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Original article

Synthesis and antitumor activity of novel 3-oxo-23-hydroxybetulinic acid derivatives

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ABSTRACT

A series of novel derivatives of 3-oxo-23-hydroxybetulinic acid was designed, synthesized, and evaluated for their antiproliferative activity against a panel of cancer cell lines (HL-60, BEL-7402, SF-763, HeLa, B16 and A375). The results indicated that majority of the derivatives exhibited more significant antitumor activity than the parent compound. In particular compound **10e** showed the most potent activity with IC_{50} values of 5.85, 6.23 and 7.22 μM against B16, SF-763 and BEL-7402 cells, respectively. Furthermore, **10e** inhibited tumor growth by 51.8% and 62.7% (w/w) in H22 and B16 xenograft mouse models, comparable to cyclophosphamide and 5-fluorouracil, respectively.

1. Introduction

Naturally occurring pentacyclic triterpenoids have been shown to exhibit a variety of biological properties and provide privileged structures for further modifications and structure activity relationships (SARs) studies [1,2]. Betulinic acid (BA, Fig. 1) in particular, has aroused great interests in the research field of cancer therapy by its high cancer cell selectivity and favorable safety profile [3–5].

Many research groups have tried to find new betulinic triterpenoids with improved pharmacological properties that would make them useful candidates for cancer treatment [3–12]. These investigations have either explored new plant species or modified the structure of known active compounds. It has been documented that 23-hydroxybetulinic acid (HBA, Fig. 1), isolated from the root of *Pulsatilla chinensis*, displayed similar antitumor activity as BA [13]. Furthermore, HBA exerted the synergistic cytotoxicity with clinically used drugs such as doxorubicin (DOX) and paclitaxel *in vitro* and *in vivo* in recent studies [14]. In addition, the HBA derivatives could inhibit the activity of a series of ATP-Binding Cassette (ABC) transporters, known as major factors contributing to multidrug-resistance (MDR) in cancer cells [15,16]. These results demonstrated that HBA derivatives are the potential therapeutic leads for future research.

Meanwhile, many attentions have been paid to the antiproliferative mechanism of HBA derivatives. Previous pharmacological studies revealed that natural betulinic derivatives BA, HBA and 3-oxo-23-hydroxybetulinic acid (3-oxo-HBA) (Fig. 1) exhibited potent apoptotic activity by increasing the formation of intracellular reactive oxidative species and reducing the mitochondrial membrane potential of murine melanoma B16 cells [13]. The telomerase may also play a critical role in HBA induced apoptosis [17]. The mitochondrion-targeted apoptosis in cancer cells along with the lower toxicity in normal cells have made HBA derivatives as new promising chemotherapeutic agents for the intervention of cancers.

To date various modifications at positions C-3, C-20 and C-28 of BA have been investigated to dissect the SARs [3,4]. Nevertheless, there are few reports about the structural modifications of HBA.

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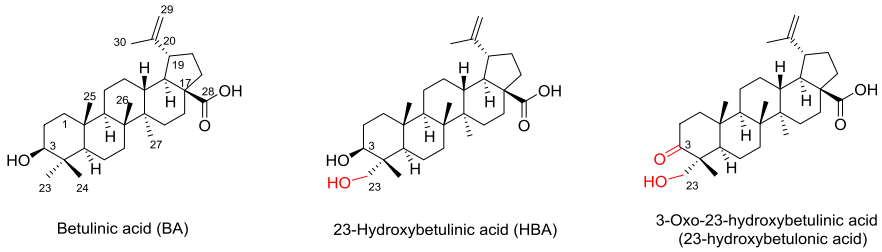


Fig. 1. Chemical structures of betulinic acid, 23-hydroxybetulinic acid and 3-oxo-23-hydroxybetulinic acid.

Previously, a set of C-17-carboxylic acid-modified 23-hydroxybetulinic acid ester and amide derivatives have been reported by our group [18,19]. The promising apoptotic activity of natural betulinic derivatives encouraged us to investigate the semi-synthesis of 3-oxo-23-hydroxybetulinic acid (**6**) from natural HBA. By utilizing the SARs of BA and HBA obtained by previous investigations, we further conducted the synthesis and anti-proliferative evaluation of novel C-28-carboxylic acid-modified 3-oxo-23-hydroxybetulinic derivatives. Moreover, the *in vivo* anti-tumor activity of the selected target compounds was evaluated against mice bearing H22 liver cancer and B16 melanoma, respectively.

2. Synthetic chemistry

3-Oxo-23-hydroxybetulinic acid was synthesized from natural HBA according to the route depicted in Scheme 1. The HBA was isolated from the roots of *Pulsatilla chinensis* (Bge) Regel [13], and characterized by ^{1}H NMR, ^{13}C NMR and high-resolution mass spectra. It was treated with BnBr and K_2CO_3 in DMF to yield 28-benzyl-23-hydroxybetulinic ester **2**, the hydroxyl group of **2** was protected using *tert*-butyldimethylsilyl chloride in the presence of DMAP to give siloxane **3**, and following oxidation of **3** with pyridinium chlorochromate afforded the ketone **4**. 3-oxo-HBA (**6**) was obtained by treatment of **4** with hydrochloric acid in acetone and subsequent debenzylation in THF with Pd/C as catalyst under atmospheric pressure of hydrogen. Furthermore, a set of C-28 substituted derivatives was synthesized as depicted in Scheme 2. The ester derivatives **7a**–**f** were synthesized by the treatment of **6** with corresponding halides under basic conditions [20]. On the other hand, the hydroxyl group of **6** was acetylated and the C-28 carboxylic group was then converted to acyl chloride intermediate **9**, which was further reacted with the corresponding amines and alcohols to provide the derivatives **10a**–**e** [21].

The previous investigations have revealed that some 3-oxime and 3-hydrazide betulinic acid derivatives showed improved anti-tumor activity [22,23]. Thus, 3-oxime- and 3-hydrazide-based 23-hydroxybetulinic derivatives (**11a**–**g**) were designed and synthesized by condensation of the 3-oxo-23-hydroxyl-17-carboxylate derivatives with hydroxylamine hydrochloride or *p*-toluenesulfonohydrazide [22] (Scheme 3).

