Synthesis and Antitumor Activity of 17-carboxylic acid Modified Amide Derivatives of 23-hydroxy betulinic acid

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Abstract: A novel series of 17-carboxylic acid modified amide derivatives of 23-hydroxy betulinic acid (1) were prepared and tested in vitro against five cell lines: A549 (human lung carcinoma), BEL-7402 (human hepatoma), SF-763 (human cerebroma), B16 (mice melanoma) and HL-60 (human leukaemia). Within this series of compounds, 4a (IC₅₀=21.08 µM in SF-763, IC₅₀=21.63 µM in HL-60), 4b (IC₅₀=28.45 µM in HL-60), 6g (IC₅₀=26.09 µM in BEL-7402) and 6g (IC₅₀=22.65 µM in HL-60) have the more potent cytotoxic activity than lead compound 1. The preliminary structure-activity relationship analysis of the C-28 amide derivatives is also discussed.

Keywords: 23-hydroxy betulinic acid; amide derivatives; structure modification; antitumor activity; structure-activity relationship.

1. Introduction

23-Hydroxy betulinic acid (1) and betulinic acid (2) have recently attracted much attention due to their antitumor activity in different cell lines in pentacyclic triterpenes kingdom [1-2]. Although many other biological activities have been reported such as antitumor, antiviral, antioxidant and so on, most research focus on their antitumor activity and have synthesized plenty of derivatives especially betulinic acid. As a good lead compound, betulinic acid showed potent antitumor activity in a series of cell lines and the mechanism of which might be related to the proliferation, migration, cell cycle and apoptosis of tumor cells [3-10]. 23-Hydroxy betulinic acid has the similar chemical structure to
betulinic acid, so we can use the experience of modification of betulinic acid to design and synthesize new 23-hydroxy betulinic acid derivatives.

In our previous study, several 23-hydroxy betulinic acid derivatives have showed more potent antitumor activity than betulinic acid and 23-hydroxy betulinic acid in different cell lines in vitro, especially compound 3 has the most potent cytotoxic activity. Preliminary structure-activity relationship displayed that the polarity and length of the chain on C-28 had an important impact on the antitumor activity [11,12]. These results motivated us to design and synthesis novel derivatives modified on 17-carboxylic acid moiety of 23-hydroxy betulinic acid.

![Figure 1. Structure of 23-hydroxy betulinic acid (1), betulinic acid (2) and the derivative of betulinic acid (3)](image)

In this paper, we report a series of new 17-carboxylic acid modified amide derivatives of 23-hydroxy betulinic acid and their antitumor activity. The preliminary structure-activity relationship is also discussed.

2. Materials and Methods

2.1. General

Melting points were obtained on a MEL-TEMP II melting-point apparatus and are uncorrected. IR were determined on the Nicolet Impact 410 or Bruker FT-IR TENSOR27 instrument. 1H-NMR spectra were recorded on a BRUKER-ACF-300 or BRUKER-ACF-500 instrument (chemical shifts are expressed as δ values relative to TMS as internal standard). ESI were recorded on an HP 1100 LC/MSD spectrometer. HR-MS were obtained using a Agilent QTOF 6520 instrument.

2.2. Synthesis

2.2.1 General procedure for synthesis of 4a-f

Ac2O (0.75mL, 7.5mmol) was added to a solution of 23-hydroxy betulinic acid (600.0mg, 1.25mmol) in dry pyridine(25mL). The mixture was stirred for a night at room temperature. After adding EtOAc (20mL), the mixture was washed with 9% HCl (30mL×3) and brine (30mL×3), dried over anhydrous Na2SO4, filtered, and evaporated to dryness. The residue was purified by crystallization in EtOAc to afford the desired ester compound as yellow powder (680.0mg, 98%).

(COCl)2 (0.1mL) was added to a solution of ester compound (500.0mg, 0.90mmol) in dry CH2Cl2 (10mL). The mixture was stirred for 4h at room temperature, evaporated to dryness, and soluted in CH2Cl2 (10mL). The solution was added dropwise to the mixture of corresponding H2NCHR1COOCH3.HCl (2 equiv) and DMAP (85.0mg, 0.696mmol) in CH2Cl2 (20mL) and stirred for 8h at the room temperature and concentrated to dryness. The mixture was diluted with EtOAc (30mL) and filtered. The filtrate was washed with 9% HCl(20mL×2), H2O(20mL×2), brine(20mL×2), dried
23-hydroxy betulinic acid

over anhydrous Na$_2$SO$_4$, filtered, and concentrated to dryness. The residue was purified by column chromatography on silica gel to afford compound 4a-f.

