ResearchGate

See discussions, stats, and author profiles for this publication at: https://www.researchgate.net/publication/274782846

Synthesis and biological evaluation of betulonic acid derivatives as antitumor agents

ARTICLE *in* EUROPEAN JOURNAL OF MEDICINAL CHEMISTRY · MAY 2015

Impact Factor: 3.45 · DOI: 10.1016/j.ejmech.2015.04.006

CITATION
1

READS
54

6 AUTHORS, INCLUDING:

Shengjie Yang
Guizhou University
17 PUBLICATIONS **81** CITATIONS
SEE PROFILE

De-yu Hu
West China Collegel of Stomatology
122 PUBLICATIONS **1,217** CITATIONS
SEE PROFILE

Min Yue
Guizhou University
74 PUBLICATIONS **1,119** CITATIONS
SEE PROFILE

Song Yang
Guizhou University
218 PUBLICATIONS **2,287** CITATIONS
SEE PROFILE

All in-text references underlined in blue are linked to publications on ResearchGate, letting you access and read them immediately.

Available from: Song Yang
Retrieved on: 13 January 2016

European Journal of Medicinal Chemistry 96 (2015) 58–65

Contents lists available at ScienceDirect

European Journal of Medicinal Chemistry

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

Original article

Synthesis and biological evaluation of betulonic acid derivatives as antitumor agents

Sheng-Jie Yang [a, b, c, 1], Ming-Chuan Liu [a, b, 1], Qi Zhao [a], De-Yu Hu [a], Wei Xue [a], Song Yang [a, *]

[a] 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
[b] Research Institute, Sinphar Tian-Li Pharmaceutical Co., Ltd, Hangzhou 311100, PR China
[c] Research Institute of Traditional Chinese Medicine, Yangtze River Pharmaceutical Group Co., Ltd, Taizhou 225321, PR China

ARTICLE INFO

Article history:
Received 29 August 2014
Received in revised form
1 April 2015
Accepted 1 April 2015
Available online 4 April 2015

Keywords:
Betulonic acid derivatives
Synthesis
Antitumor
Apoptosis
Mitochondrial pathway

ABSTRACT

Structural modification was performed at the C-28 position of betulonic acid (BetA). Twenty-five BetA derivatives were synthesized, and evaluated for their antitumor activities against MGC-803, PC3, Bcap-37, A375, and MCF-7 human cancer cell lines by MTT assay. Among the derivatives, most of the derivatives had significant antiproliferative ability (IC_{50} < 19 μM). Compound **3k**, the most active compound, showed IC_{50} values of 3.6, 5.6, 4.2, 7.8, and 5.2 μM on the five cancer cell lines respectively, and was selected to investigate cell apoptosis by subsequent florescence staining and flow cytometry analysis. The results revealed that compound **3k** could induce apoptosis in MGC-803 cell lines, and the apoptosis ratios reached 28.33% after 36 h of treatment at 10 μM. In addition, the study of cancer cell apoptotic signaling pathway indicated that the apoptosis of MGC-803 cells induced by compound **3k** could be through the mitochondrial intrinsic pathway.

© 2015 Elsevier Masson SAS. All rights reserved.

1. Introduction

Cancer is the major cause of human deaths worldwide because of its high incidence and mortality [1,2]. In order to improve the survival and quality of life of cancer patients, the need of new therapies or therapeutic combinations is very urgent all over the world [3]. Natural products have played an important role in the antitumor drug development [4,5], and about 80% of clinically antitumor agents originate from natural products [6–9]. In recent years, interest in synthetic transformations of natural products for the purpose of preclinical development is a major objective of antitumor research programs [10]. Pentacylic triterpenes are one of the most abundant natural compounds found in plant kingdom [11–13].

Betulonic acid (BetA, 3-oxo-20(29)-lupen-28-oic acid), is the one of the major effective components of many traditional Chinese medicine [14], and it can be derived rather easily from betulin by oxidation with chromium trioxide [15], and betulin forms up to 30% of the dry weight of the extractive (the bark of birch trees) [16]. BetA and its derivatives have been found to possess several medicinal properties, such as anti-inflammatory [17,18], anti-melanoma [19] and anti-viral [20,21] activity. Furthermore, BetA was also found to be as an antitumor agent. Some studies have shown that BetA could inhibit the growth of various types of human tumor cell lines, including SGC-7901, HepG-2, LNCaP, DU-145 and PC3 tumor cell lines [22,23]. The antitumor activity of BetA has attracted the attention of the pioneers who aim to develop novel antitumor agents. The structure-activity relationships (SAR) of other pentacyclic triterpene acids such as oleanolic acid (OA), ursolic acids (UA), and GA (glycyrrhetinic acid) had been established [24–26]. Interestingly, modification of C-28 carboxylic acid group of UA into amides and amines analogous led to a rise in activity [27]. The amides of BetA with esters of amino acids and with

Abbreviations: ADM, adriamycin; AO/EB, acridine orange/ethidium bromide; BetA, betulonic acid; ^{13}C NMR, ^{13}C Nuclear Magnetic Resonance; DMF, N, N-dimethylformamide; DMSO, dimethyl sulfoxide; EDCI, 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride; HCPT, 10-hydroxyl camptothecine; HOBt, 1-hydroxybenzotriazole; ^{1}H NMR, Proton Nuclear Magnetic Resonance; IR, Infra-red; MTT, 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; TUNEL, terminal deoxynucleotidyl transferase biotin-dUTP nick end labeling.

* Corresponding author. Ctr for R&D of Fine Chemicals, Guizhou University, Huaxi St., Guiyang 550025, PR China.
E-mail address: jhzx.msm@gmail.com (S. Yang).

[1] Both authors contributed equally to this work.

http://dx.doi.org/10.1016/j.ejmech.2015.04.006
0223-5234/© 2015 Elsevier Masson SAS. All rights reserved.

aliphatic and heterocyclic amines were synthesized, and were revealed valuable biological activity [20,28]. BetA and other pentacyclic triterpenes shared similar observations of SAR [29]. In the previous work, our group have synthesized novel nitrogen-containing betulinic acid derivatives. And the pharmacological evaluations revealed the target compounds had potent antitumor activity compared with betulinic acid in addition to improved aqueous solubility [30].

In view of the mentioned facts above and an attempt to discover more potent and selective antitumor agents based on BetA scaffold, a series of new BetA derivatives were also synthesized. The new products were evaluated their antitumor activities against MGC-803, PC3, Bcap-37, A375, and MCF-7 human cancer cell lines. Furthermore, the possible mechanism of MGC-803 cell growth inhibition by compound **3k** was also investigated in the present study.

2. Results

2.1. Chemistry

The BetA was derived from betulin by oxidation with Jones regent. The syntheses of BetA derivatives are summarized in Schemes 1 and 2. The structure modification of BetA used as the leading compound was done at the position C-28. BetA was reacted with 1,2-dibromoethane, 1,3-dibromopropane, or 1,4-dibromobutane in the presence of K_2CO_3 in DMF at room temperature to give the compounds **2a**–**2c** in high yield. And then, the compounds **2a**, **2b**, or **2c** were reacted with corresponding amines to yield the nitrogen-containing derivatives **3a**–**3l**, respectively (Scheme 1). Compound **2a** was treated with piperazine in DMF in the presence of K_2CO_3 at 80 °C, and reacted with aromatic or aliphatic carboxylic acids in the presence EDCI and HOBT to afford compounds **5a**–**5i** (Scheme 2). All the compounds were fully characterized by various spectroscopic methods.