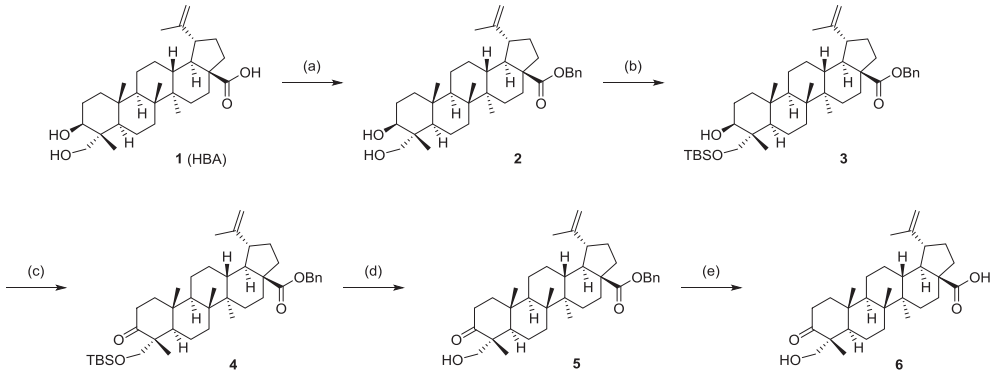
Meanwhile, to broaden the investigation of SARs, some C-2 modified derivatives were synthesized from the corresponding 3-oxo intermediates. As depicted in Scheme 4, oxidation of ketone **4** with potassium tert-butoxide in *tert*-butyl alcohol furnished C-2 enol **12** [24]. The following two steps of deprotection gave the desired enol **14**, and its acetylation with acetic anhydride afforded ester **15**. The vicinal diol **16** was prepared by reduction of enol **12** with $NaBH_4$ in CH_3OH. Subsequent deprotection produced triol **17**, and further acetylation furnished ester **18**. Hydrogenation of **18** with Pd/C in THF under H_2 gave acid **19**, which was hydrolyzed to afford the hydroxy compound **20**. Formylation of ketone **4** with HCOOEt in the presence of MeONa in CH_2Cl_2 gave hydroxymethylene-3-oxo derivative **21** [25]. The C-2 hydroxymethylated product **26** was synthesized via **22**, **23**, **24** and **25** from **21** by the similar procedure as for **20** from **12** (Scheme 4). The stereochemistry of compounds **20** and **26** was assigned by the rotating frame nuclear Overhauser effect spectroscopy (ROESY) experiment (shown in Supplementary material).

3. Results and discussion

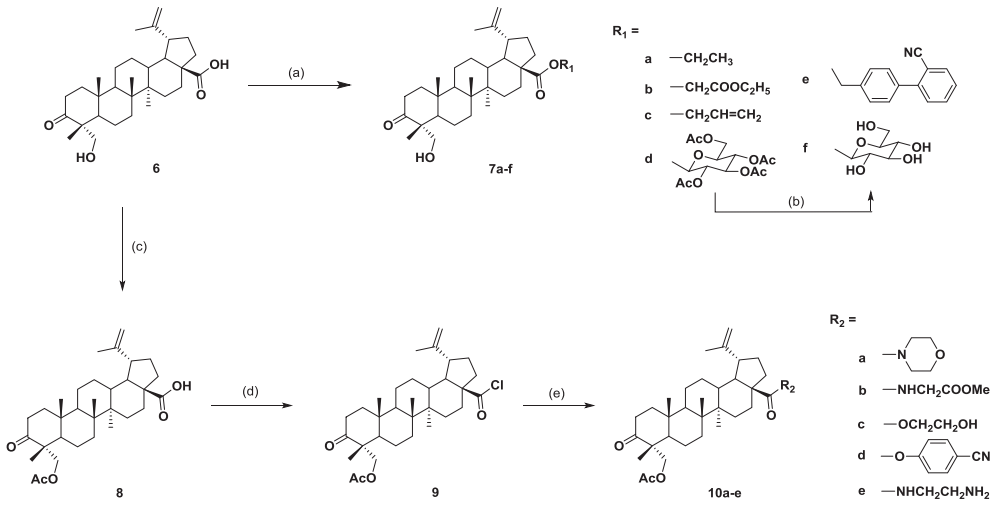
We have designed and synthesized a series of 3-oxo-23-hydroxybetulinic acid derivatives from natural 23-hydroxybetulinic acid. The *in vitro* cytotoxic activity of these novel betulinic derivatives was evaluated on five cancer cell lines (HL-60 human promyelocytic leukemia cell, BEL-7402 human hepatocellular carcinoma, HeLa human cervical adenocarcinoma, SF-763 human brain adenocarcinoma and B16 mice melanoma cells) by MTT assay with doxorubicin as the positive control. The results summarized in Table 1 are presented as the concentration of drug inhibiting 50% cell growth (IC_{50}).

The data indicated that most of the 3-oxo-HBA derivatives markedly inhibited the proliferation of cancer cells. It was also observed that like BA and HBA, 3-oxo-HBA derivatives were highly selective against melanoma (Table 1). Compound **6** displayed improved cytotoxicity in all tested cancer cell lines relative to HBA, suggesting that oxidation of the hydroxyl group at position C-3 to a ketone markedly influenced the cytotoxicity, which was consistent with the case for BA [24,26]. Comparing the activity of 3-oxo-23-acetoxy-17-carboxylate (**7a**–**f**) or amide derivatives (**10c**, **10d**) with that of semi-synthesized compound **6**, it could be found that almost all of the derivatives showed a better anti-proliferative profile than **6** except **7b**. Among them, compounds **7e**, **10a**, **10d** and **10e** were the most promising derivatives with an IC_{50} around 10 μM on all tested cell lines, and compound **10e** was about 5- to 6-fold more potent against five cancer cell lines than **HBA** and 4- to 5-fold than **6**. These findings revealed that electron-donating and/or polar substituents especially groups bearing an N atom (such as–NH_2, –CN) at the terminal of C-28 side chain would benefit the potency. The results also demonstrated that the existence of a structural constraint such as benzyl, biphenyl and phenyl at C-28 is favorable to the inhibitory activity, which is exemplified by compounds **5**, **7e**, **10d**, **17** and **23**. The obtained structure–activity relationships of C-28 position have also confirmed the results from previous comparative molecular field analysis (CoMFA), in which the contour maps illustrated that bulky and/or electron-donating groups at C-28 would be favorable for activity [27]. Considering the limited number of the C-23 modified derivatives in this study, it is hard to give a detailed SAR of this position.