Figure 2. Synthesis of 23-hydroxy betulinic acid amide derivatives

Reagents and conditions: (i) Ac$_2$O, pyridine/rt/12h, then (COCl)$_2$, CH$_2$Cl$_2$/rt/4h, then H$_2$NCHR$_1$COOCH$_3$·HCl, DMAP/rt/8h; (ii) 4N NaOH, CH$_3$OH, THF/reflux/4h; (iii) H$_2$NCHR$_2$COOCH$_3$·HCl, EDC, HOBT/rt/8h; (iv) 4N NaOH, CH$_3$OH, THF/reflux/4h.

Methyl N-[3β,23-diacetoxy-20(29)-en-28-oyl]-glycinate (4a): H$_2$NCH$_2$COOCH$_3$·HCl (217.0mg, 1.740mmol), column chromatography with petroleum ether/acetone = 5:1 (v:v). Yield: 338.0mg (62%) as a white solid, mp 109-111°C. IR (KBr, cm$^{-1}$) ν 3431, 2938, 2868, 1740, 1636, 1445, 1374, 1245, 1202, 1037, 427. $^1$H-NMR (CDCl$_3$, 500MHz) δ 0.80, 0.86, 0.93, 0.96, 1.69 (s, 3H each, 24, 25, 26, 27 and 30-CH$_3$), 2.01, 2.06 (s, 3H each, 3 and 23-OCOCH$_3$), 3.09 (m, 1H, 19-CH), 3.68, 3.84 (2H, dd, $J_A=J_B=11.6$ Hz, $J_{AB}=78.1$ Hz, 23-CH$_2$), 3.76 (s, 3H, 17-CONHCH$_2$COOCH$_3$), 4.00 (t, 2H, 17-CONHCH$_2$COOCH$_3$), 4.59, 4.73 (d, 2H, $J=68.3$ Hz, 29=CH$_2$), 4.75 (m, 1H, 3-CH), 6.05 (t, 1H, 28-CONH). MS (EI): m/z [M+H]$^+$ 628.3, [M+Na]$^+$ 650.4, [M+K]$^+$ 666.3. HR-MS (ESI, M+H) m/z: calcd for C$_{37}$H$_{57}$NO$_7$ 628.4213, found 628.4217.

Methyl N-[3β,23-diacetoxy-20(29)-en-28-oyl]-L-alaninate (4b): L-H$_2$NCH(CH$_3$)COOCH$_3$·HCl (233.0mg, 1.740mmol), column chromatography with petroleum ether/acetone = 5:1 (v:v). Yield: 302.0mg (55%) as a white solid, mp 180-182°C. IR (KBr, cm$^{-1}$) ν 3454, 3413, 2946, 2872, 2359, 1744, 1666, 1495, 1371, 1240, 1038, 887. $^1$H-NMR (CDCl$_3$, 300MHz) δ 0.80, 0.87, 0.91, 0.96, 1.69 (s,
Methyl N-[3β,23-diacetoxylup-20(29)-en-28-oyl]-L-leucinate (4c):
L-H₂NCH(2H)CH(2H)CH₂COOCH₃·HCl (315.0mg, 1.740mmol), column chromatography with petroleum ether/acetone = 5:1 (v:v). Yield: 315.0mg (53%) as a white solid, mp 108-111°C IR (KBr, cm⁻¹) ν 3417, 2953, 2870, 1743, 1664, 1510, 1469, 1371, 1246, 1163, 1037, 882. ¹H-NMR (CDCl₃, 300MHz) δ 0.80, 0.88, 0.89, 0.93, 1.69 (s, 3H each, 24, 25, 26, 27and 30-CH₃), 2.01, 2.05 (s, 3H each, 3 and 23-OCOCH₃), 3.10 (m, 1H, 19-CH), 3.68, 3.84 (2H, dd, Jₐ=Jₜ=11.6 Hz, Jₐ=48.6 Hz, 23-CH₂), 3.75 (s, 3H, 28-CONHCH(CH₃)COOCH₃), 4.56 (m, 1H, 28-CONHCH(CH₃)COOCH₃), 4.59, 4.72 (d, 2H, Jₐ=38.8 Hz, 29=CH₂), 4.74 (m, 1H, 3-CH), 6.10 (d, 1H, J=7.06, 28-CONH). MS (EI): m/z [M+H]⁺ 642.3, [M+Na]⁺ 664.3, [M+K]⁺ 680.3. HR-MS (ESI, M+H) m/z: caled for C₃₈H₅₉NO₂ 642.4370, found 642.4374.

