ChemPubSoc Europe

DOI: 10.1002/cmdc.201200559

Library Construction and Biological Evaluation of Enmein-Type Diterpenoid Analogues as Potential Anticancer Agents

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A library of promising enmein-type 14-*O*-diterpenoid derivatives was constructed from a commercially available kaurene-type oridonin by practical and efficient synthetic methods. These synthetic derivatives were evaluated for their antiproliferative activities against a set of four human cancer cell lines. The IC_{50} values are similar to or improved over those of the parent molecule and paclitaxel, the latter of which was used as a positive control. Compound **29** was further investigated for its apoptotic properties against human hepatocarcinoma Bel-7402 cells to better understand its mode of action. Moreover, compound **29** was shown to have potent antitumor activity in vivo in studies with a murine model of gastric cancer (MGC-803 mice). These results warrant further preclinical investigations of these diterpenoid-based analogues as potential novel anticancer chemotherapeutics.

Introduction

Nature is an untapped source of unique and desirable scaffolds for library construction and subsequent drug discovery.[1] Natural products (NPs) have been the major sources of chemical diversity as starting materials, driving pharmaceutical discovery over the past century. A total of 19 NP-based drugs were approved for marketing worldwide from 2005 to 2010, of which seven compounds were classified as NPs, 10 compounds as semisynthetic NPs, and two compounds as NP-derived drugs.[2] Indeed, numerous NP scaffolds have led to approved drugs or drug candidates for a range of diseases, especially anticancer agents (paclitaxel, rapamycins, epothilones).[3] The success of NPs and their semisynthetic derivatives as therapeutic agents is intrinsically linked to the fact that NPs are often referred to as evolutionarily selected "privileged structures."[1,4]

The genus *Isodon* is famous for producing bioactive diterpenoids with diverse skeletons, especially *ent*-kaurane diterpenoids.[5,6] Approximately 600 new diterpenoids with diverse skeletons have been isolated and characterized.[6] Among these, enmein-type *ent*-kaurane diterpenoids (Figure 1) with unique chemical skeletons have exhibited promising antitumor activities.[5,7] However, the structures are generally quite complex, incorporating large numbers of stereogenic centers and intricate ring systems. Therefore, preparations of NP-based libraries inevitably involve rather sophisticated and laborious synthetic sequences. Furthermore, therapeutic development of these leads is significantly impeded by the problem of large-scale compound supply.

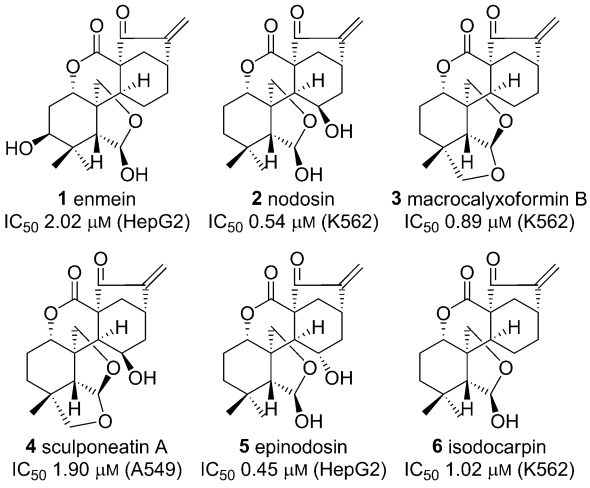

Figure 1. Reported enmein-type diterpenoids isolated from natural plant sources and their antiproliferative activities in cancer cells.

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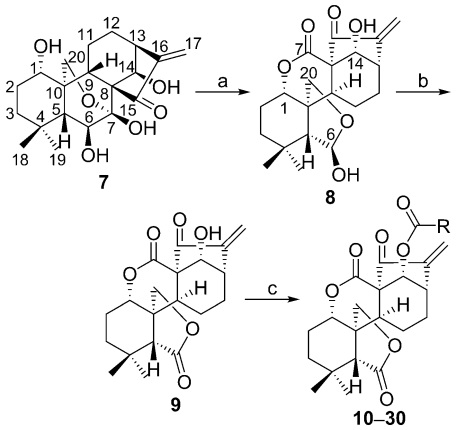
Supporting information for this article is available on the WWW under http://dx.doi.org/10.1002/cmdc.201200559.

Results and Discussion

Chemistry

While total synthesis of the required diterpenoid scaffold is a possible solution to the supply issue,[8] the structural complexity and need for multistep syntheses to make some of the templates can be prohibitive.[9] Our approach (Scheme 1) involves the use of relevant commercially available resources to acquire target NP scaffolds, thus bypassing the de novo synthetic strategy for scaffold production. Starting from commercially available oridonin (**7**), enmein-type diterpenoid analogue **8** was obtained by treatment with sodium periodate in water. Oxidation of **8** with Jones reagent at 0 °C afforded corresponding ketone **9** in total yields of 87%. The procedure is well-established, and the mechanism of structure conversion has also been studied.[10] Compound **9** provided a good building block to construct a library of 14-*O*-derivatives (**10**–**30**) of enmein-type diterpenoids with diverse chemical properties and potential anticancer activities.