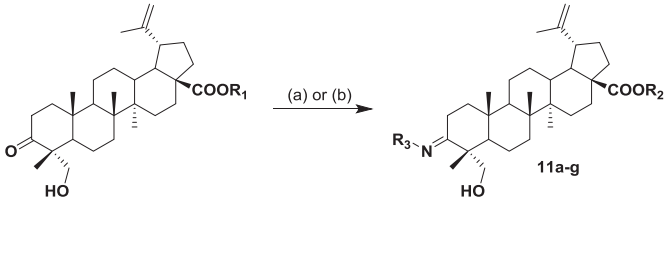
3-Oxime derivatives (**11a**–**e**) were found to be as potent as 3-oxo derivatives; the introduction of large-sized hydrazide group



Scheme 1. Semi-synthesis of 3-oxo-23-hydroxybetulinic acid from natural HBA. Reagents and conditions: (a) BrBn, K_2CO_3, DMF, rt, 12 h; (b) TBSCl, DMAP, CH_2Cl_2, rt, 4 h; (c) PCC, CH_2Cl_2, rt, 3 h; (d) 10% HCl, acetone, rt, 10 h; (e) H_2, Pd/C, THF, rt, 3 h.



Scheme 2. Synthesis of 3-oxo-23-hydroxybetulinic derivatives (**7a**–**f**, **8**, **10a**–**e**). Reagents and conditions: (a) R_1Br, K_2CO_3, DMF; (b) triethylamine, H_2O, CH_3OH/THF, rt, 2 h; (c) Ac_2O, pyridine, rt, 3 h; (d) oxalyl chloride, cat. DMF, CH_2Cl_2, rt, 3 h; (e) R_2X (X=O, NH), DMAP, CH_2Cl_2.



R_1:	
5	Bn
6	H
7a	C_2H_5
7b	$CH_2COOC_2H_5$
7c	$CH_2CH=CH_2$

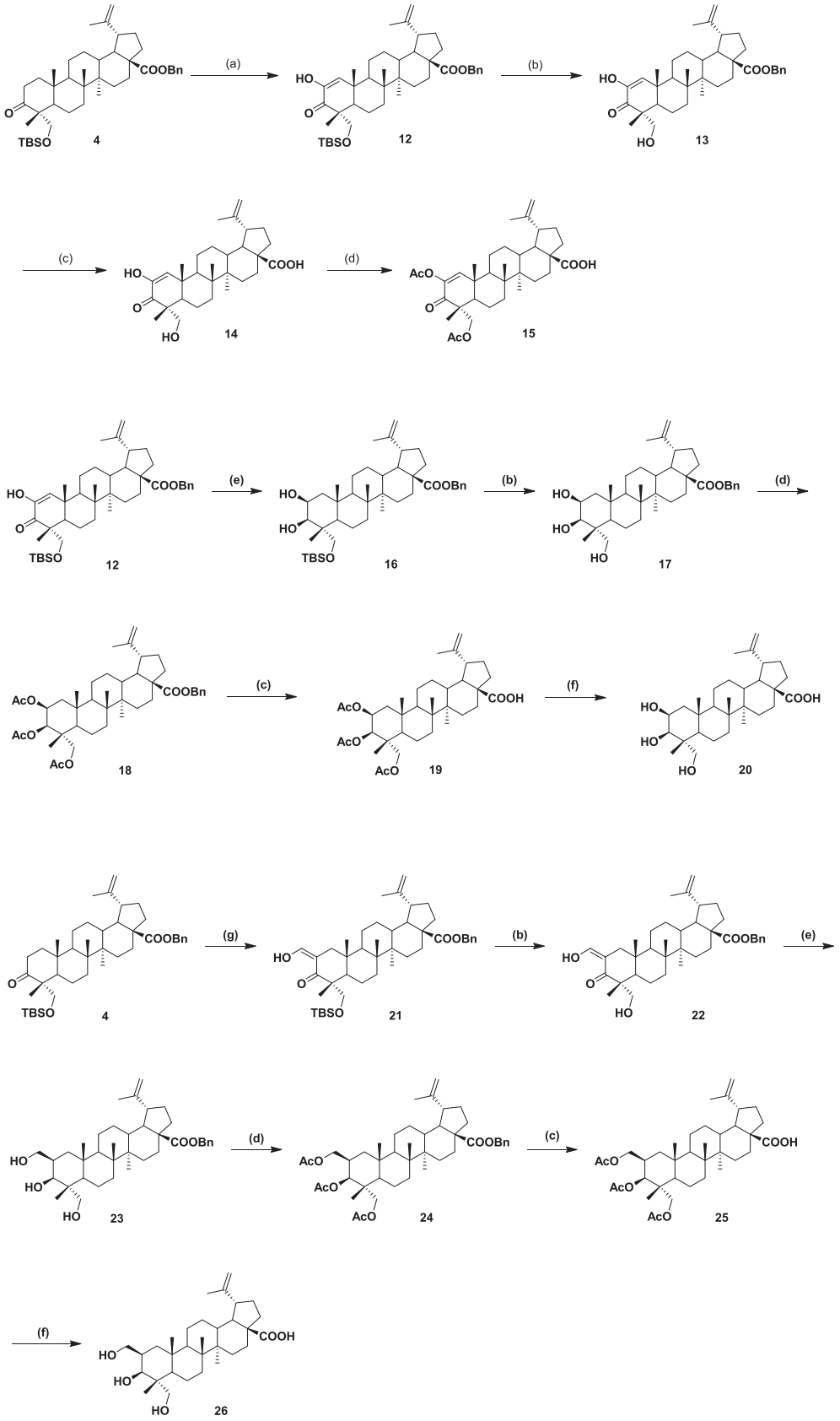
	R_2:	R_3:
a	H	OH
b	C_2H_5	OH
c	$CH_2CH=CH_2$	OH
d	$CH_2COOC_2H_5$	OH
(c) → e	CH_2COOH	OH
f	Bn	NHTs
g	H	NHTs

Scheme 3. Synthesis of 3-oxime and 3-hydrazide derivatives (**11a**–**g**). Reagents and conditions: (a) anhydrous pyridine, hydroxylamine hydrochloride, 80 °C, 2 h; (b) CH_3OH, 4-methylbenzenesulfon-hydrazide, 70 °C, 3 h; (c) NaOH, H_2O, CH_3OH/THF, rt, 1 h.

at C-3 also resulted in potent derivatives **11f** and **11g**, suggesting that bulky substitution was acceptable near the C-3 site. The impact of substitutions at C-28 on the anti-proliferative activity was consistent with that of 3-oxo-17-carboxylate derivatives.

The introduction of hydroxyl group at C-2 in compounds **20** and **26** resulted in a major fall of cytotoxicity on all the tested cell lines when compared with **HBA**, suggesting that the cytotoxic profile of 23-hydroxybetulinic derivatives may be sensitive to the polar substituents at C-2. However, the double bond between C-1 and C-2 in compounds **14** and **15** imparted potent cytotoxicity, implying that the introduction of electron-withdrawing group at C-2 of the 1-ene-3-oxo moiety may be benefit the potency. A more comprehensive structure–activity relationship was shown in Fig. 2.

