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A Novel Water-Soluble Gossypol Derivative Increases Chemotherapeutic Sensitivity and Promotes Growth Inhibition in Colon Cancer

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Compound **1** ((−)-gossypol) has been long known as a chemical anticancer agent. With its low water solubility and toxicity, it is not widely used as a commercial drug. To overcome these disadvantages, several novel derivatives of gossypol were designed, synthesized, and analyzed. One of the derivatives, compound **7** (6-aminopenicillanic acid sodium-gossypolone), was identified with great water solubility and anticancer property, suggested by inducing a dramatically decrease in Bcl-2 and Bcl-xL protein expression level found in vitro and growth inhibition of murine colon tumor in vivo. Furthermore, it was also recognized with less toxicity than compound **1** in vivo and significantly increased chemotherapeutic sensitivity against colon cancer in combination with traditional chemotherapeutic agent 5-fluorouracil. Therefore, it is concluded that compound **7** is superior to parent compound **1**, and further preclinical studies of compound **7** is necessary for colon cancer therapy.

Introduction

Gossypol, extracted from cottonseeds, was initially investigated as a candidate male contraceptive agent.[1] Recent studies have demonstrated that gossypol is able to inhibit cell growth in various carcinoma cell lines including prostate, pancreas, colon, and head and neck cancer cell lines in vitro.[2−5] It is determined that gossypol is able to interact with the BH3-binding pockets of the Bcl-2[a] and Bcl-xL proteins and inhibit their antiapoptotic function by nuclear magnetic resonance-based methods and fluorescence-polarization displacement assays.[6,7] In this regard, gossypol can promote antiproliferative and pro-apoptotic activities in cells ectopically expressing Bcl-2 and Bcl-xL proteins.

Colorectal cancer (CRC) is the third most commonly diagnosed type of cancer and the third leading cause of death from cancer among men and women in the United States.[8,9] It was estimated that 146970 new cases were diagnosed with CRC in 2009, and 49920 people died from this disease.[8] Five-year survival rate of nonmetastatic CRC patients is approximately 90%, but reduced to only 68% for regional disease such as lymph node involvement, and further declined to only 10% for distant metastases.[9] Current options for CRC treatments include surgical resection and chemotherapy. However, even with successful surgical resection and followed by chemotherapy, five-year recurrence rate is still high.[10] Therefore, the identification of novel strategies for colon cancer therapy is urgently required. It is reported that abnormal activation of Bcl-2 appears in early colorectal tumorigenesis, which inhibits apoptosis and promote tumor progression.[11,12] As mentioned above, gossypol could inhibit antiapoptotic functions of Bcl-2 and Bcl-xL and become a promising agent for colon cancer therapy.

Previous studies stated that oral delivered gossypol was used for clinical trials to treat patients with refractory metastatic breast cancer and adrenal cancer.[13,14] However, gossypol causes hepatotoxicity and GI toxicity in vivo, probably due to its reactive aldehyde groups, which makes it not suitable for clinical applications.[15−18] Several other gossypol-derived derivatives and Bcl-2 inhibitors such as *R*-(−)-gossypol acetic acid (AT-101),[19,20] apogossypol,[21] *N*-(4-(2-*tert*-butylphenylsulfonyl)phenyl)-2,3,4-trihydroxy-5-(2-isopropylbenzyl)benzamide (TW-37),[22] 4-[4-[(4′- chloro[1,1′-biphenyl]-2-yl)methyl]-1-piperazinyl]-*N*-[[4-[[(1*R*)-3-(dimethylamino)-1-[(phenylthio)methyl]propyl]amino]-3-nitrophenyl]sulfonyl]benzamide (ABT-737),[23,24] (*S*,*Z*)-2-(5-(biphenyl-4-ylmethylene)-2,4-dioxothiazolidin-3-yl)-3-phenyl-*N*-tosylpropanamide (WL-276),[25] and some apogossypol derivatives[26] also have inhibitory activities in cancer. Moreover, apogossypol induces less hepatotoxicity and GI toxicity than natural gossypol.[21] However, low water solubility of apogossypol indicates that it might be not an appropriate research target for commercial drug discovery. Great water solubility of the compounds is expected for potential drug material selection because well water-soluble compounds usually show greater pharmacokinetic properties and efficacies at lower doses compared with low water-soluble ones. Therefore, to select

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[a] Abbreviations: Bcl-2, B-cell lymphoma/leukemia-2; ^{1}H NMR, one-dimensional proton nuclear magnetic resonance spectroscopy; GI, gastrointestinal; IC_{50}, half maximal inhibitory concentration; DMSO, dimethyl sulfoxide; PBS, phosphate buffered saline; DAPI, 4′,6-diamidino-2-phenylindole; 5-FU, 5-fluorouracil; i.p., intraperitoneal.

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an appropriate potential drug against colon cancer, less toxicity and better water solubility properties of novel derivatives are required.

Combination of different chemotherapeutic agents is a conventional strategy for cancer treatment. Overexpression of antiapoptotic family members including Bcl-2, Bcl-xL, Mcl-1, and Bcl-w are associated with inhibition of apoptosis and resistance to several chemotherapeutic agents. Hence, combination Bcl-2 inhibitors with conventional chemotherapeutic agents could offer new approaches for cancer treatment. For instance, it has been observed by Kilic et al. that human nonsmall-cell lung cancer cell lines are response to gossypol alone and in combination with cisplatin.[27] Sanli et al. demonstrated that gossypol/zoledronic acid combination effectively inhibits the growth of the hormone- and drug-resistant prostate cancer cells.[28] Furthermore, Macoska et al. suggested that the addition of (−)-gossypol to conventional chemical drugs could make better efficacy for patients with advanced bladder tumors.[29] Therefore, combination of gossypol with other anticancer agents could be a feasible way for cancer therapy.

In this study, several novel derivatives of gossypol were synthesized and their efficacy was investigated in mouse and human carcinoma cell lines. One of them, compound **7** (6-aminopenicillanic acid sodium-gossypolone), with better water solubility than compound **1** ((−)-gossypol), showed significant cytotoxity against colon cancer cells with time- and dose-dependence. Furthermore, compound **7** was investigated whether it was able to induce CT26 colon cancer cell apoptosis, affect Bcl-2 and Bcl-xL protein expression, and disrupt the interactions between Bcl-2 and its family protein counterparts as a BH3 mimic. Combination of compound **7** with traditional cytotoxic anticancer drug 5-fluorouracil (5-FU) increased the inhibition of CT26 cell growth in vitro and in vivo. Moreover, compound **7** showed less toxicity than compound **1** in vivo. Taken together, the designed compound **7** could be a novel candidate agent for colon cancer therapy.

Results and Discussion

Chemistry. Compound **1** and compound **2** were synthesized as previously described.[30,31] Imino derivatives of gossypols, **3**, **4**, **5**, **6**, **8**, and **9** were synthesized by converting the aldehyde groups to the corresponding imino functions, shown in Scheme 1 in Figure 1. Compound **7** was synthesized under similar condition by condensation of gossypolone with the corresponding amine, shown in Scheme 2 in Figure 1.