Methyl N-[3β,23-diacetoxylup-20(29)-en-28-oyl]-L-phenylalaninate (4d):
L-H₂NCH(2H)CH(2H)CH₂COOCH₃·HCl (374.0mg, 1.740mmol), column chromatography with petroleum ether/acetone = 5:1 (v:v). Yield: 408.0mg (67%) as a white solid, mp 98-100°C. IR (KBr, cm⁻¹) ν 3446, 3066, 2948, 2869, 1740, 1663, 1503, 1449, 1370, 1246, 1037, 882, 700. ¹H-NMR (CDCl₃, 300MHz) δ 0.80, 0.86, 0.89, 0.93, 1.69 (s, 3H each, 24, 25, 26, 27 and 30-CH₃), 2.01, 2.05 (s, 3H each, 3 and 23-OCOCH₃), 3.03 (m, 2H, 28-CONHCH(CH₂CH₃)COOCH₃), 3.14 (m, 1H, 19-CH), 3.67, 3.86 (2H, dd, Jₐ=Jₜ=11.6 Hz, Jₐ=56.8 Hz, 23-CH₂), 3.72 (s, 3H, 28-CONHCH(CH₂CH₃)COOCH₃), 4.57, 4.69 (d, 2H, Jₐ=36.8 Hz, 29=CH₂), 4.75 (m, 2H, 3-CH and 28-CONHCH(CH₂CH₃)COOCH₃), 5.86 (d, 1H, J=7.7 Hz, 28-CONH). MS (EI): m/z [M+H]⁺ 718.4. HR-MS (ESI, M+H) m/z: caled for C₄₄H₅₈NO₄ 718.4683, found 718.4679.

Methyl N-[3β,23-diacetoxylup-20(29)-en-28-oyl]-L-lysinate (4e):
L-H₂NCH(2H)CH(2H)CH₂COOCH₃·HCl (291.0mg, 1.740mmol), column chromatography with petroleum ether/acetone = 5:1 (v:v). Yield: 297.0mg (51%) as a white solid, mp 108-110°C. IR (KBr, cm⁻¹) ν 3447, 2954, 2870, 1741, 1667, 1501, 1468, 1371, 1245, 1151, 1038, 882. ¹H-NMR (CDCl₃, 300MHz) δ 0.80, 0.87, 0.92, 0.97, 1.67 (s, 3H each, 24, 25, 26, 27 and 30-CH₃), 2.01, 2.06 (s, 3H each, 3 and 23-OCOCH₃), 3.10 (m, 1H, 19-CH), 3.68, 3.83 (2H, dd, Jₐ=Jₜ=11.3 Hz, Jₐ=45.8 Hz, 23-CH₂), 3.72 (s, 3H, 28-CONHCH(CH₂CH₃)COOCH₃), 4.57 (m, 1H, 28-CONHCH(CH₂CH₃)COOCH₃), 4.59, 4.72 (d, 2H, Jₐ=38.00 Hz, 29=CH₂), 4.76 (m, 1H, 3-CH), 6.04 (d, 1H, J=8.3 Hz, 28-CONH). MS (EI): m/z [M+H]⁺ 670.4, [M+Na]⁺ 692.4. HR-MS (ESI, M+H) m/z: caled for C₄₄H₅₈NO₄ 670.4683, found 670.4685.

Dimethyl N-[3β,23-diacetoxylup-20(29)-en-28-oyl]-L-glutamate (4f):
L-H₂NCH(2H)CH(2H)CH₂COOCH₃·HCl (367.0mg, 1.740mmol), column chromatography with petroleum ether/acetone = 3:1 (v:v). Yield: 335.0mg (54%) as a white solid, mp 82-84°C. IR (KBr, cm⁻¹) ν 3410, 2949, 2870, 1741, 1666, 1509, 1444, 1372, 1246, 1037, 883. ¹H-NMR (CDCl₃, 300MHz) δ 0.81, 0.88, 0.92, 0.97, 1.68 (s, 3H each, 24, 25, 26, 27 and 30-CH₃), 2.01, 2.06 (s, 3H each, 3 and 23-OCOCH₃), 3.07 (m, 1H, 19-CH), 3.72 (m, 7H, one of 23-CH₂ and 28-CONHCH(CH₂CH₃)COOCH₃), 3.85 (1H, d, J=11.7 Hz, 23-CH₂), 3.72 (s, 3H, 28-CONHCH(CH₂CH₃)COOCH₃), 4.56 (m, 1H, 28-CONHCH(CH₂CH₃)COOCH₃), 4.59, 4.72 (d, 2H, Jₐ=39.6 Hz, 29=CH₂), 4.74 (m, 1H, 3-CH), 6.28 (d, 1H, Jₐ=7.6 Hz, 28-CONH). MS (EI): m/z [M+H]⁺ 714.6, [M+Na]⁺ 736.6, [M+K]⁺ 752.6. HR-MS (ESI, M+H) m/z: caled for C₃₉H₅₃NO₇ 714.4581, found 714.4586.
2.2.2. General procedure for synthesis of 5a-f

Compound 4 (0.30mmol) was dissolved in CH₂OH (4mL) and THF (10mL). 4N NaOH (4mL) was added to the solution and refluxed for 4h. 9% HCl was added to the mixture until the white solid appeared, filtered, and the insoluble substance was washed with H₂O. The residue was dried to obtain the corresponding 5a-f.