Despite the promising results on *in vitro* examination of BA and its derivatives over different cancer cell lines, there is only limited *in vivo* research on mice. Therefore, in the present study, based on the significant efficiency *in vitro*, the semi-synthesized compound **6** and the most potent derivative **10e** were chosen to evaluate their antitumor activity against mice bearing H22 liver cancer and B16 melanoma to validate the potent effect of the lead and its derivative. As illustrated in Tables 2 and 3, compound **6** showed more potent anti-tumor activity (tumor inhibitory ratio of 39.5%, 48.1%) than natural HBA (tumor inhibitory ratio of 27.8%, 32.4%). In the H22 liver cancer group, **10e** exhibited stronger activity (tumor inhibitory ratio of 51.8%) than parent compound **6** (tumor inhibitory ratio of 39.5%) and similar activity to cyclophosphamide (tumor inhibitory ratio of 53.7%). In the B16 melanoma group, **10e** also



Scheme 4. Synthesis of C2-modified HBA derivatives. Reagents and conditions: (a) *t*-BuOK, O_2, *t*-BuOH, 45 °C, 3 h; (b) 10% HCl, acetone; rt, 4 h; (c) H_2, Pd/C, THF; rt, 2 h; (d) Ac_2O, pyridine, rt, 3 h; (e) $NaBH_4$, CH_3OH, rt, 3 h; (f) NaOH, CH_3OH, H_2O, rt, 2 h; (g) HCOOEt, $NaOCH_3$, CH_2Cl_2, rt, 16 h.

Table 1
Antiproliferative activity (IC_{50}, μM) of 3-oxo-23-hydroxybetulinic acid and its analogs against five cancer cell lines.

Compounds	Cell lines (IC_{50}[a], μM ± SD)				
	HL-60	BEL-7402	SF-763	HeLa	B16
[b]**HBA**	45.15 ± 4.11	39.67 ± 4.22	43.40 ± 7.20	52.39 ± 7.02	29.87 ± 3.64
5	23.74 ± 4.82	20.09 ± 5.63	19.48 ± 1.77	26.09 ± 4.05	14.09 ± 0.74
6	36.06 ± 1.75	33.78 ± 2.12	29.33 ± 1.94	39.23 ± 3.15	20.62 ± 1.02
7a	31.92 ± 2.01	30.94 ± 3.15	28.92 ± 1.67	37.08 ± 4.29	19.35 ± 1.60
7b	37.10 ± 4.02	30.73 ± 2.24	24.65 ± 3.52	40.81 ± 2.15	25.72 ± 3.14
7c	21.84 ± 3.41	19.03 ± 1.76	15.09 ± 1.42	22.78 ± 5.12	11.83 ± 1.51
7e	14.32 ± 1.10	10.35 ± 0.68	10.05 ± 1.97	15.15 ± 1.04	10.02 ± 0.97
7f	26.61 ± 5.43	22.07 ± 1.75	21.43 ± 2.06	31.03 ± 2.84	14.72 ± 2.03
8	30.22 ± 2.05	29.36 ± 4.04	28.89 ± 2.08	32.09 ± 4.71	17.06 ± 1.33
10a	12.08 ± 1.39	10.53 ± 0.84	10.07 ± 1.02	10.32 ± 1.01	8.16 ± 0.93
10b	19.23 ± 3.18	15.61 ± 2.45	13.56 ± 1.84	17.13 ± 3.68	10.71 ± 0.72
10c	31.07 ± 2.32	27.39 ± 1.40	22.09 ± 1.77	24.43 ± 3.55	18.05 ± 1.39
10d	11.55 ± 1.03	9.23 ± 1.41	9.11 ± 1.32	11.72 ± 0.60	9.02 ± 0.68
10e	8.64 ± 0.89	7.22 ± 0.84	6.23 ± 0.56	10.62 ± 1.34	5.58 ± 0.72
11a	36.85 ± 2.13	32.09 ± 2.35	31.78 ± 3.30	40.83 ± 4.69	23.76 ± 3.85
11b	31.55 ± 2.37	30.77 ± 5.23	28.56 ± 4.90	31.70 ± 3.05	17.55 ± 2.62
11c	24.14 ± 2.09	22.58 ± 0.97	15.48 ± 4.12	25.02 ± 4.03	13.09 ± 1.56
11d	33.72 ± 5.04	30.64 ± 3.18	26.09 ± 2.35	29.96 ± 2.05	17.78 ± 1.34
11e	30.53 ± 2.13	27.15 ± 4.22	17.49 ± 1.08	23.80 ± 1.12	17.40 ± 6.07
11f	29.12 ± 1.22	26.40 ± 4.79	18.35 ± 1.44	21.94 ± 3.26	16.22 ± 2.73
11g	46.31 ± 3.12	39.42 ± 5.03	25.19 ± 3.52	44.29 ± 2.07	33.26 ± 4.49
14	22.02 ± 4.37	19.55 ± 2.02	14.32 ± 3.63	20.01 ± 1.46	11.34 ± 2.61
15	29.82 ± 4.38	25.43 ± 1.89	18.94 ± 1.37	24.06 ± 7.15	15.02 ± 2.13
17	49.90 ± 3.02	39.55 ± 3.60	33.55 ± 4.93	50.44 ± 1.94	26.41 ± 5.17
18	46.15 ± 2.34	34.47 ± 4.62	32.10 ± 1.78	46.01 ± 4.05	21.62 ± 2.34
19	58.09 ± 1.15	49.55 ± 2.63	45.78 ± 3.01	59.13 ± 0.98	30.52 ± 1.83
20	66.35 ± 3.16	56.77 ± 4.20	53.90 ± 1.56	67.83 ± 4.22	36.89 ± 3.04
23	47.92 ± 4.21	41.74 ± 2.03	39.06 ± 2.07	51.36 ± 1.74	30.50 ± 2.11
25	59.85 ± 3.11	50.17 ± 1.45	46.11 ± 1.22	61.45 ± 2.19	39.04 ± 3.65
26	69.02 ± 2.16	59.14 ± 3.71	50.22 ± 3.05	70.83 ± 4.51	47.89 ± 2.13
Doxorubicin	0.17 ± 0.01	0.22 ± 0.02	0.16 ± 0.03	0.21 ± 0.03	0.18 ± 0.01

[a] IC_{50}: concentration of the tested compound that inhibits 50% of cell growth. All data are presented as means ± standard deviation of three independent experiments.
[b] HBA: 23-hydroxybetulinic acid.