Cell Viability. First, the toxic effects of these new synthesized compounds was evaluated on the cancer cell lines. These compounds at different concentration levels were incubated with several human and murine cancer cell lines for 72 h, and then the cell viability was measured by MTT assay. The results indicated that human and mouse colon cancer cell lines SW620, HCT116, and CT26 were sensitive to compound **7**, which revealed about 50% reduction in cell number within the concentration range from 6 to 18 μM (Table 1). In contrast, breast cancer cell lines MDA-MB-435, MCF-7, and 4T1 were less sensitive to these compounds, and their IC_{50} values were more than 28 μM of compound **7** treatment. Compound **7** also showed considerable cytotoxicity on murine melanoma cell line B16−F10, human lung carcinoma cell line A549, and prostate carcinoma cell line PC-3 (Table 1). These results suggested that the novel compound **7** had significant toxic effect on colon cancer. Therefore, compound **7** was chose as a candidate agent for further colon cancer therapy research.

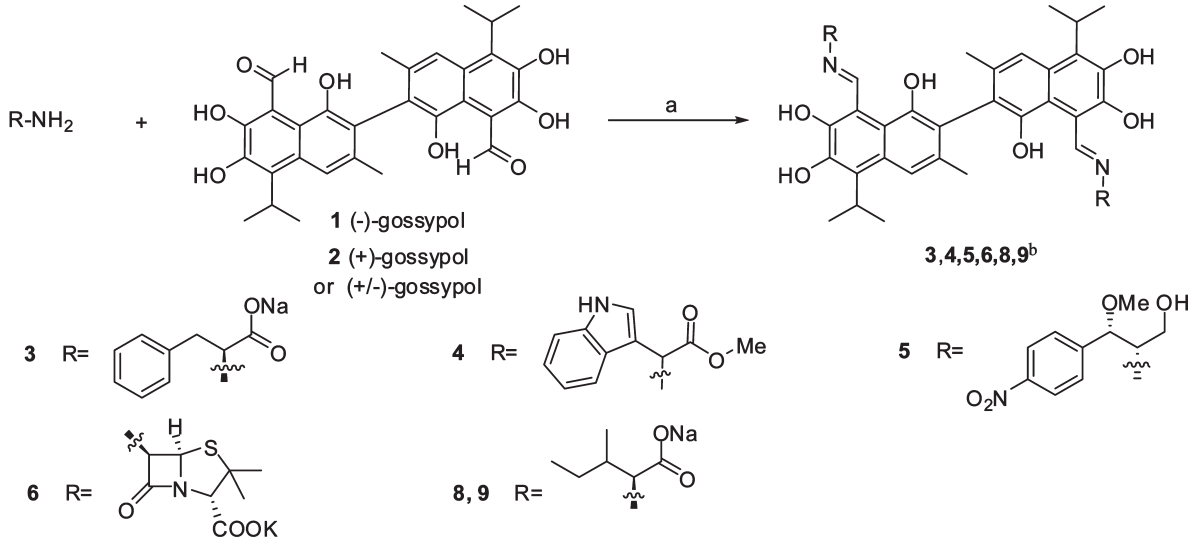
Gossypol is known to be very low-soluble in water, with less than 0.001 g dissolving in 100 g of H_2O at 37 °C. Recent studies demonstrated that the immobilization of one derivative of gossypol increases its water solubility.[32] Therefore, the solubility of compound **7** in water solution was also tested. The results indicated that compound **7** is well-soluble in water solution, with 9.25 g dissolving in 100 g of H_2O at 37 °C (Supporting Information Figure 2). Furthermore, the stability of compound **7** in biological conditions was also examined. Compound **7** was incubated in PBS buffer (pH = 7.2−7.4) or in human or mouse sera for 30 min, followed by HPLC analysis. The results indicated that compound **7** is comparatively stable after 30 min under the conditions mentioned above (Supporting Information Figure 4). On the basis of its water solubility and stability, compound **7** is expected to have better absorption, distribution, and duration time than low-solubility derivatives of gossypol in vivo.

Compound 7 Induced CT26 Colon Cancer Cell Growth Inhibition and Apoptosis. To investigate the time- and dose-dependence activity of compound **7** on colon cancer cells, a cell proliferation assay on CT26 cancer cells treated with different concentrations of compound **7** was carried out at the indicated time points. The results showed that 20 μM of compound **7** significantly inhibited CT26 cell growth (Figure 2A). After 24 h treatment with 20 μM of compound **7**, about 80% of the CT26 cells survived. With further 24 h treatment, only about 60% of the cells survived, and then followed with 3 days treatment, almost all the cells were killed. Ten μM and 5 μM of compound **7** treatments could also effectively inhibit CT26 cell growth (Figure 2A). However, 2 μM of compound **7** only had minor inhibitory effect on CT26 cells, even after 120 h treatment, more than 90% of the cells were resistant to low concentration of compound **7** (Figure 2A). Of note, at the same time points, higher doses of compound **7** exhibited more growth inhibition ability in CT26 cells. These data indicated that compound **7** was able to inhibit the growth of CT26 cells with time- and dose-dependence.

Previous studies demonstrated that gossypol is able to induce cell apoptosis.[33,34] With a query that whether compound **7** was able to trigger CT26 cell growth inhibition via inducing cell apoptosis, the following experiments were performed. After exposing the cells to different concentrations of compound **7** at assigned time points, the CT26 cells were stained with both TUNEL reaction mixture and DAPI (Figure 2B). TUNEL-positive cells were counted and represented in Figure 2C. These results indicated that compound **7** could trigger the apoptosis of CT26 cells in a time- and dose-dependent manner. It is determined that gossypol as a BH3 mimetic triggers apoptosis via regulating Bcl-2 family proteins.[29,34] Recently reported BH3 mimic Bcl-2 inhibitor ABT-737 is also known to promote various types of cancer cell growth inhibition and enhance apoptosis, such as colon cancer, small-cell lung cancer, and multiple myeloma.[23,24,35−37] In this regard, it is required to confirm whether the mechanism of apoptosis induced by compound **7** was similar to natural gossypol and ABT-737 as a BH3 mimic.

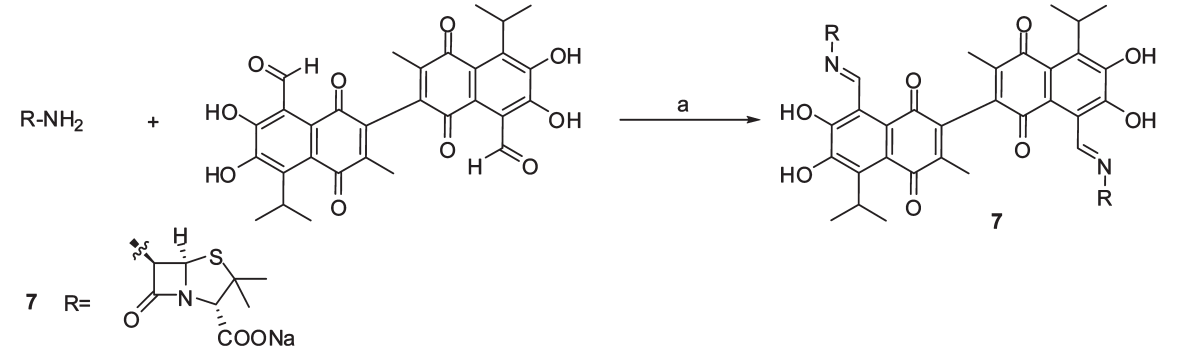
Compound 7 Decreases Bcl-2 and Bcl-xL at Protein Expression Levels and Disrupts the Interactions between Bcl-2 and its Bcl-2 Family Protein Counterparts via Binding to Their BH3 Grooves. Bcl-2 belongs to a gene family which includes

Scheme 1.