Scheme 1. Synthesis of enmein-type diterpenoid building block compound **9** and a library of 14-*O*-derivatives (**10**–**30**). *Reagents and conditions*: a) $NaIO_4$, H_2O, 24 h, RT; b) Jones reagent, acetone, 15 min, 0 °C; c) corresponding acid, EDCI, DMAP, CH_2Cl_2, 8–12 h, RT.

In vitro antiproliferative activities and structure–-activity relationships

Antiproliferative activities of enmein-type diterpenoid analogues **10**–**30** were evaluated by MTT assay against four cancer cell lines (K562, Bel-7402, CaEs-17, and MGC-803). The results are summarized in Table 1. All of the derivatives exhibited stronger antiproliferative activities than parent compound oridonin in the four cancer cell lines, and some were even more active than positive control paclitaxel (in red), especially against human hepatoma Bel-7402 cells. When the R substituent was an alkyl group (compounds **10**–**12**), improved IC_{50} values were observed against K562 (the lowest of which was 0.31 μM for **11**) and Bel-7402 cells (from 0.78 to 1.37 μM). Compounds with an aromatic group generally showed more potent antiproliferative activities. In particular, a pronounced inhibitory effect was observed for those compounds with an electron-withdrawing nitro (**16**) or chloro (**17** and **18**) group. Although fluorinated compounds comprise a substantial proportion of therapeutic drugs and impart a variety of properties to certain medicines,[11] compounds **19**–**22** exhibited only moderate IC_{50} values in vitro. Adding a linkage between the enmein-type skeleton and an aromatic group (**23**–**27**) maintained good activity in Bel-7402 cells. A similar effect was achieved in compounds for which the R group was a heterocyclic ring (**27**–**30**).

Compound **29**, with a 3-pyridyl R moiety, could react with various acids to form salts, therefore solving the critical problem of poor solubility of the diterpenoids. Thus, compound **29** was chosen for further investigation. In the MTT assay, 17 of 21 derivatives showed more promising IC_{50} values than paclitaxel against Bel-7402 cells. Consequently, this cell line was selected for intensive mechanistic studies.

Table 1. Cytotoxicity of synthetic enmein-type diterpenoid analogues **10**–**30** against various human cancer cell lines.

Compd	R	IC_{50} [μM][a]			
		K563	Bel-7402	CaEs-17	MGC-803
Paclitaxel		0.41 ± 0.02	1.89 ± 0.09	0.43 ± 0.03	0.85 ± 0.06
Oridonin		4.76 ± 0.32	7.48 ± 0.53	11.03 ± 1.02	5.69 ± 0.39
10	methyl	0.35 ± 0.05	1.37 ± 1.03	0.69 ± 0.12	1.27 ± 0.10
11	ethyl	0.31 ± 0.07	0.99 ± 0.04	0.47 ± 0.09	1.13 ± 0.02
12	cyclopentyl	1.82 ± 0.17	0.78 ± 0.06	3.77 ± 0.13	1.23 ± 0.19
13	phenyl	0.33 ± 0.02	0.79 ± 0.11	0.81 ± 0.05	0.46 ± 0.08
14	4-methylphenyl	0.52 ± 0.09	1.58 ± 0.22	1.78 ± 0.17	1.54 ± 0.19
15	4-methoxylphenyl	2.18 ± 0.11	3.93 ± 0.62	3.83 ± 0.42	3.47 ± 0.50
16	4-nitrophenyl	0.18 ± 0.10	0.82 ± 0.07	0.27 ± 0.02	0.66 ± 0.11
17	4-chlorophenyl	0.14 ± 0.08	0.89 ± 0.09	0.34 ± 0.03	0.34 ± 0.01
18	2-chlorophenyl	0.26 ± 0.03	0.72 ± 0.01	0.28 ± 0.16	0.82 ± 0.07
19	4-fluorophenyl	2.26 ± 0.17	1.95 ± 1.06	4.66 ± 0.53	1.93 ± 0.14
20	3-fluorophenyl	2.03 ± 0.21	1.25 ± 0.44	4.47 ± 0.53	1.68 ± 0.45
21	2,3,4,5-tetraflrorophenyl	2.15 ± 0.27	1.98 ± 0.80	4.07 ± 0.02	3.43 ± 0.94
22	4-trifluoromethylphenyl	0.69 ± 0.07	1.85 ± 0.12	0.93 ± 0.10	1.44 ± 0.19
23	4-fluorophenylvinyl	2.01 ± 0.35	1.08 ± 0.14	4.33 ± 0.53	1.62 ± 0.11
24	2-phenylethyl	1.90 ± 0.09	0.89 ± 0.40	3.90 ± 0.21	1.38 ± 0.31
25	4-fluorophenylmethyl	0.46 ± 0.03	1.72 ± 0.07	0.30 ± 0.10	0.87 ± 0.16
26	1-naphthylmethyl	1.65 ± 0.07	0.77 ± 0.11	3.81 ± 0.09	1.47 ± 0.16
27	3-(1*H*-indolyl)methyl	1.60 ± 0.03	0.68 ± 0.20	3.16 ± 0.13	1.12 ± 0.08
28	2-quinolyl	1.41 ± 0.18	0.63 ± 0.07	2.69 ± 0.11	0.76 ± 0.15
29	3-pyridyl	1.74 ± 0.21	0.71 ± 0.36	3.54 ± 0.27	1.16 ± 0.23
30	2-tetrahydrofuryl	2.72 ± 0.26	1.93 ± 1.04	5.41 ± 0.06	2.86 ± 0.55