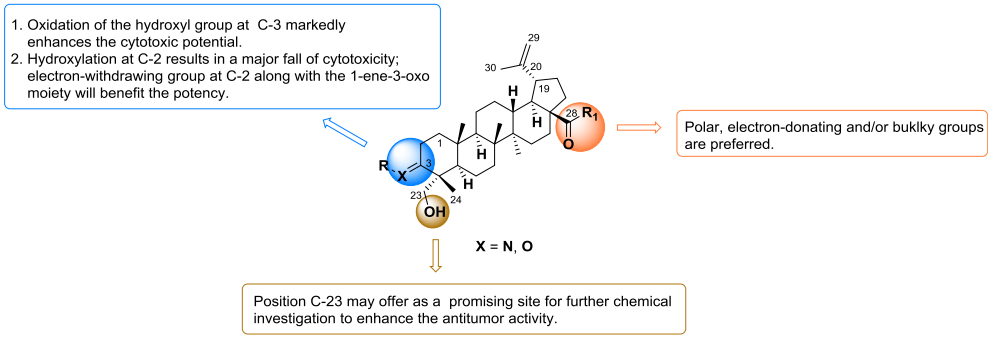


Fig. 2. Preliminary SARs of HBA derivatives.

showed more potent activity (tumor inhibitory ratio of 62.7%) than parent compound **6** (tumor inhibitory ratio of 48.1%) and comparable activity to 5-fluorouracil (tumor inhibitory ratio of 70.3%). Thus, compound **10e** is worthy of further investigation as a potential antitumor agents.

4. Conclusion

In this paper we have designed and synthesized a set of 3-oxo-23-hydroxybetulinic acid derivatives from natural 23-hydroxybetulinic acid. Among which, compounds **6**, **7e**, **10a**, **10d** and **10e** showed potent *in vitro* anti-proliferative activity. Additionally, compounds **6** and **10e** were evaluated *in vivo* against mice bearing H22 liver cancer and B16 melanoma to validate their antitumor activity. It was found that **10e** possessed significant *in vivo* antitumor activity comparable to cyclophosphamide and 5-fluorouracil, respectively. Moreover, the preliminary structure–activity relationships of these derivatives have been established. It has been observed that oxidation of the hydroxyl group at position C-3 to a ketone or oxime could greatly enhance the antitumor activity; and bulky, polar and/or electron-donating groups at position C-28 would be favorable for the activity; however, polar substituents at position C-2 caused decrease in the activity. Further intensive modifications at C-23 and the mechanism studies are currently undergoing in our laboratory, and the results will be reported in due course.

Table 2
Antitumor activity of compounds **6** and **10e** against mice bearing H22 liver cancer.

Drugs	Dose	Injection	Number of mice		Weight of mice (g)		Weight of tumor $X \pm SD$ (g)	Ratio of inhibition (%)	*P* value
			Start	End	Start	End			
Normal Saline	0.5 mL/mouse	iv	10	10	18.3 ± 1.3	27.6 ± 1.4	1.62 ± 036		
CP[a]	30 mg/kg	iv	10	10	18.1 ± 1.0	25.7 ± 1.1	0.75 ± 0.22	53.7	<0.01
HBA[b]	30 mg/kg	ip	10	10	18.4 ± 0.8	26.1 ± 1.4	1.17 ± 0.31	27.8	>0.05
6	30 mg/kg	ip	10	10	18.5 ± 1.1	26.8 ± 1.2	0.98 ± 0.14	39.5	<0.05
10e	30 mg/kg	ip	10	10	18.9 ± 1.2	27.2 ± 1.5	0.78 ± 0.29	51.8	<0.01

[a] CP: cyclophosphamide.
[b] HBA: 23-hydroxybetulinic acid.

Table 3
Antitumor activity of compounds **6** and **10e** against mice bearing B16 melanoma.

Drugs	Dose	Injection	Number of mice		Weight of mice (g)		Weight of tumor $X \pm SD$ (g)	Ratio of inhibition (%)	*P* value
			Start	End	Start	End			
Normal Saline	0.4 mL/mouse	iv	10	10	18.1 ± 1.6	27.8 ± 1.4	1.85 ± 0.34		
5-FU[a]	30 mg/kg	iv	10	10	18.2 ± 0.5	26.2 ± 3.3	0.55 ± 0.11	70.3	<0.01
HBA[b]	30 mg/kg	ip	10	10	18.3 ± 0.6	26.7 ± 1.5	1.25 ± 0.21	32.4	>0.05
6	30 mg/kg	ip	10	10	18.6 ± 0.4	27.3 ± 1.7	0.96 ± 0.08	48.1	<0.05
10e	30 mg/kg	ip	10	10	18.3 ± 0.9	26.9 ± 1.3	0.69 ± 0.13	62.7	<0.01

[a] 5-FU: 5-Fluorouracil.
[b] HBA: 23-hydroxybetulinic acid.

5. Experimental

5.1. Chemistry

Most chemicals and solvents were purchased from commercial sources. Further purification and drying by standard methods were employed when necessary. Melting points were determined on an XT-4 micro melting point apparatus and uncorrected. IR spectra were recorded in $CDCl_3$ or KBr pellets on a Nicolet Impact 410 spectrophotometer. ^1H NMR and ^{13}C NMR spectra were recorded with a Bruker AV-300 or ACF 500 spectrometer in the indicated solvents (TMS as internal standard): the values of the chemical shifts are expressed in δ values (ppm) and the coupling constants (J) in Hz. Purity of all tested compounds was ≥95%, as estimated by HPLC analysis. The major peak of the compounds analyzed by HPLC accounted for ≥95% of the combined total peak area when monitored by a UV detector at 210 nm. EI-MS spectra were recorded on an Agilent1100- LC-MSD-Trap/SL spectrometer and High-resolution mass spectra were recorded using an Agilent QTOF 6520.

All the analytical data of the compounds are shown in Supplementary material.

5.1.1. Benzyl 3,23-dihydroxy-lup-20(29)-en-28-oate (**2**)

To a solution of **1** (1.00 g, 2.12 mmol) in DMF (20 mL) was successively added K_2CO_3 (1.00 g, 7.24 mmol) and benzyl bromide (0.3 mL, 2.52 mmol). The mixture was stirred for 12 h at room temperature and then poured into water (15 mL) and extracted with ethyl acetate (30 mL × 3). The organic layer was washed with water and brine, dried over anhydrous sodium sulfate, and concentrated in vacuo. The residue was purified by chromatography on silica gel (petroleum ether-ethyl acetate 2:1) to afford compound **2** as a white solid (1.07 g, 89.9%).