N-[3β,23-dihydroxylup-20(29)-en-28-oyl]-glycine (5a): 4a (188.0mg, 0.30mmol). Yield: 133.0mg (84%) as a white solid, mp 213-215°C. IR (KBr, cm⁻¹) v 3426, 3070, 2941, 2868, 1737, 1640, 1540, 1384, 1243, 1044, 880. ¹H-NMR (DMSO, 300MHz) δ 0.52, 0.77, 0.83, 0.91, 1.62 (s, 3H each, 24, 25, 26, 27and 30-CH₃). 2.97 (m, 1H, 19-CH). 3.06, 3.31 (2H, dd, J₆=10.6 Hz, J₇=14.0 Hz, 28-CH₂), 3.55 (m, 5H, 3-CH, 3-OH, 23-OH, 28-CONHCH₂COOH). 4.53, 4.64 (d, 2H, J₆=30.9 Hz, 29-CH₂). 7.68 (t, 1H, 28-CONHCH₂COOH). MS (EI): m/z [M-H]° 528.3. HR-MS (ESI, M+H) m/z: calcd for C₄₀H₇₃NO₅ 544.4002, found 544.4006.

N-[3β,23-dihydroxylup-20(29)-en-28-oyl]-L-alanine (5b): 4b (192.0mg, 0.30mmol). Yield: 132.0mg (81%) as a white solid, mp 179-181°C. IR (KBr, cm⁻¹) v 3424, 2942, 2869, 1722, 1636, 1513, 1451, 1384, 1298, 1196, 1043, 884. ¹H-NMR (DMSO, 300MHz) δ 0.51, 0.77, 0.83, 0.91, 1.62 (s, 3H each, 24, 25, 26, 27and 30-CH₃). 3.10 (m, 2H, 19-CH and one of 23-CH₃), 3.39 (m, 2H, 3-CH and one of 23-CH₃), 4.13 (m, 2H, 3-CH, 28-CONHCH₂(CH₃)COOH). 4.32 (t, 1H, 23-OH). 4.52, 4.63 (d, 2H, J₆=33.9 Hz, 29-CH₂). 7.68 (d, 1H, J₆=7.2 Hz, 28-CONHCH₂(CH₃)COOH). MS (EI): m/z [M+Na]° 524.3. HR-MS (ESI, M+Na) m/z: calcd for C₃₉H₇₂NO₅ 544.4002, found 544.4006.

N-[3β,23-dihydroxylup-20(29)-en-28-oyl]-L-leucine (5c): 4c (205.0mg, 0.30mmol). Yield: 132.0mg (75%) as a white solid, mp 198-200°C. IR (KBr, cm⁻¹) v 3447, 2949, 2872, 1720, 1638, 1524, 1446, 1383, 1196, 1045, 882. ¹H-NMR (DMSO, 300MHz) δ 0.50, 0.77, 0.82, 0.90, 1.62 (s, 3H each, 24, 25, 26, 27and 30-CH₃). 3.00 (m, 2H, 19-CH and one of 23-CH₃), 3.39 (m, 2H, 3-CH and one of 23-CH₃), 4.10 (d, 1H, J₆=4.9 Hz, 3-OH), 4.24 (m, 1H, 28-CONHCH₂(CH₃)CH₂COOH), 4.34 (t, 1H, 23-OH). 4.52, 4.63 (d, 2H, J₆=33.4 Hz, 29=CH₂), 7.66 (d, 1H, J₆=8.2 Hz, 28-CONHCH₂(CH₃)CH₂COOH). MS (EI): m/z [M-H]° 586.4, [M+Na]° 608.4. HR-MS (ESI, M+H) m/z: calcd for C₃₉H₇₂NO₅ 586.4471, found 586.4468.

N-[3β,23-dihydroxylup-20(29)-en-28-oyl]-L-phenylalanine (5d): 4d (215.0mg, 0.30mmol). Yield: 152.0mg (82%) as a white solid, mp 180-182°C. IR (KBr, cm⁻¹) v 3425, 2942, 2869, 1718, 1639, 1522, 1451, 1386, 1199, 1044, 880, 699. ¹H-NMR (DMSO, 300MHz) δ 0.47, 0.52, 0.72, 0.81, 1.59 (s, 3H each, 24, 25, 26, 27and 30-CH₃). 2.98 (m, 4H, 19-CH and one of 23-CH₂ and 28-CONHCH₂(CH₃)H₂COOH), 3.36 (m, 2H, 3-CH and one of 23-CH₃), 4.08 (d, 1H, J₆=4.8 Hz, 3-OH), 4.31 (t, 1H, 23-OH). 4.41 (m, 1H, 28-CONHCH₂(CH₃)H₂COOH), 4.49, 4.60 (d, 2H, J₆=30.8 Hz, 29=CH₂). 7.19 (m, 5H, 28-CONHCH₂(CH₃)H₂COOH), 7.69 (d, 1H, J₆=8.8 Hz, 28-CONHCH₂(CH₃)H₂COOH). MS (EI): m/z [M-H]° 620.4, [M+Na]° 642.4. HR-MS (ESI, M+H) m/z: calcd for C₃₉H₇₂NO₅ 620.4315, found 620.4319.