2.2. Biology

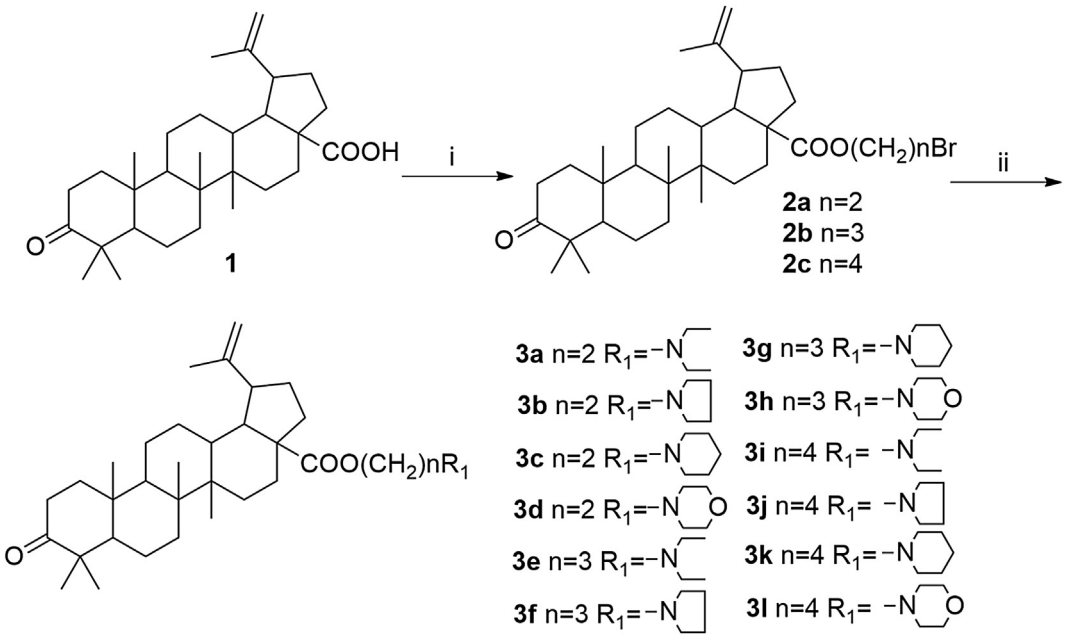
The *in vitro* antitumor activity of BetA derivatives on five different human cancer cell lines: gastric carcinoma cell MGC-803, prostate carcinoma cell PC3, breast carcinoma cell Bcap-37, malignant melanoma cell A375, and breast carcinoma cell MCF-7 cell lines were studied by MTT assay. HCPT (hydroxycamptothecine) and ADM (Adriamycin) were used as the positive controls. The negative control group was treated with culture medium containing 0.1% DMSO. All the compounds including BetA derivatives, ADM, and HCPT were dissolved in DMSO. Each experiment was repeated at least three times. The BetA derivatives showed dose dependent antitumor activity against the investigated cell lines. If IC_{50} value could not be reached at the highest concentration, then >20 was given. The IC_{50} values are summarized in Table 1.

As shown in Table 1, when the 28-COOH was only substituted with a dibromoalkane (compounds **2a**–**2c**), the effect was significantly reduced, compared with the parent BetA. The previous study in UA or BA derivatives showed that significant improvement of cell growth inhibition was not observed when the 28-COOH was just acetylated with a fatty alkyl group [30,31]. Similar results were seen in this study.

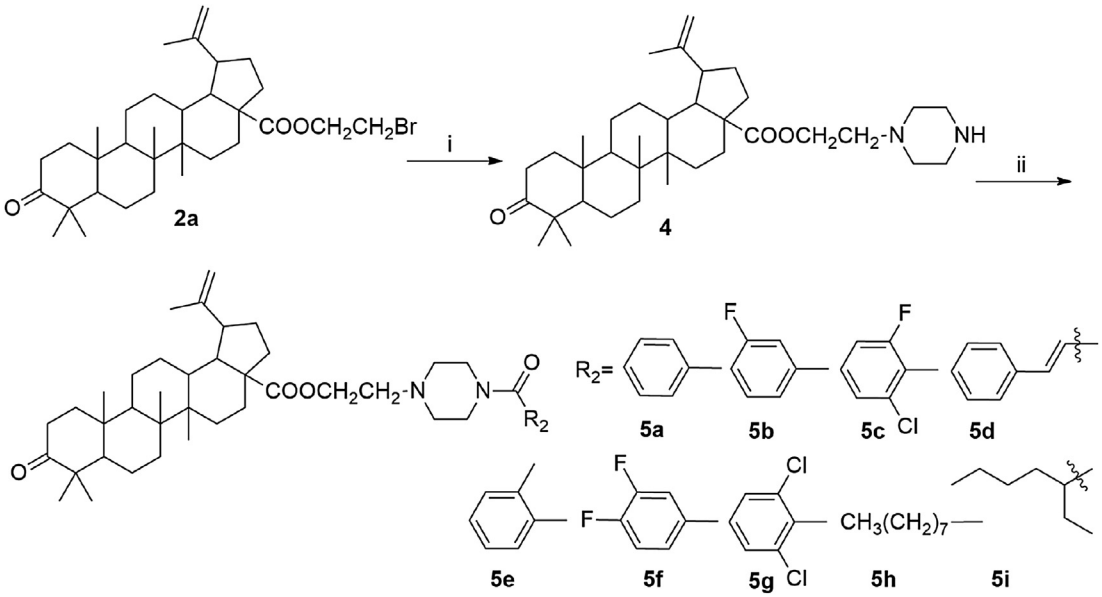
Interestingly, the most active compounds (compound **3a**–**3l**) with selected amino groups introduced were found to be two to eight times more active than BetA, IC_{50} values between 3 and 18 μM. And the activity of the compounds were increased as the length of the carbon chain was increased (4 > 3 > 2). The compound **3k**, one of the most active compounds, had the IC_{50} values of 3.6, 5.6, 4.2, 7.8, and 5.2 μM on the five cancer cell lines, respectively.

Among the derivatives substituted with acids, substituted benzoic acids (compounds **5a**–**5g**) exhibited high and moderate activities, respectively, whereas substituted alkyl carboxylic acids (compounds **5h**–**5i**) demonstrated weak antitumor activity. Compounds **5h**–**5i** did not show significant antitumor activity as reported in the literature. One reason for low activity could be due the long fatty alkyl group. The results were similar with other pentacylic triterpenes [31].

In addition, selectivity of the active compounds (**3i**, **3j**, **3k**, **5a**, **5e**, and **5f**) was also assessed on normal cell line NIH3T3. The selectivity index was calculated by IC_{50} value in normal cells divided by IC_{50} value in cancer cells, and the results are summarized in Table 2. It was observed that these compounds were 2–9



Scheme 1. Regents and conditions: (i) $BrCH_2CH_2Br$, $BrCH_2CH_2CH_2Br$, or $BrCH_2(CH_2)_2CH_2Br$, K_2CO_3, DMF, r.t.; (ii) amine, K_2CO_3, DMF, r.t..



Scheme 2. Reagents and conditions: (i) piperazine, K_2CO_3, DMF, 80 °C; (ii) EDCI, HOBT, aromatic carboxylic acids, DCM, r.t..

times more selective towards cancer cells than normal cells. That is to say that these compounds did not show significant cytotoxic effect on normal cells.

