Synthesis, Cytotoxicity and Pro-apoptosis of Novel Benzoisoindolin Hydrazones as Anticancer Agents

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A series of benzoisoindolin hydrazones as analogues of natural lignan diphyllin were synthesized and the structures of these compounds were established by ^{1}H-NMR, ^{13}C-NMR, Mass and high resolution (HR)-MS. The compounds were evaluated for *in vitro* cytotoxicity against KB, A549 and HCT-116 cancer cell lines by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Compound 4 possessed the highest growth inhibitory effect. Significant apoptosis of HCT-116 cells treated with compound 4 was observed by Hoechst33342-propidium iodide (PI) and acridine orange (AO)-ethidium bromide (EB) staining assay. Western blot analysis disclosed that compound 4 induced apoptosis *via* the mitochondrial pathway accompanied by an increased expression of Bax and a decreased expression of Bcl-2.

Key words diphyllin; synthesis; apoptosis; isoindolin; anticancer

Cancer is now the leading cause of death worldwide. There is an urgent need to develop novel agents for human cancer therapy.[1)] Because the resistance to apoptosis is a hallmark of cancer cells, the crucial approach to anticancer drug discovery is the activation of apoptotic cascades.[2)] A large number of anticancer drugs have been found to induce the apoptotic process in cancerous cells. Thus, discovery of potent apoptosis inducer for cancer treatment is an attractive and prospective strategy.

In searching for novel anticancer agents, various plants provided a large number of lead compounds for further development.[3)] Naturally occuring lignan lactones such as podophyllotoxin exhibit a wide range of pharmacological activities (Fig. 1).[4,5)] Two podophyllotoxin derivatives, etoposide and teniposide, are currently in clinical use as antineoplastic agents due to their abilities to inhibit human DNA-topoisomerase II.[6)] Structure–activity relationships (SAR) of podophyllotoxin disclose that the *trans*-fused-γ-lactone ring is not essential although it contributes to potency.[7,8)]

Diphyllin, an arylnaphthalene lignan lactone isolated from many traditional medicinal plants, has been reported to possess anticancer and antiviral activities.[9,10)] Previously, we have successfully synthesized several natural diphyllin glycosides and their analogues.[11,12)] Biological results showed that they were effective apoptosis inducers and cell proliferation inhibitors, which encouraged us to synthesize more compounds to fully discuss the SAR of diphyllin. To our best knowledge, there is no such effort addressed to modify the lactone moitey of diphyllin. On the other hand, many publications reported that the azomethine moieties showed interesting inhibitory activity against tumoral cells.[13)] It might be of great interest to combine diphyllin to azomethine groups to prepare novel derivatives. Herein, we report the synthesis, cytotoxicity and apoptosis inducing activity of these benzoisoindolin hydrazones as analogues of diphyllin (**2**—**11**, Chart 1).

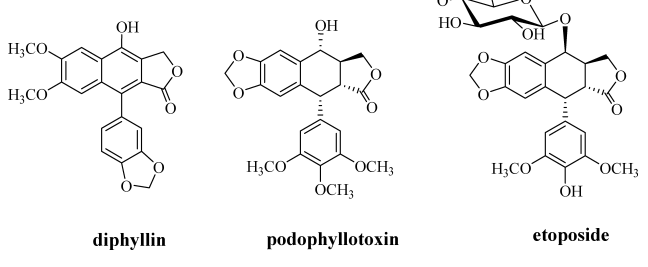

Fig. 1. Natural Antitumor Lignans and Etoposide

Results and Discussion

Synthesis of Benzoisoindolin Hydrazones The synthetic route used to synthesize title compounds is outlined in Chart 1. Diphyllin was prepared according to the procedure reported previously.[14)] Nextly, hydrazide **1** was obtained as yellow solid in 87% yield by the reaction of diphyllin with excess hydrazine hydrate in ethanol over a 6 h reflux period. The structure of compound **1** was confirmed by mass spectroscopy and ^{1}H-NMR. It gave a [M+H]-ion peak at *m*/*z* 395.3 in the electrospray ionization (ESI)-MS in accord with the molecular formula $C_{21}H_{19}N_2O_6$. The ^{1}H-NMR spectra indicated the chemical shift of the NH_2 proton at δ=4.46 ppm in the form of singlet peak. Schiff base reactions of **1** with respective aromatic aldehydes were performed in the presence of a catalytic amount of acetic acid in ethanol to give hydrazone derivatives **2**—**11** in the good yields of 79—87%.[15)]

Inhibitory Effects of Compounds 1—11 on Proliferation of Cancer Cells The title compounds **2**—**11** and intermediate **1** were evaluated for their cytotoxic activity against three cancer cell lines using a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) growth inhibition assay. Etoposide was used as a positive control. IC_{50} values were summarized in Table 1 and represent the concentration inducing a 50% decrease of cell growth after 3 d incubation.

