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Synthesis and antitumor activity of novel enediyne-linked pyrrolo[2,1-*c*][1,4]benzodiazepine hybrids

Wan-Ping Hu [a], Jium-Jia Liang [b], Chai-Lin Kao [b], You-Chiang Chen [a], Chung-Yu Chen [b], Feng-Yuan Tsai [c], Ming-Jung Wu [d,f], Long-Sen Chang [e,f], Yeh-Long Chen [b], Jeh-Jeng Wang [b,f,*]

[a] *Department of Biotechnology, College of Life Science, Kaohsiung Medical University, Kaohsiung, Taiwan*
[b] *Department of Medicinal and Applied Chemistry, Kaohsiung Medical University, Kaohsiung, Taiwan*
[c] *Division of Environmental Health and Occupational Medicine, National Health Research Institutes, Miaoli, Taiwan*
[d] *Department of Chemistry, National Sun Yat-Sen University, Kaohsiung, Taiwan*
[e] *Institute of Biomedical Science, National Sun Yat-Sen University, Kaohsiung, Taiwan*
[f] *National Sun Yat-Sen University-Kaohsiung Medical University Joint Research Center, Kaohsiung, Taiwan*

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ABSTRACT

A series of novel pyrrolo[2,1-*c*][1,4]benzodiazepine (PBD) hybrids linked with enediyne is described. These compounds were prepared by linking C-8 of DC-81 (**1**) with an enediyne (**10–16**) through carbon chain linkers to afford PBD hybrid agents **17–23** in good yields. Most of the hybrids on human cancer cell lines exhibited higher cytotoxicity, and an increase in the sub-G1 population than **1**. In a previous article, we have demonstrated that DC-81-indole conjugate agents (**3–6**) are potent inducers of cell apoptosis in melanoma. In the present article, we investigated whether DC-81-enediyne agents possess more cytotoxicity than **6** on human 293T cells. Our data revealed that treatment of 293T cells with DC-81-enediyne resulted in a significant increase of annexin V binding, caspase-3 degradation, and p53 arrest to identify apoptotic cells than **6**. These results suggest that the DC-81-enediyne agents are more efficient in inducing apoptosis than DC-81-indole in 293T cells.

1. Introduction

DNA is a target for many antitumor drugs currently used in the clinic. However, there are only a few DNA-interactive agents that bind to DNA with high sequence selectivity. The development of low molecular weight molecules with the highly sequence-selective DNA-interactive properties is a present study of interest. DC-81 (1), an antitumor antibiotic produced by *Streptomyces* species, belongs to the pyrrolo[2,1-*c*][1,4]benzodiazepines (PBDs) which are potent inhibitors of nucleic acid synthesis. Although the naturally occurring PBDs have potent anticancer activity, they have been precluded from clinical application due to side effects.[1] Therefore, conjugate agents with active moieties of known antitumor and antiviral agents are being designed and synthesized to provide highly sequence-selective DNA-interactive properties and antitumor activity (Fig. 1).[2,3] An enediyne (**7–9**, Fig. 2)[4–6] contains either DNA intercalating groups[7] or DNA minor groove binding functions[8,9] and these are potent DNA-damaging agent due to their ability to generate benzenoid diradicals.[10] These results encouraged us to design and synthesize a diversity of novel PBD conjugate agents.

In the present study, we report the design, synthesis, and biological evaluation of a homologous series of PBD-enediyne conjugates. Meikrantz and Schlegel reported the control of cell death is linked to the cell cycle.[11] Cells with a defective cell cycle are more vulnerable to some anticancer agents according to numerous preclinical studies. P53, the tumor-suppressor protein, is commonly associated with anticancer agent-induced cell cycle arrest and apoptosis.[12–15] Additionally, a major part of the phenomenon could be mediated by deregulation in cell cycle progression governed by the families of caspases, especially the caspase-3, which was the straight promoter of apoptosis.[16,17] In this regard, we have initiated experiments aimed at characterizing the above molecules in the process of apoptosis after 293T cells were treated with PBD conjugate agents.

The aim of this study was to investigate whether DC-81-enediyne conjugates possessed more cytotoxicity than DC-81-indole and verify whether hybrid agents induced antiproliferation, leading to cell growth cycle perturbation, a decrease in caspase-3 and p53 expression, and subsequent apoptotic cell death.

2. Results and discussion

2.1. Syntheses

We prepared the enediyne moieties as shown in Scheme 1. (2-Phenylethynyl)phenyl-alkyn-1-ols (**7**) were treated with carbon

* Corresponding author at present address: Department of Medicinal and Applied Chemistry, Kaohsiung Medical University, Kaohsiung, Taiwan. Tel.: +886 7 312 1101x2275; fax: +886 7 312 5339.
E-mail address: jjwang@kmu.edu.tw (J.-J. Wang).

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tetrabromide in the presence of triphenyl phosphine to give corresponding bromides **10–12** in moderate to good yields (Eq. 1, Scheme 1). While *trans*-4-alkyoxy-1-bromo-2-butenes (**13** and **14**) were prepared by the reaction of alcohols **7** with *trans*-1,4-dibrom-2-butene in basic condition with potassium iodide (Eq. 2). Under similar reaction conditions, *cis*-4-alkyoxy-1-chloro-2-butenes (**15** and **16**) were obtained in moderate yields (Eq. 3). We have previously reported the efficient synthesis of **1** in excellent yield.[18] Condensation of **1** with bromide or chloride compounds **10–16** in the presence of KI at 45 °C afforded the desired conjugate agents **17–23** in 32–83% yields (Scheme 2).

2.2. In vitro cytotoxic effects

We evaluated the cytotoxicity of the DC-81-enediyne agents **17–23** in various human cell lines by the MTT assay. As shown in Table 1, most of the conjugate agents **17–23** on human cancer cell lines exhibited higher cytotoxicity relative to **1** (DC-81). Because the agents **17–23** exhibited a higher inhibitory activity on 293T cells compared to other cell lines at a concentration of 4 μM, the 293T cells were selected as a model for further studies. The compound **18** was selected by the US National Cancer Institute for evaluation in the in vitro preclinical antitumor screening program against sixty human tumor cell lines derived from nine cancer cell types. The selected biological evaluation result for this compound is presented in Table 2. The GI_{50} value of **18** was less than 1 μM, indicating that this conjugate agent has the potency of the growth inhibition activity against cancer cell lines. Moreover, to confirm whether conjugate agent is more effective as an antiproliferative agent than **1**, **11** (enediyne) or a combination of **1** and **11**, we used conjugate agent **18** for this issue. Compound **18** exhibited a higher inhibitory activity compared to that of other agents on 293T cells (Fig. 3).

