European Journal of Medicinal Chemistry 58 (2012) 128–135

Contents lists available at SciVerse ScienceDirect

European Journal of Medicinal Chemistry

journal homepage: http://www.elsevier.com/locate/ejmech

Original article

Synthesis and cytotoxicity of novel ursolic acid derivatives containing an acyl piperazine moiety

Ming-Chuan Liu, Sheng-Jie Yang, Lin-Hong Jin, De-Yu Hu, Wei Xue, Bao-An Song*, Song Yang*

State Key Laboratory Breeding Base of Green Pesticide and Agricultural Bioengineering, Key Laboratory of Green Pesticide and Agricultural Bioengineering, Ministry of Education, Guizhou University, Guiyang 550025, PR China

ARTICLE INFO

Article history:
Received 28 March 2012
Received in revised form
29 August 2012
Accepted 30 August 2012
Available online 6 October 2012

Keywords:
Ursolic acid derivatives
Antitumor
Cytotoxicity
Apoptosis

ABSTRACT

This study designed and synthesized a series of novel ursolic acid derivatives in an attempt to develop potent antitumor agents. Their structures were confirmed using MS, IR, ^{1}H NMR and ^{13}C NMR. The inhibitory activities of the title compounds against the MGC-803 (gastric cancer cell) and Bcap-37 (breast cancer cell) human cancer cell lines were evaluated using standard MTT assay *in vitro*. The pharmacological results showed that some of the compounds displayed moderate to high levels of antitumor activities against the tested cancer cell lines and that most exhibited more potent inhibitory activities compared with ursolic acid. The mechanism of compound **4b** was preliminarily investigated by acridine orange/ethidium bromide staining, Hoechst 33258 staining, TUNEL assay and flow cytometry, which revealed that the compound can induce cell apoptosis in MGC-803 cells.

1. Introduction

Pentacyclic triterpenes exhibit various biological activities [1,2], such as anti-HIV [3], anti-inflammatory [4], antitumor [5], antioxidant [6], antifeedant [7] and antibacterial [8] activities. Ursolic acid (**UA**; 3β-hydoxy-urs-12-en-28-oic acid **1**) is a well-known pentacyclic triterpene that serves as one of the major effective components of many traditional Chinese medicines. Muto et al. [9] reported that **UA** is one of the most promising tumor-preventive medications. As an effective natural anticancer drug, **UA** has also been reported to show significant cytotoxicity against some tumor cell lines [5,10–14]. However, the low bioavailability of **UA** *in vivo* restricts its clinical application [15]. Chemical modifications in **UA** have been widely investigated in recent years to improve its antitumor activities and bioavailability [16–19]. Research has shown that keeping a polar substituent at the C-3 position is essential for the pharmacological activities of pentacyclic triterpenes [20–22] and that a hydrogen donor group at either C-3 or C-28 [19], as well as an ester group at C-3 [23], could improve the cytotoxic activity of **UA**. Liu et al. [24] found a significant improvement in cell proliferation inhibition when acyl groups were introduced at the C-28 position. Piperazine-based drug discovery has attracted considerable attention in recent years. Studies have shown that the incorporation of a piperazine moiety could occasionally provide unexpected improvements in the bioactivity of compounds [25–27].

In order to search for ursolic derivatives with high antitumor bioactivities, the present study introduced an acyl piperazine moiety to the C-28 position of **UA**. A series of **UA** derivatives containing an acyl piperazine moiety were designed and synthesized. Their antiproliferative activities *in vitro* against the MGC-803 and Bcap-37 cancer cell lines as well as the NIH3T3 (mouse fibroblast cell) normal cell line were evaluated. Results showed that the target compounds can inhibit proliferation of the two tumor cell lines at moderate to high rates. Preliminary investigation of the mode of action of compound **4b** found that it can induce cell apoptosis in MGC-823 cells with a higher apoptosis ratio compared with the positive controls **UA** and Hydroxycamptothecin (HCPT) [28,29].

Abbreviation: ADM, adriamycin; AO/EB, acridine orange/ethidium bromide; ^{13}C NMR, ^{13}C nuclear magnetic resonance; DMF, *N*,*N*-dimethylformamide; DMSO, dimethyl sulfoxide; ESI-MS, electrospray ionization mass spectrometry; HCPT, 10-hydroxyl camptothecine; ^{1}H NMR, proton nuclear magnetic resonance; IR, infrared; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; TUNEL, terminal deoxynucleotidyl transferase biotin-dUTP nick end labeling; **UA**, ursolic acid.

* Corresponding authors. Ctr for R&D of Fine Chemicals, Guizhou University, Huaxi St., Guiyang 550025, PR China. Tel.: +86 851 829 2171; fax: +86 851 829 2170.
E-mail addresses: songbaoan22@yahoo.com (B.-A. Song), jhzx.msm@gmail.com (S. Yang).

0223-5234/$ – see front matter © 2012 Elsevier Masson SAS. All rights reserved.
http://dx.doi.org/10.1016/j.ejmech.2012.08.048

2. Results and discussion

2.1. Chemistry

UA was used as the leading compound, and structure modifications were performed at the C-28 position. The synthetic pathways are shown in Scheme 1.

UA (compound **1**) was treated with ethylene dibromide in DMF for 24 h to obtain compound **2**, which was introduced with piperazine in DMF in the presence of K_2CO_3 at 80 °C, and then reacted with aromatic, heterocyclic or aliphatic amines to obtain compounds **4a**–**4w**. The target compounds were purified by flash chromatography with petroleum ether/ethyl acetate or chloroform/methanol as the eluent. The structures of title compounds were confirmed by IR, ESI-MS, ^{1}H NMR and ^{13}C NMR.

2.2. Evaluation of antitumor activities

In vitro inhibitory activities against the MGC-803 and Bcap-37 cell lines were evaluated for all the synthesized compounds, with **UA** and adriamycin (ADM) used as positive controls [30–32]. All the tested compounds were dissolved in DMSO and subsequently diluted in culture medium before treatment of cultured cells. The cells treated with 0.1% DMSO served as control. The inhibitory rates of cell viability with 10 μM concentration of each compound for 72 h and the IC_{50} values of some compounds are given in Table 1.

