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# Antiproliferative activity and apoptosis inducing effects of nitric oxide donating derivatives of evodiamine

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ABSTRACT

The first series of nitric oxide donating derivatives of evodiamine were designed and prepared. NO releasing ability of all target derivatives was evaluated in BGC-823, Bel-7402 and L-02 cells. The cytotoxicity was evaluated against three human tumor cell lines (Bel-7402, A549 and BGC-823) and normal human liver cells L-02. The nitrate derivatives **11a** and **11b** only exhibited moderate activity and furoxan-based derivatives **13a–c**, **14a** and **14b** showed promising activity. **13c** showed good cytotoxic selectivity between tumor and normal liver cells and was further investigated for its apoptotic properties on human hepatocarcinoma Bel-7402 cells. The molecular mode of action revealed that **13c** caused cell-cycle arrest at S phase and induced apoptosis in Bel-7402 cells through mitochondria-related caspase-dependent pathways.



## 1. Introduction

Natural products or their derivatives take an active role in the development of new therapeutic drugs.[1] Evodiamine (**1**, Scheme 1), a quinazolinocarboline alkaloid isolated from the fruits of *Euodia rutaecarpa*, possesses many biological effects, such as antitumor,[2–5] anti-inflammation,[6–8] antiobesity,[9–11] and so on.[12–14] Particularly, numerous studies have comprehensively demonstrated that **1** exhibited considerable cytotoxicity on a wide variety of human cancer cell lines,[15–17] and apoptosis inducing ability to suppress the proliferation of tumor cells by various mechanisms,[18–20] such as, PI3K/Akt/caspase, Fas-L/NF-κB signaling pathways,[21] caspase-dependent and -independent pathways,[22,23] and MTDH-dependent signaling pathway.[24] Though there were a great deal of reports which clarified the antiproliferation and apoptosis functions of evodiamine, it was unpractical to develop it directly as clinic agents owing to its moderate anticancer activity.[25] Besides, hepatotoxicity caused by the plant *E. rutaecarpa* had not received serious attention providing new challenges.[26] Some promising derivatives of **1** had already been reported,[27–30] including 3,4,10,13-position modified evodiamine,[27] carboxyl derivatives at position 7 targeting topoisomerase I and sirtuins,[28] a diverse library containing 11 evodiamine-inspired novel scaffolds and their derivatives as multitargeting antitumor agents,[29] hybrid molecules of 3-amino-10-hydroxylevodiamine and SAHA as triple inhibitors of topoisomerase I/II and HDAC,[30] and so on. Because of its broad-spectrum and multitargeting antitumor profile, evodiamine represented a good lead; more work of structure modification was still in urgent need to be taken out.

Nitric oxide (NO) is a small and reactive molecule, which has various physiological and biological properties.[31] High concentration of NO has shown great potential in inhibiting carcinogenesis and tumor growth by inducing tumor cell apoptosis, inhibiting tumor metastasis, and so on.[32,33] Unfortunately, the delivery of gaseous NO to tumor directly is not really effective due to its short half-life and chemical instability.[34,35] NO donors, capable of producing a sustained release with a wide range of half-time lives, and a predictable estimated dose had become useful tools to study the biological properties of NO in cells and in vivo models of carcinogenesis. Recently, great deals of NO donor hybrids spring up, which primarily served as anticancer drugs.[36–38] We were very interested in what aspects of druggability these hybrids would performance.

Inspired by the above reasons, a novel series of evodiamine derivatives bearing NO-donating groups (organic nitrate or furoxan)

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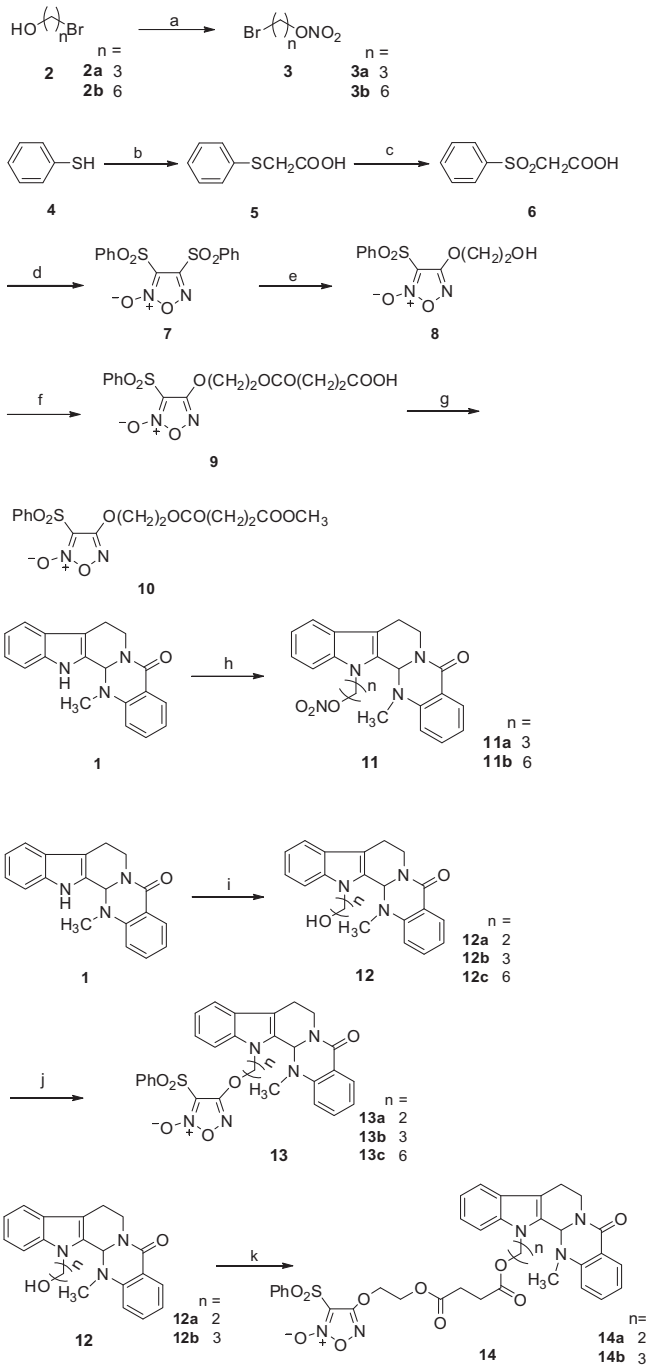