[a]Reagents and conditions: NaOH, isopropanol/methanol 1:1(v/v). (specially for **6**, we used KOH instead of NaOH).

Scheme 2.



[a]Reagents and conditions: NaOH, isopropanol/methanol 1:1(v/v).

Figure 1. Preparation of gossypol derivatives. [b]The gossypol Schiff's bases were in the imine−imine forms (except for **9**, enamine−enamine forms). Compounds **3** and **8** from racemic gossypol; **4** and **5** from **1** *R*-(−)-gossypol; **6** and **9** from **2** *S*-(+)-gossypol. Compound **7** was in the imine−imine forms.

Table 1. Cell Viability Assays[a]

	IC_{50}[b] (μM)								
compd	A549	MDA-MB435	MCF-7	SW620	HCT116	PC-3	B16-F10	4T1	CT26
1	1.32	11.78	12.58	2.90	3.33	9.14	5.54	18.78	2.21
2	33.08	33.25	21.56	17.21	21.99	22.36	17.97	21.53	13.59
3	5.38	> 40	> 40	9.76	> 40	28.45	22.40	> 40	7.36
4	> 40	> 40	> 40	> 40	> 40	> 40	> 40	> 40	> 40
5	> 40	> 40	> 40	> 40	17.69	> 40	> 40	> 40	> 40
6	> 40	> 40	> 40	23.64	> 40	> 40	> 40	> 40	> 40
7	8.77	> 40	29.06	6.56	17.35	29.46	11.05	28.25	6.59
8	14.75	> 40	28.47	22.63	34.88	> 40	19.88	> 40	18.42
9	13.22	> 40	25.80	11.85	23.44	> 40	20.14	> 40	11.92

[a] Several cancer cell lines were treated with multiple derivatives of gossypol for 72 h, and IC_{50} (μM) were calculated. The results are mean values of three independent experiments. [b] Concentration (μM) able to inhibit 50% of cell growth after 72 h of treatment.

either antiapoptotic members such as Bcl-2, Bcl-xL, Mcl-1, and A1, or cell death promoters such as Bad, Bak, Bax, Bik, and Bcl-XS.[38,39] Bcl-2 was thought as a proto-oncogene in colon carcinogenesis.[12] It is considered that gossypol is a BH3 mimic to affect the Bcl-2 family proteins.[6,7,34] Compound **7**, containing the backbone of gossypol, may have the similar mechanism as its parent compound **1**. To understand the binding mode of compound **7** to Bcl-2 family proteins, computational docking strategies were performed via AutoDock Vina.[40] The predicted docking models showed that the compound **7** bound to the BH3 pockets of Bcl-2 and Bcl-xL, and fitted comparatively well (Figure 3A−B). These docking studies indicated that compound **7** might bind to Bcl-2 and Bcl-xL as a BH3 mimic. To further investigate the binding properties of compound **7** to Bcl-xL, a competitive fluorescence polarization assay (FPA) was

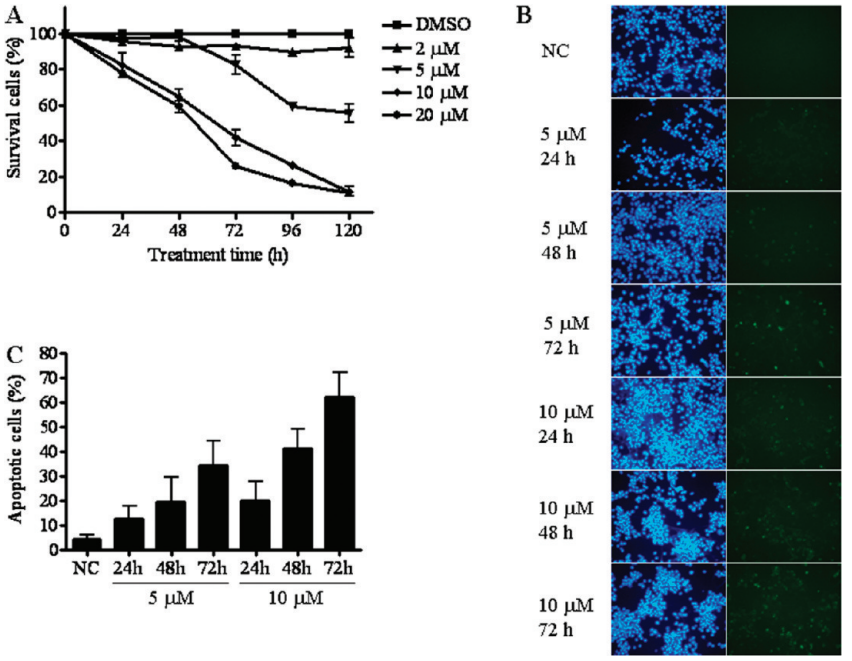


Figure 2. Compound **7** inhibited CT26 cell growth and induced apoptosis with time- and dose-dependence. (A) CT26 cells were incubated with different concentrations of compound **7** for various times as indicated and subjected to cell proliferation assay. (B,C) CT26 cells were treated with indicated concentrations of compound **7** for different hours and then were stained by TUNEL assay with DMSO treatment as negative control (NC). TUNEL-positive cells were counted and are represented in Figure 2C.