2.3. Cellular sub-G1 accumulation

Agent action resulted in cells having a hypodiploid DNA content (sub-G1 material) that is characteristic of apoptosis and reflects fragmented DNA as shown in Figure 4. Treatment of 293T cells with 4 μM agents for 24 h induced apoptosis effects in 6.3% (**control**), 10.0% (**1**), 6.3% (**17**), 14.5% (**18**), 13.2% (**19**), 13.9% (**20**), 13.6% (**21**), 15.6% (**22**), and 13.3% (**23**) of sub-G1 DNA peak. Most of the conjugate agents **17–23** were more efficient in inducing cellular sub-G1 accumulation than **1** in 293T cells.

2.4. Apoptosis detection

The changes in cellular morphology after treatment with compounds (**6** and **18**) at a concentration of 4 μM or treated with graded concentrations of **18** for 24 h is shown in Figure 5A. The apoptotic morphology was dependent on drug concentration and **18** exhibited a higher apoptotic effect compared to that of **6** on 293T. To further characterize whether compound **18**-induced cell death involved apoptosis or not, we performed a biparametric cytofluorimetric analysis using annexin V and PI double staining. Fluorescein isothiocyanate (FITC)-conjugated annexin V has been utilized to detect the externalization of phosphatidylserine that occurs at an early stage of apoptosis. Propidium iodide (PI) is used as a marker of necrosis due to cell membrane destruction.[19] The distribution of stained cells is shown in Figure 5B. At a concentration of 1 μM, compound **18** exhibited an apoptotic effect (annexin V^+/PI^-) on 293T cells, and higher concentrations induced cell death with increased cell permeability. In addition, to elucidate whether **18** induces more apoptotic cells than **6**, treatment of 293T cells with 4 μM agents for 24 h induced apoptosis effects in 2.0% (**Control**), 25.4% (**18**), and 19.4% (**6**) of annexin V-FITC cells (Fig. 5C).

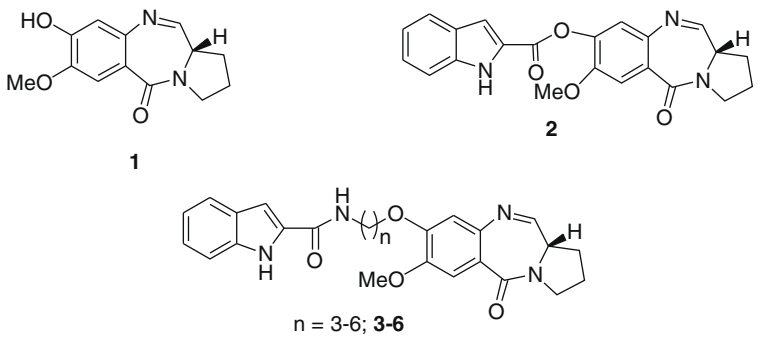


Figure 1. DC-81-indole conjugate agents.

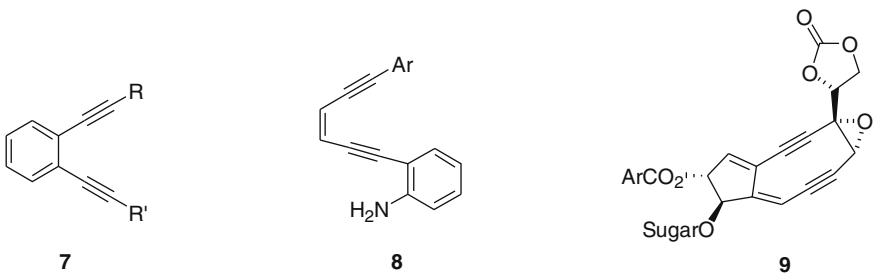
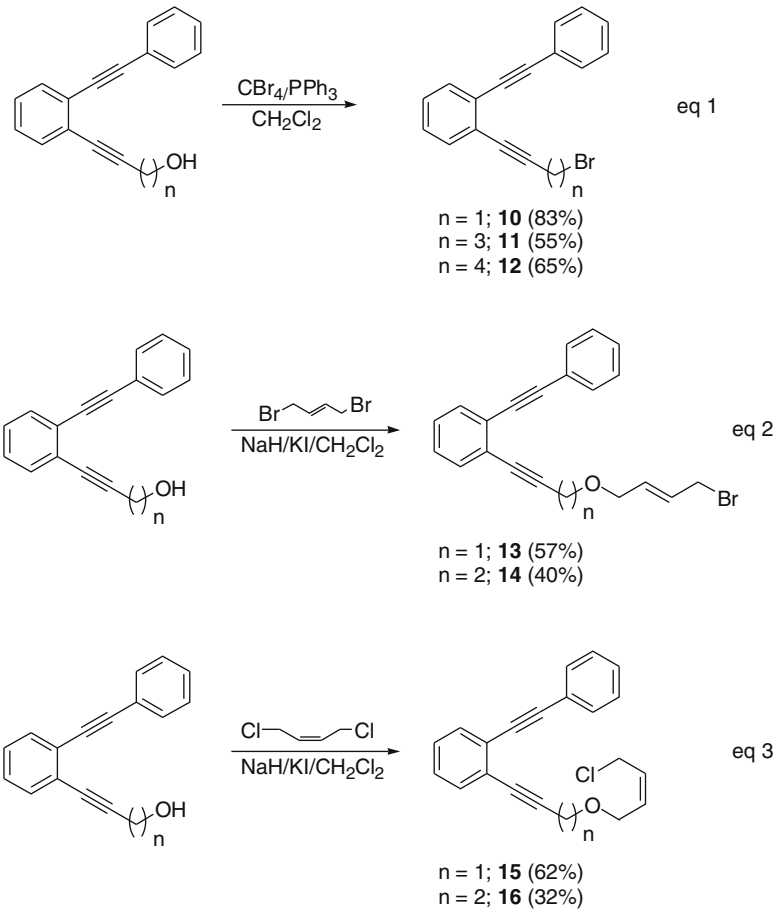


Figure 2. Enediyne analogues.



Scheme 1. Synthesis of enediyne analogues **10**–**16**.

2.5. Apoptosis-related protein expression

Caspase-3 has been shown to be one of the most important cell executioners for apoptosis.[20,21] The p53 gene is considered a tumor-suppressor gene,[22] which might be associated with the DNA repair process.[23] The expression of apoptosis-related protein was determined using immunoblotting assay. The results revealed that the expression of caspase-3 was dependent on drug concentration when 293T cells were exposed to compound **18**. An increase in p53 protein expression was observed in the groups treated with **18** at low concentrations, while DNA repair deficiency was observed at concentration higher than 2 μM. Additionally, our results also showed that **18**-treated cells exhibited significantly decreased in caspase-3 and p53 expression compared to those of **6** at a concentration of 4 μM (Fig. 6A and B).