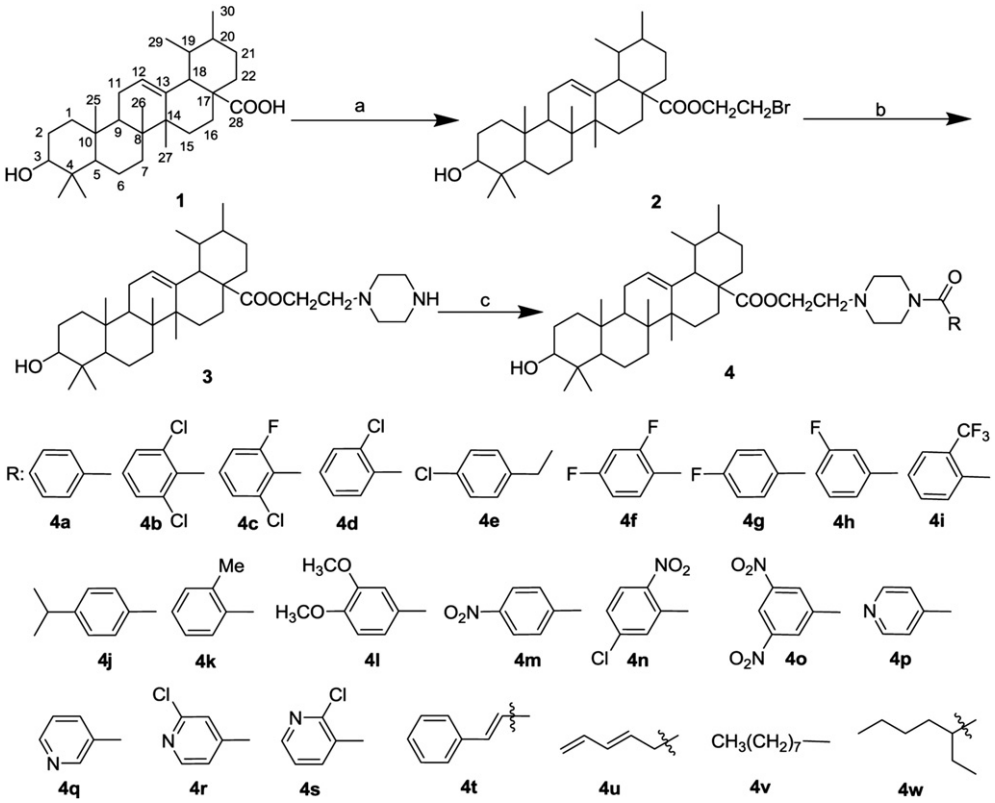
As shown in Table 1, most of the title compounds containing an acyl piperazine moiety at C-28 displayed higher inhibitory activity than **UA** against the MGC-803 and Bcap-37 cancer cell lines. The inhibitory ratios of **UA** against the MGC-803 and Bcap-37 cell lines at 10 μM were 28.9% and 11.9%, respectively, whereas those of compound **4b** were 95.6% and 76.0%, respectively. The corresponding related inhibitory ratios were 92.1% and 85.3% for compound **4c**, 93.0% and 69.1% for **4d** as well as 98.9% and 94.8% for **4k**. The IC_{50} values of **UA** against the MGC-803 and Bcap-37 cell lines were 24.32 and 28.69 μM, respectively (Table 1). The IC_{50} values against these two cell lines ranged from 2.5 to 14.01 μM for most of the title compounds, among which compounds **4b**, **4c**, **4d** and **4k** were found to have the corresponding values of 2.50 and 9.24 μM, 4.18 and 4.32 μM, 4.66 and 7.26 μM as well as 3.59 and 5.34 μM, respectively. These data indicate that the incorporation of an acyl piperazine moiety at C-28 while retaining the polar group at C-3 significantly improved the antitumor bioactivities of the compounds.

Table 1 also shows that acyl substitutions affect the antitumor bioactivities. Among the acids tested, substituted benzoic acids (compounds **4b**–**4l**) and alkyl carboxylic acids (compounds **4u**–**4w**) exhibited higher and moderate activities, respectively, whereas substituted pyridine carboxylic acids (compounds **4p**–**4s**) demonstrated weak antitumor activities. Meanwhile, halogen or methyl at the ortho position of the acyl phenyl ring favored the antitumor bioactivities. On the other hand, the introduction of a nitro group to the phenyl ring significantly reduced the activities of compounds **4m**–**4o** against the tumor cell lines.

The antiproliferative bioactivities of the title compounds against the NIH3T3 cell line were also evaluated. Most of the title compounds showed stronger antiproliferative activities against the tumor cell lines than the NIH3T3 cell line (Table 1).

2.3. Preliminary investigation of the apoptosis-inducing effect of title compound *4b*

The ability of **UA** and its derivatives to potentially induce apoptosis in certain cancer cell lines has been previously reported



Scheme 1. Synthetic route to **UA** derivatives containing acyl piperazine moieties at C-28. Reagents and conditions: (a) $Br(CH_2)_2Br$, K_2CO_3, DMF, r.t.; (b) piperidine, K_2CO_3, DMF, 80 °C; (c) EDCI, RCOOH, DCM, r.t.

Table 1
Effect of **UA** derivatives against cell viability of different cell lines.

Compound	Inhibition rates against different cells (%)[a]			IC_{50} (μM)[b]	
	MGC-803	Bcap-37	NIH3T3	MGC-803	Bcap-37
1	28.9 ± 4.9	11.9 ± 2.7	14.0 ± 8.3	24.32 ± 0.57	28.69 ± 0.35
2	39.4 ± 4.7	23.1 ± 5.2	11.0 ± 5.5	12.43 ± 0.98	NT[c]
3	86.6 ± 3.6	84.4 ± 3.0	60.1 ± 5.7	7.25 ± 0.41	7.50 ± 0.31
4a	21.4 ± 3.4	23.9 ± 2.2	19.1 ± 5.8	NT	NT
4b	95.6 ± 1.0	76.0 ± 1.5	32.1 ± 4.8	2.50 ± 0.25	9.24 ± 0.53
4c	92.1 ± 0.2	85.3 ± 3.1	24.4 ± 6.8	4.18 ± 0.33	4.32 ± 0.42
4d	93.0 ± 1.5	69.1 ± 1.6	29.8 ± 6.9	4.66 ± 0.12	7.26 ± 0.46
4e	68.6 ± 3.7	52.0 ± 4.9	2.9 ± 13.1	8.59 ± 0.53	10.00 ± 0.99
4f	32.1 ± 5.2	60.5 ± 7.3	18.1 ± 6.8	12.45 ± 0.62	8.31 ± 0.44
4g	71.8 ± 5.3	83.7 ± 0.6	71.3 ± 5.9	7.29 ± 0.68	5.12 ± 0.40
4h	45.3 ± 6.2	32.1 ± 6.3	33.5 ± 5.1	>20[d]	NT
4i	71.2 ± 6.8	38.6 ± 2.3	16.2 ± 8.4	8.72 ± 0.49	10.87 ± 0.42
4j	72.3 ± 4.6	45.5 ± 1.8	5.5 ± 12.4	5.02 ± 0.52	11.76 ± 1.00
4k	98.9 ± 0.6	94.8 ± 1.0	60.3 ± 6.5	3.59 ± 0.25	5.34 ± 0.41
4l	24.5 ± 5.7	51.8 ± 2.2	73.1 ± 2.2	NT	11.13 ± 0.55
4m	24.3 ± 8.2	19.1 ± 5.6	65.4 ± 3.8	NT	NT
4n	57.3 ± 2.0	33.2 ± 5.1	77.7 ± 6.4	9.41 ± 0.78	NT
4o	24.7 ± 7.4	10.7 ± 6.6	37.2 ± 1.2	NT	NT
4p	32.1 ± 7.0	8.3 ± 7.0	55.4 ± 6.1	13.26 ± 0.70	NT
4q	22.0 ± 5.7	24.5 ± 9.6	52.3 ± 1.8	NT	NT
4r	24.1 ± 1.1	19.0 ± 3.8	44.3 ± 4.7	NT	NT
4s	34.3 ± 4.1	29.0 ± 6.3	4.3 ± 14.1	14.01 ± 1.10	NT
4t	51.1 ± 1.9	54.4 ± 3.2	39.0 ± 8.3	12.59 ± 0.20	9.22 ± 0.40
4u	23.4 ± 2.0	14.7 ± 5.4	35.0 ± 7.1	NT	NT
4v	44.2 ± 1.1	12.5 ± 3.8	12.0 ± 9.2	>20	NT
4w	80.8 ± 4.7	68.6 ± 3.4	54.5 ± 9.3	6.89 ± 0.85	4.16 ± 0.29
HCPT[e]	29.1 ± 2.6	28.1 ± 1.0	9.3 ± 4.2	>20	>20
ADM[f]	92.1 ± 1.3	92.1 ± 1.1	99.2 ± 0.1	0.7 ± 0.2	1.28 ± 0.28