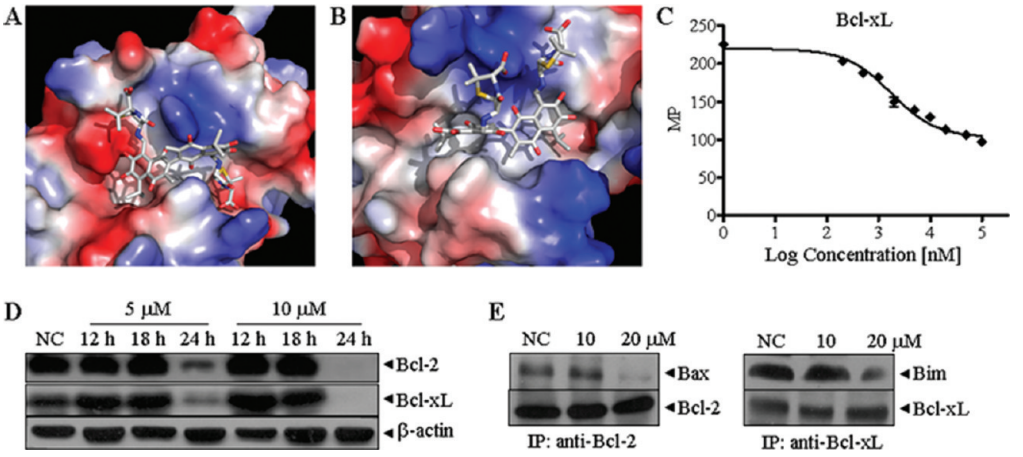


Figure 3. Compound **7** decreased Bcl-2 and Bcl-xL at protein expression levels and disrupted the interactions between Bcl-2 and its Bcl-2 family protein counterparts via binding to their BH3 grooves. (A,B) Molecular modeling studies. Stereo views of the docked structures of compound **7** in the peptide binding pocket of the Bcl-2 (Protein Data Bank (PDB) code 1YSW) (A) and Bcl-xL (PDB code 1BXL) (B). (C) 100 nM GST-Bcl-xL protein was incubated with compound **7** at various concentrations in PBS in 96-well black plates at room temperature. After 10 min incubation, 50 nM FITC-labeled Bak BH3 peptide was added and incubated for 60 min at room temperature. The polarization values were measured at excitation/emission wavelengths of 480/535 nm. (D) Compound **7** decreased Bcl-2 and Bcl-xL at protein expression levels in CT26 cells in vitro. CT26 cells were incubated with 5 and 10 μM compound **7** for the indicated times with DMSO treatment as control (NC). Cell lysates were immunoblotted against Bcl-2 and Bcl-xL with β-actin as a loading control. (E) Compound **7** inhibited the heterodimerization of Bcl-2 with Bax or Bcl-xL with Bim. CT26 cells were treated with indicated concentrations of compound **7** for 24 h, with DMSO treatment as control (NC). Cell lysates were immunoprecipitated with anti-Bcl-2 or Bcl-xL antibodies, respectively, further detected with anti-Bax or Bim antibody.

performed in which the FITC-labeled Bak BH3 peptide was displaced by compound **7**. The results indicated that compound **7** bound to Bcl-xL with an IC_{50} of 1.76 μM (Figure 3C). The docking and FPA studies showed that compound **7** was able to bind to Bcl-2 and Bcl-xL as a BH3 mimic.

Previous studies demonstrated that gossypol or its derivatives are able to decrease Bcl-2 and Bcl-xL protein expression.[19,29] Therefore, CT26 cells were treated with compound **7** and their Bcl-2 and Bcl-xL protein expression levels were examined. Five μM and 10 μM compound **7** treatments reduced the Bcl-2 and Bcl-xL protein levels in CT26 cells at 24 h (Figure 3D). These data suggested that compound **7** could bind to the BH3 grooves of Bcl-2 and Bcl-xL and inhibit their antiapoptotic functions via influencing the expression of Bcl-2 and Bcl-xL proteins in CT26 cells, similar to that of parent compound **1**. It was also supported by the molecular modeling results. However, the mRNA levels of Bcl-2 and

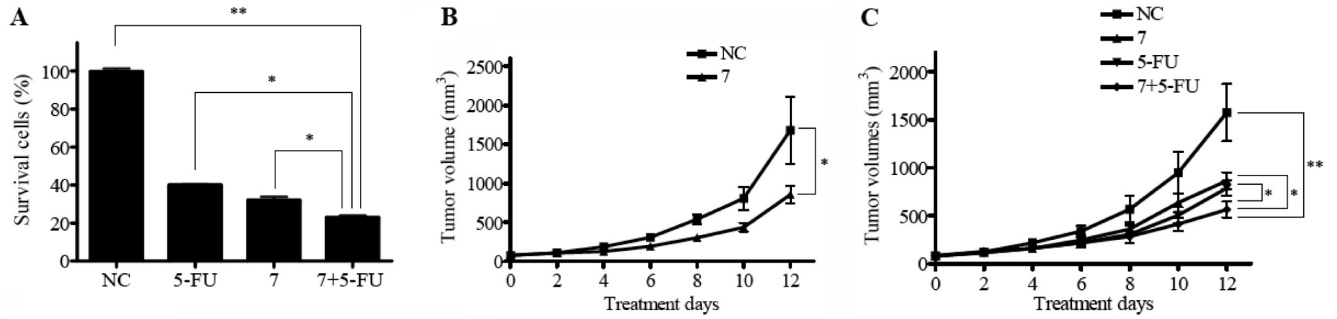


Figure 4. Combination effects of compound **7** in colon cancer in vitro and in vivo. (A) Combination of compound **7** with 5-FU significantly inhibited colon cancer. CT26 cancer cells were incubated with 10 mM compound **7**, 25 mM 5-FU alone or their combination, with DMSO treatment as control (NC) ($P < 0.01$, indicated by *; $P < 0.001$, indicated by **). (B) Compound **7** inhibited colon tumor growth in vivo. CT26 cells were subcutaneously injected into the right flanks of male BALB/c mice (6−8 weeks old). Once their tumors grew up to 60 mm^3, the mice were randomly divided into different groups ($n = 4$). Mice were given with compound **7** at the dose of 10 mmol/kg once a day in 12 consecutive days by gavage, with 10% ethanol as control (NC). Their tumors were measured by length and width every 2 days with a caliper, and tumor volumes were calculated by length × width × width × $\pi/6$ (mm^3). ($P < 0.01$, indicated by *). (C) Mice were injected with CT26 cells and divided into groups the same as (B). 5-FU was given at the dose of 10 mg/kg every two days in 12 consecutive days by ip supplemented with compound **7** at the dose shown in (A). PBS given by ip and 10% ethanol given by gavage were set as negative controls (NC). The tumors were measured and tumor volumes were calculated ($P < 0.01$, indicated by *; $P < 0.001$, indicated by **).

Bcl-xL were not significantly down-regulated by compound **7** (Supporting Information Figure 1). These data indicated that compound **7** did not down-regulate Bcl-2 and Bcl-xL at transcription levels, but at protein levels.

Zhang et al. demonstrated that gossypol could inhibit the heterodimerization of Bax with Bcl-2 or Bim with Bcl-xL.[3] On the basis of this finding, it is speculated that compound **7** might also disrupt the interactions between Bcl-2 or Bcl-xL and their pro-apoptotic counterparts. To verify this, CT26 cells were treated with indicated concentrations of compound **7** for 24 h. Cell lysates were immunoprecipitated with anti-Bcl-2 or Bcl-xL antibodies, followed by detection with Bax or Bim antibodies, respectively. As shown in Figure 3E, 20 μM of compound **7** treatment inhibited the interaction between Bcl-2 and Bax and also inhibited the binding between Bcl-xL and Bim compared with vehicle control treatment. Taken together, it is considered that compound **7** was able to decrease Bcl-2 and Bcl-xL protein expression and inhibit the heterodimerization of Bcl-2 or Bcl-xL with their pro-apoptotic counterparts.