3. Conclusions

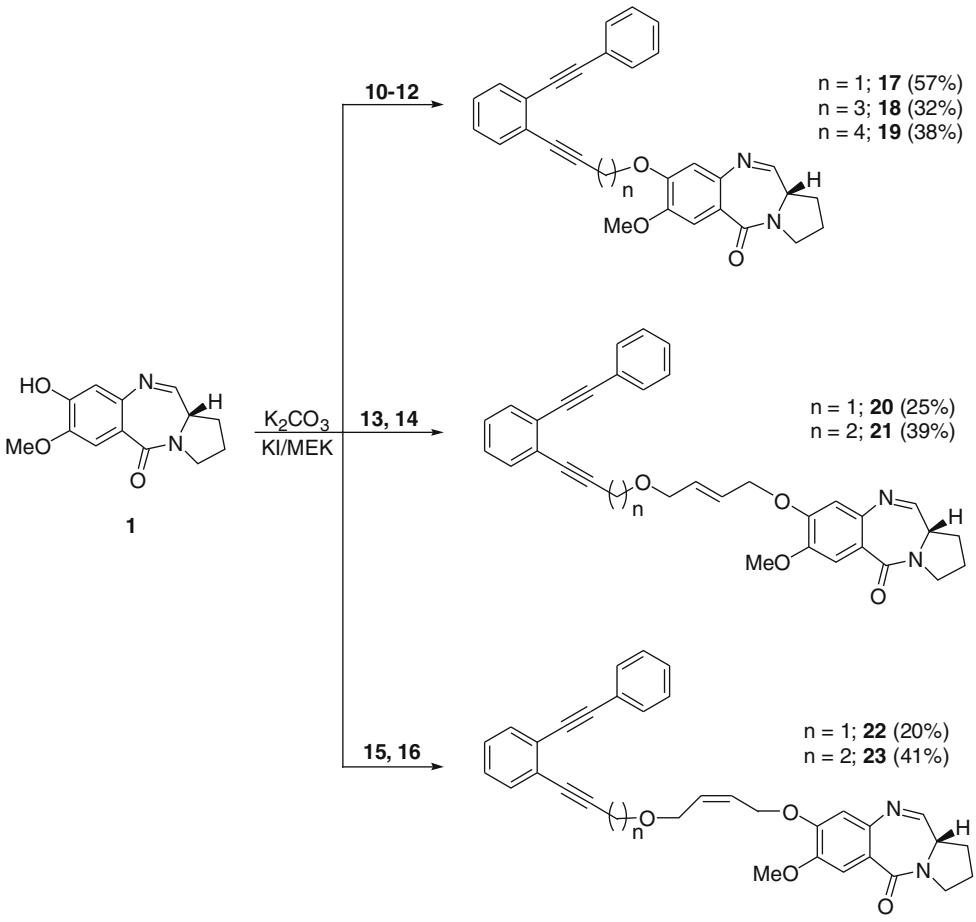
We have previously combined DC-81 and an indole 2-carbonyl moiety to synthesize DC-81-indole conjugate agents designed to have much higher sequence selectivity in DNA interactivity. We used human melanoma A2058 cells as a model as these provide the hybridizing agents that are potent inducers of cell apoptosis.[3] This novel result raised our interest and prompted us to synthesize DC-81-enediyne designed to have much more biological potency than DC-81-indole conjugate agents. Firstly, we used an MTT assay to evaluate the cytotoxicity of tested compounds in five human cell lines, A2058, A375, 293T, H1355, and MCF-7. Our results indicated that most agents are more effective as an antiproliferative agent than DC-81 (**1**), which may be associated with cellular apoptosis. In addition, most DC-81-enediynes induced a markedly increased accumulation of sub-G1 phase cells compared to that of **1** on 293T. Furthermore, compound **18** triggered more apoptotic cells than **6** as revealed by the externalization of annexin V-targeted PS residues at the periphery of the cells. It has been hypothesized that the sensitivity of tumor cells to compound **18** might be associated with the DNA repair process that involves p53. Data from Western blot analysis showed that cells treated by **18** resulted in a decrease in p53 expression than **6** at a concentration of 4 μM. Our result suggests that cells treated by **18** resulted in a decrease in p53 expression, DNA repair deficiency, DNA synthesis block, cell cycle arrest ultimately leading to 293T cell apoptosis. Moreover, we also observed that **18** exposure resulted in a degradation in caspase-3 than **6**.

The data gathered in the present study demonstrated that DC-81-enediyne agents are more efficient in inducing apoptosis than DC-81-indole in 293T cells. In this article, we highlight a novel DC-81-enediyne antitumor proliferation mechanism; this new research will be helpful in further elucidation of undiscovered biological properties of these novel antitumor DC-81-enediynes.

4. Experimental

4.1. Syntheses

^{1}H NMR and ^{13}C NMR spectra were recorded at 400 and 100 MHz, respectively, using $CDCl_3$ as a solvent. ^{1}H NMR chemical shifts are referenced to TMS or $CDCl_3$ (7.26 ppm). ^{13}C NMR was ref-



Scheme 2. Synthesis of PBD-enediyne conjugate agents **17**–**23**.

Table 1
Compounds against human-derived cancer cell lines in vitro

Survival (% control)	Compound (4 μm)							
	1	**17**	**18**	**19**	**20**	**21**	**22**	**23**
A2058 (melanoma)	63.4 ± 4.4	46.1 ± 4.8	47.2 ± 1.3	49.5 ± 2.9	43.0 ± 4.1	46.2 ± 4.9	39.1 ± 4.9	84.5 ± 5.6
A375 (melanoma)	50.2 ± 3.2	13.1 ± 6.6	22.3 ± 6.8	28.3 ± 4.8	42.9 ± 1.6	33.4 ± 2.3	33.9 ± 1.6	85.7 ± 0.7
293T (kidney)	68.9 ± 1.8	48.9 ± 1.6	28.7 ± 0.8	19.2 ± 3.5	48.4 ± 0.7	35.2 ± 1.4	44.0 ± 2.4	60.7 ± 0.2
H1355 (lung)	73.9 ± 3.6	70.9 ± 1.6	87.7 ± 0.8	75.2 ± 3.5	67.4 ± 0.7	85.2 ± 1.4	44.0 ± 2.4	71.0 ± 5.6
MCF-7 (breast)	72.3 ± 3.2	48.9 ± 1.6	49.7 ± 0.8	85.2 ± 3.5	70.4 ± 0.7	59.2 ± 1.4	44.0 ± 2.4	83.8 ± 5.6

Cells were cultured with agents at a concentration of 4 μM for 24 h before growth and viability were assessed using the MTT assay.

erenced to $CDCl_3$ (77.0 ppm). Multiplicities were determined by the DEPT sequence as s, d, t, q. Mass spectra and high resolution mass spectra (HRMS) were measured using the electron-impact (EI, 70 eV) technique by Kaohsiung Regional Instrument Center of NSC at NSYSU. Flash chromatography was carried out on Silica Gel 60 (E. Merck, 230–400 mesh).