**Scheme 1.** Synthetic routine of NO-releasing evodiamine derivatives **11**, **13** and **14**. Reagents and conditions: (a) fuming $HNO_3$, $H_2SO_4$, 0 °C, 3 h; (b) $ClCH_2COOH$, NaOH (aq), 140 °C, 2 h; (c) 30% $H_2O_2$, AcOH, rt, 3 h; (d) fuming $HNO_3$, 90 °C, 4 h; (e) $HOCH_2CH_2OH$, THF, 30% NaOH, 0 °C, 4–8 h; (f) triethylamine, succinic anhydride, DMAP, rt, 1 h; (g) $TMSCHN_2$, MeOH, 0 °C, 10 min; (h) **3**, NaH, DMF, rt, 1.5 h; (i) $HO(CH_2)_nBr$, NaH, DMF, rt, 3 h; (j) **7**, DBU, $CH_2Cl_2$, −15 °C, 3 h; (k) **9**, EDCI, DMAP, rt, 12 h.

were designed and synthesized at *N*-13. The antiproliferative activity against human hepatoma cells (Bel-7402), human non-small-cell lung cancer cells (A549), human gastric carcinoma cells (BGC-823) and human normal liver cells (L-02) was evaluated. The NO releasing property was also measured. Typical selected compound **13c** was further investigated for its apoptotic properties on human hepatocarcinoma Bel-7402 cells, in order to gain a better understanding of the mode of action. The effects of apoptosis, cell cycle arrest, and mitochondrial membrane potential were also disclosed.

## 2. Results and discussion

### 2.1. Chemistry

The procedures for the synthesis of NO-releasing evodiamine derivatives were illustrated in Scheme 1. The corresponding bromohydrin **2** was treated with fuming $HNO_3$ and concentrated $H_2SO_4$, which afforded nitrate **3**. The reaction of thiophenol **4** with chloroacetic acid in the presence of sodium hydroxide solution yielded the thiophenylacetic acid **5**, which was further oxidated with 30% $H_2O_2$ and AcOH, generating the oxidation product **6**. **6** was treated with fuming $HNO_3$ at 90 °C, which lead to **7**. 3,4-Dibenzenesulfonyl furoxan **7** was treated with ethanediol in the presence of 30% NaOH in THF to offer **8**. Treatment of **8** with triethylamine, succinic anhydride, and 4-dimethylaminopyridine (DMAP) produced the intermediate **9**. **9** was reacted with $TMSCHN_2$ to get **10**. For **11a**–**b**, the reaction of **1** with **3a** or **3b**, in the presence of NaH, was carried out in DMF. **1** was treated with bromohydrin in the presence of NaH and DMF to offer **12a**–**c**, and then **7** was added in the presence of DBU in $CH_2Cl_2$ to give evodiamine–furoxan hybrids **13a**–**c**. Intermediate **11** was reacted with **9** to afford another series of evodiamine–furoxan hybrids **14** with long linkage.

### 2.2. NO releasing ability

In order to investigate whether the evodiamine derivatives including NO donor possessed the ability to release NO and if there were any differences of NO releasing in tumor and normal cells, Griess assay was carried out in BGC-823, Bel-7402 and L-02 cell lines. As shown in Table 1, all the derivatives released more than 75 μM/L of NO at the time point of 1 h in BGC-823 and Bel-7402 cells, and less than 28.12 μM/L of NO in L-02 cells. This would be caused of special chemical environment (such as low pH) in tumor cells. In further investigation, control release of NO might be a good topic. Almost all target compounds released a little more NO in BGC-823 cells than Bel-7402 cells (except **11b**). Of all the derivatives, **11b** released the highest amount of NO of 104.18 μM/L in Bel-7402 cells at the time point of 1 h, while in L-02 cells the least NO of 6.59 μM/L was produced by **14a**.

### 2.3. Antiproliferative activity

The antiproliferative activity of target compounds was preformed on BGC-823, A549, Bel-7402, and L-02 cell lines by the standard MTT method. The results were summarized in Table 2. The nitrate derivatives **11a** and **11b** only exhibited moderate activity against Bel-7402 cells with $IC_{50}$ values of 12.82 and 28.79 μM, respectively. And shorter linkage (**11a**) was favorable. As for furoxan-based derivatives **13a**–**c**, **14a** and **14b**, they showed very promising activity against BGC-823 cells with $IC_{50}$ values ranging from 0.02 to 0.08 μM. These results revealed that the synthetic NO donating derivatives of **1** were very sensitive to BGC-823 cells. **13a** was the most potent one against A549 and Bel-7402 cells with $IC_{50}$ values of 0.23 and 0.55 μM, and other compounds were weaker than parent compound **1**. In L-02 cells, significant differences were observed that **13a** still exhibited the strongest cytotoxicity with $IC_{50}$ value of only 0.02 μM and no obvious antiproliferative activity (>100 μM) was exhibited by **13c**. **14a** with a linker of 2 carbons showed $IC_{50}$ values of 0.02, 3.04 and 1.57 μM against three tumor cell lines, respectively, which was stronger than **14b** of 3 carbons with $IC_{50}$ values of 0.06, 5.32 and 4.65 μM.