5.1.2. Benzyl 3-hydroxy-23-t-butyldimethylsilyloxy-lup-20(29)-en-28-oate (**3**)

To a solution of **2** (1.00 g, 1.78 mmol) in dichloromethane (30 mL) was added successively DMAP (300 mg, 2.46 mmol) and *tert*-Butyldimethylchlorosilane (360 mg, 2.39 mmol). The mixture was stirred for 4 h at room temperature, and then, the solvent was removed under reduced pressure. The residue was dissolved in ethyl acetate (50 mL) and washed with 10% HCl, water and brine, dried over anhydrous sodium sulfate, and concentrated in vacuo. The residue was purified by chromatography on silica gel (petroleum ether-ethyl acetate 20:1) to give compound **3** as a white solid (1.11 g, 92.2%). The crude product can be also used for the next step without further purification.

5.1.3. Benzyl 3-oxo-23-t-butyldimethylsilyloxylup-20(29)-en-28-oate (**4**)

To a solution of **3** (1.03 g, 1.52 mmol) in dry dichloromethane (30 mL) was added pyridinium chlorochromate (500 mg, 2.32 mmol). The mixture was stirred at room temperature for 3 h, then filtrated over Celite and the filtrate was concentrated in vacuo. The residue was chromatographed on silica gel (petroleum ether-ethyl acetate 30:1) to give the product as a white solid (910 mg, 88.8%).

5.1.4. Benzyl 3-oxo-23-hydroxy-lup-20(29)-en-28-oate (**5**)

Compound **4** (800 mg, 1.19 mmol) was dissolved in THF (20 mL). 10% HCl (2 mL) was added, and the reaction mixture was stirred at room temperature. After reaction completion, most of the solvent was evaporated in vacuo and diluted with ethyl acetate (30 mL). The mixture was washed with water and brine, dried over anhydrous sodium sulfate, and concentrated in vacuo. The residue was purified by chromatography on silica gel (petroleum ether-ethyl acetate 5:1) to get compound **5** as a white solid (600 mg, 90.0%).

5.1.5. Benzyl 3-oxo-23-hydroxy-lup-20(29)-en-28-oic acid (**6**)

To a solution of **5** (500 mg, 0.89 mmol) in THF (15 mL) was added 10% Pd on carbon (100 mg) at room temperature. The mixture was subjected to 1 atm of H_2 and was monitored by TLC. After most of compound **5** were consumed, filtered and the filtrate was concentrated in vacuo. The residue was chromatographed on silica gel

(petroleum ether-acetone 4:1) to give compound **6** (300 mg, 71.6%) as a white solid.

*5.1.6. Ethyl 3-oxo-23-hydroxy-lup-20(29)-en-28-oate (**7a**)*

To a solution of **6** (100 mg, 0.21 mmol) in DMF (8 mL) was successively added K_2CO_3 (100 mg, 0.72 mmol) and bromoethane (35 mg, 0.32 mmol). The mixture was stirred at room temperature for 12 h and then poured into water (5 mL) and extracted with ethyl acetate (30 mL). The organic layer was washed with water and brine, dried over anhydrous sodium sulfate, and concentrated in vacuo. The residue was purified by chromatography on silica gel (petroleum ether-ethyl acetate 4:1) to afford the target compound as a white solid (88 mg, 84.1%).

Compounds **7b**–**e** were obtained by using the similar synthetic procedure of compound **7a**.

*5.1.7. β-D-Glucopyranosyl 3-oxo-23-hydroxy-lup-20(29)-en-28-oate (**7f**)*

To a solution of **7d** (50 mg, 0.06 mmol) in THF (5 mL) and CH_3OH (3 mL) was added triethylamine (1 mL) and water (1 mL). The mixture was stirred for 4 h at room temperature, and then diluted with ethyl acetate (15 mL), washed with water and brine. The organic layer was dried over anhydrous Na_2SO_4, and concentrated in vacuo. The residue was purified over silica gel (petroleum ether-acetone 1:1) to afford **7f** as a white solid (22 mg, 56.4%).

*5.1.8. 3-Oxo-23-acetoxy-lup-20(29)-en-28-oic acid (**8**)*

To a solution of **6** (500 mg, 1.06 mmol) in pyridine (10 mL) was added acetic anhydride (2 mL). After reaction completion, the reaction mixture was diluted with ethyl acetate (25 mL). The organic layer was washed with 10% HCl, water and brine, dried over anhydrous Na_2SO_4, and concentrated in vacuo. The residue was chromatographed on silica gel (petroleum ether-acetone 6:1) to afford compound **8** as a white solid (490 mg, 90.0%).

*5.1.9. 3-Oxo-23-acetoxy-lup-20(29)-en-28-oic chloride (**9**)*

To a solution of compound **8** (515 mg, 1.00 mmol) in dry dichloromethane (40 mL) was added oxalyl chloride (0.6 mL, 3.18 mmol) and dry DMF (0.1 mL). The solution was stirred at room temperature for 4 h. After quenching the reaction, the solution was concentrated to give compound **9** (about 0.5 g) as yellow foam.

*5.1.10. N-[3-oxo-23-acetoxy-lup-20(29)-en-28-oyl]-morpholine (**10a**)*

To a solution of compound **9** (110 mg, 0.21 mmol) in dry dichloromethane (8 mL) was added morpholine (0.1 mL, 1.15 mmol). The solution was stirred at room temperature for 2 h. At which time the reaction was completed, the solution was concentrated, the residue was dissolved in ethyl acetate (15 mL), and the organic layer was separated and washed by brine, dried with anhydrous Na_2SO_4. Filtered, the filtrate was concentrated and chromatographed over silica gel (petroleum ether-ethyl acetate 4:1) to give **10a** (89 mg, 72.9%) as white foam.

Compounds **10b**–**e** were obtained by using the similar synthetic procedure of compound **10a**.

*5.1.11. General procedure for preparation of **11a**–**d***

To a solution of **6** or **7a**–**c** (500 mg) in pyridine (6 mL) was added hydroxylamine hydrochloride (50 mg, 0.72 mmol), the mixture was stirred at room temperature for 4 h at 80 °C. After cooling, it was diluted with ethyl acetate (15 mL), washed with 10% HCl, water and brine, dried over anhydrous sodium sulfate. Filtered, the filtrate was concentrated and chromatographed on silica gel to afford the target compounds.

