$ and evaporated under reduced pressure. The crude product was purified by alumina chromatography with a gradient elution of EtOAc:*n*-hexane (1:2, v:v) to afford a white solid **22** (yield; 23.8%). TLC (EtOAc:*n*-hexane = 1:2, v:v), $R_f = 0.38$.

With the same method described above, products **21–24** were obtained.

4.2. Biological assays

4.2.1. Cytotoxicity assay [18,19]

All experimental procedures followed the NCI's protocol based on the Sulforhodamine B (SRB) method. Briefly, tumor cells were cultured to maintain logarithmic growth by changing the medium 24 h before cytotoxicity assay. On the day of the assay, the cells were harvested by trysinisation, counted, diluted in media and added to 96-well plates. The concentrations of tumor cells used were 5×10^3 (A549, HCT15), 1×10^4 (SK-MEL-2), and 2×10^4 cells/well (SK-OV-3). The cells were then preincubated for 24 h in 5% CO_2 incubator at 37 °C. The compounds dissolved in DMSO were added to the wells in six 3-fold dilutions starting from the highest concentrations, and incubated for 48 h in 5% CO_2 incubator at 37 °C. The final DMSO concentration was <0.5%. At the termination of the incubation, the culture medium in each well was removed, and the cells were fixed with cold 10% trichloroacetic acid (TCA) for 1 h at 4 °C. The microplates were washed, dried, and stained with 0.4% SRB in 1% acetic acid for 30 min at room temperature. The cells were washed again and the bound stain was solubilized with 10 mM tris base solution (pH 10.5), and the absorbances were measured spectrophotometrically at 520 nm on a microtiter plate reader (Molecular Devices, Sunnyvale, CA). The data were transformed into MS Excel format and survival fractions were calculated by regression analysis (plotting the cell viability versus the concentration of the test compound). The EC_{50} values represent the concentrations of the compounds that inhibit 50% of cell growth. All data represent the average values for a minimum of three wells.

4.2.2. Topoisomerase I inhibitory assay [20]

The topoisomerase I inhibitory activity was carried out as following: The activity of DNA topoisomerase I was determined by measuring the relaxation of supercoiled DNA pBR322. For measurement of topoisomerase I activity, the reaction mixture was comprised of 35 mM tris-HCl (pH 8.0), 72 mM KCl, 5 mM $MgCl_2$, 5 mM DTT, 5 mM spermidine, 0.01% bovine serum albumin (BSA), 1 μg pBR322, and 2 U DNA topoisomerase I, and the prepared compounds in a final volume of 10 μL. The reaction mixture was incubated at 37 °C for 30 min. The reactions were terminated by adding dye solution comprising 1% SDS, 0.02% bromophenol blue and 50% glycerol. The mixture was applied to 1% agarose gel and electrophoresed for 1 h with a running buffer of tris-acetate EDTA. The gel was stained with ethidium bromide.

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