[a] Inhibitory percentages of cells treated with 10 μM concentration of each compound for 72 h.
[b] Agent concentration (μM) that inhibited cell growth by 50% at 72 h after treatment.
[c] Not tested.
[d] Inhibition (50%) was higher than 20 μM.
[e] Hydroxycamptothecin, positive control.
[f] Adriamycin, positive control.

[16–18,33]. In the present study, compound **4b** was selected and its mechanism of growth inhibition of MGC-803 cells was evaluated.

2.3.1. Fluorescence staining

Changes in the morphological character of MGC-803 cells were investigated using acridine orange (AO)/ethidium bromide (EB) and Hoechst 33258 staining under fluorescence microscopy to determine whether the growth inhibitory activity of the selected compound was related to the induction of apoptosis.

2.3.1.1. AO/EB staining. AO is a vital dye that can stain nuclear DNA across an intact cell membrane, whereas EB can only stains cells that had lost their membrane integrity. Thus, after simultaneous treatment with AO and EB, live cells will be uniformly stained as green (in the web version) and early apoptotic cells will be densely stained as green yellow or show green yellow fragments (in the web version), whereas late apoptotic cells will be densely stained as orange or display orange fragments and necrotic cells will be stained as orange with no condensed chromatin. The cytotoxicity of compound **4b** at the concentration of 5 μM against MGC-803 cells from 12 to 48 h was detected by AO/EB staining, with HCPT and **UA** used as positive controls at 10 μM against MGC-803 cells for 48 h. The results are given in Fig. 1.

Fig. 1 shows that the cells treated with **4b** from 12 to 48 h and **UA** for 48 h had changed in MGC-803 cells. The nuclei stained as yellow green or orange, and the morphology showed pycnosis, membrane blebbing and cell budding. These phenomena are associated with cell apoptosis.

Green live MGC-803 cells with normal morphology were seen in the negative control group. Green yellow or orange dots were detected in the HCPT after 48 h. However, cells under **UA** treatment changed only minimally and pycnosis could only be seen after 48 h. Cells treated with compound **4b** displayed yellow dots as well as cell budding in MGC-803 cells at 24 h and stained green 48 h after treatment. Moreover, the emerging orange dots at 48 h also showed typical late apoptotic cells.

In conclusion, the cells presented with an apoptotic morphology. The nearly complete absence of red cells in compounds **4b** and **UA** indicated that it was associated with very low cytotoxicity. These findings show that compounds **4b** and **UA** could induce apoptosis with low cytotoxicity.

2.3.1.2. Hoechst 33258 staining. Hoechst 33258 is a membrane-permeable blue fluorescent dye that stains the cell nucleus. Live cells with uniformly light blue nuclei were observed under fluorescence microscope after treatment with Hoechst 33258, whereas apoptotic cells had bright blue nuclei because of karyopyknosis and chromatin condensation; the nuclei of dead cells could not be stained. MGC-803 cells treated with compound **4b** at 5 μM from 12 to 48 h were stained with Hoechst 33258. HCPT and **UA** were used as positive controls at 10 μM for 48 h. The results are shown in Fig. 2.

Fig. 2 shows that cells treated with the negative control DMSO were normally blue (in the web version). On the other hand, the cell nuclei of HCPT (positive control)-treated cells appeared to be compact and condensed. **UA** had no obvious morphological changes, but most cell nuclei appeared to be highly condensed (brightly stained). For **4b** treatment, the cells exhibited strong blue fluorescence and revealed typical apoptotic morphology after 12, 24 and 48 h. These findings demonstrate that compounds **4b** and **UA** induced apoptosis against MGC-803 cell lines, consistent with the results for AO/EB double staining.

2.3.2. TUNEL assay

TUNEL (terminal deoxynucleotidyl transferase biotin-dUTP nick end labeling) is a popular method for identifying apoptotic cells in situ by detecting DNA fragmentation. Due to degradation of DNA caused by the activation of Ca/Mg-dependent endonucleases in apoptotic cells, DNA cleavage occurred and led to strand breakage within the DNA. These strand breaks of cleaved DNA could be identified by terminal deoxynucleotidyl transferase that catalyzed the addition of biotin-dUTP. The biotin-labeled cleavage sites were then detected by reaction with streptavidin–HRP and visualized by diaminobenzidine, as indicated by a brown color (in the web version). The results are illustrated in Fig. 3.

Fig. 3 shows that cells in the negative group (DMSO treatment) did not appear as brown precipitates (Fig. 3, 1). The cells treated with compound **4b** at different concentrations (Fig. 3, 4–6), **UA** (Fig. 3, 3) and HCPT (positive control; Fig. 3, 2) appeared as brown precipitate. Therefore, we further concluded that compound **4b** induced apoptosis against MGC-803. The results were identical with the previous experiment.