N-[3β,23-dihydroxylup-20(29)-en-28-oyl]-L-valine (5e): 4e (201.0mg, 0.30mmol). Yield: 140.0mg (82%) as a white solid, mp 175-177°C. IR (KBr, cm⁻¹) v 3429, 3177, 2943, 2870, 1727, 1640, 1500, 1460, 1384, 1196, 1043, 882. ¹H-NMR (DMSO, 300MHz) δ 0.50, 0.77, 0.82, 0.90, 1.62 (s, 3H each, 24, 25, 26, 27and 30-CH₃). 2.98 (m, 2H, 19-CH and one of 23-CH₃), 3.41 (m, 2H, 3-CH and one of 23-CH₃), 4.06 (m, 2H, 3-OH and 28-CONHCH₂(CH₃)H₂COOH), 4.35 (t, 1H, 23-OH), 4.52, 4.63 (d, 2H, J₆=32.9 Hz, 29=CH₂), 7.40 (d, 1H, J₆=8.4 Hz, 28-CONHCH₂(CH₃)H₂COOH),
12.35 (s, 1H, 28-CONHCH(CH(2)CH3)COOH). MS (EI): m/z [M+H]+ 572.3, [M+Na]+ 594.3. HR-MS (ESI, M+H) m/z: calcd for C30H35NO7 572.4315, found 572.4313.

**N-[3β,23-dihydroxy-yl-20(29)-en-28-oyl]-L-glutamic acid (5f):** 4f (214.0mg, 0.30mmol). Yield: 128.0mg (71%) as a white solid, mp 188-190°C. IR (KBr, cm-1) ν 3447, 2944, 2870, 1717, 1640, 1512, 1450, 1386, 1198, 1041, 884. 1H-NMR (DMSO, 300MHz) δ 0.51, 0.77, 0.83, 0.91, 1.62 (s, 3H each, 24, 25, 26, 27 and 30-CH3), 2.98 (m, 2H, 19-CH and one of 23-CH2), 3.40 (m, 2H, 3-CH and one of 23-CH2), 4.14 (m, 2H, 3-OH and 28-CONHCH(CH2CH2COOH)COOH), 4.33 (t, 1H, 23-CH2), 4.52, 4.63 (d, 2H, J=33.4 Hz, 29=CH), 7.66 (d, 1H, J=7.7 Hz, 28-CONHCH(CH2CH2COOH)COOH). MS (EI): m/z [M+H]+ 602.4, [M+Na]+ 624.4, [M+K]+ 640.3. HR-MS (ESI, M+H) m/z: calcd for C36H57NO7 616.4213, found 616.4211.

**2.2.3. General procedure for synthesis of 6a-g**

Corresponding compound 5 (0.20mmol) in CH2Cl2 (10mL) was added to H2NCHR′COOCH3.HCl (1.2equiv) in CH2Cl2 (10mL) in the absence of EDC (0.40mmol) and HOBT (0.50mmol). The mixture was stirred for 8h at the room temperature and washed with H2O (20mL×2), brine (20mL×2), dried over anhydrous Na2SO4, filtered, and concentrated to dryness. The residue was purified by column chromatography on silica gel to afford compound 6a-g.

**Methyl N′-[N-[3β,23-dihydroxy-yl-20(29)-en-28-oyl]-2-aminoethanoyl]-glucinate (6a):** 5a (106.0mg, 0.20mmol), H2NCH2COOCH3.HCl (30.0mg, 0.24mmol). column chromatography with petroleum ether /acetone = 2:1 (v:v). Yield: 96.0mg (80%) as a white solid, mp213-215°C. IR (KBr, cm-1) ν 3417, 3072, 2968, 1749, 1526, 1444, 1376, 1211, 1043, 883, 567. 1H-NMR (CDCl3, 300MHz) δ 0.69, 0.85, 0.88, 0.96, 1.68 (s, 3H each, 24, 25, 26, 27 and 30-CH3), 3.08 (m, 1H, 19-CH), 3.40, 3.70 (2H, dd, J=10.3 Hz, J=89.0 Hz, 23-CH2), 3.61 (m, 1H, 3-CH), 3.75 (s, 3H, 28-CONHCH2CONHCH2COOCH3), 4.00 (m, 2H, 28-CONHCH2CONHCH2COOCH3 and 28-CONHCH2CONHCH2COOCH3), 4.59, 4.73 (d, 2H, J=40.9 Hz, 29=CH2), 6.33 (t, 1H, 28-CONHCH2CONHCH2COOCH3), 6.66 (t, 1H, 28-CONHCH2CONHCH2COOCH3). MS (EI): m/z [M+H]+ 601.3, [M+Na]+ 623.4. HR-MS (ESI, M+H) m/z: calcd for C36H55N2O6 601.4217, found 601.4214.