**Table 1**
NO releasing ability (μM/L) of target derivatives[a]

| Compd | BGC-823 | Bel-7402 | L-02 |
|---|---|---|---|
| **11a** | 83.79 ± 3.58 | 78.49 ± 1.82 | 19.37 ± 0.98 |
| **11b** | 99.96 ± 1.90 | 104.18 ± 5.30 | 16.39 ± 0.80 |
| **13a** | 78.75 ± 2.86 | 77.46 ± 2.21 | 18.50 ± 1.85 |
| **13b** | 83.64 ± 2.77 | 83.10 ± 1.10 | 16.37 ± 1.20 |
| **13c** | 87.35 ± 2.57 | 82.17 ± 1.83 | 25.98 ± 2.14 |
| **14a** | 93.49 ± 3.32 | 88.71 ± 2.72 | 6.59 ± 0.41 |
| **14b** | 97.57 ± 2.85 | 97.05 ± 2.02 | 17.98 ± 1.57 |

[a] Results are expressed as mean ± SD of three independent experiments.

This trend reversed in L-02 cells. For **13a–c**, the linkage of 2 carbons (**13a**) was more beneficial to antiproliferative activity than 3 carbons (**13b**). **13c** with the linkage of 6 carbons showed $IC_{50}$ values between those of **13a** and **13b** against tumor cells, and good selectivity between tumor and normal liver cells. These results confirmed with previous reports[39,40] that some NO donating derivatives showed less cytotoxicity to normal cells. So it was selected for further mechanism study to search for a potential safer chemotherapy agent.

### 2.4. Effect of cell cycle

Cell cycle arrest was an important sign for inhibition of proliferation and the series of events that took place in a cell leading to its division and duplication (replication). Some NO donating hybrids exhibited cell cycle arrest properties.[40] To determine whether the suppression of cell growth by **13c** was caused by cell-cycle effect, the DNA content of cell nuclei was detected in Bel-7402 cells by flow cytometry. As shown in Figure 1, the cells in $G_1$, S and $G_2$ phase of control group accounted for 43.13%, 33.70% and 23.17%, respectively. After cells were treated with compound **13c** at concentrations of 1, 2, and 3 μM for 24 h, the ratio of $G_1$ phase were almost not changed. The cells of S phase increased to 39.34%, 47.32% and 51.51%, respectively, confirming that Bel-7402 cells were arrested at S phase.

### 2.5. Induction of apoptosis

Apoptosis was a process of programmed cell death and cancer cells usually had an abnormal ability of proliferation mainly due to the defective apoptosis. Thus, activation of apoptosis could reduce accumulation of cancer cells. High levels of NO could act as a tumor cell apoptosis inducer,[36,41] in order to examine the influence of **13c** on apoptosis, an annexin V-FITC/propidium iodide (PI) binding assay was carried out. The Bel-7402 cells were treated with variable concentrations of **13c** (1, 2, and 3 μM). The percentage of apoptotic cells was shown in Figure 2. After 72 h treatment, the observed apoptotic cells were 19.63%, 36.38% and 52.57% (early and late stage apoptosis) at the indicated concentrations, and the control group displayed 9.21%, indicating the remarkable effect of compound **13c** to induce apoptosis in a concentration-dependent manner.

**Table 2**
The antiproliferative activity ($IC_{50}$[a] μM) of NO-releasing evodiamine derivatives[b]

| Compd | BGC-823 | A549 | Bel-7402 | L-02 |
|---|---|---|---|---|
| **1** | 1.16 ± 0.22 | 0.98 ± 0.28 | 1.23 ± 0.21 | 0.34 ± 0.11 |
| **9** | 17.96 ± 0.58 | 27.85 ± 0.28 | 21.45 ± 1.03 | 38.73 ± 1.55 |
| **10** | 21.37 ± 0.83 | 24.73 ± 0.28 | 27.86 ± 0.76 | 43.27 ± 1.48 |
| **11a** | >100 | >100 | 12.82 ± 0.07 | >100 |
| **11b** | >100 | >100 | 28.79 ± 1.09 | >100 |
| **13a** | 0.02 ± 0.01 | 0.23 ± 0.03 | 0.55 ± 0.14 | 0.02 ± 0.01 |
| **13b** | 0.08 ± 0.02 | 5.85 ± 1.01 | 6.21 ± 0.17 | 2.35 ± 0.12 |
| **13c** | 0.07 ± 0.01 | 2.31 ± 0.21 | 2.10 ± 0.15 | >100 |
| **14a** | 0.02 ± 0.01 | 3.04 ± 0.10 | 1.54 ± 0.07 | 17.10 ± 0.58 |
| **14b** | 0.06 ± 0.01 | 5.32 ± 1.02 | 4.65 ± 0.29 | 6.38 ± 0.72 |

[a] $IC_{50}$: concentration that inhibits 50% of cell growth.
[b] Results are expressed as the mean ± SD of three independent experiments.

### 2.6. Effect of mitochondrial depolarization

Apoptosis played an important role in cancer, since its induction in tumor cells was essential for successful treatment and mitochondria was essential in the propagation of apoptosis. On the other hand, NO was known to induce apoptosis by activating the intrinsic mitochondria-related pathway, which mainly resulted in the loss of mitochondrial membrane potential ($\Delta\psi_m$).[40,42] We therefore studied the effects of **13c** on mitochondria-mediated apoptosis in Bel-7402 cells. After being exposed to different concentrations of **13c** (0, 1, 2, and 3 μM) for 72 h, $\Delta\psi_m$ values were determined by flow cytometry analysis (Fig. 3) using the lipophilic mitochondrial probe JC-1. 4.26%, 13.50%, 26.57%, and 44.37% apoptotic cells were observed, respectively. These results demonstrated that incubation with **13c** increased the number of cells with collapsed mitochondrial membrane potentials at low concentrations and in dose-dependent manner.

### 2.7. Effect of apoptosis-related proteins

During the process of apoptosis, apoptotic signals could result in the release of cytochrome C from mitochondria to cytoplasm, a down-regulation of Bcl2, and an up-regulation of Bax. Caspases also played an important role in the apoptotic signaling network, and apoptotic pathways depended on activation of caspases for the final execution of apoptosis. Therefore, the effect of apoptosis-related proteins by **13c** was carried out by western blotting analysis. It was found that **13c** could up-regulate proapoptotic caspase 3, caspase 8, caspase 9, Bax, FAS and cytochrome-C, and down-regulate anti-apoptotic Bcl-2 expression (Fig. 4).