2.3. Preliminary investigation of the apoptosis-inducing effect of title compound *3k*

To determine whether the growth inhibitory activity of the selected compound (compound **3k**) were related to the induction of apoptosis, the morphological character changes of MGC-803 cells were investigated using the AO/EB, Hoechst 33258 staining under fluorescence microscopy.

The morphologic changes in the cell after treatment with compound **3k** were assessed by fluorescene microscopy after staining with AO/EB. AO is a vital dye and will stain both live and dead cells, but EB will stain only cells that have lost membrane integrity. The stained cells revealed four different types under a fluorescence microscope: living cells (normal green nucleus), early apoptotic (bright green nucleus with condensed or fragmented chromatin), late apoptotic (orange-stained nuclei with chromatin condensation or fragmentation), and necrotic cells (uniformly orange-stained cell nuclei) [32]. With HCPT as positive control at 10 μM for 48 h, the compound **3k** at 5 μM against MGC-803 cells from 12 to 48 h was detected *via* AO/EB staining. As can be seen in Fig. 1**A**, the cells treated with compound **3k** from 12 to 48 h had changed. Yellow and orange dots (in the web version) in MGC-803 cells showed early and late apoptotic cells, and the appearance of little red cells indicated that compound **3k** was low cytotoxicity. Therefore, it can be concluded that compound **3k** could induce apoptosis without any significant cytotoxicity.

Hoechst 33258 staining was also used to investigate the apoptosis induction on cells. Live cells with uniformly light blue nuclei were treated with Hoechst 33258 and observed under a fluorescence microscope [33]. Hoechst 33258 staining showed apoptosis in all four types of cells, which was characterized by cytoplasmic and nuclear shrinkage, chromatin condensation and apoptosis body [34]. With HCPT as positive control at 10 μM for 48 h, the compound **3k** at 5 μM against MGC-803 cells from 12 to 48 h was detected *via* Hoechst 33258 staining. As can be seen in

Table 1
Antitumor activity in a panel of various human cancer cell lines *in vitro*.

Compound	IC_{50} in μM for cancer cell lines				
	MGC-803	PC3	Bcap-37	A375	MCF-7
1	17.7 ± 0.3	13.9 ± 0.7	25.7 ± 0.4	28.9 ± 0.7	18.2 ± 0.3
2a	>20	>20	>20	>20	>20
2b	>20	>20	>20	>20	>20
2c	>20	>20	>20	>20	>20
3a	11.7 ± 1.1	10.1 ± 0.7	15.6 ± 0.8	14.3 ± 1.4	12.8 ± 0.3
3b	10.8 ± 0.1	12.3 ± 0.3	10.2 ± 0.9	9.7 ± 0.3	9.4 ± 0.4
3c	12.5 ± 1.5	18.8 ± 0.2	16.9 ± 1.3	17.1 ± 0.1	16.0 ± 0.2
3d	9.4 ± 0.3	11.3 ± 0.7	8.8 ± 0.4	10.5 ± 0.6	10.1 ± 0.7
3e	6.8 ± 0.4	7.0 ± 0.6	6.1 ± 0.3	9.3 ± 0.2	7.3 ± 0.6
3f	7.4 ± 0.1	7.6 ± 0.8	9.2 ± 0.4	8.3 ± 0.4	8.4 ± 0.3
3g	6.3 ± 0.3	5.7 ± 0.3	9.5 ± 0.4	9.5 ± 0.6	6.6 ± 0.4
3h	5.6 ± 0.3	6.6 ± 0.8	7.2 ± 0.8	8.6 ± 0.4	9.3 ± 0.2
3i	3.8 ± 0.1	4.4 ± 0.8	5.2 ± 0.2	9.2 ± 0.7	7.9 ± 0.5
3j	4.0 ± 0.1	6.7 ± 0.4	4.7 ± 0.8	8.5 ± 0.7	5.4 ± 0.2
3k	3.6 ± 0.1	5.6 ± 0.3	4.2 ± 0.1	7.8 ± 0.4	5.2 ± 0.5
3l	5.6 ± 0.3	7.2 ± 0.7	5.2 ± 0.5	6.8 ± 0.5	8.5 ± 0.6
5a	5.3 ± 0.3	4.1 ± 0.6	7.4 ± 0.4	6.3 ± 0.5	4.7 ± 0.4
5b	4.9 ± 0.2	6.8 ± 0.5	6.4 ± 0.1	7.6 ± 0.3	5.4 ± 0.2
5c	5.3 ± 0.2	9.3 ± 0.2	7.5 ± 0.4	8.4 ± 0.2	8.1 ± 0.1
5d	13.4 ± 0.1	11.0 ± 0.6	9.4 ± 0.5	9.3 ± 0.4	7.3 ± 0.4
5e	4.5 ± 0.3	8.7 ± 0.8	6.4 ± 0.4	4.5 ± 0.9	5.4 ± 1.0
5f	4.4 ± 0.2	4.6 ± 0.7	3.9 ± 0.8	7.2 ± 0.4	3.7 ± 0.7
5g	16.6 ± 0.4	14.1 ± 0.5	>20	17.4 ± 0.1	10.8 ± 1.2
5h	>20	>20	>20	>20	17.6 ± 0.1
5i	13.4 ± 0.9	>20	18.7 ± 1.2	>20	15.4 ± 0.7
HCPT	29.1 ± 2.6	34.5 ± 1.5	28.1 ± 1.0	27.8 ± 1.2	48.2 ± 0.4
ADM	0.7 ± 0.2	0.6 ± 0.1	1.2 ± 0.2	1.0 ± 0.6	1.35 ± 0.1

Table 2
Selectivity index of the compounds towards tumor cells.

Compound	MGC-803	PC3	Bcap-37	A375	MCF-7
3i	6.15	5.32	4.50	2.54	2.96
3j	7.18	4.28	6.11	3.38	5.31
3k	7.38	4.75	6.33	3.41	5.11
5a	7.62	9.85	5.46	6.41	8.60
5e	4.69	2.43	3.30	4.69	3.91
5f	4.20	4.02	4.74	2.57	5.00

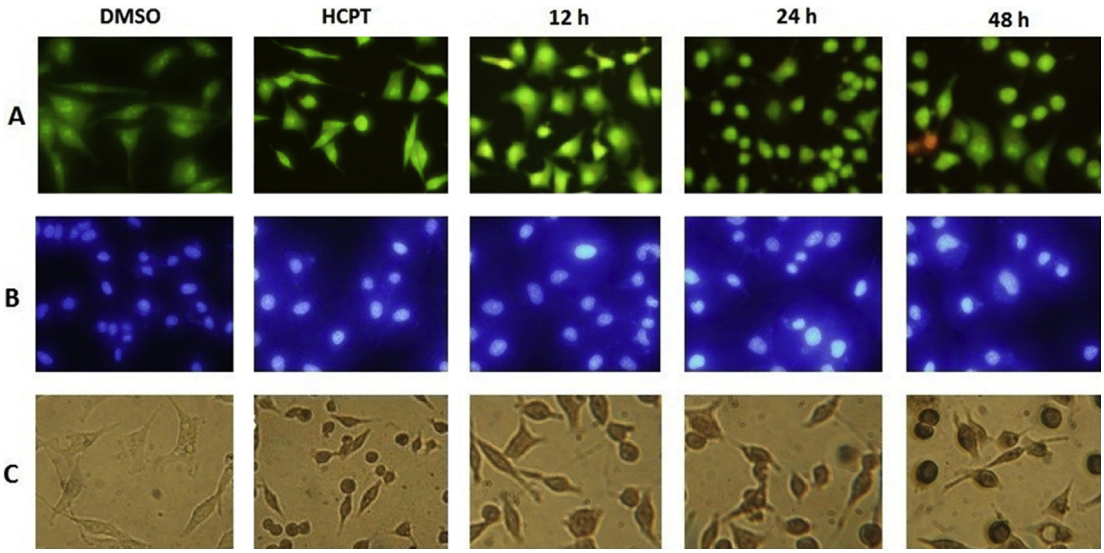


Fig. 1. Apoptosis induction studies of compound **3k**. (A) AO\EB staining. (B) Hoechst 33258 staining. (C) TUNEL assay.