In general, these derivatives displayed potent antiproliferative activity against three cancer cell lines. These results suggested that lactone ring was not essential to diphyllin to keep potency. Substituting lactone ring with isoindolin hydrazones did not show substantial loss in their antiproliferative. The hydrazone **4** was the most effective derivative of all the newly synthesized compounds, showing IC_{50} values in the

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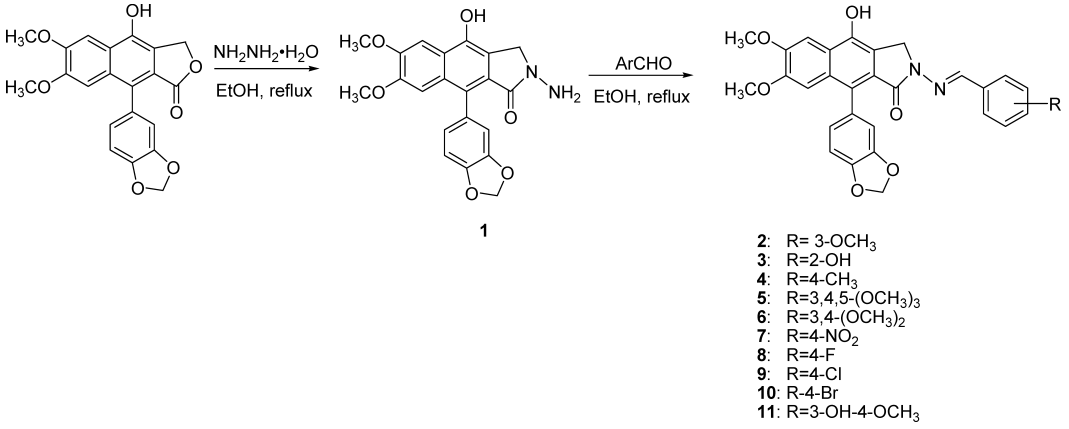

Chart 1. Synthesis of Benzoisoindolin Hydrazones

Table 1. Antiproliferative Activity against Cancer Cell Lines of Hydrazones **1**—**11** and Etoposide

Compound	IC_{50} (μM)		
	KB	HCT-116	A549
1	>50	5.3	>50
2	28.7	10.0	>50
3	>50	5.8	>50
4	7.2	5.6	10.4
5	>50	>50	>50
6	>50	19.8	17.4
7	>50	19.8	>50
8	>50	>50	35.5
9	10.4	>50	>50
10	16.6	24.3	>50
11	>50	>50	>50
Diphyllin	12.4	48.1	>50
Etoposide	11.3	13.5	>50

range of 5.6—10.4 μM in three cancer cell lines. Compound **4** has a methyl group at 4-position of benzaidehyde skeleton showed the best antiproliferative activities. The results showed that compounds **1** and **3** were highly active only against HCT-116 cell line. However, the precurosor diphyllin showed lower activities, which suggestted there is different modes of action of these two compounds.

Induction of Apoptosis in HCT-116 Cells by Compound 4 On the basis of the above results, further biological evaluations have been focused on compound **4**. HCT-116 cells were stained with Hoechst33342-propidium iodide (PI) and acridine orange-ethidium bromide (AO-EB) to verify the type of cell death. Without compound **4** treatment, the nuclei of control cells showed uniform blue fluorescence, which indicated the cells were healthy and the nuclei were intact. However, after treatment with compound **4** for 72 h, most of nucleis were stained pale red by PI, which indicated cells were apoptotic (Fig. 2). These results showed that cells death occurred primarily through apoptosis.