2.3.3. Flow cytometry

The apoptosis ratios induced by compound **4b** in tumor cells were quantitatively assessed by flow cytometry. In the early stages of apoptosis, phosphatidylserine was translocated from within the cell membrane to its outer part. Annexin V, a calcium-dependent phospholipid-binding protein associated with a high affinity for phosphatidylserine, was used to detect early apoptotic cells. Propidine iodide (PI) is a red fluorescent dye that stains cells that had lost their membrane integrity. Cells stained with Annexin V-FITC and PI were classified as necrotic cells (B1; $Annexin^{-}/PI^{+}$), late

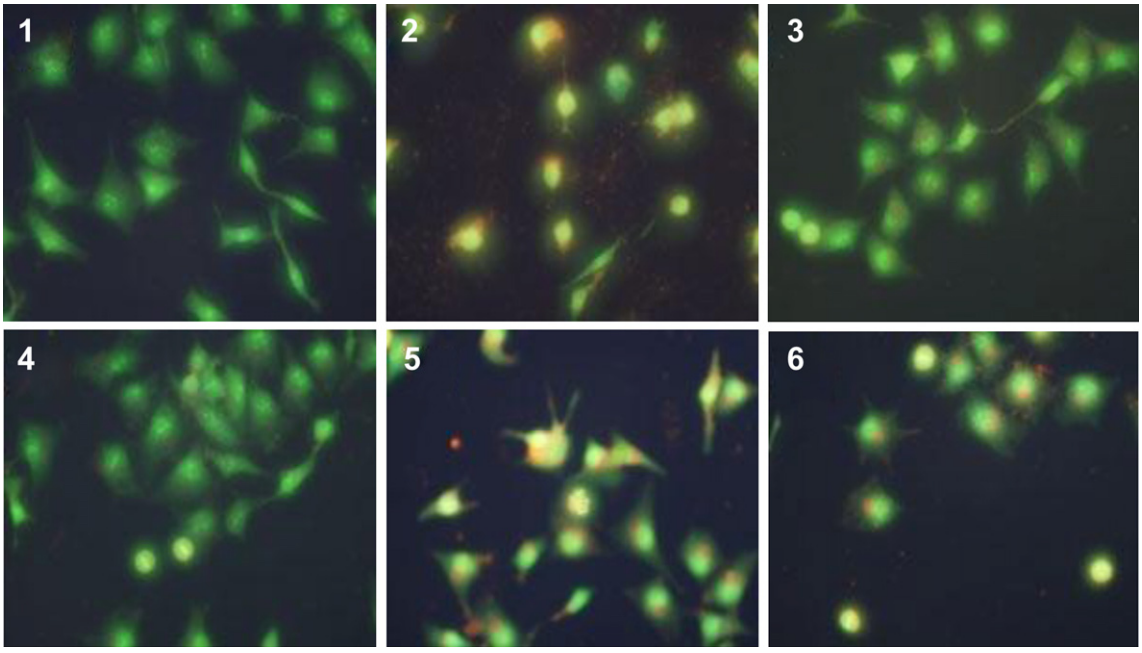


Fig. 1. AO/EB staining of compound **4b** in MGC-803 cells. (2,3) Respectively HCPT and **UA** (10 μM each) as positive for 48 h (4–6) treatment with compound **4b** (5 μM) for respectively 12 h, 24 h and 48 h.

apoptotic cells (B2; Annexin$^+$/PI$^+$), intact cells (B3; Annexin$^-$/PI$^-$) or early apoptotic cells (B4; Annexin$^+$/PI$^-$). The results are given in Fig. 4.

Fig. 5 shows that compound **4b** (10 μM) could induce apoptosis in MGC-803 cells. Apoptosis ratios (including the early and late apoptosis ratios) for compound **4b** were obtained after 36 h of treatment at a concentration of 10 μM, with the highest apoptosis ratio being 33.35%. Furthermore, as shown in Fig. 5, the apoptosis of MGC-803 cells treated with compound **4b** increased gradually in a time-dependent manner; meanwhile, the apoptosis ratios of compound **4b** measured at different time points (23.57%, 28.01% and 33.35% at 12, 24 and 36 h, respectively) were higher than those of HCPT (17.37%, 18.66% and 24.95%, respectively).

3. Conclusions

UA is an important natural product with antitumor bioactivity. In this study, a series of **UA** derivatives containing an acyl piperazine moiety were designed and synthesized, and their cell growth inhibition activities against the MGC-803 and Bcap-37 cell lines were evaluated using MTT assay. The corresponding IC_{50} values of compounds **4b**, **4c**, **4d** and **4k** against MGC-803 and Bcap-37 were 2.50 ± 0.25 and 9.24 ± 0.53 μM, 4.18 ± 0.33 and 4.32 ± 0.42 μM, 4.66 ± 0.12 and 7.26 ± 0.46 μM as well as 3.59 ± 0.25 and 5.34 ± 0.41 μM, respectively. Incorporation of an acyl piperazine moiety at C-28 while retaining the polar group at C-3 significantly improved the antitumor bioactivities of the compounds. Moreover,

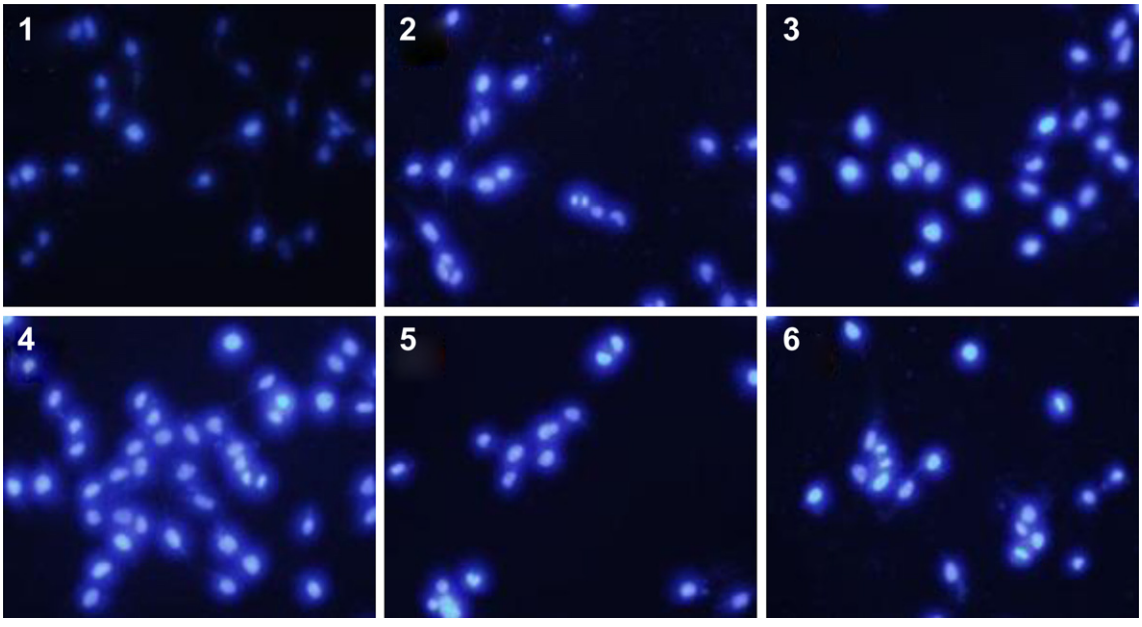


Fig. 2. Hoechst 33258 staining of compound **4b** in MGC-803 cells. (2,3) Respectively HCPT and **UA** (10 μM each) as positive for 48 h (4–6) treatment with compound **4b** (5 μM) for respectively 12 h, 24 h and 48 h.