Fig. 1**B**, the cells of the negative group (DMSO) were normal blue. However, the cells of HCPT group appeared compact condensed, and crescent-shaped. The cells exhibited strong blue fluorescence, revealing the typical apoptosis characteristics. The cells treated with compound **3k** from 12 to 48 h had changed. The cell nuclei appeared to be highly condensed and crescent-shaped; indicating that compound **3k** could induce apoptosis against MGC-803 cell lines. These results were identical with the previous AO/EB double staining.

In addition, TUNEL is a popular method for identifying apoptotic cells by detection of DNA fragmentation [33]. The cells were observed that where brown precipitate was the result of positive apoptosis. With HCPT as positive control at 10 μM for 48 h, the compound **3k** at 5 μM against MGC-803 cells from 12 to 48 h was detected *via* TUNEL assay. As can be seen in Fig. 1**C** (in the web version), the cells of the negative group (DMSO) did not appear as brown precipitates, whereas the other groups, namely, HCPT, appeared as brown precipitates. The cells treated with compound **3k** from 12 to 48 h had changed. Therefore, it can be further concluded that compound **3k** induced apoptosis against MGC-803 cells. The results were identical with the previous experiment.

The apoptosis ratios induced by compound **3k** in tumor cells were quantitatively assessed by FCM. In early apoptotic cells, the membrane phospholipid phosphatidylserine (PS) is translocated from the inner to the outer leaflet of the plasma membrane, thereby exposing PS to the external cellular environment. Annexin V is a 35–36 kDa Ca^{2+} dependent phospholipid-binding protein that has a high affinity for PS, and is used to detect early apoptotic cells. PI (Propidine Iodide) was a red fluorescent dye and stained cells that had lost membrane integrity. So, the different periods of apoptotic cells could be distinguished when Annexin V matched with PI: the upper left quadrant contains the necrotic cells ($Annexin^{-}/PI^{+}$); the upper right quadrant contains late apoptotic cells ($Annexin^{-}/PI^{+}$); the lower left quadrant contains the intact cells ($Annexin^{-}/PI^{+}$); the lower right quadrant contains the early apoptotic cells ($Annexin^{-}/PI^{+}$) [35]. As shown in Fig. 2, with HCPT as positive control, compound **3k** (10 μM) could induce apoptosis of MGC-803 cells, and highest apoptosis ratios, 28.33% for compound **3k**, were obtained after 36 h of treatment at a concentration of 10 μM. Furthermore, as shown in Fig. 3 the apoptosis of MGC-803 cells which treated with compound **3k** increased gradually in a time-dependent manner.

2.4. The study of cancer cell apoptotic signaling pathway

p53 can initiate apoptosis, the programmed cell death, if DNA damage proves to be irreparable, and it is able to reduce bcl-2 and promote Bax expression [36]. Thus, cytochrome *c*, which is a component of the electron transport chain in mitochondria, will be released [37]. This release of cytochrome *c* in turn activates caspase 9, a cysteine protease. And then, caspase 9 can activate caspase 3 to induce cell apoptosis. As shown in Fig. 4**A**, the expression of p53 and Bax, was significantly promoted [38]. At the same time, the treatment of compound **3k** also resulted in a significant activation of caspase 3/9 (Fig. 4**B**). These findings indicated that compound **3k** could induce apoptosis of MGC-803 cells through the mitochondrial intrinsic pathway, involving suppression of the expressions of p53, Bax, caspase 9 and caspase 3.

3. Conclusions

In conclusion, we have described the synthesis of BetA derivatives and their *in vitro* antitumor activity on MGC-803, PC3,

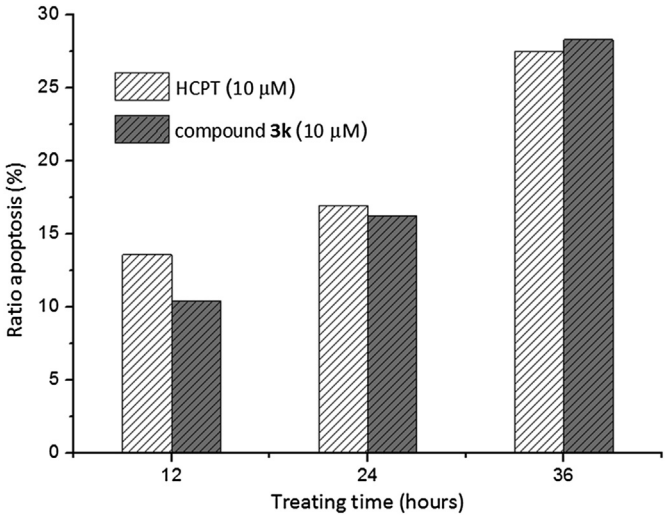


Fig. 2. The apoptosis ratios, including the early (B4) and late apoptosis ratios (B2).

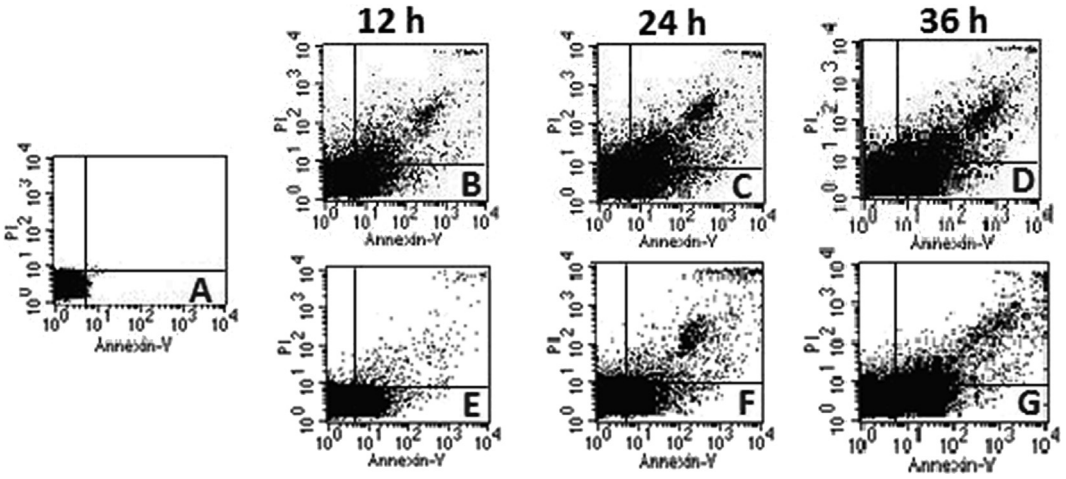


Fig. 3. Annexin V/PI dual staining of MGC-803 cell lines. A: negative control; B, C, and D: cells treated with HCPT (10 μM) as positive control; E, F, and G: cells treated with compound **3k** (10 μM).