Combination of Compound 7 with Traditional Cytotoxic Anticancer Agent 5-Fluorouracil Enhances the Growth Inhibition in Colon Cancer in Vitro and in Vivo. Clinical data presents that combination therapies are often successful in the treatment of cancer. Combination of two chemical drugs could overcome a series of problems in individual administration. But only a fraction of the combinations of typical chemical agents were proved to be effective. Thus, it is necessary to develop new chemical combination therapies for cancer patients. Mego et al. found that gossypol was a potential telomerase inhibitor, and the use of gossypol combination with other anticancer chemotherapeutic drugs could lead to effective therapies for cancers.[41] Combination therapies of gossypol and conventional chemical drugs have been proved to be more effective for patients with several types of tumors.[27−29] Moreover, another BH3 mimic compound ABT-737 in combination with commercial anticancer drugs could also inhibit cancer cell growth.[35,42,43] In the view of these findings, the anticancer property of compound **7** alone or in combination with traditional anticancer drugs against CT26 colon cancer cells was examined in vitro. 5-Fluorouracil (5-FU) is widely used for colon cancer chemotherapy, therefore, it was selected for our drug combination study. The combination of 10 μM compound **7** and 25 μM 5-FU was considerably more effective than compound **7** or 5-FU alone in CT26 cell growth inhibition ($P < 0.01$) (Figure 4A). These results indicated that combination of compound **7** with traditional cytotoxic anticancer drug 5-FU increased chemotherapeutic sensitivity against colon cancer cell growth compared with compound **7** or 5-FU alone.

Previous data demonstrated that compound **7** has anticancer effects in vitro, and then its function was investigated in vivo. CT26 tumor volumes were significantly decreased with compound **7** treatment at the dose of 10 μmol/kg per day in 12 consecutive days by gavage compared with control groups ($P < 0.01$) (Figure 4B). The results showed that compound **7** triggered colon cancer growth inhibition in vivo. On the basis of the combination effects of compound **7** and 5-FU in vitro, CT26 tumor-bearing mice were treated with 10 μmol/kg compound **7** per day in combination with 10 mg/kg 5-FU treatment every two days for 12 consecutive days. The results indicated that compound **7** in combination with 5-FU showed additional anticancer efficacy compared with compound **7** or 5-FU treatment alone ($P < 0.01$) (Figure 4C). This data suggested that the combination of compound **7** with 5-FU offered a new strategy for colon cancer chemotherapy.

Compound 7 is Less Toxic than Compound 1. Previous studies determined that apogossypol which lacks two reactive aldehyde groups than gossypol is less toxic when orally delivered at 120 μmol/kg daily for three weeks.[21] Therefore, the toxicity of compound **1** and compound **7** at the same dose (120 μmol/kg) was further studied for consecutive days by gavage, used 10% ethanol as vehicle control. After the treatments, gossypol-induced damage in the compound **1**-treated mice was accumulated. At day 8, the first compound **1**-treated mouse was dead. The second one out of six mice was dead at day 12 (Figure 5A). Yet, all the compound **7**-treated mice were still alive at day 12. The mice treated with compound **1** also showed remarkable weight loss ($P < 0.01$), while the body weights of compound **7**-treated mice did not significantly decline (Figure 5B), as did the mice treated with vehicle control. Furthermore, compound **7** did not remarkably induce damage on small intestine compared with vehicle control, while compound **1** did (Figure 5C). According to

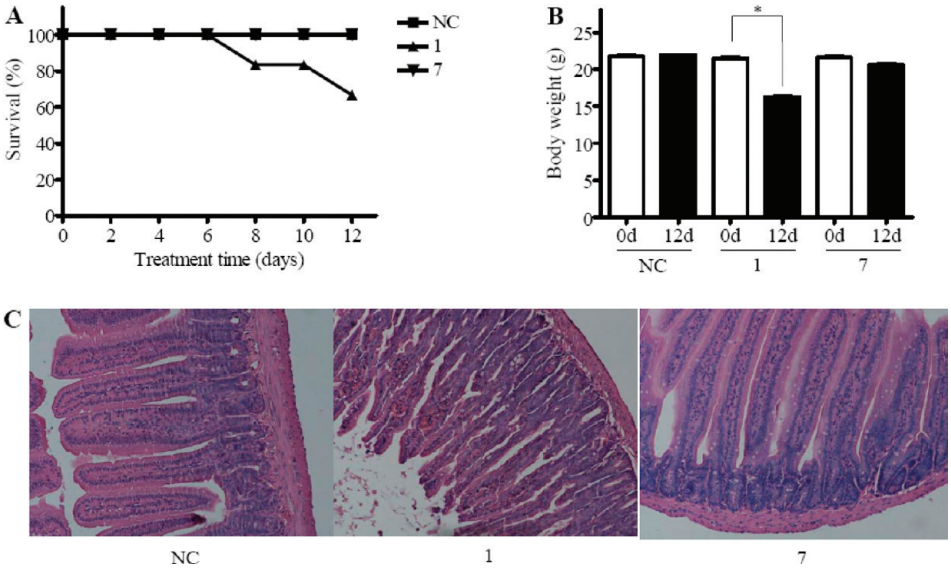


Figure 5. In vivo toxicity studies of compound **7** versus **1** in normal mice. Male BALB/c mice (6−8 weeks old) (n = 6 per group) were orally delivered vehicle control (NC), 120 μmol/kg compound **1**, or 120 μmol/kg compound **7** once a day in 12 consecutive days. (A) Percentage survival curves of mice with different treatments are shown. (B) Body weights of the mice after treatments are shown ($P < 0.01$, indicated by *). (C) The small intestine tissue sections were stained with hematoxylin and eosin (H&E) and examined using a light microscope. Data are representative of three experiments.

these maximum tolerated dose (MTD) studies, compound **7** had been proved that it was less toxic than compound **1** in vivo. In the reason of the reduced toxicity of compound **7**, it is possible to give animals with higher doses of compound **7** compared with natural gossypol for greater anticancer activities, alone or in combination with other commercial drugs against cancer. Taken together, we developed a novel combination chemotherapeutic strategy with low toxicity against colon cancer, which could be targeted for clinical applications in future.

Conclusion

Gossypol is known to be able to trigger apoptosis. However, natural gossypol is low-soluble and might cause hepatotoxicity and GI toxicity in vivo. Therefore, it is not suitable for clinical applications. In this study, we synthesized several novel derivatives of gossypol and test their anticancer efficacies in mouse and human carcinoma cell lines. One of them, compound **7**, showed greater water solubility than natural gossypol. It was found that compound **7** significantly induced CT26 colon cancer cell apoptosis with time- and dose-dependence. Furthermore, compound **7** could decrease Bcl-2 and Bcl-xL protein expression and disrupt the interactions between Bcl-2 and its Bcl-2 family protein counterparts via binding to their BH3 grooves, but the mRNA levels of Bcl-2 and Bcl-xL were not significantly affected. Besides, combination of compound **7** with traditional cytotoxic anticancer drug 5-fluorouracil (5-FU) increased their chemosensitivity in CT26 colon cancer in vitro and in vivo. Moreover, compound **7**-treated mice lived longer and caused reduced damage on small intestine than compound **1**-treated ones, and the body weights of compound **7**-treated mice did not significantly declined compared with compound **1**-treated ones. These data indicated that compound **7** was less toxic than compound **1** in vivo. In conclusion, compound **7** is a novel candidate agent for colon cancer therapy, superior to parent compound gossypol, suggesting that further clinical studies of compound **7** is necessary to be investigated.