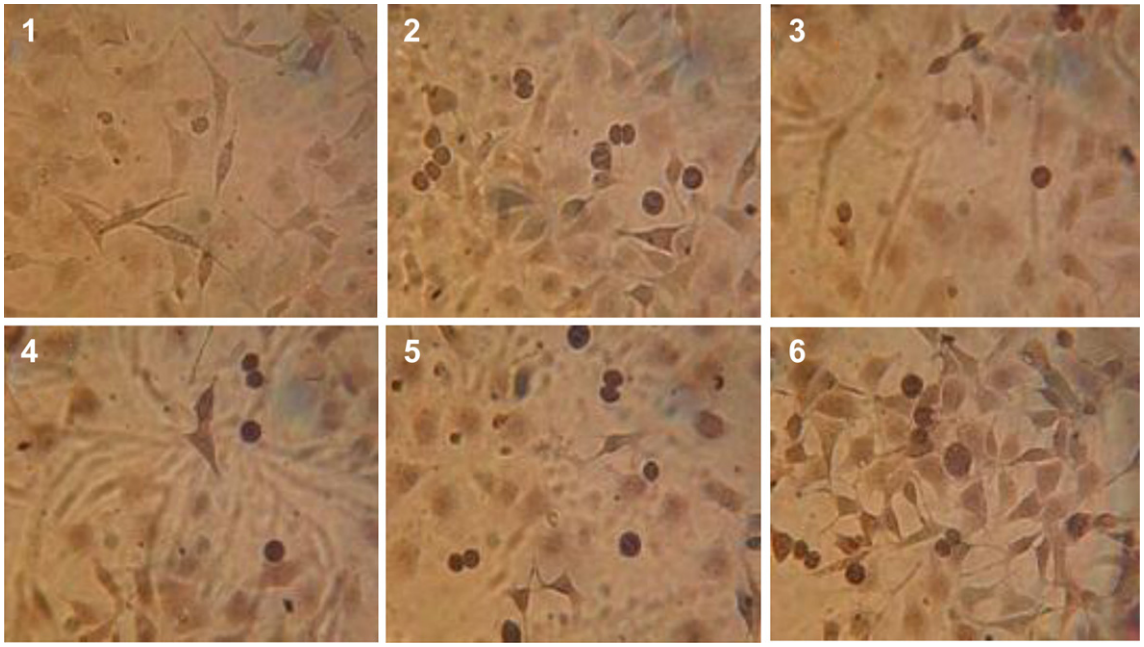


Fig. 3. TUNEL assay of compound **4b** in MGC-803 cells for 24 h (2,3). Respectively HCPT and **UA** (10 μM each) as positive for 24 h (4–6) treatment with compound **4b** at 2.5 μM (4), 5 μM (5) and 10 μM (6) for 24 h.

most of the title compounds showed lower antiproliferative effects against NIH3T3 cells than the tumor cells. The apoptosis-inducing activity of compound **4b** in MGC-803 cells was investigated by AO/EB staining, Hoechst 33258 staining, TUNEL assay and flow cytometry. This compound clearly demonstrated cell apoptosis-inducing effects, with its inducing ratio being higher than the ratios observed for the positive control HCPT and **UA**. Further studies of the specific mechanisms of these compounds in human malignant tumors are currently underway.

4. Experimental

4.1. General

UA with more than 90% purity was purchased from Hainan Super Biotech Co., Ltd. Reagents of analytical grade were obtained from Yuda Chemistry Co., Ltd., and used without further purification unless otherwise noted. Silica gel (200–300 mesh) used in column chromatography was provided by Tsingtao Marine Chemistry Co., Ltd. IR spectra were recorded on a Bruker VECTOR22 spectrophotometer in KBr disks. ESI-MS spectra were determined with Agilent 1100 IC/MDS Trap XCT and are reported as *m/z*. ^{1}H NMR and ^{13}C NMR were performed on a JEOL-ECX500 spectrometer at 22 °C, with TMS as the internal standard.

4.2. Synthesis

4.2.1. General procedure for compounds *2*, *3* and *4a–4w*

UA (1 mmol) and K_2CO_3 (2 mmol) were added to DMF (15 mL) and stirred at room temperature for 30 min, after which bromoalkane or dibromoalkane (4 mmol) was added and KI (0.5 mmol) was dripped into the mixture. After being stirred for another 12 h, the reaction mixture was poured onto 100 mL of distilled water and partitioned with ethyl acetate (3 × 20 mL). The organic layer was washed with saturated sodium chloride, dried over Na_2SO_4 and purified *via* silica gel column chromatography with petroleum ether/ethyl acetate to obtain compound **2**.

Compound **2** (1 mmol) and K_2CO_3 (2 mmol) were added to DMF (15 mL) and stirred at room temperature for 10 min, and then piperazine (5 mmol) was dripped into the mixture, which was stirred at 80 °C for 5 h. After cooling to room temperature, the reaction mixture was poured onto 100 mL of distilled water and partitioned with ethyl acetate (3 × 20 mL). The organic layer was washed with saturated sodium chloride, dried over Na_2SO_4 and purified *via* silica gel column chromatography with chloroform/methanol (10:1, v/v) to obtain compound **3**.

Compound **3** (1 mmol) and amine compounds (1.2 mmol) and HATU (1.2 mmol) or EDCI (1.2 mmol) and HOBt (1.2 mmol) were added to DCM (10 mL) containing Et_3N (0.5 mmol); the mixture was then stirred at room temperature for 6–12 h. After the reaction was completed, the mixture was poured onto 100 mL of distilled water and partitioned with ethyl acetate (3 × 50 mL). The target compounds were purified on a flash column with chloroform/methanol (20:1, v/v) to yield compounds **4a–4w**. The structures were confirmed by IR, ESI-MS, ^{1}H NMR and ^{13}C NMR (see Supporting information).

*4.2.1.1. (2-(Piperazin-1-yl)ethyl) 3-hydroxy-urs-12-en-28-oate (**3**).* According to the general procedure, compound **2** was treated with piperazine and then purified on silica gel column using chloroform/methanol (10:1, v/v) to obtain compound **3**, R_f [petroleum ether/ethyl acetate (3:1, v/v)] = 0.31.