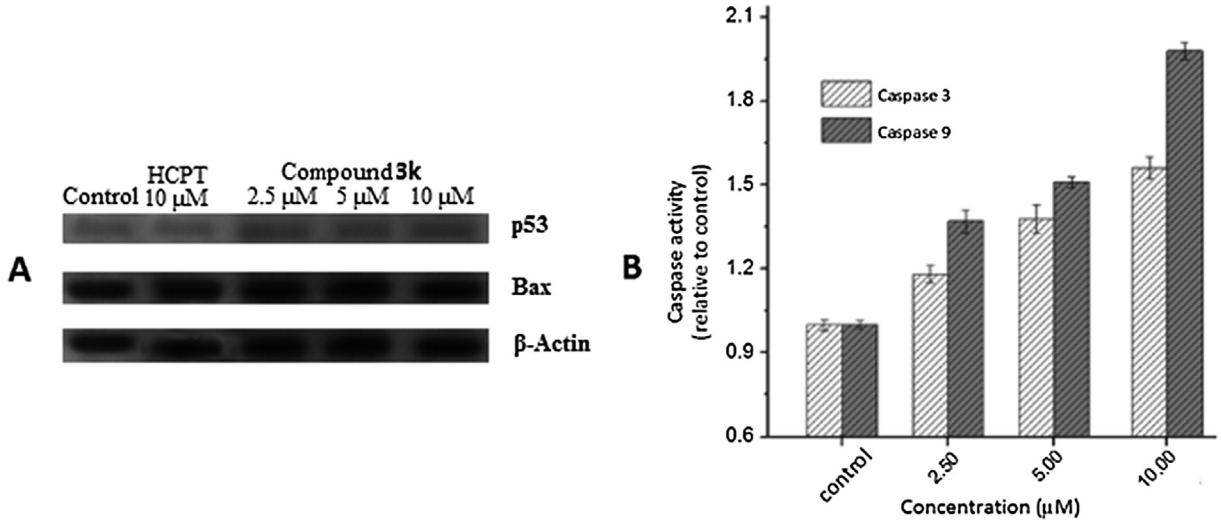


Fig. 4. Expression levels of caspases, p53, and Bax. A: western blot analysis of p53 and Bax in MGC-803 cells; B: activation of caspase 3/9 in MGC-803 cells.

Bcap-37, A375, and MCF-7 human cell lines. The pharmacological results showed that most of the compounds displayed moderate to high levels of antitumor activity, against the five cancer cell lines and that most exhibited more potent inhibitory activity compared with the parent BetA. Meanwhile, the results also indicated that (1) Significant improvement of the cell growth inhibition was achieved when amino groups were introduced at the C-28 position. (2) Substituted benzoic acids (compounds **5a**–**5g**) displayed moderate to high inhibitory activity, whereas substituted alkyl carboxylic acids (compounds **5h**–**5i**) demonstrated weak antitumor activity. (3) When the 28-COOH was only substituted with a dibromoalkane (compounds **2a**–**2c**), the effect was significantly reduced.

Moreover, the compound **3k**, one of the most active compounds, had the IC_{50} values of 3.6, 5.6, 4.2, 7.8, and 5.2 μM on the five cancer cell lines respectively, and it was selected to analyze the mechanism by subsequent florescence staining and flow cytometry analysis. The preliminary mechanism indicated that compound **3k** could induce apoptosis in MGC-803 cell lines, and the apoptosis ratio reached 28.33% after 36 h of treatment at 10 μM, higher than the ratios observed for the positive control HCPT. Anything else, compound **3k** can induce apoptosis of MGC-803 cells through the mitochondrial intrinsic pathway, involving suppression of the expressions of p53, Bax, caspase 9 and caspase 3. These suggest that BetA derivatives could possess a strong potential for development as a preventive and therapeutic agent against human carcinomas. Further study is needed to investigate the active BetA derivatives and the underlying mechanism. And this study also provides a very powerful incentive for further research on the chemical modification and structure-activity relationships of BetA and other triterpenoid acids.

4. Experimental

4.1. General

Betulin with more than 98% purity was purchased from Zhejiang Tiancao Biotech Co., Ltd. Reagents of analytical grade were obtained from Yuda Chemistry Co., Ltd., and used without further purification unless otherwise noted. Infrared spectra were recorded on a Bruker VECTOR22 spectrometer in KBr disks. ^{1}H-NMR and ^{13}C-NMR were recorded using a JEOL-ECX500 spectrometer at 22 °C, with tetramethylsilane as the internal standard and $CDCl_3$ as the solvent.

Column chromatography was performed using silica gel (200–300 meshes) (Qingdao Marine Chemistry Co., Qingdao, China).

4.2. Synthesis

4.2.1. General procedure for compounds (**3a-3l**)

BetA (1 mmol) and K_2CO_3 (2 mmol) were added to DMF (25 mL) and stirred at room temperature for 10 min, after which 1,2-dibromoethane, 1,3-dibromopropane, or 1,4-dibromobutane (4 mmol) was added. After being stirred for another 24 h, the reaction mixture was poured onto 100 mL of distilled water and partitioned with DCM (3 × 25 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 compounds **2a**–**2c**. The compounds **2a**, **2b** or **2c** (1 mmol) and K_2CO_3 (2 mmol) were assed to DMF (10 mL) and stirred at room temperature for 10 min, after saturated nitrogen heterocyclic rings (2 mmol) was added. After being stirred for 24 h, the reaction mixture was poured into the 100 mL distilled water and partitioned with DCM (3 × 30 mL). The organic layer was washed with saturated sodium chloride, dried over Na_2SO_4, and purified *via* silica gel column chromatography with $CHCl_3$/MeOH (10:1, v/v) to obtain compounds **3a**–**3l**.

*4.2.1.1. (4-(piperidin-1-yl)butyl) 3-oxo-20(29)-lupen-28-oate (**3k**).* According to the general procedure, compound **2a** was treated with 1,4-dibromobutane, and then purified on silica gel column using petroleum ether/ethyl acetate (15:1, v/v) to get compound **3k**, R_f [petroleum ether/ethyl acetate (3:1, v/v)] = 0.24.