## 3. Conclusion

In summary, several NO-donor/evodiamine hybrids were designed and synthesized. Of which, most compounds showed significant antitumor activity against three selected human tumor cell lines (BGC-823, A549 and Bel-7402). The selectivity of antiproliferative activity could be observed since some derivatives showed no obvious cytotoxicity against normal human liver L-02 cells. The NO-releasing ability of all the synthetic NO-donor/evodiamine hybrids was measured by Griess assay. Interestingly, all the target compounds released much higher amount of NO in Bel-7402 cells than in L-02 cells. High NO releasing ability should, at least to some extent, contribute to the strong cytotoxicity. **13c** with $IC_{50}$ values of 0.07, 2.31 and 2.10 μM against BGC-823, A549 and Bel-7402 cell lines, respectively, and almost no antiproliferative activity ($IC_{50}$ >100 μM) against L-02 cells, was further investigated for its apoptotic properties on human hepatocarcinoma Bel-7402 cells in order to gain a better understanding of the mode of action. The results revealed that **13c** caused cell-cycle arrest of S phase and induced apoptosis in Bel-7402 cells through mitochondria-related caspase-dependent pathways. These stimulated our great interest in the possibility to develop new antitumor agents from NO-donor/evodiamine hybrids.

## 4. Experimental

### 4.1. Chemistry

NMR spectra were recorded with a Bruker ARX-400 NMR spectrometer in the indicated solvents (TMS): the values of the

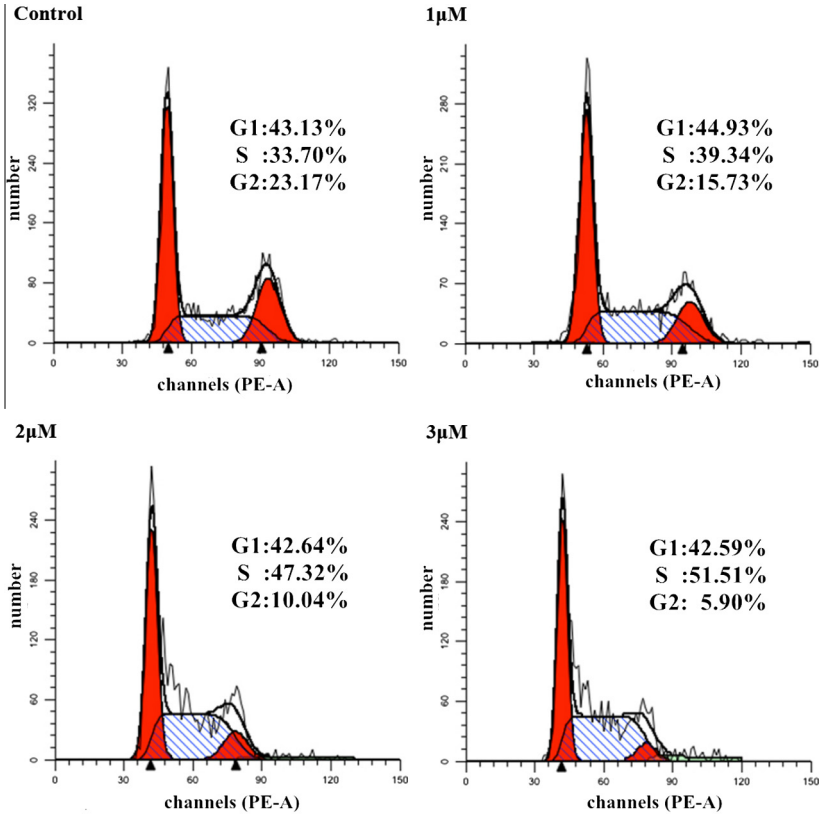


**Figure 1.** The influence of Bel-7402 cell cycle by compound **13c**. Bel-7402 cells were incubated with the indicated concentrations of **13c** for 24 h before staining with PI. Cellular DNA content for cell-cycle distribution analysis was measured by flow cytometry. The diagrams show the distribution of cells according to their DNA content; the inserts give the percentages of cells in various cell-cycle phases.

chemical shifts are expressed in $\delta$ values (ppm) and the coupling constants ($J$) in Hz. Melting points were taken on an XT-4 micro melting point apparatus and uncorrected. Mass spectra were obtained using Agilent 1100 Ion trap mass spectrometer. HR-MS were carried out with Agilent Q-TOF B.05.01 (B5125.2). All commercially available solvents and reagents were used without further purification.

### 4.2. Synthesis of 10

Compound **9** (13 mg, 0.34 mmol) was dissolved in 6 mL anhydrous MeOH at °C. $TMSCHN_2$ (about 0.6 mL) was added dropwise until no air bubble was produced anymore. The reaction mixture was concentrated in vacuo and subjected to silica gel column chromatography (PE/EA 5:1 v/v) to give **10** (13 mg).

Compound **10**, colorless oil, 98% yield: $^1$H NMR (400 MHz, DMSO-$d_6$), $\delta$ (ppm): 7.73–8.02 (5H, m, H-Ph), 4.39–4.61 (4H, m, –$OCH_2CH_2O$–), 3.57 (3H, s, O-$CH_3$), 2.57–2.61 (4H, m, –CO-$CH_2CH_2$-CO–); $^{13}$C NMR (100 MHz, DMSO-$d_6$), $\delta$ (ppm): 172.35, 171.84, 158.70, 137.20, 136.16, 129.99, 129.99, 128.34, 128.34, 110.48, 69.27, 61.53, 51.47, 28.52, 28.33; ESI-MS $m/z$ 423.0 $[M+Na]^+$.