**Methyl N′-[N-[3β,23-dihydroxy-yl-20(29)-en-28-oyl]-2-aminoethanoyl]-L-phenylalaninate (6b):** 5a (106.0mg, 0.20mmol), H2NCH(CH2CH2H)COOCH3.HCl (52.0mg, 0.24mmol). column chromatography with petroleum ether /acetone =1:1 (v:v). Yield: 105.0mg (76%) as a white solid, mp182-184°C. IR (KBr, cm-1) ν 3474, 3419, 2953, 2866, 1747, 1638, 1502, 1452, 1383, 1045, 882, 699, 616. 1H-NMR (CDCl3, 300MHz) δ 0.68, 0.85, 0.88, 0.95, 1.67 (s, 3H each, 24, 25, 26, 27and 30-CH3), 3.12 (m, 3H, 19-CH and 28-CONHCH2CONHCH2CH2CH2H), 3.40 (1H, d, J=10.3 Hz, one of 23-CH3), 3.65 (m, 2H, 3-CH and one of 23-CH3), 3.72 (s, 3H, 28-CONHCH2CONHCH2CH2CH2H), 3.89 (m, 2H, 28-CONHCH2CONHCH2CH2CH2H), 4.59, 4.72 (d, 2H, J=41.1 Hz, 29=CH2), 4.85 (m, 1H, 28-CONHCH2CONHCH2CH2CH2H), 6.17 (t, 1H, 28-CONHCH2CONHCH2CH2CH2H), 6.27 (d, 1H, J=7.5 Hz, 28-CONHCH2CONHCH2CH2CH2H), 7.19 (m, 5H, 28-CONHCH2CONHCH2CH2H). MS (EI): m/z [M+H]+ 691.3, [M+Na]+ 713.3, [M+K]+ 729.3. HR-MS (ESI, M+H) m/z: calcd for C46H62N2O6 691.4686, found 691.4683.

**Methyl N′-[N-[3β,23-dihydroxy-yl-20(29)-en-28-oyl]-2-aminoethanoyl]-L-leucinate (6c):** 5a (106.0mg, 0.20mmol), H2NCH(CH2CH2CH2CH2CH2H)COOCH3.HCl (40.0mg, 0.24mmol). column chromatography with petroleum ether /acetone =1:1 (v:v). Yield: 96.0mg (73%) as a white solid,
Methyl N'-[3β,23-dihydroxylup-20(29)-en-28-oyl]-2-amino-2-benzyl-ethanoyl]-L-leucinate (6d): 5d (124.0mg, 0.20mmol), H₂NCH₂CH₂CH₂CH₂CH₂COOCH₃, HCl (40.0mg, 0.24mmol). column chromatography with petroleum ether /EtOAc =1:2 (v:v). Yield: 103.0mg (69%) as a white solid, mp146-148°C. IR (KBr, cm⁻¹) ν 3104, 2981, 1655, 1497, 1369, 1202, 1149, 1046, 879, 695. ¹H-NMR (DMSO, 300MHz) δ 0.44, 0.51, 0.72, 0.79, 1.58 (s, 3H each, 24, 25, 26, 27 and 30-CH₃), 2.95 (m, 4H, 19-CH and one of 23-CH₂), 3.37 (2H, m, 3-CH and one of 23-CH₂), 3.62 (s, 3H, 28-CONHCH₂CH₂CH₂COOCH₃), 4.04 (br, 1H, 3-CH₂), 4.33 (m, 2H, 23-OH and 28-CONHCH₂CH₂CH₂COOCH₃), 4.49 (m, 1H, 28-CONHCH₂CH₂CH₂COOCH₃), 4.9 (m, 2H, 28-CONHCH₂CH₂CH₂COOCH₃), 7.63 (d, 1H, J=8.5 Hz, 28-CONHCH₂CH₂CH₂COOCH₃), 7.98 (d, 1H, J=7.9 Hz, 28-CONHCH₂CH₂CH₂COOCH₃), MS (EI): m/z [M+Na]⁺ 769.5. HR-MS (ESI, M+H) m/z: cale for C₃₉H₄₃N₂O₈ 747.5312, found 747.5314.