The results obtained from the AO-EB staining are as shown in Fig. 2. Viable cells with intact DNA and nucleus showed homogeneous bright green. Early apoptotic cells showed green nuclei, but perinuclear chromatin condensation was visible as bright green patches or fragments. Late apoptotic cells DNA were fragmented and stained orange. It was

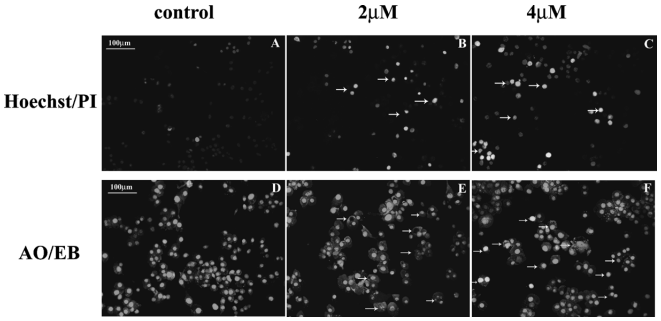

Fig. 2. Morphological Study of HCT-116 Cells Treated with Compound **4** in 2 μM, 4 μM Respectively for 72 h by Hoechst33342-PI, AO/EB Double Staining

Hoechst/PI (A—C): bright blue cells showed early apoptotic, pale red cells were later apoptotic. AO/EB (D—F): vacuolation and DNA fragmentation was obvious, orange cells indicate later apoptosis.

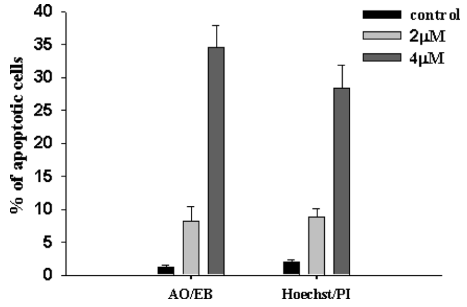

Fig. 3. Bar Diagram for Comparison of Percent Cells in Apoptosis as Revealed by AO/EB, Hoechst/PI Staining Followed by Manual Counting in Different Fields of Vision for 3 Times

clear that most of cells treated with compound **4** exhibited typical characteristics of apoptotic cells like plasma membrane blebbing. This result also indicates that cells death occurred primarily through apoptosis. Data collected from the manual counting of cells with normal and apoptotic nuclear features are shown in Fig. 3.

Effect of Compound 4 on Apoptosis-Related Proteins Apoptosis is usually controlled by two major pathway, the mitochondrial pathway and membrane death receptor pathway.[16] To reveal the possible mechanisms responsible for the apoptotic effects of compound **4**, we nextly evaluate its effects on the expression level of a series of proteins associated with apoptosis. Bcl-2 can prevent the release of cytochrome

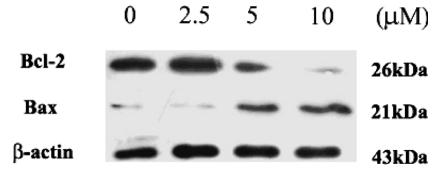

Fig. 4. Western Blot Analysis of Protein Levels of Bcl-2, Bax in A549 Cells Treated with Compound **4**

Compound **4** treated cell extracts were obtained and equal amounts of protein were resolved by SDS-PAGE. Separated protein bands were transferred onto PVDF membranes and incubated with specific primary and secondary antibodies, respectively. Western blots were developed with enhanced chemiluminescence reagent. *β*-Actin was used normalize total proteins.

c from the mitochondria during apoptosis mediated by the mitochondrial pathway. In contrast, Bax can induce the release of cytochrome *c* from the mitochondria.[17] The present results revealed that compound **4** induced apoptosis *via* the mitochondrial pathway accompanied by an increased expression of Bax and a decreased expression of Bcl-2 (Fig. 4).

Conclusion

In the present study, we have described the preparation and identification of benzoisoindolin hydrazones from diphyllin in two steps. These derivatives displayed potent cytotoxic activity against three human cancer cell lines. Compound **4** showed the highest cytotoxic potency. Apoptosis of HCT-116 cells induced by compound **4** was observed by Hoechst33342-PI staining assay, AO-EB staining assay and Western blot analysis. Compound **4** induced apoptosis *via* the mitochondrial pathway accompanied by an increased expression of Bax and a decreased expression of Bcl-2. Our preclinical data indicate that compound **4** is a potential therapeutic agent for cancer.

Experimental

Syntheses Solvents were purified in the usual way. TLC was performed on precoated Merck silica Gel 60 F_{254} plates. Flash chromatography was performed on silica gel (100—200 mesh, Qingdao, China). ^{1}H- and ^{13}C-NMR spectra were taken on a Bruker 300 MHz spectrometer with tetramethylsilane (TMS) as an internal standard, and chemical shifts were recorded in values. The high resolution spectra were obtained on a Q-TOF Global Mass (ESI-MS).