*5.1.12. Carboxymethyl 3-hydroxyimino-23-hydroxy-lup-20(29)-en-28-oate (**11e**)*

To a solution of **11d** (40 mg, 0.07 mmol) in THF (5 mL) and CH_3OH (3 mL) was added 4 N NaOH (0.5 mL) aqueous solution, the resulting solution was stirred at room temperature for 1 h. The mixture was diluted with ethyl acetate (15 mL), washed with water and brine, dried over anhydrous sodium sulfate. Filtered, the filtrate was concentrated in vacuo. The residue was chromatographed over silica gel (dichloromethane-methanol 40:1) to afford **11e** as a white solid (10 mg, 26.3%).

*5.1.13. General procedure for preparation of **11f**–**g***

To a solution of **5** or **6** (500 mg) in CH_3OH (8 mL) was added corresponding hydrazine compounds (2 equiv.) and sodium acetate (3 equiv.), the mixture was refluxed for 3 h. The reaction mixture was cooled to room temperature and diluted with ethyl acetate (15 mL), the organic layer was 10% HCl, water and brine, dried over anhydrous sodium sulfate. Filtered, the filtrate was concentrated and chromatographed over silica gel to afford the target compounds.

*5.1.14. Benzyl 2-hydroxy-3-oxo-23-t-butyldimethylsilyloxy-lup-1,20(29)-dien-28-oate (**12**)*

To a solution of **4** (1.00 g, 1.48 mmol) in *tert*-butanol (30 mL) was added potassium *tert*-butoxide (0.5 g, 4.45 mmol) and bubbled with O_2 for 6 h at room temperature. The reaction mixture was concentrated under reduced pressure and diluted with ethyl acetate, washed with water and brine, dried over anhydrous sodium sulfate. Filtered, the filtrate was concentrated and chromatographed over silica gel (petroleum ether-ethyl acetate 30:1) to afford **12** as a white solid (860 mg, 84.4%).

*5.1.15. Benzyl 3-oxo-2,23-dihydroxy-lup-1,20(29)-dien-28-oate (**13**)*

To a solution of **12** (800 mg, 1.16 mmol) was dissolved in THF (30 mL) was added 10% HCl (2 mL). The mixture was stirred for 2 h at room temperature. Most of the solvent was evaporated in vacuo, ethyl acetate was added. The organic layer was washed with water and brine, dried over anhydrous sodium sulfate, and concentrated in vacuo. The residue was purified over silica gel (petroleum ether-ethyl acetate 4:1) to give compound **13** as a white solid (570 mg, 85.5%).

*5.1.16. 3-Oxo-2,23-dihydroxy-lup-1,20(29)-dien-28-oic acid (**14**)*

To a solution of **13** (500 mg, 0.87 mmol) in THF (15 mL) was added 10% Pd on carbon (100 mg) at room temperature. The mixture was subjected to 1 atm of H_2 and was monitored by TLC. The mixture was filtered after most of compound **13** was consumed, and the filtrate was concentrated, chromatographed over silica gel (petroleum ether-acetone 5:1) to give **14** as a white solid (300 mg, 71.2%).

*5.1.17. 3-Oxo-2,23-diacetoxy-lup-1,20(29)-dien-28-oic acid (**15**)*

To a solution of **14** (100 mg, 0.21 mmol) in dry pyridine (8 mL) was added acetic anhydride (0.50 mL, 5.29 mmol). The reaction mixture was stirred at room temperature for 12 h. The mixture was then diluted with EtOAc (15 mL), washed with water and brine, dried over Na_2SO_4, and concentrated in vacuo. The crude product was purified by column chromatography (petroleum ether-acetate 6:1) to afford compound **15** as a white amorphous powder (95 mg, 67.7%).

*5.1.18. Benzyl 2,3-dihydroxy-23-t-butyldimethylsilyloxy-lup-20(29)-en-28-oate (**16**)*

To a solution of **12** (800 mg, 1.20 mmol) in THF (10 mL) and CH_3OH (15 mL) cooled at 0 °C was added sodium borohydride (100 mg, 2.7 mmol). The reaction mixture was then stirred at room temperature for 3 h, quenched with water (1 mL) and diluted with EtOAc (20 mL). The organic layer was washed with water and brine, dried over Na_2SO_4, and concentrated in vacuo. The crude product was purified by column chromatography (petroleum ether-acetate 10:1) to afford the product **16** as a white powder (580 mg, 69.8%).

*5.1.19. Benzyl 2,3,23-trihydroxy-lup-20(29)-en-28-oate (**17**)*

To a solution of **16** (550 mg, 0.79 mmol) was dissolved in THF (30 mL), 10% HCl (2 mL) was added and the resulting mixture was stirred at room temperature. After reaction completion, most of the solvent was evaporated in vacuo and diluted with ethyl acetate (30 mL). The organic layer was washed with water and brine, dried over anhydrous sodium sulfate, and concentrated in vacuo. The crude product was purified over silica gel (petroleum ether-acetone 4:1) to give compound **17** as a white solid (310 mg, 67.8%).

*5.1.20. Benzyl 2,3,23-triacetoxy-lup-20(29)-en-28-oate (**18**)*

To a solution of **17** (250 mg, 0.43 mmol) in dry pyridine (10 mL) was added acetic anhydride (0.8 mL, 8.46 mmol). The reaction mixture was stirred at room temperature for 12 h. The mixture was diluted with EtOAc (25 mL), washed with 10% HCl and brine, dried over Na_2SO_4, and concentrated in vacuo. The crude product was purified by column chromatography (petroleum ether-acetate 7:1) to afford the product **18** as a white amorphous powder (220 mg, 72.6%).

*5.1.21. 2,3,23-Triacetoxy-lup-20(29)-en-28-oic acid (**19**)*

To a solution of **18** (150 mg, 0.21 mmol) in THF (10 mL) was added 10% Pd on carbon (30 mg) at room temperature. The mixture was subjected to 1 atm of H_2 and was monitored by TLC. The mixture was filtered after most of compound **18** was consumed, and the filtrate was concentrated, chromatographed over silica gel (petroleum ether-acetone 5:1) to give **19** as a white solid (80 mg, 62.0%).

*5.1.22. 2,3,23-Diacetoxy-lup-20(29)-en-28-oic acid (**20**)*

To a solution of **19** (50 mg, 0.081 mmol) in THF (5 mL) and CH_3OH (3 mL) was added 4 N NaOH (1 mL) aqueous solution, the resulting solution was stirred at room temperature for 2 h. The mixture was diluted with ethyl acetate (15 mL), washed with water and brine, dried over anhydrous sodium sulfate. Filtered, the filtrate was concentrated and chromatographed over silica gel (dichloromethane-methanol 40:1) to afford **20** as a white solid (16 mg, 40.8%).