Yield: 72.8%; pale yellow oil; IR (KBr): ν_{max} 3441, 2938, 2877, 1689, 1640, 880 cm^{-1}; ^1H NMR ($CDCl_3$, 500 MHz) δ: 4.67 (1H, s, Hb-29), 4.54 (1H, s, Ha-29), 4.04 (2H, m, CH_2), 2.94 (3H, m, H-2, 19), 2.44 (4H, m, CH_2), 2.38 (2H, m, CH_2), 1.63 (3H, s, CH_3), 1.58 (2H, m, CH_2), 1.41 (4H, m, CH_2), 1.01 (3H, s, CH_3), 0.96 (3H, s, CH_3), 0.91 (3H, s, CH_3), 0.89 (3H, s, CH_3), 0.86 (3H, s, CH_3); ^{13}C NMR ($CDCl_3$, 125 MHz) δ: 218.2 (C-3), 176.1 (C-28), 150.5 (C-20), 109.7 (C-29), 63.7 (CH_2), 58.6 (CH_2), 56.5 (C-17), 54.9 (C-5), 54.3 (CH_2), 49.9 (C-9), 49.4 (C-18), 47.4 (C-4), 47.0 (C-19), 42.5 (C-14), 40.6 (C-8), 39.7 (C-1), 38.4 (C-13), 37.0 (C-22), 36.9 (C-10), 34.2 (C-2), 33.7 (C-7), 32.1 (C-16), 30.6 (C-21), 29.7 (C-15), 26.8 (C-23), 26.7 (CH_2), 25.6 (C-12), 25.4 (CH_2), 24.1 (CH_2), 22.9 (CH_2), 21.5 (C-11), 21.1 (C-24), 19.7 (C-6), 19.4 (C-30), 16.0 (C-26), 15.9 (C-25), 14.7 (C-27).

4.2.2. General procedure for compounds (**5a**–**5i**)

Compound **2a** (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 DCM (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 $CHCl_3$/MeOH (10:1, v/v) to obtain compound **4**. Compound **4** (1 mmol), amine compounds (1.2 mmol), EDCI (1.2 mmol) and HOBt (1.2 mmol) were added to DCM (25 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 **5a**–**5i**.

*4.2.2.1. (2-(4-benzoylpiperazin-1-yl)ethyl) 3-oxo-20(29)-lupen-28-oate (**5a**).* According to the general procedure, compound **4** was treated with aromatic acids and then purified on silica gel column using chloroform/methanol (10:1, v/v) to obtain compound **5a**, R_f [chloroform/methanol (5:1, v/v)] = 0.28.

Yield: 71.1%; colorless oil; IR (KBr): ν_{max} 3444, 2931, 2869, 1713, 1644, 881, 755, 701 cm^{-1}; ^1H NMR ($CDCl_3$, 500 MHz) δ: 7.37 (5H, s, PhH), 4.69 (1H, s, Hb-29), 4.57 (1H, s, Ha-29), 4.19 (2H, t, J = 5 Hz, CH_2), 3.43 (2H, brs, H in piperazine), 2.97 (3H, m, H-2, 19), 2.64 (2H, t, J = 6.5 Hz, CH_2), 2.46 (2H, m, H in piperazine), 2.38 (2H, m, H in piperazine), 1.67 (3H, s, CH_3), 1.04 (3H, s, CH_3), 0.99 (3H, s, CH_3), 0.94 (3H, s, CH_3), 0.92 (3H, s, CH_3), 0.89 (3H, s, CH_3); ^{13}C NMR ($CDCl_3$, 125 MHz) δ: 218.2 (C-3), 175.9 (C-28), 170.4 (C═O), 150.5 (C-20), 135.8 (C), 129.8 (CH), 128.6 (CH), 127.1 (CH), 109.8 (C-29), 60.8 (CH_2), 56.8 (CH_2), 55.6 (C-17), 55.0 (C-5), 53.7 (CH_2), 53.1 (CH_2), 49.9 (C-9), 49.3 (C-18), 47.8 (CH_2), 47.4 (C-4), 47.0 (C-19), 42.5 (C-14), 42.2 (CH_2), 40.7 (C-8), 39.7 (C-1), 38.4 (C-13), 36.9 (C-22), 36.9 (C-10), 34.2 (C-2), 33.7 (C-7), 32.1 (C-16), 30.7 (C-21), 29.7 (C-15), 26.6 (C-23), 25.5 (C-12), 21.5 (C-11), 21.1 (C-24), 19.7 (C-6), 19.4 (C-30), 16.0 (C-26), 15.9 (C-25), 14.7 (C-27).

4.3. Cell lines and culture

MGC-803, PC3, Bcap-37, A375, and MCF-7 cell lines were obtained from the Institute of Biochemistry and Cell Biology, China Academy of Science. MGC-803 is gastric cancer cell line, PC3 is prostate cancer cell line, A375 is malignant melanoma cell line, Bcap-37, and MCF-7 are breast cancer cell lines. The entire cancer cell lines were maintained in the RPMI 1640 medium. They were supplemented with 10% heat-inactivated fetal bovine serum (FBS) in a humidified atmosphere of 5% CO_2 at 37 °C. All cell lines were maintained at 37 °C in a humidified 5% carbon dioxide and 95% air incubator.

4.4. MTT assays

All compounds were dissolved in DMSO and subsequently diluted in the culture medium before treatment of the cultured cells. When the cells were 80–90% confluent, they were harvested by treatment with a solution containing 0.25% trypsin, thoroughly washed and resuspended in supplemented growth medium. Cells (2×10^3/well) were seeded in 96-well plates overnight. The tested compounds at different concentrations were added into wells and cells were treated at 37 °C for 72 h. In parallel, the cells treated with 0.1% DMSO served as negative control and ADM (Adriamycin) as positive control. Then MTT (5 mg/ml, in PBS) was added into each well and cultured for another 4 h. The MTT-formazan formed by metabolically viable cells was dissolved in 100 μL of SDS for 12 h. The absorbance was then measured at 595 nm with a microplate reader, which is directly proportional to the number of living cells in culture. The percentage cytotoxicity was calculated using the formula. IC_{50} values were calculated according to the dose-dependent curves [39].

4.5. AO/EB staining

The cells (5×10^4 cell/mL in a volume of 0.6 mL) were seeded in 6-well tissue culture plates. Following incubation, the medium was removed and replaced with fresh medium plus 10% FBS and then supplemented with compounds. After the treatment period, cells were washed with 1 × PBS once, and the cover slip with monolayer cells was inverted on the glass slide with 20 μL of AO/EB stain (100 μg/mL) [40]. The fluorescence was read using an IX71SIF-3 fluorescence microscope.

4.6. Hoechst 33258 staining

The cells (5×10^4 cell/mL in a volume of 0.6 mL) were seeded in 6-well tissue culture plates. Following incubation, the medium was

removed and replaced with fresh medium plus 10% FBS and then supplemented with compounds (5 μmol/L). After the treatment period, cells were washed with 1 × PBS twice, and the cells were fixed in 4% paraformaldehyde for 10 min. The cells were washed twice with PBS, and were consequently stained with 0.5 mL of Hoechst 33258 staining solution for 5 min. Then the wells were washed twice, (3 min/once) with 1 × PBS after staining. Later, coverslips having cells were covered the glass slide, detected by IX71SIF-3 fluorescence microscope at 350 nm excitation and 460 nm emissions [41].

4.7. TUNEL assay

The cells (5×10^4 cell/mL in a volume of 0.6 mL) were seeded in 6-well tissue culture plates. Following incubation, the medium was removed and replaced with fresh medium plus 10% FBS and then supplemented with compounds. TUNEL assays were performed using a colorimetric TUNEL apoptosis assay kit according to the manufacturer's instructions. (1) After the treatment period, cells were washed with 1 × PBS and fixed in 4% paraformaldehyde for 40 min. The cells were washed once with PBS, and were consequently permeabilized with immunol staining wash buffer for 2 min on ice. (2) The cells were rewashed once with PBS, and were consequently 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. (3) The cells were incubated with 2 μL of TdT-enzyme and 48 μL of Biotin-dUTP per specimen for 60 min at 37 °C. The cells were terminated for 10 min, and were consequently incubated with streptavidin-HRP (50 μL per specimen) conjugate diluted at 1:50 in sample diluent for 30 min (4) The cells were washed three times with PBS, and were consequently incubated with diaminobenzidine solution (200 μL per specimen) for 10 min. At last, the cells were rewashed twice with PBS, and were consequently imaged under an XDS-1B inverted biological microscope [42].