[a] IC_{50}: concentration required to inhibit cell growth by 50% relative to untreated control cells; results are expressed as the mean ± SD of three independent experiments. Human cell lines: leukemia (K562); hepatoma (Bel-7402); esophageal cancer (CaEs-17); gastric cancer (MGC-803).

Cell-cycle analysis

To determine whether the suppression of cell growth by enmein-type diterpenoids is caused by a cell-cycle effect, we detected the DNA content of cell nuclei by flow cytometry

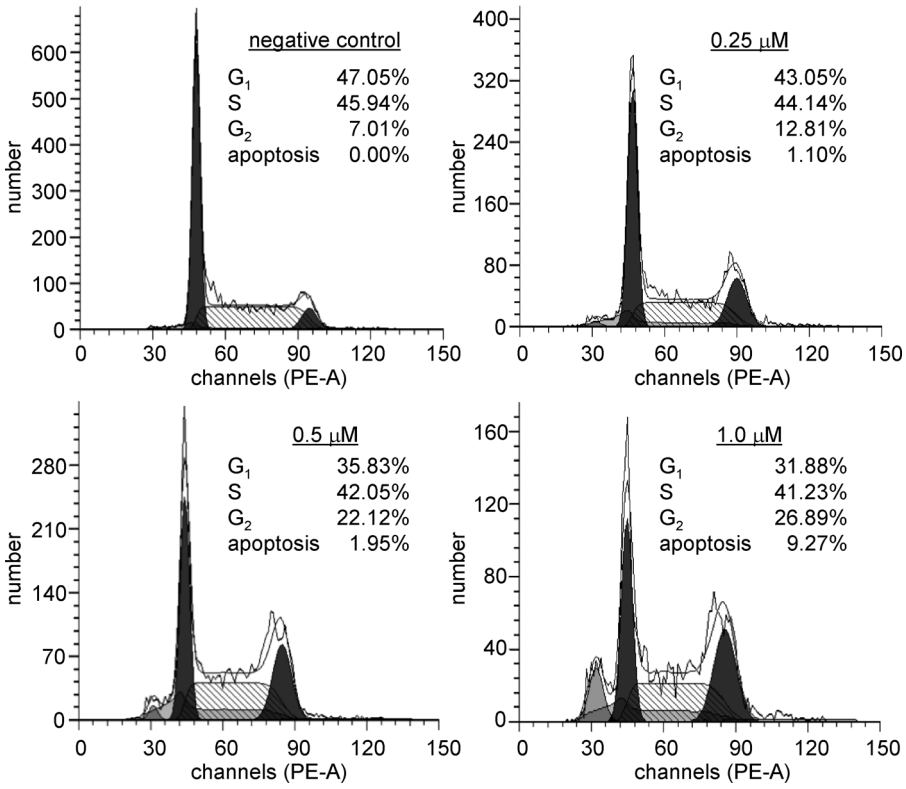


Figure 2. Influence of the Bel-7402 cell cycle by enmein-type compound **29**. Bel-7402 cells were incubated with the indicated concentrations of **29** for 48 h before staining with PI. Cellular DNA content for cell-cycle distribution analysis was measured by flow cytometry. The diagrams show the distribution of cells according to their DNA content; inserts give the percentages of cells in various cell-cycle phases.

(Figure 2). Bel-7402 cells were treated with compound **29** at concentrations of 0.25, 0.5, and 1.0 μM, which resulted in accumulation of 12.81, 22.12, and 26.89% of cells at the G_2/M phase, respectively, compared with the untreated cells. The influence of cell-cycle progression at low micromolar concentrations of enmein-type diterpenoid analogues makes these compounds promising agents for use in combination with anticancer drugs acting at different stages of the cell cycle.