Experimental Section

General Synthetic Procedures. Unless otherwise indicated, all reagents were obtained from GL Biochem (Shanghai) Ltd. and used without further purification. Optical rotations were recorded on Perkin-Elmer 341MC instrument. All final compounds were purified to >95% purity, as determined by a HPLC Breeze from Perkin-Elmer using an Dikma C-18 5 μM 4.6 mm ×150 mm reverse phase column. Chiral HPLC analysis was performed on a Chiralpak AD-H analytical column using i-PrOH/hexane/TFA = 30:70:0.1 as an eluent (0.9 mL/min), detected at 254 nm. ^{1}H NMR and ^{13}C NMR spectra were recorded on a Bruker AMX-300. The chemical shifts are expressed in ppm (δ), and coupling constants are given in Hz. Low-resolution mass spectra were obtained on a Shimadzu LCMS-2010EV.

(−)-Gossypol [(−)-1,1′,6,6′,7,7′-Hexahydroxy-5,5′-diisopropyl-3,3′-dimeth- yl-2,2′-binaphthyl-8,8′-dicarbaldehyde] (1) and (+)-Gossypol [(+)-1,1′, 6,6′,7,7′-Hexahydroxy-5,5′-diisopropyl-3,3′-dimethyl-2,2′-binaphthyl-8,8′-dicarbaldehyde] (2). Compounds **1** and **2** were synthesized as previously described.[30,31] Briefly, (Rg, S) L-Trp-OMe-(−)-gossypol (200 mg, 0.22 mmol) was dissolved in 20 mL of ether and 2 mL of glacial acetic acid under stirring at 50 °C under nitrogen, then 60 μL of concentrated hydrochloric acid (37%) was added and the mixture was stirred for additional 1 h. After the white precipitate was filtered off, the filtrate was washed with distilled water until the pH was near 7. Subsequently, the solvent was removed at reduced pressure and the residue was dried to give compound **1** as orange crystalline solid. Yield, 108 mg (95%); $[\alpha]^{25}_D$ −387.6142 (c 0.05, $CHCl_3$) (lit. $[\alpha]^{29}$ −363.6, c 0.2, CH_3OH); HPLC: R_t = 6.173 min (ee >99%, i-PrOH/hexane = 30:70 and 0.1% TFA). ESI-MS $[M - H]^+$: 517.1. ^{1}H NMR (300 MHz, $CDCl_3$) δ 14.98 (s, OH), 11.10 (s, 2H), 7.77 (s, 2H), 6.36 (s, OH), 6.07 (s, OH), 3.90 (m, 2H), 2.14 (s, 6H), 1.54 (d, J = 7.5 Hz, 12H).

Compound **2**, orange crystalline solid, was obtained from (Sg, S) L-Trp-OMe-(+)-gossypol (200 mg, 0.22 mmol) by following the same procedure described for preparation of compound **1**. Yield, 102 mg (90%); $[\alpha]^{25}_D$ +386.44 (c 0.05, $CHCl_3$) (lit. $[\alpha]^{19}$ +348.25, c 0.05, CH_3OH); HPLC: R_t = 26.79 min (ee >99%, *i*-PrOH/hexane = 30:70 and 0.1% TFA). ESI-MS $[M - H]^+$: 517.1. ^{1}H NMR (300 MHz, $CDCl_3$) δ 14.42 (s, OH), 11.11 (s,

2H), 7.78 (s, 2H), 6.39 (s, OH), 5.99 (s, OH), 3.89 (m, 2H), 2.15 (s, 6H), 1.54 (d, J = 6.9 Hz, 12H).

L-Phe-ONa-(±)-gossypol [L-Phenylalanine sodium-(±)-gossypol] (3). NaOH (8 mg, 0.2 mmol) was dissolved in 6 mL of anhydrous methanol and isopropyl alcohol[1:5(v/v)] under stirring, then L-phenylalanine (33 mg, 0.2 mmol) was added and the reaction mixture was heated at 60 °C for 0.5 h (the pH was near 8). After that, (±)-gossypol (52 mg, 0.1 mmol) was added and the solution was stirred at 65−70 °C for 3 h to generate a yellow precipitate. The precipitate was collected by filtration, washing with isopropyl alcohol, and drying under vacuum. Compound **3** (55 mg, 64.3%) was obtained as yellow powder. ^{1}H NMR (300 MHz, CD_3OD) δ 9.45 (s, 2H), 9.26 (s, 2H), 7.50 (s, 2H), 7.49 (s, 2H), 7.23−7.00 (m, 20H), 4.19 (dd, J = 9.3, 3.6 Hz, 2H), 4.13 (dd, J = 9.3, 3.6 Hz, 2H), 3.74 (m, 4H), 3.39 (dd, J = 13.5, 3.6 Hz, 4H), 3.07 (dd, J = 13.5, 9.3 Hz, 2H), 2.97 (dd, J = 13.5, 9.3 Hz, 2H), 1.95 (s, 12H), 1.51 (d, J = 6.3 Hz, 12H), 1.49 (d, J = 6.9 Hz, 12H). ^{13}C NMR (75 MHz, CD_3OD) δ 175.2, 175.2, 172.0, 171.9, 161.5, 149.5, 149.4, 146.9, 137.2, 131.5, 131.4, 129.4, 129.3, 129.1, 128.5, 128.2, 128.2, 128.0, 128.0, 127.2, 127.2, 126.3, 126.3, 117.5, 117.4, 116.8, 116.7, 115.4, 103.5, 103.4, 67.2, 67.1, 63.4, 40.9, 40.5, 27.2, 23.9, 19.4, 19.1, 19.0.

2-Amino-2-(1*H*-indol-3-yl) Acetate Methyl-(−)-gossypol (4). 2-Amino-2-(1H-indol-3-yl) acetate methyl (41 mg, 0.2 mmol) was dissolved in methanol under stirring at room temperature, and (−)-gossypol (52 mg, 0.1 mmol) was added, followed by heating at 50−54 °C. The progress of the reaction was monitored by TLC. After 2−3 h, the crude product was purified by chromatography (silica gel; PE/AcOEt 2:1) to afford **4** (62 mg, 69.7%). ^{1}H NMR (300 MHz, $CDCl_3$) δ 9.64 (d, J = 7.8 Hz, 1H), 9.60 (d, J = 7.5 Hz, 1H), 8.30 (br s, OH), 7.95 (br s, NH), 7.64 (m, 2H), 7.52 (s, 2H), 7.08−7.36 (m, 6H), 5.53(m, 1H), 5.55 (m, 1H), 5.37 (br s, OH), 5.32 (br s, OH), 3.76(s, 3H), 3.78 (s, 3H), 3.66 (m, 2H), 1.99 (s, 3H), 2.02 (s, 3H), 1.50 (d, J = 6.6 Hz, 12H). ^{13}C NMR (75 MHz, CD_3OD) δ 173.5, 170.2, 161.2, 149.1, 147.1, 136.4, 132.2, 129.1, 128.0, 128.0, 125.0, 124.1, 123.0, 120.7, 118.8, 118.2, 116.0, 114.6, 111.7, 104.0, 58.8, 58.7, 53.1, 27.5, 20.3, 20.3, 20.0.