4.8. Flow cytometry analysis

Prepared MGC-803 cells (1×10^6/mL) were washed twice with cold PBS and then re-suspended gently in 500 μL binding buffer. Thereafter, cells were stained in 5 μL Annexin V-FITC and shaked well. Finally, 5 μL PI was added to these cells and incubated for 20 min in a dark place, analyzed by FACS Calibur, Becton Dickinson.

4.9. Western blotting analysis

Cells were collected after treatment with compound **3k** at 2.5, 5, and 10 μM for 12 h, respectively. Western blotting analysis was performed as previously described [43], using the following antibodies at dilutions of 1: 500 to 1:1000: anti-p53, anti-Bax, and anti-β actin.

4.10. Caspase 3 enzyme assay

Cells were collected after treatment with compound **3k** at 2.5, 5, and 10 μM for 12 h, respectively. Prepared MGC-803 cells (1×10^6/mL, 5 ml) were washed twice with cold PBS. Then, 100 μL of lysis buffer was added to the cells for 25 min on ice and centrifuged at 18,000 g for 15 min. 80 μL of reaction buffer and 10 μL of Ac-DEVED-*p*NA were added to 10 μL of supernatant liquid. After incubating at 37 °C for 2–3 h in darkness, the absorbance was measured at 405 nm, with the lysis buffer and reaction buffer as control.

4.11. Caspase 9 enzyme assay

Cells were collected after treatment with compound **3k** at 2.5, 5, and 10 μM for 12 h, respectively. Prepared MGC-803 cells (1×10^6/mL, 5 mL) were washed twice with cold PBS. Then, 100 μL of lysis buffer was added to the cells for 25 min on ice and centrifuged at 16,000 g for 15 min. 80 μL of reaction buffer and 10 μL of Ac-LEHD-pNA were added to 10 μL of supernatant liquid. After incubating at 37 °C for 2–3 h in darkness, the absorbance was measured at 405 nm, with the lysis buffer and reaction buffer as control.

4.12. Statistical analysis

All statistical analyses were performed using SPSS 10.0, and the data were analyzed using one-way ANOVA. The mean separations were performed using the least significant difference method. Each experiment was performed in triplicate, and all experiments were run thrice and yielded similar results. Measurements from all the replicates were combined, and the treatment effects were analyzed.

Conflicts of interest

None.

Acknowledgments

The authors wish to thank the National Nature Science Foundation of China (Grant Nos. 21172048, 21372052), Key Technologies R&D Program (No. 2011BAE06B02), the Research Project of Chinese Ministry of Education (No. 213033A), and Guizhou Key S&T Project (Nos. [2012]6012, [2011]3016, [2010]3052) for the financial support.

Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.ejmech.2015.04.006.

References

[1] G. Danaei, S.V. Hoorn, A.D. Lopez, C.J. Murray, M. Ezzati, Causes of cancer in the world: comparative risk assessment of nine behavioural and environmental risk factors, Lancet 366 (2005) 1784–1793.
[2] C.D. Mathers, D. Loncar, Projections of global mortality and burden of disease from 2002 to 2030, Plos Med. 3 (2006) 2011–2029.
[3] K. Sikora, S. Advani, V. Koroltchouk, I. Magrath, L. Levy, H. Pinedo, G. Schwartsmann, M. Tattersall, S. Yan, Essential drugs for cancer therapy: a World Health Organization consultation, Ann. Oncol. 10 (1999) 385–390.
[4] S. Dev, Impact of natural products in modern drug development, India J. Exp. Biol. 48 (2010) 191–198.
[5] A.D. Kinghorn, Y.W. Chin, S.M. Swanson, Discovery of natural product anticancer agents from biodiverse organisms, Curr. Opin. Drug Discov. Devel 12 (2009) 189–196.
[6] M. Shoeb, Anticancer agents from medicinal plants, Bangl J. Pharmacol. 1 (2006) 35–41.
[7] D.R.A. Mans, A.B. Da Rocha, G. Schwartsmann, Anti-cancer drug discovery and development in Brazil: targeted plant collection as a rational strategy to acquire candidate anti-cancer compounds, Oncologist 5 (2000) 185–198.
[8] D.J. Newman, G.M. Gragg, K.M. Snader, The influence of natural products upon drug discovery, Nat. Prod. Rep. 17 (2000) 215–234.
[9] T.W. Hodges, C.F. Hossain, Y.P. Kim, Y.D. Zhou, D.G. Nagle, Molecular-targeted antitumor agents: the saururus cernuus dineolignans manassantin B and 4-*O*-demethylmanassantin B are potent inhibitors of hypoxia-activated HIF-1, J. Nat. Prod. 67 (2004) 767–771.
[10] D.G. Nagle, Y.D. Zhou, F.D. Mora, K.A. Mohammed, Y.P. Kim, Mechanism targeted discovery of antitumor marine natural products, Curr. Med. Chem. 11 (2004) 1725–1756.
[11] Z. Skrzypek, H. Wysokińska, Sterols and triterpenes in cell culture of *Hyssopus officinalis* L. Z Naturforsch C 58 (2003) 308–312.
[12] S. Jäger, H. Trojan, T. Kopp, M.N. Laszczyk, A. Scheffler, Pentacyclic triterpene distribution in various plants - rich sources for a new group of multi-potent plant extracts, Molecules 14 (2009) 2016–2031.