4.1.1. 1-(3-Brom-prop-1-ynyl)-2-phenylethynyl-benzene (10)

To the solution of 3-(2-phenylethynyl-phenyl)-prop-2-yn-1-ol (500 mg, 1.7 mmol) in anhydrous CH_2Cl_2 (30 mL) was added CBr_4 (1.68 g, 5.1 mmol) at 0 °C. After 10 min of being stirred at the same conditions, PPh_3 (888 mg, 3.4 mmol) was added to the reaction then, the ice bath was removed and the reaction was stirred at the ambient temperature for additional 6 h. The reaction was quenched with ethanol (3 mL) then extracted with ethyl acetate three times. The combined organic layer was washed with brine, dried over $MgSO_4$, and concentrated to give crude product. The crude mixture was subjected to chromatography (silica gel: 40–63 μM, hexane) to give desired product **10** (83%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.62–7.58 (m, 2H), 7.54–7.52 (m, 1H), 7.48–7.45 (m, 1H), 7.39–7.25 (m, 5H), 4.25 (s, 2H); ^{13}C NMR (100 MHz) δ 132.1, 131.9, 131.8, 128.58, 128.51, 128.3, 127.9, 126,2, 124.6, 123.1, 93.7, 88.1, 87.8, 85.4, 15,1. HRMS (EI *m/z*) for $C_{17}H_{11}Br$, calcd 294.0044, found 294.0046.

4.1.2. 1-(5-Brom-pent-1-ynyl)-2-phenylethynyl-benzene (11)

5-(2-Phenyl ethynyl-phenyl)-pent-4-yn-1-ol (500 mg, 1.9 mmol) was subjected to the same procedure which was used to produce **10** afford desired product **11** with 55% yield. ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.55–7.49 (m, 3H), 7.43–7.41 (m, 1H), 7.36–7.32 (m, 3H), 7.26–7.21 (m, 2H), 3.58 (t, J = 6 Hz, 2H), 2.68 (t, J = 6 Hz, 2H), 2.13 (quintet, J = 6 Hz, 2H). ^{13}C NMR (100 MHz) δ 131.9, 131.8,

Table 2
Cytotoxic activity of **18** from the NCI's in vitro selected human cancer cell lines

Panels/cancer cell lines	GI_{50} (μm)
Leukemia	
CCRF-CEM	0.422
MOLT-4	0.648
PRMI-8226	0.285
Colon cancer	
SW-620	0.478
Melanoma	
SK-MEL-2	0.924
Ovarian cancer	
IGROVI	0.549
OVCAR-3	0.520
OVCAR-8	0.394
Renal cancer	
CAKI-1	0.485
RXF393	0.686
Breast cancer	
MCF7	0.305
MDA-MB-435	0.468
BT-549	0.641
T-47D	0.779

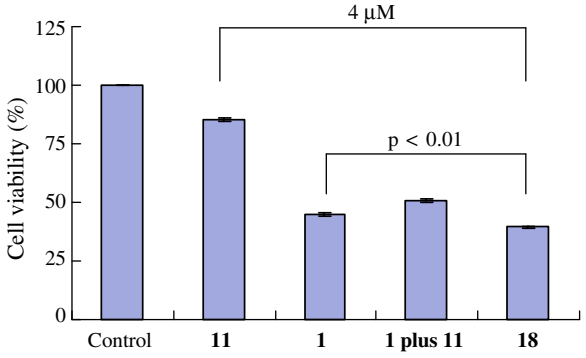


Figure 3. Cell viability of compounds tested against 293T cells (same experimental conditions as in Table 1).

131.6, 128.4, 128.3, 127.9, 127.5, 126,0, 125.6, 123.2, 92.9, 92.3, 88.3, 80.4, 32.4, 31.5, 18.3. HRMS (ESI *m/z*) for $C_{19}H_{15}Br$, calcd 322.0357, found 322.0358.

4.1.3. 1-(6-Brom-hex-1-ynyl)-2-phenylethynyl-benzene (12)

6-(2-Phenyl ethynyl-phenyl)-hexa-5-yn-1-ol (500 mg, 1.92 mmol) was subjected to the same procedure which was used to produce **10** afford desired product **12** with 65% yield. ^{1}H NMR (400 MHz,$CDCl_3$) δ 7.56–7.48 (m, 3H), 7.43–7.33 (4H, m), 7.27–7.22 (m, 2H), 3.34 (t, *J* = 7 Hz, 2H), 2.54 (t, *J* = 7 Hz, 2H), 2.05 (quintet, *J* = 7 Hz, 2H), 1.78 (quintet, *J* = 7 Hz, 2H); ^{13}C NMR (100 MHz) δ 131.8, 131.8, 131.6, 128.4, 128.3, 128.0, 127.4, 126,2, 125.6, 123.2, 93.7, 92.8, 88.4, 80.1, 33.4, 31.6, 27.1, 18.8. HRMS (EI *m/z*) for $C_{20}H_{17}Br$, calcd 336.0514, found 336.0512.

4.1.4. 1-[3-((*E*)-4-Bromo-but-2-enyloxy)-prop-1-ynyl]-2-phenylethynyl-benzene (13)

To the solution of 3-(2-phenylethynyl-phenyl)-prop-2-yn-1-ol (200 mg, 0.86 mmol) in anhydrous CH_2Cl_2 (30 mL) was added NaH (60%, 34 mg, 1.44 mmol) at 0 °C. After 10 min of stirring, the ice bath was removed. To the resulting solution was added *trans*-1,4-dibromo-but-2-ene (553 μμL, 2.59 mmol) and KI (43 mg, 0.26 mmol). After 10 min of stirring at ambient temperature, the resulting solution was heated at reflux temperature for 6 h. The reaction was quenched with iced water and extracted with ethyl acetate (30 mL). The organic layer was washed with brine, dried over $MgSO_4$, and concentrated to give crude product. The crude mixture was purified over chromatography (silica gel: 40–63 μM, CH_2Cl_2/hexane = 1:10) to give desired product (57%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.56–7.28 (m, 9H), 5.94–5.75 (m, 2H), 4.47 (s, 2H), 4.18 (d, *J* = 6.8 Hz, 2H), 3.89 (d, *J* = 6.8 Hz, 2H); ^{13}C NMR (100 MHz) δ 131.9, 131.8, 131.7, 130.9, 129.2, 128.5, 128.4, 128.2, 127.9, 125.9, 125.0, 123.1, 93.3, 88.9, 88.0, 85.4, 68.7, 58.2, 31.8.

4.1.5. 1-[4-((*E*)-4-Bromo-but-2-enyloxy)-but-1-ynyl]-2-phenylethynyl-benzene (14)

4-(2-Phenylethynyl-phenyl)-but-3-yn-1-ol (200 mg, 0.81 mmol) and *trans*-1,4-dibromo-but-2-ene (521 μμL, 2.44 mmol) were subjected to the same procedure which was used to produce **13** afford desired product **11** with 40% yield. ^{1}H NMR(400 MHz, $CDCl_3$) δ 7.56–7.46 (m, 4H), 7.38–7.32 (m, 3H), 7.27–7,22 (m, 2H), 5.96–5.88 (m, 1H), 5.83–5.57 (m, 1H), 4.01 (dd, *J* = 1, 6 Hz, 2H), 3.91 (d, *J* = 7 Hz, 2H), 3.67 (t, *J* = 7.2 Hz, 2H), 2.79 (t, *J* = 7.2 Hz, 2H); ^{13}C NMR (100 MHz) δ 131.8, 131.7, 131.6, 130.4, 130.0, 128.6, 128.3, 128,3, 127,9, 126.0, 125.7, 123.3, 92.9, 90.9, 88.4, 80.4, 70.2, 68.2, 31.9, 21.0.