(Rg, S, S) 2-Amino-3-methoxy-3-(4-nitrophenyl)propan-1-ol-(−)-gossypol (5). (S, S) 2-amino-3-methoxy-3-(4-nitrophenyl) propan-1-ol hydrochloride (39 mg, 0.15 mmol) and NaOH (6 mg, 0.15 mmol) were dissolved in 5 mL of anhydrous methanol under stirring at room temperature (the pH was near 8). Gossypol (39 mg, 0.075 mmol) was added later, and the mixture was heated at 50−54 °C for 5 h. The crude product was purified by chromatography (silica gel; PE/acetone 4:1) to afford compound **5** (36 mg, 51.4%). ESI-MS $[M - H]^+$: 933.3. ^{1}H NMR (300 MHz, $CDCl_3$) δ 13.45 (m, NH), 9.31 (d, J = 9 Hz, 2H), 8.17 (d, J = 7.8 Hz, 4H), 7.93 (br s, OH), 7.54 (s, 2H), 7.49 (d, J=7.8 Hz, 4H), 5.36 (br s, OH), 4.62 (br s, OH), 3.86 (m, 2H), 3.73 (m, 4H), 3.44 (m, 2H), 3.31 (s, 6H), 2.00 (s, 6H), 1.49 (d, J= 7.8 Hz, 12H). ^{13}C NMR (75 MHz, $CDCl_3$) δ 172.9, 163.4, 149.5, 147.8, 146.6, 146.0, 131.9, 128.2, 128.0, 127.9, 127.9, 123.3, 123.3, 117.8, 117.1, 115.1, 103.6, 81.0, 68.1, 61.5, 56.8, 27.2, 19.4, 19.4, 18.8.

6-APA-K-(+)-gossypol [6-Aminopenicillanic acid potassium-(+)-gossypol] (6). 6-APA (43 mg, 0.2 mmol) and KOH (11 mg, 0.2 mmol) were dissolved in 6 mL of anhydrous methanol and isopropyl alcohol [1:1(v/v)] under stirring at 0 °C under nitrogen (the pH was near 6). Subsequently, (+)-gossypol (52 mg, 0.1 mmol) was added and the solution was stirred at room temperature for 5 h to give a yellow precipitate. The precipitate was collected by filtration, washing with isopropyl alcohol and drying under vacuum. Compound **6** was obtained as yellow powder in 42 mg (42.4%). ^{1}H NMR (300 MHz, CD_3OD) δ 9.86 (s, 2H), 7.55 (s, 2H), 5.66 (br s, 2H), 5.41 (br s, 2H), 4.30 (br s, OH), 3.82(m, 2H), 2.03 (s, 6H), 1.73 (s, 6H), 1.58 (s, 6H), 1.44 (br d, 12H).

6-APA-Na-gossypolone [6-Aminopenicillanic Acid Sodium-gossypolone] (7). 6-APA (39 mg, 0.25 mmol) and NaOH (10 mg, 0.25 mmol) were dissolved in 6 mL of anhydrous methanol and isopropyl alcohol [1:1(v/v)] under stirring at 0 °C under nitrogen (the pH was near 6). Gossypolone (48 mg, 0.13 mmol) was added later, and the solution was stirred at room temperature for 5 h to generate a red precipitate. The precipitate was collected by filtration, washing with isopropyl alcohol and drying under vacuum. Compound **7** was obtained as red powder in 64 mg (48.4%). ^{1}H NMR (300 MHz, CD_3OD) δ 9.54 (s, 2H), 9.53 (s, 2H), 5.65 (d, J = 6 Hz, 2H), 5.66 (d, J = 5.7 Hz, 2H), 5.44−5.46 (m, 4H), 4.28(br s, OH), 4.25 (br s, OH), 3.99 (m, 4H), 1.96(s, 6H), 1.98 (s, 6H), 1.66(s, 6H), 1.66 (s, 6H), 1.59 (s, 12H), 1.44 (d, J = 6.9 Hz, 12H), 1.43 (d, J = 7.2 Hz, 12H). ^{13}C NMR (75 MHz, CD_3OD) δ 187.3, 187.3, 185.4, 185.3, 172.8, 171.2, 171.1, 167.4, 167.2, 161.8, 161.5, 151.7, 151.6, 145.9, 145.8, 138.3, 138.3, 135.4, 135.4, 126.6, 126.5, 125.8, 125.7, 110.2, 73.3, 70.7, 70.5, 66.8, 66.7, 64.5, 63.4, 31.2, 28.3, 26.3, 23.9, 19.3, 19.1, 19.0, 13.4, 13.2. Anal. Calcd for $C_{46}H_{44}N_4Na_2O_{14}S_2 \cdot 3CH_3OH/H_2O$: C, 51.75; H, 5.50; N, 4.93. Found: C, 51.23; H, 5.06; N, 4.87.

L-Iso-ONa-(±)-gossypol [L-Isolencine sodium-(±)-gossypol] (8). NaOH (9 mg, 0.225 mmol) was dissolved in 6 mL of anhydrous methanol and isopropyl alcohol [1:5(v/v)] under stirring, and L-isolencine (29 mg, 0.225 mmol) was added and heated at 60 °C for 0.5 h (the pH was near 9). Subsequently, (±)-gossypol (58 mg, 0.112 mmol) was added and the solution was stirred at 65−70 °C for 3 h to afford a yellow precipitate. The precipitate was collected by filtration, washing with isopropyl alcohol and drying under vacuum. Compound **8** was obtained as yellow powder in 52 mg (58.9%). ^{1}H NMR (300 MHz, CD_3OD) δ 9.78 (s, 2H), 7.54 (s, 2H), 3.85 (d, J = 7.5 Hz, 2H), 3.77 (m, 2H), 2.06 (m, 2H), 2.02 (s, 6H), 1.65 (m, 2H), 1.50 (br d, 12H), 1.24 (m, 2H), 1.00 (2 × br d, 6H), 0.94 (br t, 6H). ^{13}C NMR (75 MHz, $CDCl_3$) δ 175.6, 175.5, 171.7, 161.8, 149.7, 147.1, 131.6, 128.2, 127.5, 127.4, 117.9, 117.7, 117.0, 115.8, 103.8, 63.4, 38.9, 38.8, 27.2, 24.1, 24.1, 19.6, 19.2, 15.3, 15.3, 10.8.

(Sg, S) L-Iso-ONa-(+)-gossypol [L-Isolencine sodium-(+)-gossypol] (9). NaOH (13 mg, 0.325 mmol) was dissolved in 6 mL of anhydrous methanol and isopropyl alcohol [1:5(v/v)] under stirring, and L-isolencine (42 mg, 0.325 mmol) was added and heated at 60 °C for 0.5 h (the pH was near 8−9). After that, (+)-gossypol (85 mg, 0.164 mmol) was added and the solution was stirred at 65−70 °C for 3 h to generate a yellow precipitate. The precipitate was collected by filtration, washing with isopropyl alcohol and drying under vacuum. Compound **9** was obtained as yellow powder in 119 mg (92.1%). ^{1}H NMR (300 MHz, CD_3OD) δ 9.76 (s, 2H), 7.53 (s, 2H), 3.82(d, J = 2.4 Hz, 2H), 3.75 (m, 2H), 2.03 (m, 2H), 2.03 (s, 6H), 1.50 (t, J = 6.9 Hz, 12H), 1.00 (d, J = 6.6 Hz, 6H), 0.93 (t, J = 7.2 Hz, 6H). ^{13}C NMR (75 MHz, CD_3OD) δ 175.4, 171.8, 161.8, 149.7, 147.0, 131.7, 128.1, 127.3, 117.8, 116.9, 115.7, 103.7, 63.4, 38.8, 27.2, 24.6, 24.2, 24.0, 19.5, 19.2, 15.3, 10.7.