4′-(3″,4″-Methylenedioxyphenyl)-6′,7′-dimethoxyl-1′-hydroxyl-benzyl-2,3-dihydro-isoindol-1-one-hydrazine (1) To a stirred solution of diphyllin (190 mg, 0.5 mmol) and in EtOH (30 ml) was added hydrazine hydrate (2 ml, 80%, 32 mmol). The mixture was maintained under reflux for 6 h, until TLC indicated the end of reaction. The reaction mixture was cooled and the solid was collected by filtration and washed with cool ethanol to afford a pale yellow solid **1** (170 mg, 87%). ^{1}H-NMR (DMSO-d_6) δ: 7.59 (1H, s), 6.96 (1H, d, J=7.8 Hz), 6.91 (1H, s), 6.78 (1H, d, J=1.5 Hz), 6.69 (1H, dd, J=7.8, 1.5 Hz), 6.08 (2H, s), 4.81 (2H, s), 4.46 (2H, s), 3.91 (3H, s), 3.62 (3H, s). MS (ESI) m/z: 395 (M^+). High resolution (HR)-ESI-MS (positive) m/z: 417.1066 $[M+Na]^+$ (Calcd for $C_{21}H_{18}N_2O_6Na$: 417.1063).

The hydrazones **2**—**11** were prepared as the following method: a solution of 6 mmol of substituted aldehydes in ethanol was added to a solution of 5 mmol of **1** in 50 ml of ethanol. The mixture was refluxed on a water bath for 2—2.5 h. After cooling, the precipitate was filtered, dried and recrystallized from ethanol.

***N*-(3-Methoxybenzylidenyl)-4′-(3″,4″-methylenedioxyphenyl)-6′,7′-dimethoxyl-1′-hydroxyl-benzyl-2,3-dihydro-isoindol-1-hydrazone (2)** White solid, yield 82%. ^{1}H-NMR (DMSO-d_6) δ: 8.15 (1H, s), 7.66 (1H, s), 7.34—7.33 (3H, m), 7.02 (1H, s), 6.99 (1H, s), 6.95 (1H, s), 6.85 (1H, d, J=1.5 Hz), 6.75 (1H, dd, J=7.8, 1.5 Hz), 6.10 (2H, d, J=1.5 Hz), 4.85 (2H, s), 3.93 (3H, s), 3.79 (3H, s), 3.64 (3H, s). ^{13}C-NMR (DMSO-d_6) δ: 163.9, 160.0, 150.4, 149.8, 147.1, 146.8, 145.9, 143.2, 136.7, 130.4, 130.1, 129.7, 128.9, 124.7, 124.2, 123.1, 120.4, 116.3, 115.8, 111.7, 111.6, 108.1, 106.0, 101.4, 56.5, 56.0, 55.5, 45.8. MS (ESI) m/z: 513 (M^+). HR-ESI-MS (positive) m/z: 535.1488 $[M+Na]^+$ (Calcd for $C_{29}H_{24}N_2O_7Na$ 535.1481). 152 °C turn red, then decomposed.

***N*-(2-Hydroxybenzylidenyl)-4′-(3″,4″-methylenedioxyphenyl)-6′,7′-dimethoxyl-1′-hydroxyl-benzyl-2,3-dihydro-isoindol-1-hydrazone (3)** White solid, yield 83%. ^{1}H-NMR (DMSO-d_6) δ: 8.28 (1H, s), 7.59 (1H, s), 7.56 (1H, dd, J=8.1, 1.5 Hz), 7.26 (1H, t, J=7.8 Hz), 6.98 (1H, d, J=7.8 Hz), 6.94—6.90 (3H, m), 6.84 (1H, d, J=1.5 Hz), 6.73 (1H, dd, J=8.1, 1.5 Hz), 6.08 (2H, s), 4.78 (2H, s), 3.89 (3H, s), 3.60 (3H, s). ^{13}C-NMR (DMSO-d_6) δ: 163.6, 157.4, 150.4, 149.8, 147.1, 146.8, 145.8, 144.1, 131.6, 130.1, 130.0, 129.6, 129.5, 129.1, 124.2, 124.1, 123.1, 119.9, 119.3, 116.8, 115.4, 111.7, 108.1, 105.9, 101.4, 55.9, 55.5, 44.6. MS (ESI) m/z: 499 (M^+). HR-ESI-MS (positive) m/z: 521.1324 $[M+Na]^+$ (Calcd for $C_{28}H_{22}N_2O_7Na$ 521.1325). 251 °C turn red, then decomposed.