Induction of apoptosis

To clarify whether the loss of cancer cell viability promoted by enmein-type diterpenoids is associated with apoptosis, an annexin V–FITC/propidium iodide (PI) binding assay was performed. Bel-7402 cells were treated with vehicle alone (controls) or with various concentrations (0.25, 0.5, or 1.0 μM) of compound **29** for 48 h, then were stained with annexin V–FITC and PI. In the early stages of apoptosis, phosphatidylserine was translocated from within the cell membrane to outside of it. Annexin V, a calcium-dependent phospholipid-binding protein characterized by a high affinity for phosphatidylserine, was used to detect early apoptotic cells. PI is a red fluorescent dye that stains cells that have lost their membrane integrity. Cells stained with annexin V–FITC and PI were classified as necrotic cells (annexin$^-$/PI$^+$), late apoptotic cells (annexin$^+$/PI$^+$), intact cells (annexin$^-$/PI$^-$), or early apoptotic cells (annexin$^+$/PI$^-$). Percentages of apoptotic Bel-7402 cells were determined by flow cytometry. As shown in Figure 3, compound **29** exhibited potent dose-dependent activity in the induction of apoptosis. Treatment of Bel-7402 cells with compound **29** at 0.25, 0.5, and 1.0 μM for two days resulted in 16.6, 24.9, and 33.5% apoptotic cells (early and late), as compared with 8.3% in an untreated vehicle control, indicating that compound **29** was able to induce apoptotic cell death in Bel-7402 cells. During the process of apoptosis, apoptotic signals can result in the loss of mitochondrial membrane potential. To determine plausible path-

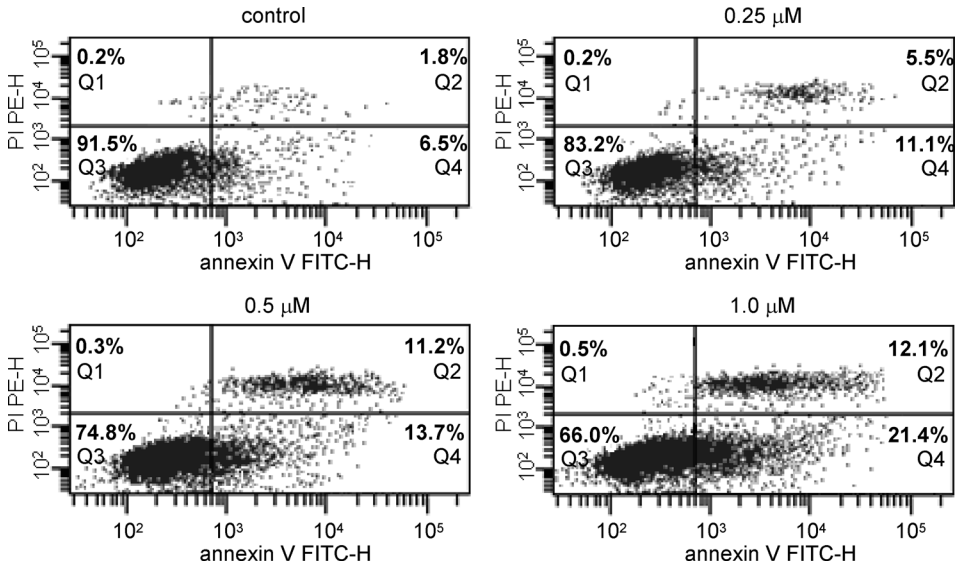


Figure 3. Apoptosis ratio detection of compound **29**. Bel-7402 cells were incubated with the indicated concentrations of **29** for 48 h before staining with annexin V–FITC and PI, followed by flow cytometric analysis.

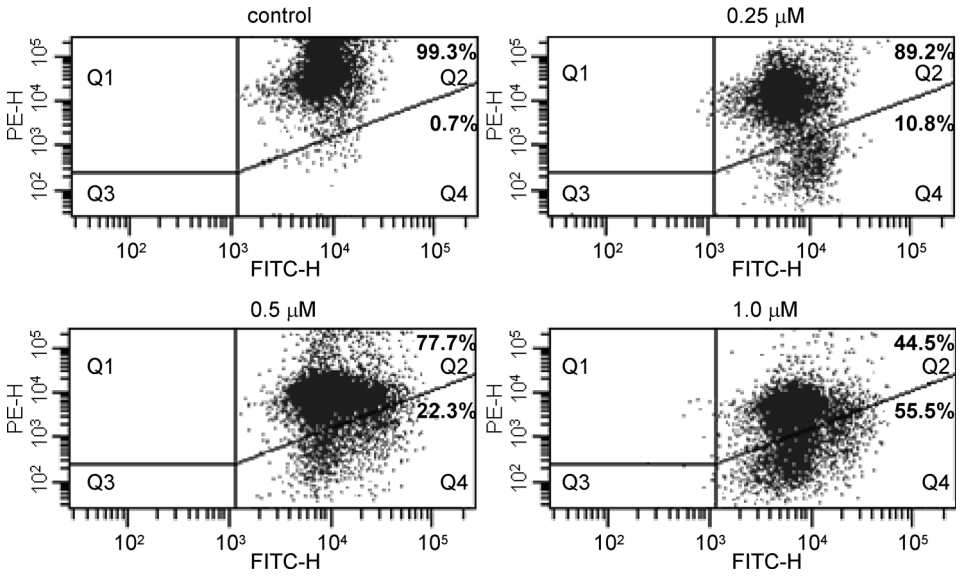


Figure 4. Effect of compound **29** on the mitochondrial membrane potentials of Bel-7402 cells. Bel-7402 cells were incubated with the indicated concentrations of **29** for 48 h prior to staining with JC-1.