*5.1.23. Benzyl 2-hydroxymethylene-3-oxo-23-t-butyldimethylsilyloxy-lup-20(29)-en -28-oate (**21**)*

To a solution of **4** (1.50 g, 2.22 mmol) in dry dichloromethane (30 mL) was added sodium methoxide (300 mg, 5.55 mmol) and ethyl formate (1.5 mL), the mixture was stirred for 16 h at room temperature, then diluted with ethyl acetate (40 mL). The organic layer was washed with water and brine, dried over Na_2SO_4, and concentrated in vacuo. The crude product was purified by column chromatography (petroleum ether-acetate 6:1) to afford the product **21** as a white powder. The crude product can be also used for the next step without further purification.

*5.1.24. Benzyl 2-hydroxymethyl-3-hydroxy-23-t-butyldimethylsilyloxy-lup-20(29)-en-28-oate (**22**)*

To a solution of **21** (600 mg, 0.85 mmol) was dissolved in THF (30 mL), 10% HCl (2 mL) was added. After reaction completion, most of the solvent was evaporated in vacuo and diluted with ethyl acetate (30 mL). The mixture was washed with water and brine, dried over anhydrous sodium sulfate, and concentrated in vacuo. The residue was purified over silica gel (petroleum ether-acetone 4:1) to give compound **22** as a white solid (343 mg, 68.6%).

*5.1.25. Benzyl 2-hydroxymethyl-3,23-dihydroxy-lup-20(29)-en-28-oate (**23**)*

To a solution of **22** (600 mg, 1.02 mmol) in THF (15 mL) and CH_3OH (10 mL) cooled at 0 °C was added sodium borohydride (150 mg, 3.96 mmol). The reaction mixture was stirred at room temperature for 3 h. The mixture was then quenched with water (1 mL) and diluted with EtOAc (30 mL), washed with water and brine, dried over Na_2SO_4, and concentrated in vacuo. The crude product was purified by column chromatography (petroleum ether-acetone 2:1) to afford compound **23** as a white powder (370 mg, 61.2%).

*5.1.26. Benzyl 2-acetoxymethyl-3,23-diacetoxy-lup-20(29)-en-28-oate (**24**)*

To a solution of **23** (280 mg, 0.47 mmol) in dry pyridine (10 mL) was added acetic anhydride (0.8 mL). The reaction mixture was stirred at 60 °C for 8 h. The mixture was then diluted with EtOAc (25 mL), washed 10% HCl, water and brine, dried over Na_2SO_4, and concentrated in vacuo. The crude product was purified by column chromatography (petroleum ether-acetate 5:1) to afford the product **24** as a white amorphous powder (220 mg, 75.7%).

*5.1.27. 2-Acetoxymethyl-3,23-diacetoxy-lup-20(29)-en-28-oic acid (**25**)*

To a solution of **24** (150 mg, 0.24 mmol) in THF (10 mL) was added 10% Pd on carbon (30 mg) at room temperature. The mixture was subjected to 1 atm of H_2 and was monitored by TLC. The mixture was filtered when most of compound **24** was consumed, and the filtrate was concentrated, chromatographed over silica gel (petroleum ether-ethyl acetate 3:1) to give **25** as a white solid (70 mg, 51.6%).

*5.1.28. 2-Hydroxymethyl-3,23-dihydroxy-lup-20(29)-en-28-oic acid (**26**)*

To a solution of **25** (50 mg, 0.077 mmol) in THF (5 mL) and CH_3OH (3 mL) was added 4 N NaOH (1 mL) aqueous solution, the resulting solution was stirred at room temperature for 2 h. The mixture was then diluted with ethyl acetate (15 mL), washed with water and brine, dried over anhydrous sodium sulfate. Filtered, the filtrate was concentrated and chromatographed over silica gel (dichloromethane-methanol 30:1) to afford **26** as a white solid (21 mg, 54.3%).

5.2. In vitro anti-proliferative activity

5.2.1. Cell lines and culture conditions

HL-60 (human leukemia), BEL-7402 (human hepatoma) and SF-763 (human cerebroma) originally obtained from American type of cell culture collection (ATCC), USA and stock was maintained in laboratory; B16 (mice melanoma) and HeLa (human cervical adenocarcinoma) were obtained from Chinese Academy of Sciences Committee Type Culture Collection. All cell lines were cultured in RPMI 1640 (Gibco) containing 10% fetal bovine serum (Gibco) and 1% penicillin streptomycin (Gibco) at 37 °C in the presence of 5% CO_2.

5.2.2. Antiproliferative assay

The cytotoxic activity of the compounds was determined using MTT assay. Cells were seeded in 96-well micro culture plates incubated for 24 h at 37 °C in CO_2 incubator. All of the reported betulinic derivatives were dissolved in DMSO while the positive control doxorubicin was dissolved in PBS. These tested compounds at different concentrations were added into wells and cells were treated at 37 °C for 72 h. Then MTT (5 mg/mL, in PBS) was added into each well and cultured for another 4 h. The optical density was detected in a microplate reader at 570 nm. IC_{50} values were calculated according to the dose-dependent curves [21].

5.3. In vivo antitumor assays

Institute of Cancer Research (ICR) female mice with body weight of 18–22 g was transplanted with H22 and B16 cell subcutaneously into the right oxter according to protocols of tumor transplant research. After 7 d of tumor transplantation, mice were weighed, and each model group was at random divided into 5 groups, each of which had 10 mice in H22 and B16 group. The groups with 23-hydroxybetulinic acid (HBA), **6** and **10e** were administered intraperitoneally 30 mg/kg in a vehicle of 1% DMSO/2% poloxamer/97% saline, respectively. The positive control group was treated with cyclophosphamide (30 mg/kg) in H22 group and 5-fluorouracil (30 mg/kg) in B16 group through intravenous injection in a vehicle of 1% DMSO/2% poloxamer/97% saline. The negative control group received 0.9% normal saline through intravenous injection. All of the test compounds were given through injections after 7 days of tumor transplantation (or inoculation). Treatments were done at a frequency of intravenous or intraperitoneal injection one dose per day for a total of 25 consecutive days. After the treatments, all of the mice were killed and weighed simultaneously, and then tumors were segregated and weighed [21]. Tumor inhibitory ratio was calculated by the following formula and perform *T* test:

$$\text{Tumor inhibitory ratio (\%)} = (1 - \text{average tumor weight of treated group} / \text{average tumor weight of control group}) \times 100\%$$

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.ejmech.2014.09.058.

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