***N*-(4-Methylbenzylidenyl)-4′-(3″,4″-methylenedioxyphenyl)-6′,7′-dimethoxyl-1′-hydroxyl-benzyl-2,3-dihydro-isoindol-1-hydrazone (4)** White solid, yield 87%. ^{1}H-NMR (DMSO-d_6) δ: 8.10 (1H, s), 7.63 (1H, s), 7.60 (2H, s), 7.24 (1H, s), 7.21 (1H, s), 6.98 (1H, d, J=8.1 Hz), 6.90 (1H, s), 6.82 (1H, d, J=1.5 Hz), 6.72 (1H, dd, J=7.8, 1.5 Hz), 6.08 (2H, s), 4.77 (2H, s), 3.90 (3H, s), 3.60 (3H, s), 2.29 (3H, s). ^{13}C-NMR (DMSO-d_6) δ: 163.9, 150.4, 149.8, 147.1, 146.7, 145.7, 143.7, 140.1, 132.5, 130.1, 129.8, 129.7, 128.9, 127.5, 124.8, 124.2, 123.1, 115.6, 111.7, 108.1, 106.1, 101.4, 101.3, 56.0, 55.5, 45.4, 21.5. MS (ESI) m/z: 497 (M^+). HR-ESI-MS (positive) m/z: 519.1522 (Calcd for $C_{29}H_{24}N_2O_6Na$ 519.1532). 139 °C turn red, then decomposed.

***N*-(3,4,5-Trimethoxybenzylidenyl)-4′-(3″,4″-methylenedioxyphenyl)-6′,7′-dimethoxyl-1′-hydroxyl-benzyl-2,3-dihydro-isoindol-1-hydrazone (5)** White solid, yield 80%.^{1}H-NMR (DMSO-d_6) δ: 8.11 (1H, s), 7.64 (1H, s), 7.07 (2H, s), 6.99 (1H, d, J=8.1 Hz), 6.93 (1H, s), 6.84 (1H, d, J=1.5 Hz), 6.73 (1H, dd, J=8.1, 1.5 Hz), 6.08 (2H, d, J=3.9 Hz), 4.80 (2H, s), 3.91 (3H, s), 3.81 (6 H, s), 3.68 (3H, s), 3.62 (3H, s). ^{13}C-NMR (DMSO-d_6) δ: 163.9, 153.6, 150.4, 149.9, 147.1, 146.8, 145.8, 143.6, 139.5, 130.7, 130.1, 129.7, 128.9, 124.7, 124.2, 123.1, 115.8, 111.7, 108.1, 106.0, 104.8, 101.5, 101.3, 60.6, 56.4, 56.3, 56.0, 55.5, 45.6. MS (ESI) m/z: 573 (M^+). HR-ESI-MS (positive) m/z: 595.1708 (Calcd for $C_{31}H_{28}N_2O_9Na$ 595.1693). 155 °C turn red, then decomposed.

***N*-(3,4-Dimethoxybenzylidenyl)-4′-(3″,4″-methylenedioxyphenyl)-6′,7′-dimethoxyl-1′-hydroxyl-benzyl-2,3-dihydro-isoindol-1-hydrazone (6)** White solid, yield 79%.^{1}H-NMR (DMSO-d_6) δ: 8.06 (1H, s), 7.60 (1H, s), 7.34 (1H, d, J=1.8 Hz), 7.19 (1H, dd, J=8.4, 1.5 Hz), 6.98 (1H, d, J=1.8 Hz), 6.95 (1H, s), 6.90 (1H, s), 6.81 (1H, d, J=1.8 Hz), 6.71 (1H, dd, J=7.8, 1.5 Hz), 6.07 (2H, d, J=3.3 Hz), 4.76 (2H, s), 3.89 (3H, s), 3.75 (6H, s), 3.59 (3H, s). ^{13}C-NMR (DMSO-d_6) δ: 163.8, 150.9, 150.3, 149.8, 149.4, 147.1, 146.7, 145.9, 143.6, 130.1, 129.6, 128.7, 127.9, 124.9, 124.2, 123.0, 122.1, 115.7, 111.9, 111.8, 108.7, 108.1, 105.9, 101.4, 101.3, 56.1, 56.0, 55.7, 55.5, 45.5. MS (ESI) m/z: 543 (M^+). HR-ESI-MS (positive) m/z: 565.1573 (Calcd for $C_{30}H_{26}N_2O_8Na$ 565.1587). 151 °C turn red, then decomposed.