ways by which **29** triggered cell apoptosis, Bel-7402 cells were incubated with different concentrations (0, 0.25, 0.5, 1.0 μM) of **29** for 48 h prior to staining with the lipophilic mitochondrial probe 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolcarbocyanine iodide (JC-1). The number of cells with collapsed mitochondrial membrane potentials in different groups of cells were determined by flow cytometry analysis (Figure 4), yielding 0, 8.5, 17.6, and 45.6% apoptotic cells, respectively. These studies showed that incubation with **29** increased the number of cells with collapsed mitochondrial membrane potentials.

Effect of apoptosis-related proteins

In general, all apoptotic pathways depend on activation of caspases for the final execution of apoptosis. Therefore, it might be reasonable to first look into caspases as a sign of apoptosis. Within this context, the assessment of the activities of caspases 3, 8, and 9 in Bel-7402 cells showed strong activation after 48 h treatment with compound **29** in a concentration-dependent manner. Further western blotting analysis revealed that incubation with **29** dramatically increased the relative levels of pro-apoptotic cytochrome *c*, Bax, and Fas expression, but decreased the levels of anti-apoptotic Bcl2 expression (Figure 5), thus suggesting the mitochondrial pathway for activation of this important mechanism of tumor cell death.

Antitumor activity in vivo

Based on the in vitro results and intensive mechanistic studies, we further tested the antitumor activity of compound **29** in vivo

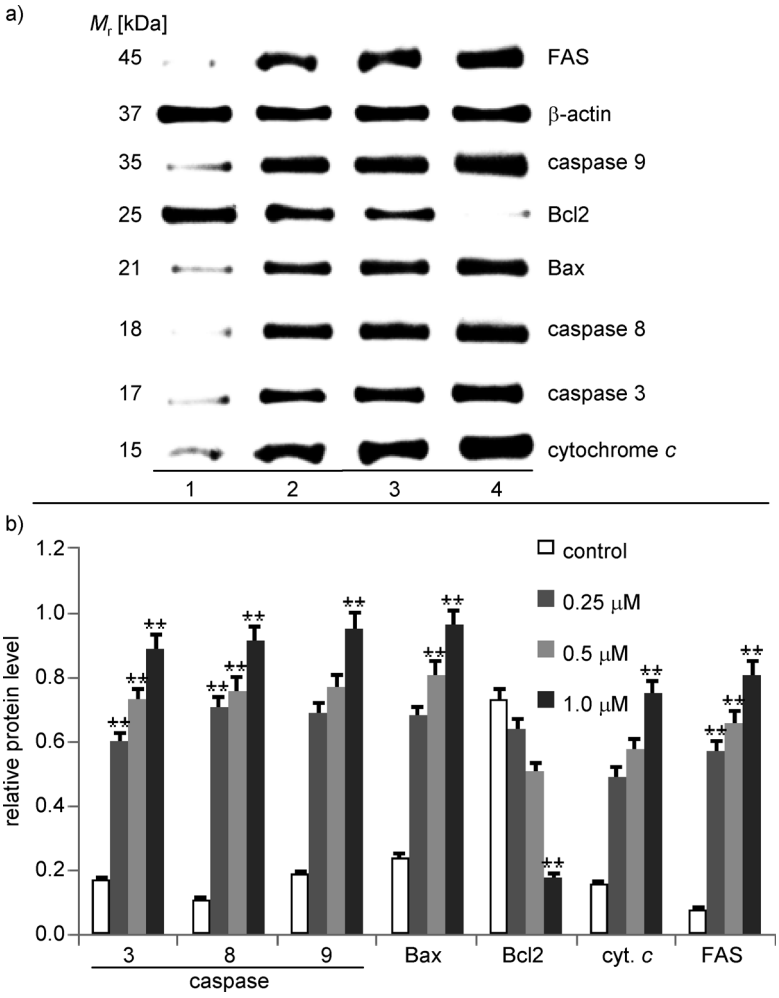


Figure 5. a) Western blot analysis of compound **29** on the expression of apoptosis-related proteins in Bel-7402 cells. Lane 1: negative control, 2: 0.25 μM **29**, 3: 0.5 μM **29**, 4: 1.0 μM **29**; b) quantitative analysis showing the levels of each protein relative to control β-actin: $^{*}p < 0.05$, $^{**}p < 0.01$ vs. vehicle control.