Cell Culture. Murine colon carcinoma cell line CT26, breast carcinoma cell line 4T1, and melanoma cell line B16−F10 were maintained in RMPI-1640 medium (HyClone, Logan, UT) supplemented with 10% fetal bovine serum (FBS, HyClone), 100 units/mL penicillin, and 100ug/mL streptomycin (Invitrogen, Carlsbad, CA). Human colorectal carcinoma cell lines HCT116 and SW620, lung carcinoma cell line A549, breast carcinoma cell lines MDA-MB-435, and MCF-7 and prostate carcinoma cell line PC-3 were cultured in Dulbecco's Modified Eagle's Medium (HyClone) supplemented with 10% FBS, penicillin and streptomycin in a 5% CO_2 humidified atmosphere at 37 °C.

Cell Viability. All of the synthesized compounds were dissolved in DMSO. Different types of cancer cell lines were plated into 96-well plates at a density of 5×10^3 cells/100 μL medium per well. After adherence, cells were treated with various concentrations of synthesized compounds individually or in combination with 5-fluorouracil (Wako Pure Chemical Industries, Osaka, Japan) for 72 h, with DMSO as negative controls. At

the end of treatment, the tetrazolium compound, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT, Sigma-Aldrich, St. Louis, MO), was added and then incubated for 4 h at 37 °C in the dark. The formazan crystals were dissolved by DMSO, and the absorbance was recorded using an ELISA plate reader.

Cell Proliferation Assay. CT26 cells were plated into 96-well plates at a density of 2×10^3 cells/100 μL medium per well. After adherence, cells were treated with compound **7** for indicated concentrations and times, and cell viability was measured by the Cell Titer 96 Aqueous One Solution cell proliferation kit (Promega, Madison, WI) according to the manufacturer's instructions.

TUNEL Assay. Apoptosis was detected by In Situ Cell Death Detection Kit (Roche Applied Science, Mannheim, Germany) according to the manufacturer's instructions. Briefly, cells were washed twice with PBS after treatment, followed by fixed with 4% paraformaldehyde (Sigma-Aldrich) in PBS for 1 h at room temperature. After washing with PBS, cells were treated in permeabilization solution for 2 min on ice, washed with PBS, and then incubated in TUNEL reaction mixture for 1 h at 37 °C in the dark. DAPI were used for cell nuclear staining. Samples can be analyzed under a fluorescence microscope. Five random fields were counted and averaged for statistical analysis.

Molecular Modeling Studies. We used the conformation of antiapoptotic proteins Bcl-2 (PDB code 1YSW) and Bcl-xL in complex with a Bak-peptide (PDB code 1BXL) and performed the docking studies with the software AutoDock Vina (http://vina.scripps.edu/).[40]

Fluorescence Polarization Assays (FPAs). FP assays were performed as previously described.[44] Briefly, FITC-labeled Bak BH3 peptide (GQVGRQLAIIGDDINR) was synthesized then purified by HPLC. Then 100 nM GST-Bcl-xL protein was incubated with compound **7** at various concentrations in PBS in 96-well black plates at room temperature for 10 min, and then 50 nM FITC-labeled Bak BH3 peptide was added and incubated for 60 min at room temperature. The polarization values were measured at excitation/emission wavelengths of 480/535 nm with a multilabel plate reader.

Immunoblot Analysis. Cell lysates were equally loaded to 10% SDS−polyacrylamide gels, electrophoresed, and transferred to nitrocellulose membranes (Amersham Bioscience, Buckinghamshire, UK). Membranes were blocked for 1 h in TBS-Tween-20 containing 5% nonfat milk and then incubated with primary antibodies against Bcl-2 (Cell Signaling, Beverly, MA), Bcl-xL (Cell Signaling), and β-actin (Santa Cruz Biotechnology, Santa Cruz, CA) at 4 °C overnight. After being washed 3 times, the blots were incubated with horseradish peroxidase (HRP)-linked secondary antibodies (Jackson ImmunoResearch Laboratories, West Grove, PA) at room temperature for 1 h. The blots were visualized by SuperSignal West Pico Chemiluminescent Substrate (Pierce Biotechnology, Rockford, IL) according to the manufacturer's instructions. All experiments were repeated three times with similar results.

co-IP Assay. Cells were treated with compound **7** as indicated concentrations and times. After treatment, cells were lysed in 2 × 1% Triton X-100 lysis buffer for 30 min at 4 °C. After centrifugation, cell lysates were incubated with antibodies against Bcl-2 (Cell Signaling) or Bcl-xL (Cell Signaling) together with protein A-Agarose resin (Pierce Biotechnology) overnight at 4 °C. The immunoprecipitates were eluted by the 2 × sodium dodecyl sulfate (SDS) sample buffer and then detected for Bax or Bim with antibodies from Cell Signaling by Western blots.

Animal Tumor Model Studies. Male BALB/c mice were purchased from Shanghai Laboratory Animal Center and care of the mice was in accordance with the Guidelines of Shanghai Second Medical University for the Use of Animals in Research. CT26 cells (5×10^5/0.1 mL) were subcutaneously injected into the right flanks of BALB/c mice (6−8 weeks old). Once their tumors grew up to 60 mm^3, the mice were randomly divided into different groups, with four mice in each group. Compound **7** solution was prepared in 10% ethanol. Mice were given with compound **7** at the dose of 10 μmol/kg once a day in 12 consecutive days, with 10% ethanol as a negative control by gavage, and 5-fluorouracil was given at the dose of 10 mg/kg every two days in 12 consecutive days by i.p., with PBS as a negative control. The tumors were measured by length and width every 2 days. Tumor volumes were calculated by length × width × width × π/6.

In Vivo Toxicity Studies. Compounds **7** and **1** were prepared in 10% ethanol. Male BALB/c mice (6−8 weeks old) were given compound **7** or **1** at the dose of 120 μmol/kg once a day in 12 consecutive days, with 10% ethanol as control by gavage ($n = 6$). At the end of the 12-day treatments, mice were weighted and sacrificed. The small intestine tissue samples were embedded in paraffin and cut into 5 μm sections. The sections were stained with hematoxylin and eosin (H&E) and examined using a light microscope.

Statistical Analysis. The Student's t test was used to compare the difference between two groups. A value of $P < 0.05$ was regarded as indicating a significant difference.

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Supporting Information Available: Chemical data for key compounds, quantitative polymerase chain reaction (QPCR) methods, and results. This material is available free of charge via the Internet at http://pubs.acs.org.

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