### 4.3. General procedure to synthesize 11

Concentrated $H_2SO_4$ (1 mL) was added into the fuming $HNO_3$ (0.84 mL) at 0 °C and stirred for 10 min. Dichloromethane (DCM, 10 mL) and 1-bromo-3-propanol (0.9 mL, 10 mmol) were put in dropwise. The reaction mixture was stirred at room temperature for another 3 h, then poured into 10 mL of $H_2O$, and extracted with DCM (10 mL × 3). The organic layer was combined, washed with saturated NaCl solution, dried over anhydrous $Na_2SO_4$, and concentrated in vacuo to give the crude product **3a**. **3b** was obtained according to similar procedures. No further purification was needed for the next step.

Compound **1** (91 mg, 0.3 mmol) was mixed with **3a** or **3b** (0.4 mmol), and NaH (10 mg, 0.36 mmol) in 5 mL of anhydrous DMF and stirred at room temperature for 1.5 h. The mixture was poured into 5 mL of $H_2O$, and extracted with ethyl acetate (EA, 5 mL × 3). The organic layer was combined, washed with brine, dried over anhydrous $Na_2SO_4$, and concentrated in vacuo. The crude product was purified by column chromatography (petroleum ether, PE/acetone 2:1 v/v) to give **11a** or **11b**.

#### 4.3.1. Compound 11a

Yellow oil, 85% yield: $^1$H NMR (400 MHz, $CDCl_3$), $\delta$ (ppm): 7.22–7.68 (8H, m, H-1–4, 9–12), 6.00 (1H, br s, H-13b), 4.96 (1H, m, H-15a), 4.59–4.49 (3H, m, H-7b, 15b, 17a), 4.36 (1H, m, H-17b), 3.17 (1H, m, H-7a), 3.01 (1H, m, H-8b), 2.88 (1H, m, H-8a), 2.38 (3H, s, –$NCH_3$), 2.29–2.41 (2H, m, $H_2$-16); $^{13}$C NMR (100 MHz, $CDCl_3$), $\delta$ (ppm): 164.53, 150.88, 137.28, 133.12, 129.01, 128.20, 125.88, 124.69, 124.27, 123.37, 123.17, 120.05, 119.29, 113.99, 109.32, 70.36, 68.14, 50.83, 40.28, 36.57, 27.49, 20.37; ESI-MS $m/z$ 407.2 $[M+H]^+$; HR-MS (ESI, M+Na) $m/z$: calcd for $C_{22}H_{22}N_4NaO_4$: 429.1533, found 429.1529.

#### 4.3.2. Compound 11b

Yellow oil, 87% yield: $^1$H NMR (400 MHz, $CDCl_3$), $\delta$ (ppm): 6.05–7.71 (8H, m, H-1–4, 9–12), 6.05 (1H, br s, H-13b), 4.24–5.21 (6H, m, $H_2$-7, $H_2$-15, $H_2$-20), 2.97–3.51 (4H, m, $H_2$-8, $H_2$-16), 2.40 (3H, s, –$NCH_3$), 1.75–1.98 (6H, m, $H_2$-17, $H_2$-18, $H_2$-19); $^{13}$C NMR (100 MHz, $CDCl_3$), $\delta$ (ppm): 164.65, 150.98, 137.22, 133.03, 129.11, 128.36, 125.82, 124.38, 124.24, 123.14, 122.74, 119.70,

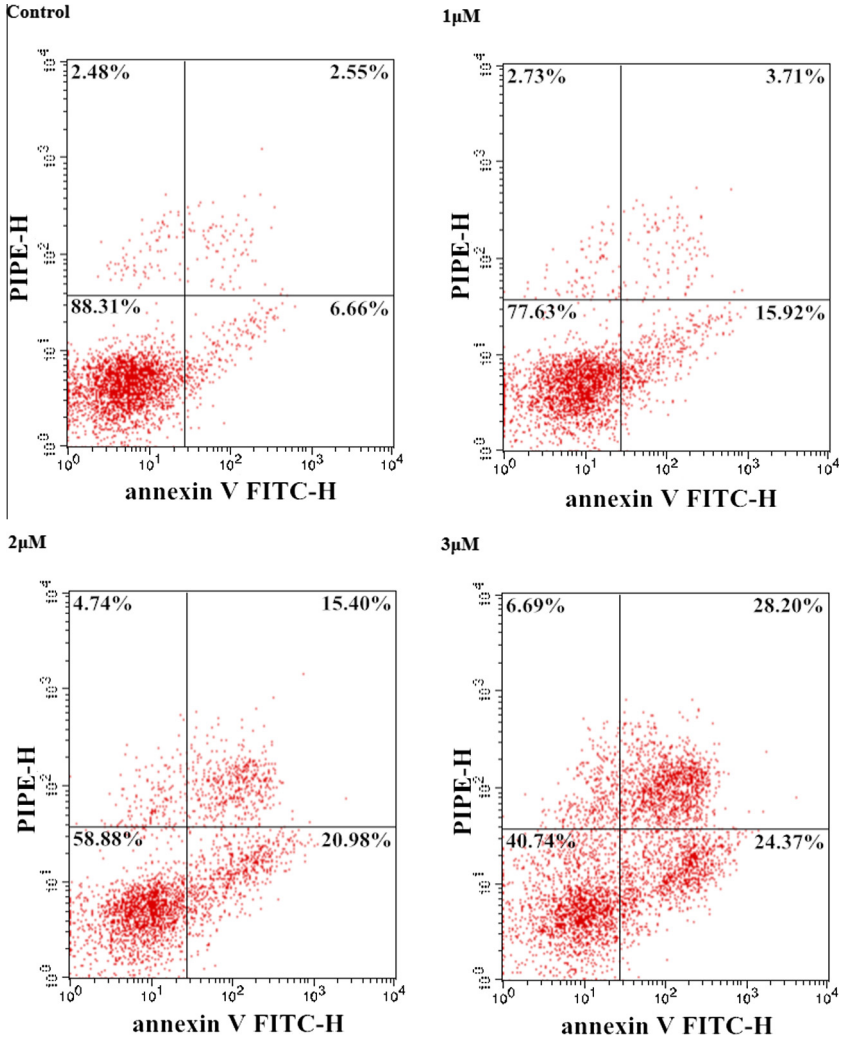


**Figure 2.** Apoptosis inducing effect of **13c** in Bel-7402 cells. Bel-7402 cells were incubated with the indicated concentrations of **13c** for 72 h before staining with annexin V-FITC and PI, followed by flow cytometric analysis. The inserts give the percentages of cells in each quadrant.