Yield: 55.5%; colorless oil; ESI-MS: 569$[M + H]^+$; IR (KBr, cm^{-1}) *v*: 3269, 3163, 2941, 2868, 1730, 1463, 1377, 1220, 1029; ^{1}H NMR ($CDCl_3$, 500 MHz): 5.23 (1H, s, H-12), 4.14 (2H, t, J = 5 Hz, CH_2), 3.20 (1H, dd, J = 10 Hz, 10 Hz, H-3), 2.89 (4H, t, J = 5 Hz, H in piperazine), 2.65 (2H, t, J = 5 Hz, CH_2), 2.48 (4H, brs, H in piperazine), 2.22 (1H, d, J = 10 Hz, H-18), 1.07 (CH_3, s, H-27), 0.98 (CH_3, s, H-25), 0.94 (CH_3, s, H-26), 0.93 (CH_3, s, H-24), 0.91 (CH_3, s, H-23), 0.86 (CH_3, d, J = 10 Hz, H-30), 0.77 (CH_3, d, J = 10 Hz, H-29); ^{13}C NMR ($CDCl_3$, 125 MHz): 177.4 (C-28), 138.1 (C-13), 125.6 (C-12), 78.9 (C-3), 61.8 (CH_2), 57.2 (CH_2), 55.2 (C-5), 54.7 (CH_2), 52.9 (C-18), 48.0 (C-17), 47.6 (C-9), 46.1 (CH_2), 42.1 (C-14), 39.6 (C-8), 39.1 (C-19), 38.9 (C-1), 38.8 (C-4), 38.6 (C-20), 37.0 (C-10), 36.7 (C-22), 33.1 (C-7), 30.7 (C-21), 28.2 (C-23), 28.0 (C-15), 27.3 (C-2), 24.2 (C-16), 23.6 (C-11), 23.3 (C-27), 21.2 (C-30), 18.3 (C-6), 17.2 (C-26), 17.0 (C-29), 15.7 (C-25), 15.5 (C-24).

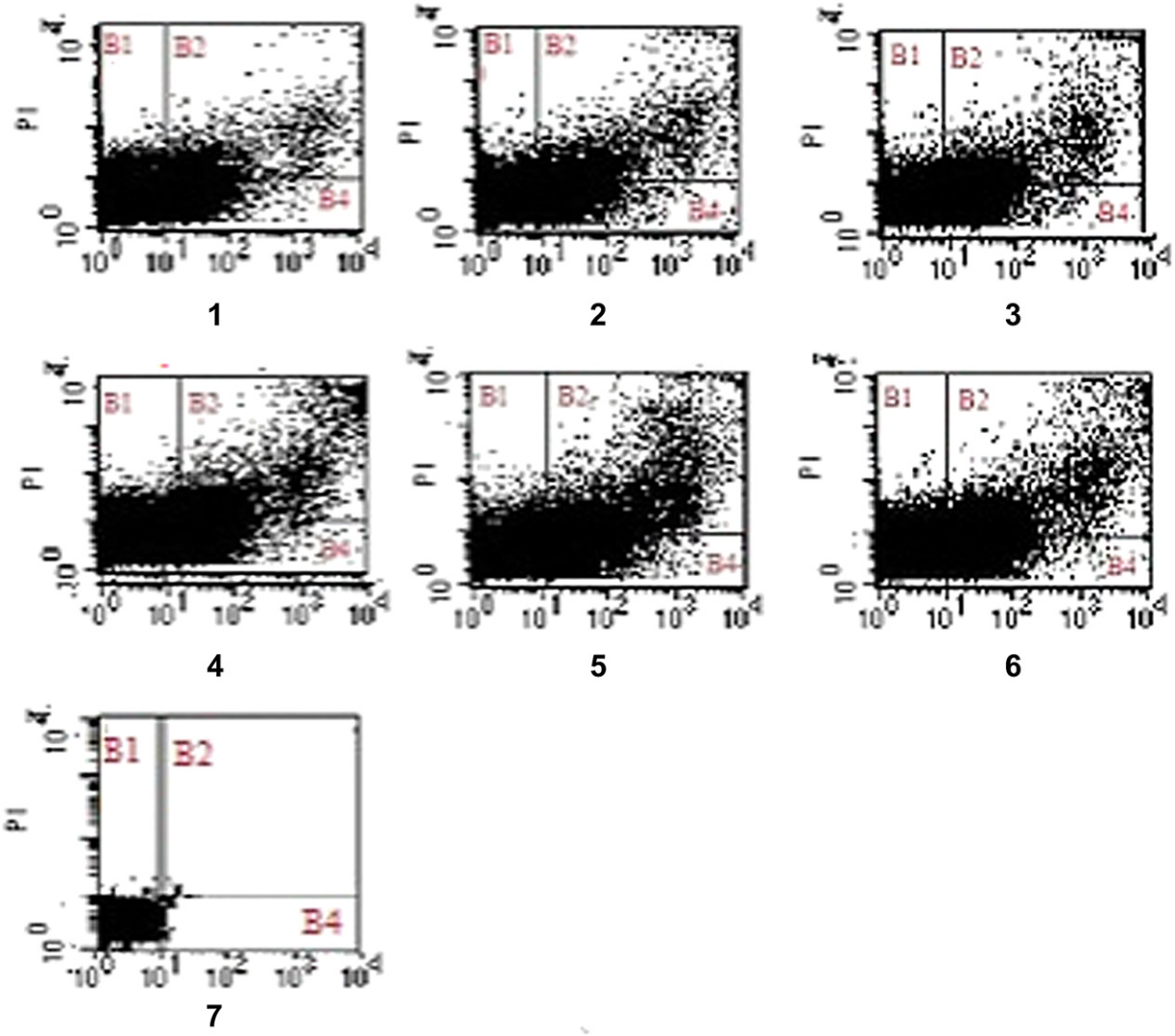


Fig. 4. Apoptosis ratio detection of compound **4b** by Annexin V/PI assay. (1–3) Cells were treated with HCPT at 10 μM for 12 h, 24 h, and 36 h, respectively. (4–6) Cells were treated with compound **4b** at 10 μM for 12 h, 24 h, and 36 h, respectively. (7) Cells were treated with 0.1% DMSO for 36 h.

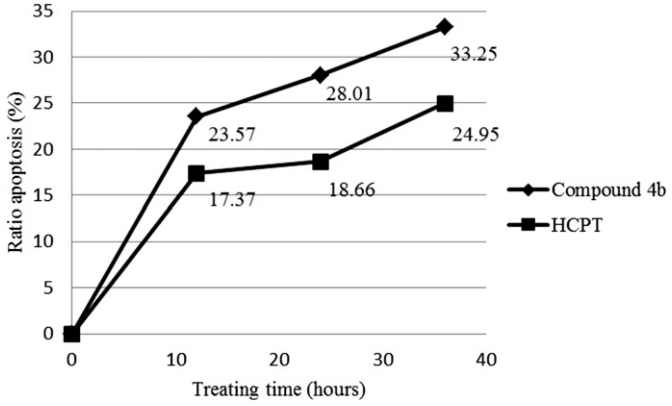


Fig. 5. Apoptosis ratios of MGC-803 cells treated with compound **4b** (10 μM) and HCPT (10 μM) as assessed by flow cytometry at 12, 24 and 36 h. The apoptosis ratio includes the early (B4) and late (B2) apoptosis ratios.