4.1.6. 1-[3-((*Z*)-4-Chloro-but-2-enyloxy)-prop-1-ynyl]-2-phenylethynyl-benzene (15)

3-(2-Phenylethynyl-phenyl)-prop-2-yn-1-ol (200 mg, 0.86 mmol) and *cis*-1,4-dichloro-but-2-ene (322 μμL, 2.59 mmol) were subjected to the same procedure which was used to produce **13** afford desired produce **15** with 62% yield.^{1}H NMR(400 MHz, $CDCl_3$) δ *7.56–7.53* (m, 3H), 7.50–7.48 (m, 1H), 7.38–7.27 (m, 5H), 5.81–5.69 (m, 2H), 4.48 (s, 2H), 4.28 (d, *J* = 6 Hz, 2H), 4.06 (d, *J* = 7 Hz, 2H); ^{13}C NMR (100 MHz) δ 132.0, 131.8, 131.7, 129.8, 129.2, 128.5, 128.4, 128.3, 128.0, 125.9, 125.0, 123.1, 93.4, 88.8, 88.1, 85.4, 64.4, 58.2, 39.1. HRMS (ESI *m/z*) for $C_{21}H_{17}ClONa$, calcd 343.0866, found 343.0863.

4.1.7. 1-[4-((*Z*)-4-Chloro-but-2-enyloxy)-but-1-ynyl]-2-phenylethynyl-benzene (16)

4-(2-Phenylethynyl-phenyl)-but-3-yn-1-ol (100 mg, 0.41 mmol) and *cis*-1,4-dichloro-but-2-ene (152 μμL, 1.22 mmol) were subjected to the same procedure which was used to produce **13** afford desired product **16** with 32% yield.^{1}H NMR (400 MHz, $CDCl_3$) δ 7.57–7.49 (m, 3H), 7.44–7.42 (m, 1H), 7.38–7,33 (m, 3H), 7.27–7,22 (m, 2H), 5.79–5.68 (m, 2H),4.10 (q, 4H), 3.67 (t, *J* = 7 Hz, 2H), 2.79 (t, *J* = 7 Hz, 2H); ^{13}C NMR (100 MHz) δ 131.8, 131.64, 131.64, 130.6, 128.4,128.3, 128.31, 127,9, 127.5, 126.0, 125.7, 123.3, 92.9, 90.9, 88.4, 80.3, 68.8, 66.0, 39.1, 21.0.

4.1.8. 7-Methoxy-8-[3-(2-phenylethynyl-phenyl)-prop-2-ynyloxy]-1,2,3,11a-tetrahydro-benzo[*e*]pyrrolo[1,2-*a*][1,4]diazepin-5-one (17)

To the solution of **10** (120 mg, 0.406 mmol) in anhydrous MEK (5 mL) was added KI (40 mg, 0.24 mmol) and irritated in sonicator for 5 min. To the resulting mixture was added the solution of **1** (100 mg, 0.41 mmol) and K_2CO_3 (168 mg, 1.21 mmol) in anhydrous butanone (25 mL). After the addition, the resulting solution was heated to 45 °C for 24 h. The reaction quenched by iced water. The resulting mixture was extracted with ethyl acetate. The organic layer was washed with brine, dried over $MgSO_4$ to give a crude product which was further purified over chromatography (silica gel, 40–63 μM, CH_2Cl_2/MeOH = 150:1) to give desired product (**17**) with 57% yield. ^{1}H NMR (400 MHz, $CDCl_3$) δ *7.60–7.30* (11H, m), 7.10 (s, 1H), 5.13 (dd, *J* = 7, 16 Hz, 2H), 3.95 (s, 3H), 3.82–3.76 (m, 1H), 3.60–3.52 (m, 2H), 2.28–2.23 (m, 2H), 2.06–2.00 (m,

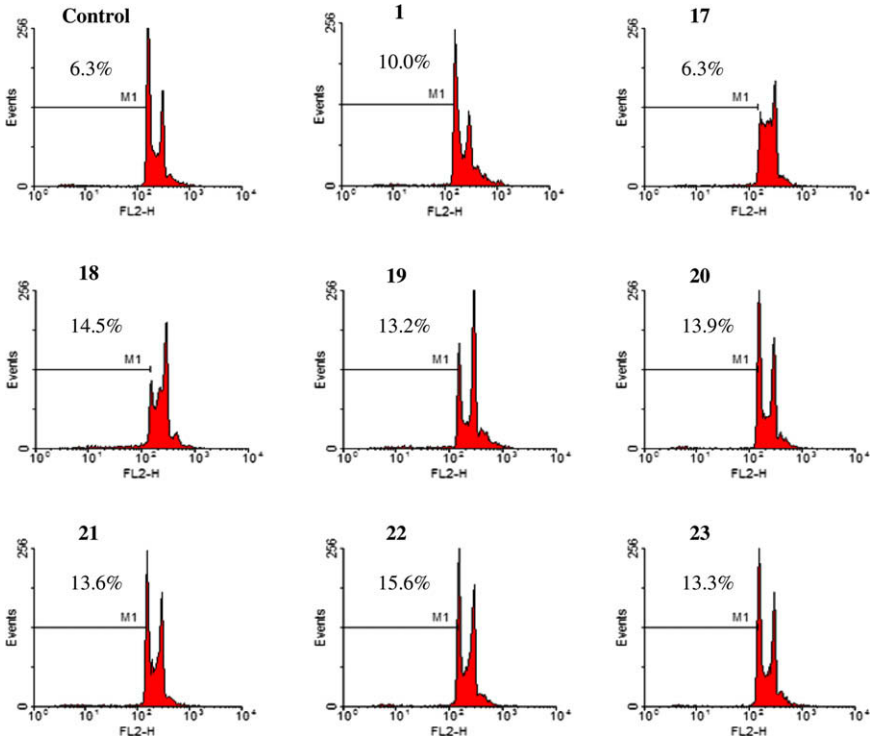


Figure 4. Effect of compound tested on the cellular sub-G1 content. 293T cells were treated with treated with 4 μM agents for 24 h and stained with PI. Approximately 10,000 cells from each group were analyzed with the FACScan flow cytometer. Data represent the percentage of cell counts and display sub-G1.

2H); ^{13}C NMR (100 MHz) δ 164.5, 162,4, 149.4, 147.8, 140.5, 132.2, 131.8, 131.2 131.8, 131.7, 128.4, 128.3, 128.2, 127.8, 125.9, 124.6, 123.1, 121.0 111.7, 111.5, 93.5, 87.8, 87.0, 86.6, 57.5, 56.1, 53.5, 46.6, 29.6, 24.1. HRMS (ESI *m/z*) for $C_{30}H_{25}N_2O_3$, calcd 461.1865, found 461.1868.