119.17, 113.26, 109.79, 73.16, 68.18, 43.81, 39.41, 36.57, 30.07, 27.74, 26.82, 25.60, 20.47; ESI-MS *m/z* 449.2 $[M+H]^+$; HR-MS (ESI, M+K) *m/z*: calcd for $C_{25}H_{28}KN_4O_4$: 487.1742, found 487.1753.

### 4.4. General procedure to synthesize 13

Compound **1** (91 mg, 0.3 mmol) was mixed with NaH (10 mg, 0.36 mmol), bromohydrin (0.6 mmol) in 5 mL of anhydrous DMF and stirred at room temperature for 3 h. The mixture was poured into 10 mL of $H_2O$, and extracted with EA (10 mL × 3). The organic layer was combined, washed with water and saturated NaCl solution sequentially, dried over anhydrous $Na_2SO_4$, and concentrated in vacuo. The crude **12a–c** were got. **7** was obtained from thiophenol (**4**) in a three-step sequence according to the literature.[36–38] **12** (0.3 mmol) was mixed with **7** (219.6 mg, 0.6 mmol), and DBU (58.5 mL, 0.4 mmol) in 5 mL of DCM and stirred at -15 °C for 3 h. The reaction mixture was then poured into 10 mL of $H_2O$, and extracted with DCM (10 mL × 3). The organic layer was combined, washed with water and brine, sequentially, dried over anhydrous $Na_2SO_4$, and concentrated in vacuo. The crude product was purified by column chromatography (PE/EA 5:1–2:1 v/v) to give **13**.

#### 4.4.1. Compound 13a

Yellow oil, 76% yield: $^1$H NMR (400 MHz, $CDCl_3$), $\delta$ (ppm): 7.23–8.15 (13H, m, H-1–4, 9–12, –Ph), 6.33 (1H, br s, H-13b), 5.19–5.27 (1H, m, H-15a), 4.90–4.93 (1H, m, H-15b), 4.63–4.77 (3H, m, H-7b, $H_2$-16), 3.30 (1H, m, H-7a), 3.04 (1H, m, H-8b), 2.92 (1H, m, H-8a), 2.42 (3H, s, $-NCH_3$); $^{13}$C NMR (100 MHz, $CDCl_3$), $\delta$ (ppm): 164.55, 158.65, 150.92, 137.92, 137.12, 135.59, 132.94, 129.73 (×2), 129.21, 128.33 (×2), 127.64, 126.35, 124.67, 123.47, 123.31, 120.35, 119.37, 114.08, 110.52, 109.60, 69.77, 68.10, 60.52, 42.28, 39.15, 36.74, 20.41; ESI-MS *m/z* 572.2 $[M+H]^+$; HR-MS (ESI, M+H) *m/z*: calcd for $C_{29}H_{26}N_5O_6S$: 572.1598, found 572.1576.

#### 4.4.2. Compound 13b

Yellow oil, 80% yield: $^1$H NMR (400 MHz, $CDCl_3$), $\delta$ (ppm): 7.16–8.14 (13H, m, H-1–4, 9–12, –Ph), 5.98 (1H, br s, H-13b), 4.42–4.91 (6H, m, $H_2$-7, $H_2$-15, $H_2$-17), 3.21 (1H, m, H-8b), 3.03 (1H, m, H-8a), 2.92–3.03 (2H, m, $H_2$-16), 2.40 (3H, s, $-NCH_3$). $^{13}$C NMR (100 MHz, $CDCl_3$), $\delta$ (ppm): 164.74, 159.04, 151.07, 138.17, 137.36, 135.75, 133.01, 129.77 (×2), 129.07, 128.68 (×2), 128.64, 125.88, 124.22, 123.11, 122.76, 119.76, 119.15, 113.27, 110.63, 109.87, 68.68, 67.88, 60.53, 40.72, 39.48, 30.27, 28.95, 20.51; ESI-MS *m/z* 586.2 $[M+H]^+$; HR-MS (ESI, M+H) *m/z*: calcd for $C_{30}H_{28}N_5O_6S$: 586.1755, found 586.1722.

#### 4.4.3. Compound 13c

Yellow oil, 78% yield: $^1$H NMR (400 MHz, $CDCl_3$), $\delta$ (ppm): 7.16–8.15 (13H, m, H-1–4, 9–12, –Ph), 5.98 (1H, br s, H-13b), 4.16–4.99 (4H, m, $H_2$-15, $H_2$-20), 3.16–3.38 (2H, m, $H_2$-7), 2.89–3.05 (2H, m,