by performing an assay in mice with gastric cancer (MGC-803). Oridonin and compound **29** were administered intraperitoneally 40 mg kg^{-1} in a vehicle of 1% DMSO, 2% poloxamer, and 97% saline. The positive control group was treated with paclitaxel (10 mg kg^{-1}) through intravenous injection. The negative control group received 0.9% normal saline through intraperitoneal injection. Results are shown in Table 2. Compound **29** exhibited a stronger antitumor activity (tumor inhibitory ratio of 64.28%) than that of parent compound oridonin (37.30%) and only slightly less potent than that of paclitaxel (71.43%) in MGC-803 mice. Thus, compound **29** is worthy of further investigation as a potential anticancer drug candidate.

Table 2. Antitumor activity of compound **29** in mice with MGC-803 gastric cancer.[a]

Compd	Mouse weight [g]		Tumor weight [g]	Inhib. [%]
	Start	End		
Saline (control)	15.41 ± 0.55	23.45 ± 1.19	1.26 ± 0.24	–
Paclitaxel	15.37 ± 0.47	23.98 ± 1.87	0.36 ± 0.15	71.43*
Oridonin	15.49 ± 0.35	22.69 ± 1.35	0.79 ± 0.09	37.30**
29	15.45 ± 0.89	23.27 ± 1.23	0.45 ± 0.11	64.28*

[a] Number of mice in each group was 10; all mice completed treatment; weights given are the mean ± SD. *$p < 0.01$, **$p < 0.05$.

Conclusions

The findings arising from the studies described above open a possible approach to the development of novel enmein-type diterpenoid analogues as potential anticancer agents. In this effort, a novel semisynthetic library of enmein-type diterpenoid derivatives with potent in vitro and in vivo antitumor activities was prepared from commercially available starting materials. Furthermore, the molecular mode of action revealed that derivative **29** caused cell-cycle arrest and induced apoptosis in Bel-7402 cells through oxidative-stress-triggered mitochondria-related caspase 3-, 8-, and 9-dependent pathways. It is expected that the mechanistic and biological studies described, together with our previous reports of different kinds of diterpenoids,[10,12] could expedite the development of new diterpenoid-based therapeutic agents for clinical cancer intervention.

Experimental Section

Chemistry

General: All commercially available solvents and reagents were used without further purification. Melting points were taken on an XT-4 micro melting point apparatus and uncorrected. IR spectra (KBr) were recorded on a Nicolet Impact 410 instrument (KBr pellet). ^{1}H and ^{13}C NMR spectra were recorded with a Bruker AV-300 or ACF-500 spectrometer in the indicated solvents (TMS as internal standard); chemical shifts (δ) are expressed in ppm, and coupling constants (J) are reported in Hz. Mass spectra were obtained with an FTMS-2000 instrument. HRMS data were collected with an Agilent QTOF 6520.

Compound 8: Compound **7** (364 mg, 1 mmol) was dissolved in H_2O (10 mL). $NaIO_4$ (3.16 g, 14.8 mmol) was added to this solution, and the mixture was stirred at room temperature for 24 h and extracted with CH_2Cl_2. The CH_2Cl_2 layer was washed with brine, dried over anhydrous Na_2SO_4, filtered, and evaporated to give compound **8** (360 mg, 99%) as a white powder; mp: 183–185 °C; ^{1}H NMR ([D$_6$]DMSO, 500 MHz): $\delta = 6.18$ (1H, d, $J = 3.3$ Hz, 6-OH), 5.92 (1H, s, 17-CH$_2$), 5.52 (1H, s, 17-CH$_2$), 5.38 (1H, d, $J = 2.1$ Hz, 14-OH), 5.17 (1H, d, $J = 2.1$ Hz, 6-CH), 4.67 (1H, s, 14-CH), 4.65–4.63 (1H, m, 1-CH), 3.87, 3.58 (each 1H, dd, $J_A = J_B = 9.1$ Hz, 20-CH$_2$), 2.95 (1H, d, $J = 9.1$ Hz, 13-CH), 2.49–2.40 (2H, m, 12-CH$_2$), 1.70–1.67 (2H, m, 2-CH$_2$), 1.40–1.36 (2H, m, 11-CH$_2$), 1.29–1.25 (1H, m, 9-CH), 0.93 (3H, s, 18-CH$_3$), 0.88 ppm (3H, s, 19-CH$_3$); ^{13}C NMR ([D$_6$]DMSO, 300 MHz): $\delta = 200.30$, 166.88, 150.42, 118.59, 100.73, 72.81, 71.28, 62.18, 54.99, 53.78, 49.86, 47.67, 43.07, 36.44, 32.67, 30.70, 29.55, 23.22, 22.82, 18.75 ppm; IR (KBr): $\tilde{\nu}_{max} = 3450$, 1754, 1645 cm^{-1}; MS(ESI) m/z: 747.4 $[2M+Na]^+$; HRMS (ESI, $M+H$) m/z calcd for $C_{20}H_{27}O_6$: 363.1802, found: 363.1799.