4.1.9. 7-Methoxy-8-[4-(2-phenylethynyl-phenyl)-but-3-ynyl-oxy]-1,2,3,11a-tetrahydro-benzo[*e*]pyrrolo[1,2-*a*][1,4]diazepin-5-one (18)

Compound **1** (100 mg, 0.41 mmol) and **11** (125 mg, 0.41 mmol) were subjected to the same procedure which was used to produce **17** afford desired product **18** with 32% yield. ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.64 (d, J = 4 Hz, 1H), 7.52–7.49 (m, 4H), 7.44–7.41(m, 1H), 7.31–7.23 (m, 5H), 6.80 (s, 1H), 4.26–4.20 (m, 2H), 3.93 (s, 3H), 3.84–3.78 (m, 1H), 3.68–3.48 (m, 2H), 2.74 (t, J = 7 Hz, 2H), 2.31–2.27 (m, 2H), 2.23–2.16 (m, 2H), 2.08–2.01(m, 2H); ^{13}C NMR (100 MHz) δ 162.3, 150.7, 147.8, 140.6, 131.9, 131.7, 131.6, 128.3, 128.2, 127.9, 127.4, 126.2, 125.6, 123.3, 120.2, 111.6, 110.6, 93.2, 93.2, 88.4, 80.1, 67.5, 56.1, 53.7, 46.6, 29.6, 28.7, 24.2, 16.4. HRMS (ESI *m/z*) for $C_{32}H_{29}N_2O_3$, calcd 489.2178, found 489.2180.

4.1.10. 7-Methoxy-8-[5-(2-phenylethynyl-phenyl)-pent-4-ynyl-oxy]-1,2,3,11a-tetrahydro-benzo[*e*]pyrrolo[1,2-*a*][1,4]diazepin-5-one (19)

Compound **1** (100 mg, 0.41 mmol) and **12** (131 mg, 0.41 mmol) were subjected to the same procedure which was used to produce **17** afford desired product **19** with 38% yield.^{1}H NMR (400MHz, $CDCl_3$) δ 7.64 (d, J = 4 Hz, 1H), 7.52–7.49 (m. 4H), 7.43–7.40 (m, 1H), 7.32–7.23 (m, 5H), 6.75 (s, 1H), 4.04–4.20 (m, 2H), 3.93 (s, 3H), 3.84–3.78 (m, 1H), 3.72–3.68 (m, 1H), 3.61–3.54 (m, 1H), 2.59 (t, J = 7 Hz, 2H), 2.34–2.28 (m, 2H), 2.10–2.00 (m, 5H), 1.86–1.79 (m, 2H); ^{13}C NMR (100 MHz) δ 164.6, 162,3, 150.7, 147.8, 140.6, 131.9, 131.7, 131.6, 128.3, 128.2, 127.9, 127.4, 126.2, 125,6, 123.3, 120.2, 111.6, 110.6, 94.1, 92.8, 88.5, 80.1, 68.3, 56.1, 53.7, 46.6, 29.6, 28.1, 25.2, 24.1, 16.4. HRMS (EI *m/z*) for $C_{33}H_{31}N_2O_3$, calcd 503.2335, found 503.2332.

4.1.11. 7-Methoxy-8-[6-(2-phenylethynyl-phenyl)-hex-5-ynyl-oxy]-1,2,3,11a-tetrahydro-benzo[*e*]pyrrolo[1,2-*a*][1,4]diazepin-5-one (20)

Compound **1** (100 mg, 0.41 mmol) and **13** (137 mg, 0.41 mmol) were subjected to the same procedure which was used to produce **17** afford desired product **20** with 25% yield. ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.66 (d, J = 4 Hz, 1H), 7.56–7.46 (m, 5H), 7.36–7.25 (m, 5H), 6.78 (s, 1H), 5.99–5.86 (m, 2H), 4.64–4.54 (m, 2H), 4.47–4.54 (m, 2H), 4.21 (d, J = 5 Hz, 2H), 3.94 (s, 3H), 3.87–3.79 (m, 1H), 3.73–3.69 (m, 1H), 3.61–3.54 (m, 1H), 2.32–2.28 (m, 2H), 2.08–2.00 (m, 2H); ^{13}C NMR (100 MHz) δ 164.6, 162.4, 150.2, 147.8, 140.5, 131.9, 131.7, 131.7, 130.3, 128.4, 128.3, 128.1, 127.9, 127.3, 125.9, 125.1, 123.1, 120.5, 111.6, 110.8, 93.3, 89.1, 88.1, 85.0, 69.1, 68.6, 58.1, 56.1, 53.7, 46.6, 29.6, 24.2. HRMS (EI *m/z*) for $C_{34}H_{31}N_2O_2$, calcd 531.2284, found 531.2284.

4.1.12. 7-Methoxy-8-{(*E*)-4-[3-(2-phenylethynyl-phenyl)-prop-2-ynyloxy]-but-2-enyloxy}-1,2,3,11a-tetrahydro-benzo[*e*]pyrrolo[1,2-*a*][1,4]diazepin-5-one (21)

Compound **1** (100 mg, 0.41 mmol) and **14** (147 mg, 0.41 mmol) were subjected to the same procedure which was used to produce **17** afford desired product **21** with 39% yield. ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.65 (d, J = 4.4 Hz, 1H), 7.56–7.46 (m, 5H), 7.36–7.25 (m, 5H), 6.78 (s, 1H), 5.99–5.89 (m, 2H), 4.66–4.57 (m, 2H), 4.05 (d, J = 4.8Hz, 2H), 3.94 (s, 3H), 3.87–3.79 (m, 1H), 3.73–3.69 (m, 3H), 3.61–3.54 (m, 1H), 2.89 (t, J = 7 Hz, 2H), 2.31–2.27 (m, 2H), 2.08–2.02 (m, 2H); ^{13}C NMR (100 MHz) δ 164.6, 162.4, 150.2, 147.8, 140.5, 131.9, 131.8, 131.7, 130.3, 128.4, 128.3, 127.9, 127.4, 126.9, 126.1, 125,7, 125,7, 123.4, 120.4, 111.6, 110.8, 93.0, 91.0, 88.4, 80.4, 70.6, 68.7, 56.1, 53.7, 46.6, 29.6, 24.2, 21.1. HRMS (ESI *m/z*) for $C_{35}H_{33}N_2O_4$, calcd 545.2440, found 545.2443.