*4.2.1.2. (2-(4-(2,6-Dichlorobenzoyl)piperazin-1-yl)ethyl)3-hydroxy-urs-12-en-28-oate (**4b**).* According to the general procedure, compound **3** was treated with aromatic amines and then purified on silica gel column using chloroform/methanol (10:1, v/v) to obtain compound **4b**, R_f [chloroform/methanol (5:1, v/v)] = 0.22.

Yield: 51.5%; yellow colorless oil; ESI-MS: 741[M + H]$^+$; IR (KBr, cm^{-1}) *v*: 3444, 2924, 2868, 1718, 1637, 1429, 1139, 794, 754; ^{1}H NMR ($CDCl_3$, 500 MHz): 7.34–7.28 (3H, m, PhH), 5.22 (1H, s, H-12), 4.12 (2H, t, J = 7.5 Hz, CH_2), 3.84 (2H, m, H in piperazine), 3.23 (2H, t, J = 7.5 Hz, H in piperazine), 3.16 (1H, d, J = 5 Hz, H-3), 2.65 (2H, t, J = 5 Hz, CH_2), 2.61 (2H, m, H in piperazine), 2.49 (2H, brm, H in piperazine), 2.21 (1H, d, J = 10 Hz, H-18), 1.07 (CH_3, s, H-27), 0.98 (CH_3, s, H-25), 0.94 (CH_3, s, H-26), 0.93 (CH_3, s, H-24), 0.90 (CH_3, s, H-23), 0.86 (CH_3, d, J = 5 Hz, H-30), 0.77 (CH_3, d, J = 15 Hz, H-29); ^{13}C NMR ($CDCl_3$, 125 MHz): 177.4 (C-28), 161.9 (C), 159.7 (C), 157.7 (C), 138.1 (C-13), 131.9 (CH_2), 130.8 (CH_2), 125.6 (C-12), 124.3 (C), 114.5 (CH_2), 79.0 (C-3), 61.7 (CH_2), 56.5 (CH_2), 55.2 (C-5), 53.4 (CH_2), 52.9 (C-18), 52.8 (CH_2), 48.1 (C-17), 47.5 (C-9), 46.6 (CH_2), 42.1 (C-14), 41.8 (CH_2), 39.6 (C-8), 39.1 (C-19), 38.9 (C-1), 38.8 (C-4), 38.6 (C-20), 37.0 (C-10), 36.8 (C-22), 33.1 (C-7), 30.7 (C-21), 28.2 (C-23), 28.0 (C-15), 27.2 (C-2), 24.3 (C-16), 23.6 (C-11), 23.3 (C-27), 21.2 (C-30), 18.3 (C-6), 17.2 (C-26), 17.0 (C-29), 15.7 (C-25), 15.5 (C-24).

4.3. Cell lines and culture

The MGC-803, Bcap-37 and NIH3T3 cell lines used in this study were all obtained from the Institute of Biochemistry and Cell Biology, China Academy of Sciences. MGC-803 is stomach cancer, Bcap-37 is breast cancer and NIH3T3 is normal mouse fibroblast. The MGC-803 and Bcap-37 cell lines were maintained in RPMI 1640 medium, whereas the NIH3T3 cell line was maintained in DMEM medium; all were supplemented with 10% heat-inactivated fetal bovine serum in a humidified atmosphere of 5% CO_2 at 37 °C.

4.4. MTT assay against cancer cell viability

All tested compounds were dissolved in DMSO and subsequently diluted in culture medium before treatment of cultured cells. Tested cells were plated in 96-well plates at a density of 2×10^3 cells/well per 100 μL of the proper culture medium and treated with the compounds at 1–20 μM for 72 h. In parallel, the cells treated with 0.1% DMSO served as negative control and those treated with ADM as positive control. Finally, 100 μL of MTT was added, and the cells were incubated for 4 h. The MTT formazan formed by metabolically viable cells was dissolved in 100 μL of SDS for 12 h. Absorbance, which is directly proportional to the number of living cells in culture, was then measured at 595 nm with a microplate reader (Model 680; BIO-RAD). The percentage of cell viability inhibition was calculated using the following formula:

$$\%\ \text{viability inhibition} = \frac{(\text{Control abs} - \text{Blank abs}) - (\text{Test abs} - \text{Blank abs})}{(\text{Control abs} - \text{Blank abs})} \times 100$$

4.5. AO/EB staining

Cells were seeded at a concentration of 5×10^4 cell/mL in a volume of 0.6 mL on a sterile cover slip in six-well tissue culture plates. Following incubation, the medium was removed and replaced with fresh medium plus 10% fetal bovine serum and supplemented with compounds (10 μM). After the treatment period, the cover slip with monolayer cells was inverted on a glass slide with 20 μL of AO/EB stain (100 μg/mL). Fluorescence was read on an IX71SIF-3 fluorescence microscope (OLYMPUS Co., Japan).

4.6. Hoechst 333258 staining

Cells grown on a sterile cover slip in six-well tissue culture plates were treated with compounds for a certain range of time. The culture medium containing compounds was removed, and the cells were fixed in 4% paraformaldehyde for 10 min. After being washed twice with PBS, the cells were stained with 0.5 mL of Hoechst 33258 (Beyotime) for 5 min and then again washed twice with PBS. The stained nuclei were observed under an IX71SIF-3 fluorescence microscope using 350 nm excitation and 460 nm emission.

4.7. TUNEL assay

TUNEL assays were performed with a colorimetric TUNEL apoptosis assay kit (Beyotime) according to the manufacturer's instructions. Cells grown in six-well culture clusters were treated as in the mitochondrial depolarization assay. In short, MGC-803 or Bcap-37 cells grown in six-well tissue culture plates were washed with PBS and fixed in 4% paraformaldehyde for 40 min. After washing once with PBS, cells were permeabilized with Immunol staining wash buffer (Beyotime) for 2 min on ice. Cells were washed once with PBS again and incubated in 0.3% H_2O_2 in methanol at room temperature for 20 min to inactivate the endogenous peroxidases, after which the cells were washed thrice with PBS. Thereafter, the cells were incubated with 2 μL of terminal deoxynucleotidyl transferase enzymes and 48 μL of biotin-dUTP per specimen for 60 min at 37 °C. After termination for 10 min, cells were incubated with streptavidin–HRP (50 μL per specimen) conjugate diluted at 1:50 in a diluent of streptavidin–HRP for 30 min. After washing thrice with PBS, cells were incubated with diaminobenzidine solution (200 μL per specimen) for 10 min. This was again followed by washing with PBS two times, and the result was imaged under an XDS-1B inverted biological microscope (Chongqing Photoelectric Device Co.).

4.8. Flow cytometry

Prepared MGC-803 cells (1×10^6 cells/mL) were washed twice with cold PBS and then resuspended gently in 500 μL of binding buffer. Thereafter, cells were stained in 5 μL of Annexin V-FITC and shaken well. Finally, the cells were mixed with 5 μL of PI, incubated for 20 min in the dark and subsequently analyzed using FACSCalibur (Becton Dickinson).