***N*-(4-Nitrobenzylidenyl)-4′-(3″,4″-methylenedioxyphenyl)-6′,7′-dimethoxyl-1′-hydroxyl-benzyl-2,3-dihydro-isoindol-1-hydrazone (7)** Yellow solid, yield 87%. ^{1}H-NMR (DMSO-d_6) δ: 8.27 (1H, s), 8.24 (1H, s), 8.20 (1H, s), 7.95 (1H, s), 7.92 (1H, s), 7.63 (1H, s), 7.01 (1H, d, J=7.8 Hz), 6.92 (1H, s), 6.85 (1H, s), 6.75 (1H, d, J=8.1 Hz), 6.11 (2H, s), 4.83 (2H, s), 3.91 (3H, s), 3.63 (3H, s). ^{13}C-NMR (DMSO-d_6) δ: 164.1, 150.6, 149.9, 148.0, 147.2, 146.8, 146.0, 141.6, 140.9, 130.0, 129.7, 129.3, 128.2, 124.5, 124.3, 124.2, 123.4, 115.9, 111.7, 108.1, 106.1, 101.5, 101.4, 56.0, 55.6, 45.6. MS (ESI) m/z: 528 (M^+). HR-ESI-MS (positive) m/z: 550.1235 (Calcd for $C_{28}H_{21}N_3O_8Na$ 550.1226). 165 °C turn red, then decomposed.

***N*-(4-Fluorobenzylidenyl)-4′-(3″,4″-methylenedioxyphenyl)-6′,7′-dimethoxyl-1′-hydroxyl-benzyl-2,3-dihydro-isoindol-1-hydrazone (8)** White solid, yield 83%. ^{1}H-NMR (DMSO-d_6) δ: 8.14 (1H, s), 7.78—7.73 (2H, m), 7.61 (1H, s), 7.29—7.23 (2H, m), 6.97 (1H, d, J=7.8 Hz), 6.90 (1H, s), 6.82 (1H, d, J=1.8 Hz), 6.71 (1H, dd, J=7.8, 1.5 Hz), 6.11 (2H, s), 4.78 (2H, s), 3.89 (3H, s), 3.60 (3H, s). ^{13}C-NMR (DMSO-d_6) δ: 165.0, 163.9, 150.3, 149.8, 147.1, 146.8, 145.9, 131.9, 130.1, 129.7, 129.5, 129.4, 128.9, 124.7, 124.2, 123.1, 116.5, 116.2, 115.7, 111.7, 108.1, 106.0, 101.4, 101.3, 56.0, 55.5, 45.5. MS (ESI) m/z: 501 (M^+). HR-ESI-MS (positive) m/z: 523.1281 (Calcd for $C_{28}H_{21}N_2O_6FNa$ 523.1281). 157 °C turn red, then decomposed.

***N*-(4-Chlorobenzylidenyl)-4′-(3″,4″-methylenedioxyphenyl)-6′,7′-dimethoxyl-1′-hydroxyl-benzyl-2,3-dihydro-isoindol-1-hydrazone (9)** White solid, yield 84%. ^{1}H-NMR (DMSO-d_6) δ: 8.12 (1H, s), 7.73 (1H, s), 7.71 (1H, s), 7.62 (1H, s), 7.50 (1H, s), 7.47 (1H, s), 6.97 (1H, d, J=7.8 Hz), 6.90 (1H, s), 6.82 (1H, s), 6.72 (1H, d, J=7.8Hz), 6.08 (2H, s), 4.80 (2H, s), 3.89 (3H, s), 3.60 (3H, s). ^{13}C-NMR (DMSO-d_6) δ: 163.9, 150.3, 149.8, 147.1, 146.8, 145.9, 142.0, 134.6, 134.2, 130.1, 129.7, 129.3, 128.9, 124.5,

124.2, 123.1, 115.7, 111.7, 108.1, 105.9, 101.4, 56.0, 55.5, 45.5. MS (ESI) *m/z*: 517 (M^+). HR-ESI-MS (positive) *m/z*: 539.0986 (Calcd for $C_{28}H_{21}N_2O_6$ NaCl 539.0982). 149 °C turn red, then decomposed.