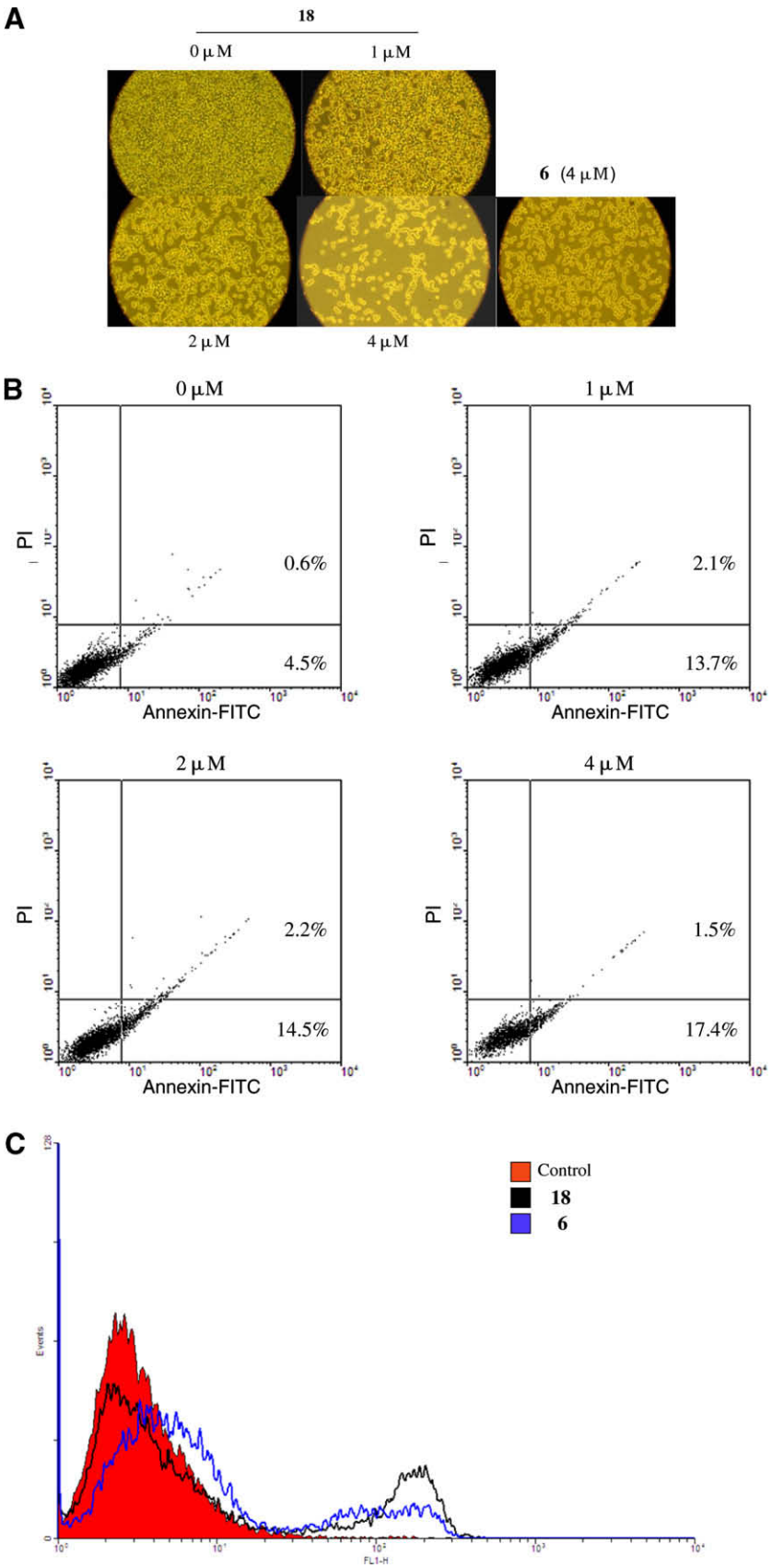


Figure 5. Compound **18** induces 293T cell apoptosis. (A) Morphological changes of 293T cells treated with compound (**6**, **18**) at a concentration of 4 μM or treated with graded concentrations of **18** for 24 h. (B) Dot plots for 293T cells treated with graded concentrations of **18** for 24 h and then stained with PI and an annexin V-FITC conjugate specifically detecting the exposure of PS residues at the cell surface. (C) To elucidate whether **18** induces more apoptotic cells than **6**, cells were cultured with 4 μM agents for 24 h, then stained with annexin V-FITC and approximately 10,000 cells from each group were analyzed by FACS.

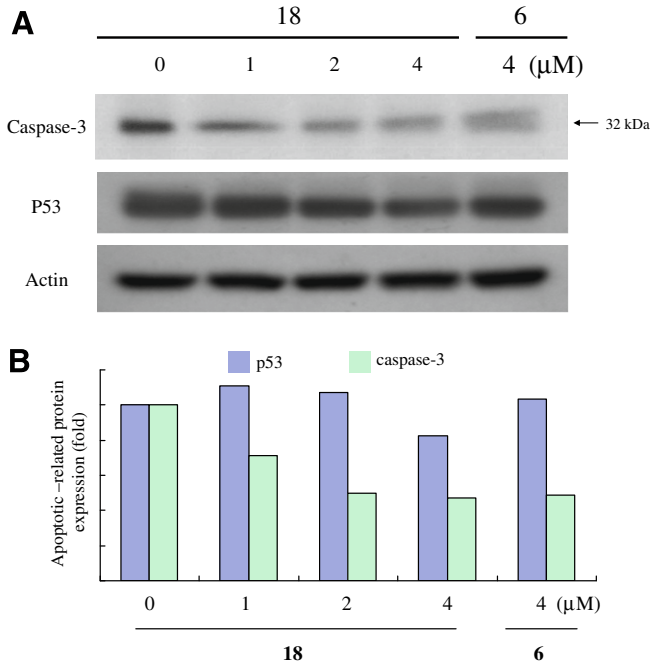


Figure 6. Immunoblot analysis showed the effect of compound on the degradation of apoptotic molecules. (A) After exposure to different concentration of **18** or incubated with 4 μM **6** for 24 h, cell lysates were collected and immunoblotted with specific antibodies as indicated. For the internal control, the same amounts of protein extract were also probed with antibody against actin. (B) The pattern of the caspase-3 and p53 by 293T cells treated with compound (**6**, **18**).

4.1.13. 7-Methoxy-8-{(*Z*)-4-[3-(2-phenylethynyl-phenyl)-prop-2-ynyloxy]-but-2-enyloxy}-1,2,3,11a-tetrahydro-benzo[*e*]pyrrolo[1,2-*a*][1,4]diazepin-5-one (22)

Compound **1** (100 mg, 0.41 mmol) and **15** (130 mg, 0.41 mmol) were subjected to the same procedure which was used to produce **17** afford desired product **22** with 20% yield. ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.62 (d, J = 4 Hz, 1H), 7.57–7.44 (m, 5H), 7.34—7.22 (m, 5H), 6.77 (s, 1H), 5.92–5.76 (m, 2H), 4.70 (d, J = 6 Hz, 2H), 4.50–4.46 (m, 2H), 4.31 (d, J = 6 Hz, 2H), 3.92 (s, 3H), 3.87–3.77 (m, 1H), 3.67–3.53 (m, 2H), 2.32–2.25 (m, 2H), 2.08–2.00 (m, 2H); ^{13}C NMR (100 MHz) δ 164.6, 162,2, 150.1, 147.8, 140.6, 131.9, 131.7, 131.6, 129.4, 128.5, 128.3, 128.2, 128.1, 127.9, 125,9, 125,0, 123.1, 120.4, 111.5, 110.8, 93.4, 88.9, 88.0, 85.3, 65.3, 65.0, 58.2, 56.1, 53.6, 46.6, 29.6, 24.1. HRMS (EI m/z) for $C_{34}H_{31}N_2O_4$, calcd 531.2284, found 531.2283.