Methyl N'-[3β,23-dihydroxylup-20(29)-en-28-oyl]-2-amino-2-benzyl-ethanoyl]-glucinate (6e): 5d (124.0mg, 0.20mmol), H₂NCH₂COOCH₃, HCl (30.0mg, 0.24mmol). column chromatography with petroleum ether / acetone =1:1 (v:v). Yield: 106.0mg (77%) as a white solid, mp135-137°C. IR (KBr, cm⁻¹) ν 3424, 2943, 2867, 1751, 1664, 1512, 1497, 1452, 1374, 1206, 1046, 882, 702. ¹H-NMR (DMSO, 300MHz) δ 0.42, 0.51, 0.72, 0.78, 1.58 (s, 3H each, 24, 25, 26, 27 and 30-CH₃), 2.86 (m, 2H, 28-CONHCH₂CH₂CH₂COOCH₃), 3.05 (m, 2H, 19-CH and one of 23-CH₃), 3.36 (m, 2H, 23-OH and one of 23-CH₃), 3.63 (s, 3H, 28-CONHCH₂CH₂CH₂COOCH₃), 3.73 (m, 2H, 28-CONHCH₂CH₂CH₂COOCH₃), 4.08 (d, 1H, J=4.8 Hz, 3-CH₂), 4.31 (t, 1H, 23-OH), 4.52 (m, 1H, 28-CONHCH₂CH₂CH₂COOCH₃), 4.9 (m, 2H, 28-CONHCH₂CH₂CH₂COOCH₃), 7.20 (m, 5H, 28-CONHCH₂CH₂CH₂COOCH₃), 7.65 (d, 1H, J=8.3 Hz, 28-CONHCH₂CH₂CH₂COOCH₃), 8.11 (t, 1H, 28-CONHCH₂CH₂CH₂COOCH₃), MS (EI): m/z [M+H]⁺ 691.3, [M+Na]⁺ 713.4, [M+K]⁺ 729.4. HR-MS (ESI, M+H) m/z: cale for C₃₉H₄₃N₂O₈ 747.5312, found 747.5314.

Methyl N'-[3β,23-dihydroxylup-20(29)-en-28-oyl]-2-amino-2-(2'-isobutyl)-ethanoyl]-glucinate (6f): 5e (117.0mg, 0.20mmol), H₂NCH₂COOCH₃, HCl (30.0mg, 0.24mmol). column chromatography with petroleum ether / acetone =3:2 (v:v). Yield: 93.0mg (71%) as a white solid, mp160-162°C. IR (KBr, cm⁻¹) ν 3415, 2950, 2873, 1756, 1636, 1520, 1466, 1383, 1207, 1045, 886.¹H-NMR (DMSO, 300MHz) δ 0.50, 0.77, 0.82, 0.85, 1.62 (s, 3H each, 24, 25, 26, 27 and 30-CH₃), 3.02 (2H, m, 3-CH and one of 23-CH₂), 3.40 (2H, m, 3-CH and one of 23-CH₂), 3.60 (s, 3H, 28-CONHCH₂CH₂CH₂COOCH₃), 3.83 (m, 2H, 28-CONHCH₂CH₂CH₂COOCH₃), 4.10 (d, 1H, J=4.8 Hz, 3-CH₂), 4.34 (m, 2H, 23-OH and 28-CONHCH₂CH₂CH₂COOCH₃), 4.52, 4.64 (d, 2H, J=34.9 Hz, 29=CH₂), 7.54 (d, 1H, J=8.5 Hz, 28-CONHCH₂CH₂CH₂COOCH₃), 8.00 (t, 1H, 28-CONHCH₂CH₂CH₂COOCH₃), MS (EI): m/z [M+H]⁺ 657.5, [M+Na]⁺ 679.5, [M+K]⁺ 695.5. HR-MS (ESI, M+H) m/z: cale for C₃₉H₄₃N₂O₈ 657.4843, found 657.4848.
Dimethyl N'-[N-3β, 23- dihydroxy]-20(29)-en-28-oyl]-2- amino-2- benzyl-ethanoyl]-L-glutamate (6g): 5d (124.0mg, 0.20mmol), H₂NCH(CH₂CH₂COOCH₃)COOCH₃, HCl (51.0mg, 0.24mmol). column chromatography with petroleum ether / acetone = 2:1(v:v). Yield: 104.0mg (63%) as a white solid, mp 138-140°C. IR (KBr, cm⁻¹) v 3424, 2923, 2852, 1741, 1632, 1554, 1447, 1376, 1217, 1168, 1048, 737. ¹H-NMR (DMSO, 300MHz) δ 6.52, 0.78, 0.81, 0.84, 0.98 (s, 3H each, 24, 25, 26, 27and 30-CH₃), 2.96 (m, 4H, 28-CONHCH(CH₂CH₂H₂)CONHCH(CH₂CH₂COOCH₃)COOCH₃ and 19-H and one of 23-CH₂), 3.37 (m, 2H, 3-CH and one of 23-CH₂), 3.58, 3.64 (s, 3H each, 28-CONHCH(CH₂CH₂H₂)CONHCH(CH₂CH₂COOCH₃)COOCH₃), 4.12 (d, 1H, J=4.6 Hz, 3-ΟH), 4.36 (m, 4H, 28-CONHCH(CH₂CH₂H₂)CONHCH(CH₂CH₂COOCH₃)COOCH₃ and 29=CH₂), 7.19 (m, 6H, 28-CONHCH(CH₂CH₂H₂)CONHCH(CH₂CH₂COOCH₃)COOCH₃), 8.28 (d, 1H, J=7.3 Hz, 28-CONHCH(CH₂CH₂H₂)CONHCH(CH₂CH₂COOCH₃)COOCH₃). MS (EI): m/z [M+H]+ 777.6, [M+Na]+ 799.6, [M+K]+ 815.6. HR-MS (ESI, M+H) m/z: calcd for C₆₈H₇₅N₆O₈ 777.5054, found 777.5051.