***N*-(4-Bromobenzylidenyl)-4′-(3″,4″-methylenedioxyphenyl)-6′, 7′-dimethoxyl-1′-hydroxyl-benzyl-2,3-dihydro-isoindol-1-hydrazone (10)** White solid, yield 81%. ^{1}H-NMR (DMSO-d_6) δ: 8.09 (1H, s), 7.65—7.58 (5H, m), 6.97 (1H, d, J=8.1 Hz), 6.90 (1H, s), 6.82 (1H, d, J=1.5 Hz), 6.71 (1H, dd, J=7.8, 1.5 Hz), 6.07 (2H, s), 4.77 (2H, s), 3.89 (3H, s), 3.60 (3H, s). ^{13}C-NMR (DMSO-d_6) δ: 163.9, 150.4, 149.8, 147.1, 146.8, 145.9, 142.1, 134.5, 132.2, 130.1, 129.7, 129.2, 128.9, 124.6, 124.2, 123.3, 123.1, 115.7, 111.7, 108.1, 106.0, 101.4, 56.0, 55.5, 45.5. MS (ESI) *m/z*: 561 (M^+). HR-ESI-MS (positive) *m/z*: 583.0494 (Calcd for $C_{28}H_{21}N_2O_6$NaBr 583.0481). 159 °C turn red, then decomposed.

***N*-(3-Hydroxy-4-methoxybenzylidenyl)-4′-(3″,4″-methylenedioxyphenyl)-6′,7′-dimethoxyl-1′-hydroxyl-benzyl-2,3-dihydro-isoindol-1-hydrazone (11)** White solid, yield 80%. ^{1}H-NMR (DMSO-d_6) δ: 8.03 (1H, s), 7.62 (1H, s), 7.23 (1H, d, J=1.8 Hz), 7.10 (1H, dd, J=8.7, 1.8 Hz), 6.98 (1H, d, J=3.3 Hz), 6.95 (1H, d, J=3.6 Hz), 6.90 (1H, s), 6.81 (1H, d, J=1.5 Hz), 6.71 (1H, dd, J=8.1, 1.8 Hz), 6.07 (2H, s), 4.79 (2H, s), 3.90 (3H, s), 3.77 (3H, s), 3.60 (3H, s). ^{13}C-NMR (DMSO-d_6) δ: 163.7, 150.3, 150.0, 149.8, 147.2, 147.1, 146.7, 145.9, 144.0, 130.2, 129.7, 128.7, 128.2, 125.0, 124.2, 123.0, 120.6, 115.7, 112.9, 112.3, 111.7, 108.1, 106.0, 101.4, 56.5, 56.0, 55.5, 45.6. MS (ESI) *m/z*: 529 (M^+). HR-ESI-MS (positive) *m/z*: 551.1440 (Calcd for $C_{29}H_{24}N_2O_8$Na 551.1430). 129 °C turn red, then decomposed.

Cell Culture Three human cell lines, KB (oral squamous), A549 (lung), HCT-116 (colon) were cultured on RPMI-1640 medium supplemented with fetal bovine serum (10%), penicillin (100 U/ml) and streptomycin (100 μg/ml) in 25 cm^2 culture flasks at 37 °C in a humidified atmosphere with 5% CO_2.

Cell Viability Cell viability was assessed by the MTT assay. Cells were harvested from the culture during the exponential growth phase, and seeded into multiwell culture plates at 5×10^4—1×10^5 cells/ml in fresh medium. After overnight growth, cells were treated with compounds (predissolved in dimethyl sulfoxide (DMSO)) at selected concentrations for a period of 3 d. The medium was then discarded and replaced with MTT dye. Plates were incubated at 37 °C for 4 h. The resulting formazan crystals were solubilized in Lysis buffer (sodium dodecyl sulfate (SDS) 10 g, *N*,*N*-dimethylformamide (DMF) 25 ml, H_2O 25 ml, acetic acid 1 ml, pH 4.7), and the optical density was read at 570 nm with a microplate reader (Biotek synergy 2).

Fluorescence Morphological Examination Apoptotic morphology was studied by staining the cells with acridine orange (AO) and ethidium bromide (EB).[18] Cells were washed three times with PBS after being incubated with compound **4** in 2 μM, 4 μM for 72 h, and were then stained with AO and EB for 3 min. Stained cells were viewed under a fluorescence microscope (Leica, German) with 200× magnification.