4.9. Statistical analysis

All statistical analysis was performed with SPSS Version 10. Data was analyzed by one-way ANOVA. Mean separations were performed using the least significant difference method. Each experiment was replicated thrice, and all experiments yielded similar results. Measurements from all the replicates were combined, and treatment effects were analyzed.

Authors contributions

Ming-Chuan Liu synthesized the compounds and carried out most of the bioassay experiments. Sheng-Jie Yang did part of the bioassay experiments. Lin-Hong Jin took part in the compound structural elucidation and bioassay experiments. De-Yu Hu carried out some structure elucidation experiments. Wei Xue assisted in structural elucidation experiments. Prof. Bao-An Song and Song Yang are the co-corresponding authors for this work.

Conflicts of interest

None.

Acknowledgments

This work was supported by the National Key Program for Basic Research (Grant No. 2010CB 126105), the National Basic Research Preliminary Program of China (Grant No. 2010CB134504) and the

National Natural Science Foundation of China (Grant Nos. 21132003 and 21172048).

Appendix A. Supporting information

Supporting information related to this article can be found at http://dx.doi.org/10.1016/j.ejmech.2012.08.048.

References

[1] A. Vachalkova, Z. Ovesna, K. Horvathova, D. Tothova, Minireview Neoplasma 55 (2004) 327–333.
[2] P. Yogeeswari, D. Sriram, Curr. Med. Chem. 12 (2005) 657–666.
[3] C.M. Ma, N. Nakamura, M. Hattori, H. Kakuda, J.C. Qiao, H.L. Yu, J. Nat. Prod. 63 (2000) 238–242.
[4] S.J. Tsai, M.C. Yin, J. Food Sci. 73 (2008) 174–178.
[5] J. Li, W.J. Guo, Q.Y. Yang, World J. Gastroenterol. 8 (2002) 493–495.
[6] A.A. Ramos, C.P. Wilson, A.R. Collins, Mutat. Res. 692 (2010) 6–11.
[7] U.V. Mallavadhani, M.S. Anita, J. Agric. Food Chem. 51 (2003) 1952–1955.
[8] M. Mitsuo, O. Yoshiharu, I. Koji, J. Agric. Food Chem. 53 (2005) 2312–2315.
[9] Y. Muto, M. Ninomia, H.J. Fujiki, Jpn. J. Clin. Oncol. 20 (1990) 219–224.
[10] D.K. Kim, J.H. Baek, C.M. Kang, M.A. Yoo, J.W. Sung, D.K. Kim, H.Y. Chung, N.D. Kim, Y.H. Choi, S.H. Lee, K.W. Kim, Int. J. Cancer 87 (2000) 629–636.
[11] P.O. Harmand, R. Duval, C. Delage, A. Simon, Int. J. Cancer 114 (2005) 1–11.
[12] D. Anderson, J.J. Liu, A. Nilson, R.D. Duan, Anticancer Res. 23 (2003) 3317–3322.
[13] Y.H. Choi, J.H. Baek, M.A. Yoo, H.Y. Chung, N.D. Kim, K.W. Kim, Int. J. Oncol. 17 (2000) 565–571.
[14] M. Kanjoormana, G. Kuttan, Integr. Cancer Ther. 9 (2010) 224–235.
[15] B. Saraswat, P.K. Visen, D.P. Agarwal, Phytother. Res. 14 (2000) 163–166.
[16] J.W. Shao, Y.C. Dai, J.P. Xue, J.C. Wang, F.P. Lin, Y.H. Gao, Eur. J. Med. Chem. 46 (2011) 2652–2661.
[17] K.K. Bai, F.L. Chen, Y. Zhou, Y.Q. Zheng, Y.N. Li, Y.H. Guo, Bioorg. Med. Chem. 19 (2011) 4043–4050.
[18] H.Y. Tu, A.M. Huang, B.L. Wei, K.H. Gan, T.C. Hour, S.C. Yang, Y.S. Pu, C.N. Lin, Bioorg. Med. Chem. 17 (2009) 7265–7274.
[19] C.M. Ma, S.Q. Cai, J.R. Cui, R.Q. Wang, P.F. Tu, M. Hattori, M. Daneshtalab, Eur. J. Med. Chem. 40 (2005) 582–589.
[20] Y.Q. Meng, Y.L. Song, Z.K. Yan, Y. Xia, Molecules 15 (2010) 4033–4040.
[21] J. Heather, T. Honda, Bioorg. Med. Chem. Lett. 7 (1997) 1769–1772.
[22] Y.Q. Meng, D. Liu, L.L. Cai, Bioorg. Med. Chem. 17 (2009) 848–854.
[23] Y. Kashiwada, J.I. Chiyo, Y. Keshiro, T. Nagao, H. Okabe, L.M. Cosentino, K. Fowke, S.L.M. Natschke, K.H. Lee, Chem. Pharm. Bull. 48 (2000) 1387–1390.
[24] D. Liu, Y.Q. Meng, J. Zhao, L.G. Chen, Chem. Res. Chin. Univ. 24 (2008) 42–46.
[25] L.L. Rokosz, C.Y. Huang, J.C. Reader, T.M. Stauffer, D. Chelsky, N.H. Sigal, A.K. Ganguly, J.J. Baldwin, Bioorg. Med. Chem. Lett. 15 (2005) 5537–5543.
[26] J.J. Chen, M. Lu, Y.K. Jing, J.H. Dong, Bioorg. Med. Chem. 14 (2006) 6539–6547.
[27] S. Zeng, W. Liu, F.F. Nie, Q. Zhao, J.J. Rong, J. Wang, L. Tao, Q. Qi, N. Lu, Z.Y. Li, Q.L. Guo, Biochem. Biophys. Res. Commun. 385 (2011) 551–556.
[28] Y.H. Hsing, L.F. Liu, Cancer Res. 48 (1998) 1722–1726.
[29] A. Lansiaux, M. Facompre, N. Wattez, M.P. Hildebrand, C. Bal, Mol. Pharmacol. 60 (2001) 450–461.
[30] S. Cai, S.W. Sun, H.N. Zhou, X.G. Kong, T.J. Zhu, J. Nat. Prod. 74 (2011) 1106–1110.
[31] J.Z. Liu, B.A. Song, H.T. Fan, P.S. Bhadury, W.T. Wan, Eur. J. Med. Chem. 45 (2010) 5108–5122.
[32] Y.M. Xie, Y. Deng, X.Y. Dai, J. Liu, L. Ouyang, Y.Q. Wei, Molecules 16 (2011) 2516–2526.
[33] E. Kassi, Z. Papoutsi, H. Pratsinis, N. Aligiannis, M. Manoussakis, P. Moutsatsou, J. Cancer Res. Clin. 133 (2007) 493–500.