Compound 9: Compound **8** (72 mg, 0.2 mmol) was dissolved in acetone. Jones reagent was added dropwise to this solution until a red color persisted. The mixture was stirred at 0 °C for 15 min. The mixture was then diluted with H_2O and extracted with CH_2Cl_2. The extract was washed with brine, dried over anhydrous Na_2SO_4, filtered, and evaporated to give compound **9** (63 mg, 87%) as a white powder; mp: 248–250 °C; ^{1}H NMR ([D$_6$]DMSO, 300 MHz): $\delta = 5.95$ (1H, s, 17-CH$_2$), 5.57 (1H, s, 17-CH$_2$), 5.53 (1H, d, $J = 3.5$ Hz, 14-OH), 4.79–4.76 (1H, m, 14-CH), 4.76–4.72 (1H, m, 1-CH), 4.51, 3.69 (each 1H, dd, $J_A = J_B = 9.9$ Hz, 20-CH$_2$), 3.00 (1H, d, $J = 9.6$ Hz, 13-CH), 1.08 (3H, s, 18-CH$_3$), 0.95 ppm (3H, s, 19-CH$_3$); ^{13}C NMR ([D$_6$]DMSO, 300 MHz): $\delta = 200.25$, 175.45, 166.31, 149.84, 119.45, 73.78, 71.03, 70.98, 61.66, 50.19, 47.42, 45.77, 42.87, 35.50, 32.66, 31.83, 29.09, 23.42, 22.87, 18.15 ppm; IR (KBr): $\tilde{\nu}_{max} = 3508$, 1785, 1751, 1645 cm^{-1}; MS(ESI) m/z: 361.1 $[M+H]^+$; HRMS (ESI, $M+H$) m/z calcd for $C_{20}H_{25}O_6$: 361.1646, found: 361.1647.

General procedure for the synthesis of enmein-type diterpenoids 10–30: Compound **9** (72 mg, 0.2 mmol) was mixed with the corresponding acid (0.24 mmol), EDCI (0.4 mmol), and DMAP (0.02 mmol) in 15 mL CH_2Cl_2. After stirring at room temperature for 8–12 h, the mixture was poured into 15 mL 10% HCl and extracted with CH_2Cl_2 (3×10 mL). The organic layer was combined, washed sequentially with H_2O and saturated NaCl solution, dried over anhydrous Na_2SO_4, and concentrated in vacuo. The crude product was purified by column chromatography (MeOH/CH_2Cl_2 1:100 v/v) to give the title compounds. Data for compounds **10**–**28** and **30** are listed in the Supporting Information.

Compound 29: Yellow powder (41 mg, 46%): mp: 150–152 °C; ^{1}H NMR ($CDCl_3$, 300 MHz): $\delta = 9.12$ (1H, s, Ar-H), 8.77 (1H, s, Ar-H), 8.19 (1H, d, $J = 7.9$ Hz, Ar-H), 7.39–7.28 (1H, m, Ar-H), 6.25 (1H, s, 17-CH$_2$), 6.09 (1H, s, 14-CH), 5.68 (1H, s, 17-CH$_2$), 4.66–4.62 (1H, m, 1-CH), 4.38, 4.11 (each 1H, dd, $J_A = J_B = 10.6$ Hz, 20-CH$_2$), 3.37 (1H, d, $J = 9.3$ Hz, 13-CH), 1.22 (3H, s, 18-CH$_3$), 1.07 ppm (3H, s, 19-CH$_3$); ^{13}C NMR ($CDCl_3$): $\delta = 196.92$, 175.11, 165.63, 164.53, 153.87, 151.00, 146.65, 137.38, 124.87, 123.33, 121.74, 74.58, 74.31, 71.20, 59.37, 50.72, 47.53, 45.99, 40.41, 36.28, 32.99, 32.14, 29.49, 23.54, 23.01, 19.04 ppm; IR (KBr): $\tilde{\nu}_{max} = 2925$, 1762, 1731, 1647, 1591, 1282, 1188, 1023, 916, 736, 701 cm^{-1}; MS(ESI) m/z: 446.2 $[M+H]^+$; HRMS (ESI, $M+H$) m/z calcd for $C_{26}H_{28}NO_7$: 466.1860, found: 466.1856.