4.1.14. 7-Methoxy-8-{(*E*)-4-[4-(2-phenylethynyl-phenyl)-but-3-ynyloxy]-but-2-enyloxy}-1,2,3,11a-tetrahydro-benzo[*e*]p- yrrolo-[1,2-*a*][1,4]diazepin-5-one (23)

Compound **1** (100 mg, 0.41 mmol) and **16** (135 mg, 0.41 mmol) were subjected to the same procedure which was used to produce **17** afford desired product **23** with 41% yield. ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.64 (d, J = 4.4 Hz, 1H), 7.55–7.48 (m, 4H), 7.43–7.41 (m, 1H), 7.36—7.31 (m, 3H), 7.25–7.23 (m, 2H), 6.80 (s, 1H), 5.89–5.77 (m, 2H), 4.78–4.71 (m, 2H), 4.16(d, J = 5.6 Hz, 2H), 3.93 (s, 3H), 3.87–3.78 (m, 1H), 3.71–3.65 (m, 3H), 3.60–3.53 (m, 1H), 2.80 (t, J = 7.2 Hz, 2H), 2.30–2.25 (m, 2H), 2.07–2.00 (m, 2H); ^{13}C NMR (100 MHz) δ 164.5, 162.4, 150.1, 147.7, 140.5, 131.8, 131.61, 131.61, 130.1, 128.3, 128.2, 127.9, 127.5, 127.4, 126,0, 125.6, 123.3, 120.4, 111.5, 110.7, 92.9, 91.0, 88.3, 80.3, 68.8, 66.8, 65.0, 56.1, 53.6, 46.6, 29.5, 24.1, 21.0. HRMS (ESI m/z) for $C_{35}H_{33}N_2O_4$, calcd 545.2440, found 545.2438.

4.2. Cell culture

Five human cell lines, A2058 (melanoma), A375 (melanoma), 293T (kidney), H1355 (lung) and MCF-7 (breast) purchased from American Type Culture Collection (Manassas, VA), were maintained in DMEM (A2058, A375, 293T) or RPMI1640 (H1355, MCF-7) medium supplemented with 10% FCS and 100 U/mL penicillin G and 100 μg/mL streptomycin sulfate (Gibco, BRL) at 37 °C in a humidified atmosphere containing 5% CO_2.

4.3. Cell viability

Cell viability was assessed by the MTT assay, a mitochondrial function assay based on the ability of viable cells to reduce the redox indicator MTT to insoluble formazan crystals by mitochondrial dehydrogenase. Briefly, cells were seeded in a 96-well plate at the cell density of 2500 cells/well. After an overnight incubation, the cells were treated with compounds at 4 μM and incubated for 24 h. The medium was then discarded and replaced with 10 μL of MTT dye. Plates were incubated at 37 °C for 2 h. The resulting formazan crystals were solubilized in 100 μL DMSO, and the optical density was read at 540 nm with a microplate reader (MRX-II, Dynex technology, Chantilly, VA).

4.4. Sub-G1 region analysis

293T cells were treated with compounds at 4 μM for 24 h. Cells were harvested by trypsinization and centrifugation. Cell pellets were resuspended in 50% cold ethanol and fixed at −20 °C. After fixation, cells were washed once with cold PBS and incubated in 0.5 mL of PBS containing 100 μg/mL RNase A for 20 min at 37 °C. Cells were harvested by centrifugation at 400g for 5 min, and 250 μL of PBS containing 50 μg/mL propidium iodide (PI) was added to the pellet. Thirty minutes later, the DNA contents of 10,000 events were measured by FACSscan flow cytometer (Elite ESP, Beckman Coulter, Brea, CA). Histograms were analyzed using Windows Multiple Document Interface software (WinMDI). Cells with DNA content less than that in untreated cells in G0/G1 were considered apoptotic.

4.5. Morphology observation

239T (5×10^5 cells/well) seeded on 6 well plate. After grow one day, treated with compound **6** or **18** for 24 h. Take photos by using microscope at 200× phase.

4.6. Annexin V and PI binding assay

To assess the simultaneous observation of early phase of apoptotic and necrotic features, 293T cells were treated with different agents at 4 μM for 24 h. Cells were harvested by trypsinization and centrifugation and measured by cytometry by adding annexin V-FITC to 10^6 cells per sample according to the manufacturer's specifications (Bender MedSystems, Vienna, Austria). Simultaneously, the cells were stained with PI. Flow cytometry data were analyzed by the WinMDI software.

4.7. Protein extraction and western blot analysis

Total cell extracts from cultured 293T cells were obtained by lysing the cells in ice-cold RIPA buffer (1 × PBS, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS) containing 100 μg/mL PMSF, 2 μg/mL aprotinin, 2 μg/mL leupeptin and 100 μg/mL NaF. After centrifugation at 14,000g for 30 min, protein in the supernatants was quantified by Bradford method (Bio-Rad). Forty micrograms of pro-

tein per lane was applied in 10% SDS-poly-acrylamide gel. After electrophoresis, protein was transferred from the gel to polyvinylidene difluoride (PVDF) membrane (Millipore, Bedford, MA). The membranes were blocked at room temperature for 1 h in PBS + 0.1% Tween 20 (PBS-T) containing 5% skim milk. After being briefly rinsed with PBS-T, the membrane was incubated with primary antibody at room temperature for 2 h or at 4 °C overnight. Rabbit polyclonal antibodies against CPP32 (H-277) and mouse monoclonal antibody P53 (DO-1) were purchased from Santa Cruz Biotechnology. Mouse monoclonal antibody against actin was purchased from Chemicon Int. Inc. (Temecula, CA). The membrane was incubated with the corresponding horseradish peroxidase-labeled secondary antibody (Santa Cruz Biotechnology) at room temperature for 1 h. Membranes were washed with PBS-T four times for 15 min and the protein blots were visualized with the Western Lightning Chemiluminescence Reagent Plus (Perkin Elmer Life Sciences, Boston, MA). The relative amounts of specific proteins were quantified by densitometry scanning of X-ray films, and analyzed by the Eagle Eye Image System (Stratagene, La Jolla, CA).

4.8. Statistical analysis

All results were expressed as means values ± standard deviation (SD) and analyzed by using the statistical analysis system (SPSS, SPSS Inc., Chicago, IL). Differences among groups were analyzed by Student's t-test. P values <0.05 were considered as significant for all statistical tests.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmc.2008.12.036.

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