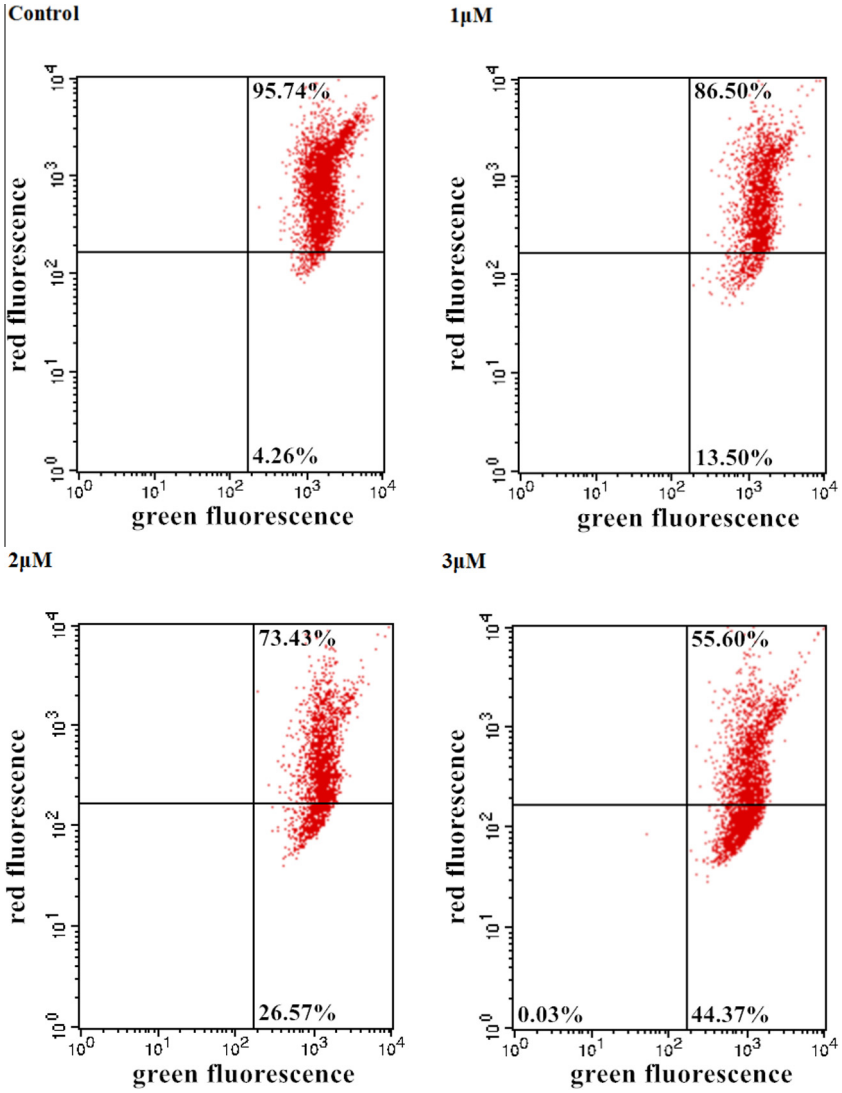


**Figure 3.** Effect of **13c** on the mitochondrial membrane potentials in Bel-7402 cells. Bel-7402 cells were incubated with the indicated concentrations of **13c** for 72 h prior to staining with JC-1. The inserts give the percentages of cells in each quadrant.

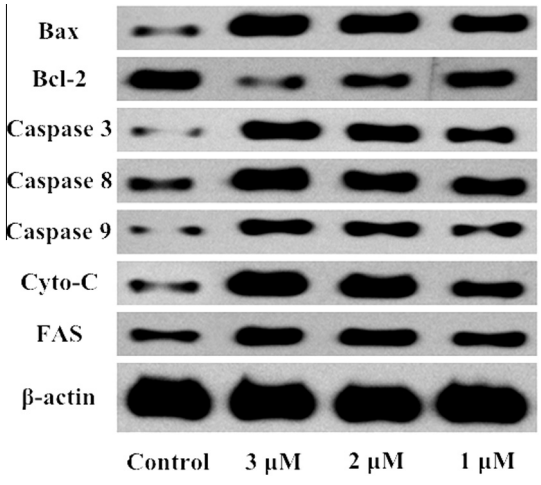


**Figure 4.** Effect of **13c** on the expression of apoptosis-related proteins in Bel-7402 cells. Bel-7402 cells were incubated with different doses of **13c** for 72 h. The target proteins in the membranes were probed with monoclonal antibodies.

$H_2$-8), 2.41 (3H, s, $-NCH_3$), 1.55–1.87 (8H, m, $H_2$-16, $H_2$-17, $H_2$-18, $H_2$-19); $^{13}C$ NMR (100 MHz, $CDCl_3$), $\delta$ (ppm): 163.53, 158.83, 150.73, 137.20, 136.79, 136.04, 132.87, 129.95 (×2), 128.23 (×2), 128.09, 127.57, 125.48, 125.26, 123.59, 122.16, 119.10, 118.74, 112.15, 110.41, 110.06, 71.36, 69.81, 66.88, 48.60, 43.25, 29.42, 29.08, 27.68, 25.89, 24.70, 19.86; ESI-MS *m*/*z* 650.3 $[M+Na]^+$; HR-MS (ESI, M+H) *m*/*z*: calcd for $C_{33}H_{34}N_5O_6S$: 628.2224, found 628.2180.

### 4.5. General procedure to synthesize 14

To a solution of **9** (61 mg, 0.16 mmol) in 10 mL of anhydrous DCM, **12** (60 mg, 0.17 mmol), EDCI (93 mg, 0.6 mmol), and catalytic amount of DMAP were added. The reaction solution was stirred for 12 h at room temperature. The mixture was poured into 10 mL of $H_2O$, and extracted with DCM (10 mL × 3). The organic layer was combined, washed with saturated NaCl solution, dried over anhydrous $Na_2SO_4$, and concentrated in vacuo. The crude product was purified by column chromatography (PE/EA 2:1 v/v).

#### 4.5.1. Compound 14a

Yellow oil, 7% yield: $^1$H NMR (400 MHz, $CDCl_3$), $\delta$ (ppm): 7.18–8.14 (13H, m, H-1–4, 9–12, –Ph), 6.01 (1H, br s, H-13b), 4.43–4.94 (8H, m, $H_2$-15, $H_2$-16, $H_2$-21, $H_2$-22), 3.01–3.24 (2H, m, $H_2$-7), 2.50–2.92 (6H, m, $H_2$-8, $H_2$-18, $H_2$-19), 2.39 (3H, s, $-NCH_3$); $^{13}$C NMR (100 MHz, $CDCl_3$), $\delta$ (ppm): 171.70, 171.66, 163.43, 158.67, 148.41, 139.77, 137.22, 137.17, 136.11, 132.94, 129.96, 129.96, 128.32, 128.32, 128.06, 127.58, 125.47, 125.37, 122.37, 119.42, 118.77, 112.83, 110.46, 110.19, 69.21, 67.08, 63.00, 61.48, 46.50, 42.05, 40.06, 36.03, 28.44, 28.28, 19.84; ESI-MS *m/z* 738.2 $[M+Na]^+$; HR-MS (ESI, M+Na) *m/z*: calcd for $C_{35}H_{33}N_5NaO_{10}S$: 738.1840, found 738.1850.