[13] A.A.L. Gunatilaka, Triterpenoids and steroids of Sri Lankan plants: a review of occurrence and chemistry, J. Natl. Sci. Coun. Sri Lanka 14 (1986) 1–54.
[14] K.H. Lee, S. Morris-Natschke, K. Qian, Y. Dong, X. Yang, T. Zhou, E. Belding, S.F. Wu, K. Wada, T. Akiyama, Recent progress of research on herbal products used in traditional Chinese medicine: the herbs belonging to the divine husbandman's herbal foundation canon, J. Tradit. Complem. Med. 2 (2012) 6–26.
[15] A.V. Symon, N.N. Veselova, A.P. Kaplun, N.K. Vlasenkova, G.A. Fedorova, A.I. Liutik, G.K. Gerasimova, V.I. Shvrts, Synthesis and antitumor activity of cyclopropane derivatives of betulinic and betulonic acids, Russ. J. Bioorg. Chem. 31 (2005) 320–325.
[16] P. Silhár, S. Alakurtti, K. Čapková, F. Xiaochuan, C.B. Shoemaker, J. Yli-kauhaluoma, K.D. Janda, Synthesis and evaluation of library of betulin derivatives against the botulinum neurotoxin A protease, Bioorg. Med. Chem. Lett. 21 (2011) 2229–2231.
[17] A.I. Govdi, I.V. Sorokina, T.G. Tolstikova, S.F. Vasilevsky, G.A. Tolstikov, Synthesis and biological activity of novel acetylene betulonic acid derivatives, Chem. Sust. Dev. 18 (2010) 397–402.
[18] S.F. Vasilevsky, A.I. Govdi, E.E. Shults, M.M. Shakirov, I.V. Sorokina, T.G. Tolstikova, D.S. Baev, G.A. Tolstikov, I.V. Alabugin, Efficient synthesis of the first betulonic acid-acetylene hybrids and their hepatoprotective and anti-inflammatory activity, Bioorg. Med. Chem. 17 (2009) 5164–5169.
[19] D.Z.L. Bastos, I.C. Pimentel, D.A. de Jesus, B.H. de Oliveira, Biotransformation of betulinic and betulonic acids by fungi, Phytochemistry 68 (2007) 834–839.
[20] O.B. Flekhter, E.I. Boreko, L.R. Nigmatullina, E.V. Tret'yakova, N.I. Pavlova, L.A. Baltina, S.N. Nikolaeva, O.V. Savinova, V.F. Eremin, F.Z. Galin, G.A. Tolstikov, Synthesis and antiviral activity of betulonic acid amides and conjugates with amino acids, Russ. J. Bioorg. Chem. 30 (2004) 80–88.
[21] P. Coric, S. Turcaud, F. Souquet, L. Briant, B. Gay, J. Royer, N. Chazal, S. Bouaziz, Synthesis and biological evaluation of a new derivative of bevirimat that targets the gag CA-SP1 cleavage site, Eur. J. Med. Chem. 62 (2013) 453–465.
[22] X. Zhang, H. Li, Y. Jin, G. Fang, Effects of betulonic acid on SGC-7901, HepG-2 and mice of bearing S_{180} tumor cells, Nat. Prod. Res. Dev. 21 (2009) 766–770.
[23] S. Yang, Q. Zhao, H. Xiang, M. Liu, Q. Zhang, W. Xue, B. Song, S. Yang, Antiproliferative activity and apoptosis-inducing mechanism of constituents from *Toona sinensis* on human cancer cells, Cancer Cell. Int. 13 (2013) 12.
[24] R.C. Santos, J.A. Salvador, S. Marín, M. Cascante, J.N. Moreira, T.C. Dinis, Synthesis and structure-activity relationship study of novel cytotoxic carbamate and *N*-acylheterocyclic bearing derivatives of betulin and betulinic acid, Bioorg. Med. Chem. 18 (2010) 4385–4396.
[25] D. Emmerich, K. Vanchanagiri, L.C. Baratto, H. Schmidt, R. Paschke, Synthesis and studies of anticancer properties of lupane-type triterpenoid derivatives containing a cisplatin fragment, Eur. J. Med. Chem. 75 (2014) 460–466.
[26] S. Schwarz, B. Siewert, N.M. Xavier, A.R. Jesus, A.P. Rauter, R. Csuk, A "natural" approach: synthesis and cytoxicity of monodesmosidic glycyrrhetinic acid glycosides, Eur. J. Med. Chem. 72 (2014) 78–83.
[27] K.K. Bai, Z. Yu, F.L. Chen, F. Li, W.Y. Li, Y.H. Guo, Synthesis and evaluation of ursolic acid derivatives as potent cytotoxic agents, Bioorg. Med. Chem. Lett. 22 (2012) 2488–2493.
[28] O.B. Flekhter, E.I. Boreko, E.V. Tret'iakova, N.I. Pavlova, L.A. Baltina, S.N. Nikolaeva, O.V. Savinova, V.F. Eremin, F.Z. Galin, G.A. Tolstikov, Synthesis and antiviral activity of amides and conjugates of betulonic acid with amino acids, Russ. J. Bioorg. Chem. 30 (2004) 89–98.
[29] Y. Liu, W.X. Lu, M.C. Yan, Y. Yu, T. Ikejima, M.S. Cheng, Synthesis and tumor cytotoxicity of novel amide derivatives of β-hederin, Molecules 15 (2010) 7871–7883.
[30] S. Yang, N. Liang, H. Li, W. Xue, D. Hu, L. Jin, Q. Zhao, S. Yang, Design, synthesis and biological evaluation of novel betulinic acid derivatives, Chem. Cent. J. 6 (2012) 141.
[31] J.W. Shao, Y.C. Dai, J.P. Xue, J.C. Wang, F.P. Lin, Y.H. Guo, *In vitro* and *in vivo* anticancer activity evaluation of ursolic acid derivatives, Eur. J. Med. Chem. 46 (2011) 2652–2661.
[32] F. Attari, H. Sepehri, L. Delphi, B. Goliaei, Apoptotic and necrotic effects of pectic acid on rat pituitary GH3/B6 tumor cells, Iran. Biomed. J. 13 (2009) 229–236.
[33] M.C. Liu, S.J. Yang, L.H. Jin, D.Y. Hu, Z.B. Wu, S. Yang, Chemical constituents of the ethyl acetate extract of *Belamcanda chinensis* (L.) DC roots and their antitumor activities, Molecules 17 (2012) 6156–6169.
[34] Y.L. Wu, B. Sun, X.J. Zhang, S.N. Wang, H.Y. He, M.M. Qiao, J. Zhong, J.Y. Xu, Growth inhibition and apoptosis induction of sulindac on human gastric cancer cells, World J. Gastroenterol. 7 (2001) 796–800.
[35] B. Schutte, R. Nuydens, H. Geerts, F. Ramaekers, Annexin V binding assay as a tool to measure apoptosis in differentiated neuronal cells, J. Neurosci. Meth. 86 (1998) 63–69.
[36] R. Kumariya, A.K. Barui, S. Singh, p53-Cells' inbuilt mechanism to inhibit cancer through apoptosis, Cancer Sci. Ther. 4 (2012) 368–370.
[37] J. Cai, J. Yang, D.P. Jones, Mitochondrial control of apoptosis: the role of cytochrome c, Biochim. Biophys. Acta 1366 (1998) 139–149.
[38] X. Jiang, X. Wang, Cytochrome c-mediated apoptosis, Annu. Rev. Biochem. 73 (2004) 87–106.
[39] P. Lan, J. Wang, D.M. Zhang, C. Shu, H.H. Cao, P.H. Sun, X.M. Wu, W.C. Ye, W.M. Chen, Synthesis and antiproliferative evaluation of 23-hydroxybetulinic acid derivatives, Eur. J. Med. Chem. 46 (2011) 2490–2502.
[40] D. Ribble, N.B. Goldstein, D.A. Norris, Y.G. Shellman, A simple technique for quantifying apoptosis in 96-well plates, BMC Biotechnol. 5 (2005) 12.
[41] G.Y. Wang, J.W. Zhang, Q.H. Lü, R.Z. Xu, Q.H. Dong, Berbamine induces apoptosis in human hepatoma cell line SMMC7721 by loss in mitochondrial transmembrane potential and caspase activation, J. Zhejiang Univ. Sci. B 8 (2007) 248–255.
[42] X. Xu, X. Gao, L. Jin, P.S. Bhadury, K. Yuan, D. Hu, B. Song, S. Yang, Anti-proliferation and cell apoptosis inducing bioactivities of constituents from *Dysosma versipellis* in PC3 and Bcap-37 cell lines, Cell. Div. 6 (2011) 14.
[43] J.Q. Yu, Y. Yin, J.C. Lei, X.Q. Zhang, W. Chen, C.L. Ding, S. Wud, X.Y. He, Y.W. Liu, G.L. Zou, Activation of apoptosis by ethyl acetate fraction of ethanol extract of *Dianthus superbus* in HepG2 cell line, Cancer Epidemiol. 36 (2012) e40–e45.