Hoechst 33342-PI staining was carried out according to the procedure of literature.[19] Cells were treated with compound **4** in 2 μM, 4 μM for 72 h. Then stained by incubating in PBS containing 500 nM Hoechst 33342 and 500 nM propidium iodide (PI) at 37 °C for 3 min in the dark. Then washed cells with PBS three times. Stained cells were viewed under a fluorescence microscope (Leica, German) with 200× magnification.

Western Blot Analysis Cells were exposed to various concentrations of **4** for 72 h. Cell lysates with identical amounts of protein were fractionated and transferred to PVDF membranes (Millipore Corp., U.S.A.). The polyvinylidene difluoride (PVDF) membrane was incubated in blocking buffer (TBS containing 0.1% Tween 20 and 5% nonfat milk) for 1 h at room temperature. Then the membrane was incubated with the appropriate primary antibody overnight at 4 °C or 2 h at room temperature with gentle shaking. The membrane was washed thrice with rinsing buffer for 15 min and then incubated with the corresponding peroxidase-conjugated secondary antibody for 1 h at room temperature. After repeating the washes in triplicate, the protein of interest was detected by enhanced chemiluminescence reagents from EC3 imaging system (UVP, U.S.A.).

Statistical Analyses Data were presented as means±S.E. and analyzed by SPSS software. Picture were processed with Photoshop software. Mean values were obtained from at least three indepent experiments.

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References

1) Jemal A., Murray T., Ward E., Smuels A., Tiwari R. C., Ghafoor A., Feuer E. J., Thun M. J., *CA Cancer J. Clin.* **55**, 10—30 (2005).
2) Fesik S. W., *Nat. Rev. Cancer*, **5**, 876—885 (2005).
3) Cragg G. M., Grothaus P. G., Newman D. J., *Chem. Rev.*, **109**, 3012—3043 (2009).
4) Magedov I. V., Manpadi M., Slambrouck S., Steelant F. A., Rozhkova E., Przhevalskii, Rogelj N. M. S., Kornienko A., *J. Med. Chem.*, **50**, 5183—5192 (2007).
5) Fukamiya N., Lee K. H., *J. Nat. Prod.*, **49**, 348—350 (1986).
6) Hande K. R., *Eur. J. Cancer*, **34**, 1514—1521 (1998).
7) Gordaliza M., Caorral J. M., Angels C. M., Lopez V. M. L., Garcia P. A., San F. A., Garcia G. M. D., *Bioorg. Med. Chem. Lett.*, **5**, 2465—2468 (1995).
8) Imperio D., Pirali T., Galli U., Pagliai F., Cafici L., Canonico P. L., Sorba G., Genazzani A. A., Tron G. C., *Bioorg. Med. Chem.*, **15**, 6748—6757 (2007).
9) Beers S. A., Imakura Y., Dai H. J., Li D. H., Cheng Y. C., Lee K. H., *J. Nat. Prod.*, **51**, 901—905 (1988).
10) Day S. H., Lin Y. C., Tsai M. L., *J. Nat. Prod.*, **65**, 379—381 (2002).
11) Zhao Y., Lu Y. P., Zhu L., *J. Carbohydr. Chem.*, **27**, 113—119 (2008).
12) Zhao Y., Li Y. X., *Chin. J. Chem.*, **25**, 679—682 (2007).
13) Abdel H. O. M., Abdel L. N. A., Mohamed T. K., Ahmeda E. Y., Maher T., *Eur. J. Med. Chem.*, **44**, 2967—2974 (2009).
14) Charlton J. L., Oleschuk C. J., Chee G. L., *J. Org. Chem.*, **61**, 3452—3457 (1996).
15) Bedia K., Elcin O., Seda U., Fatma K., Nathlay S., Sevim R., Dimalgo A., *Eur. J. Med. Chem.*, **41**, 1253—1261 (2006).
16) Hegardt C., Andersson G., Stina M. O., *Exp. Cell Res.*, **266**, 333—341 (2001).
17) Liu J., Li Y., Ren W., Hu W. X., *Cancer Lett.*, **242**, 133—140 (2006).
18) Srinivasan B., Wang Z. H., Brun-Zinkernagel A. M., Collier R. J., Black R. A., Frank S. J., Barker P. A., Roquea R. S., *Mol. Cell. Neurosci.*, **36**, 449—461 (2007).
19) Ding L., Liu B., Qi L. L., Zhou Q. Y., Hou Q., Li J., Zhang Q., *Toxicology in Vitro*, **23**, 408—417 (2009).