#### 4.5.2. Compound 14b

Yellow oil, 5% yield: $^1$H NMR (400 MHz, $CDCl_3$), $\delta$ (ppm): 7.39–8.36 (13H, m, H-1–4, 9–12, –Ph), 5.98 (1H, br s, H-3), 4.50–4.91 (10H, m, $H_2$-7, $H_2$-15, $H_2$-17, $H_2$-22, $H_2$-23), 2.90–3.30 (8H, m, $H_2$-8, $H_2$-16, $H_2$-19, $H_2$-20), 2.39 (3H, s, $-NCH_3$). $^{13}$C NMR (100 MHz, $CDCl_3$), $\delta$ (ppm): 171.86, 171.81, 163.54, 158.29, 148.44, 139.74, 136.74, 136.14, 131.74, 131.61, 129.98, 128.32, 127.60, 127.60, 126.14, 125.47, 125.47, 123.35, 121.69, 120.16, 118.77, 118.60, 118.48, 113.53, 111.48, 110.88, 69.27, 67.41, 61.57, 42.12, 40.06, 38.08, 29.79, 29.03, 28.44, 18.57. ESI-MS *m/z* 752.2 $[M+Na]^+$; HR-MS (ESI, M+Na) *m/z*: calcd for $C_{36}H_{35}N_5NaO_{10}S$: 752.1997, found 752.2105.

### 4.6. MTT assay

Cytotoxicity of all the tested compounds against Bel-7402, A549, BGC-823 and L-02 cells was determined by MTT assay.[43,44] The assay was performed in 96-well plates. Cells were added to each well and incubated for 24 h at 37 °C in a humidified atmosphere of 5% $CO_2$. Then cells were incubated in the presence or absence of test compounds. After 72 h, 20 μL of MTT solution (5 mg/mL) per well was added to each cultured medium, which was incubated for another 4 h. Then, DMSO (150 μL) was added to each well and the plates were shaken for 10 min at room temperature. After 10 min, the OD of each well was measured on a Microplate Reader (BIO-RAD) at the wavelength of 570 nm. In these experiments, the negative reference agent was 0.1% DMSO; evodiamine and 5-Fu were used as the positive reference.

### 4.7. Griess assay

NO-release data were acquired for test compounds using the Griess reaction in Bel-7402 and L-02 cells according to the manufacturer's instructions (S0024, Beyotime, China). Briefly, cells were treated with 100 μM of each compound for 150 min. Subsequently, the cells were harvested and their cell lysates were prepared and then mixed with Griess reagent for 10 min at 37 °C, followed by measurement at 540 nm by a microplate reader. The cells treated with 0.4% DMSO in medium were used as negative controls for the background levels of nitrite production, while sodium nitrite at different concentrations was prepared as the positive control for the establishment of a standard curve.[37,45]

### 4.8. Cell cycle study

Cell cycle effect was assessed by flow cytometry with PI (KGA511, KeyGEN Biotech, Nanjing, China). Bel-7402 cells were plated in 6-well plates and incubated at 37 °C for 24 h. Cells were then incubated with **13c** at a certain concentration. After 48 h, cells were centrifuged and fixed in 70% ethanol at 4 °C overnight and resuspended in PBS containing 100 μL RNase A and 400 μL PI. Cellular DNA content, for cell cycle distribution analysis, was measured using a flow cytometer (FACS Calibur Becton–Dickinson).[46,47]

### 4.9. Analysis of cellular apoptosis

Apoptosis was analyzed using Annexin-V and PI double staining by flow cytometry according to the manufacturer's instructions (KGA1024, KeyGEN Biotech, Nanjing, China) in order to detect apoptotic cells.[48,49] The Bel-7402 cells were seeded in 6-well plates to grow overnight, and then treated with or without **13c** at indicated concentrations for 72 h. Cells were then washed twice in PBS and resuspended in Annexin V binding buffer. Annexin V-FITC was then added and the mixture was incubated for 15 min under dark conditions at 25 °C. PI was added just prior to acquisition. The percentage of cells positive for PI and/or Annexin V-FITC was reported inside the quadrants.

### 4.10. Mitochondrial membrane potential assay

Briefly, Bel-7402 cells were incubated with the **13c** or vehicle for 72 h, and then washed with PBS and stained with JC-1 dye under dark conditions according to the manufacturer's instruction (KGA601, KeyGEN Biotech, Nanjing, China). The percentage of cells with healthy or collapsed mitochondrial membrane potentials was monitored by flow cytometry analysis.[48,50]

### 4.11. Western blot analysis

Bel-7402 cells were incubated with different doses of **13c** for 72 h. The cells were harvested and lysed using lysis buffer, and the solution was centrifuged. Then the protein concentrations were determined, and individual cell lysates (50 mg per lane) were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (10% gel, SDS–PAGE) and transferred onto nitrocellulose membranes. After being blocked with 5% fat-free milk, the target proteins in the membranes were probed with monoclonal anti-Bax (KGA714, KeyGEN Biotech, Nanjing, China), anti-Bcl 2 (KGA715), anti-caspase 3 (KGA717), anti-caspase 9 (KGA720), anti-cyto C (KGA723) and anti-β-actin antibodies (KGA731), respectively. The bound antibodies were detected by horseradish peroxidase (HRP) conjugated second antibodies and visualized using an enhanced chemiluminescent reagent.[47,51]

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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.2016.05.001.

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