Biological evaluations

In vitro MTT assays: The MTT assay was employed in an in vitro cytotoxicity assay, which was performed in 96-well plates. K562 cells at the log phase of their growth cycle (5×10 cells mL^{-1}) were added to each well (100 μL per well) and treated in three replicates at various concentrations of the samples (0.39–100 μg mL^{-1}). The cells were then incubated for 24 h at 37 °C in a humidified atmosphere of 5% CO_2. After 72 h, 20 μL of MTT solution (5 mg mL^{-1}) per well was added to each cultured medium before incubating for another 4 h. DMSO was then added to each well (150 μL per well). After 10 min at room temperature, the OD of each well was measured using a microplate reader (Bio-Rad 550) at a wavelength of 490 nm. In these experiments, the negative reference agent was 0.1% DMSO, and paclitaxel was used as the positive reference at a concentration of 10 μg mL^{-1}. The same method was used for CaEs-17, Bel-7402, and MGC-803 cell lines.

Cell-cycle analyses: Bel-7402 cells were plated in six-well plates (5.0×10^3 cells per well) and incubated at 37 °C for 24 h. Exponentially growing cells were then incubated with test compound at various concentrations. Untreated cells and cells treated with DMSO alone were included as controls. After 48 h treatment, cells were centrifuged and fixed in 70% EtOH at 4 °C overnight, and were subsequently resuspended in PBS containing 100 μL RNase A and 400 μL PI. Cellular DNA content was measured for cell-cycle distribution analysis using a flow cytometer (FACSCalibur, Becton–Dickinson).

Analyses of cellular apoptosis: Bel-7402 cells were incubated with the compounds as described above. Cells were treated with tested compound for 48 h, and apoptosis was analyzed using annexin V and PI double staining by flow cytometry, according to the manufacturer's instructions, in order to detect apoptotic cells.

Mitochondrial membrane potential assays: Bel-7402 cells were cultured overnight and incubated in triplicate with test compounds (0.25, 0.5, and 1.0 μM) or vehicle for 48 h. The cells were stained with JC-1 dye, according to the manufacturer's instructions (KGA601, Keygen). The percentage of cells with healthy or collapsed mitochondrial membrane potentials was monitored by flow cytometry analysis ($\lambda_{ex}=488$ nm, $\lambda_{em}=530$ nm).

Western blot assays: Bel-7402 cells were incubated in triplicate with different doses of **29** for 48 h, then the cells were harvested and lysed using lysis buffer, and the solution was centrifuged. After protein concentrations were determined, individual cell lysates (50 μg per lane) were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (10% gel, SDS-PAGE) and transferred onto nitrocellulose membranes. After being blocked with 5% fat-free milk, the target proteins in the membranes were respectively probed with monoclonal anti-Bax (KGA714), anti-Bcl2 (KGA715), anti-caspase 3 (KGA717), anti-caspase 9 (KGA720), anti-cyt. *c* (KGA723), and anti-β-actin antibodies (KGA731, KeyGEN). The bound antibodies were detected by horseradish peroxidase (HRP)-conjugated second antibodies and visualized using an enhanced chemiluminescent reagent. The relative levels of each signaling event to control β-actin were determined by densitometric scanning.

In vivo antitumor activity: Female mice from the Institute of Cancer Research (ICR) with body weights of 12–16 g were transplanted with MGC-803 cells subcutaneously into the right oxter according to tumor transplant research protocols. After 7 d of tumor transplantation, mice in the MGC-803 group were weighed and divided into four groups at random. Oridonin and compound **29** were administered intraperitoneally 40 mg kg^{-1} to two different groups in a vehicle of 1% DMSO, 2% poloxamer, and 97% saline. The positive control group was treated with paclitaxel (10 mg kg^{-1}) through intravenous injection in a vehicle of 1% DMSO, 2% poloxamer, and 97% saline. The negative control group received 0.9% normal saline through intraperitoneal injection. All test compounds were given through injections after 7 d of tumor transplantation (or inoculation). Treatments were given at a frequency of one dose of intravenous or intraperitoneal injection per day for a total of 25 consecutive days. After the treatments, all of the mice were killed and weighed simultaneously, and the tumors were segregated and weighed. Ratio of inhibition of tumor (%) = (1 − average tumor weight of treated group/average tumor weight of control group) × 100%. Female Balb/c mice (12–16 g) were purchased from Shanghai SLAC Laboratory Animals Co. Ltd. (China). All procedures were performed following institutional approval in accordance with the NIH Guide for the Care and Use of Laboratory Animals.

Acknowledgements

This study was supported by grants from the National Natural Science Foundation (No. 30973610), the Specialized Research Fund for the Doctoral Program of Higher Education (No. 20100096110001), the Project for Research and Innovation of Graduates in Universities of Jiangsu Province (CXZZ11-0800), the Fundamental Research Funds for the Central Universities (JKY2011030) and the Key Fund of Ministry of Education of China (No. 108069).

Keywords: antitumor agents · apoptosis · cell cycle · enmein-type diterpenoids · oridonin

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Received: December 3, 2012
Revised: February 26, 2013
Published online on March 20, 2013