2.2.4. General procedure for synthesis of 7a-g

Compound 6 (0.10mmol) was dissolved in CH₃OH (4mL) and THF (4mL). 4N NaOH (2mL) was added to the solution and refluxed for 4h. 9% HCl was added to the mixture until the white solid appeared, filtered and the insoluble substance was washed with H₂O. The residue was dried to obtain the corresponding 7a-f.

N'-[N-3β, 23-dihydroxy]-20(29)-en-28-oyl]-2-ami nocyanoethyl]-L-glucose (7a): 6a (60.0mg, 0.10mmol). Yield: 50.0mg (85%) as a white solid, mp 255°C (dec). IR (KBr, cm⁻¹) v 3416, 2929, 2867, 1730, 1640, 1530, 1450, 1383, 1197, 1044, 882, 622. ¹H-NMR (DMSO, 500MHz) δ 0.51, 0.77, 0.82, 0.87, 1.62 (s, 3H each, 24, 25, 26, 27and 30-CH₃), 2.97 (m, 1H, 19-CH), 3.05, 3.70 (d, 1H, J=8.9 Hz, one of 23-CH₂), 3.40 (m, 2H, 3-CH and one of 23-CH₂), 3.67 (m, 4H, 28-CONHCH₂CONHCH₂COOH), 4.10 (d, 1H, J=89.0 Hz, 3-ΟH), 4.33 (t, 1H, 23-ΟH), 4.52, 4.64 (d, 2H, J=58.8 Hz, 29=CH₂), 6.77 (t, 2H, 28- CONHCH₂CONHCH₂COOH). MS (ESI): m/z [M+H]+ 587.4, [M+Na]+ 609.5. HR-MS (ESI, M+H) m/z: calcd for C₄₃H₄₃N₅O₆ 587.4060, found 587.4065.

N'-[N-3β, 23-dihydroxy]-20(29)-en-28-oyl]-2-ami nocyanoethyl]-L-phenylalanine (7b): 6b (69.0mg, 0.10mmol). Yield: 55.0mg (82%) as a white solid, mp 158-160°C. IR (KBr, cm⁻¹) v 3401, 2924, 2854, 1731, 1644, 1585, 1447, 1334, 1125, 1048, 879, 611. ¹H-NMR (DMSO, 300MHz) δ 0.51, 0.76, 0.81, 0.86, 1.62 (s, 3H each, 24, 25, 26, 27and 30-CH₃), 2.95 (m, 4H, 19-CH and 28-CONHCH₂CONHCH₂COOH and one of 23-CH₂), 3.36 (m, 2H, 3-CH and one of 23-CH₂), 3.63 (d, 2H, J=5.7 Hz, 28-CONHCH₂CONHCH₂(CH₂CH₂H₂)COOH), 4.09 (d, 1H, J=5.2 Hz, 3-ΟH), 4.32 (t, 1H, 23-ΟH), 4.43 (m, 1H, 28-CONHCH₂CONHCH₂(CH₂CH₂H₂)COOH), 4.52, 4.64 (d, 2H, J=34.9 Hz, 29=CH₂), 7.24 (m, 5H, 28-CONHCH₂CONHCH₂(CH₂CH₂H₂)COOH), 7.69 (t, 1H, 28-CONHCH₂CONHCH₂(CH₂CH₂H₂)COOH), 7.89 (d, 1H, J=7.8 Hz, 28-CONHCH₂CONHCH₂(CH₂CH₂H₂)COOH). MS (ESI): m/z [M+H]+ 767.4, [M+Na]+ 699.5. HR-MS (ESI, M+H) m/z: calcd for C₄₃H₄₃N₅O₆ 767.4530, found 767.4533.

N'-[N-3β, 23-dihydroxy]-20(29)-en-28-oyl]-2-ami nocyanoethyl]-L-leucine (7c): 6c (66.0mg, 0.10mmol). Yield: 54.0mg (84%) as a white solid, mp 242-244°C. IR (KBr, cm⁻¹) v 3415, 2923, 2869, 1724, 1638, 1519, 1467, 1385, 1271, 1156, 1045, 882, 612. ¹H-NMR (DMSO, 300MHz) δ 0.51, 0.77, 0.82, 0.88, 1.62 (s, 3H each, 24, 25, 26, 27and 30-CH₃), 2.95 (m, 1H, 19-CH), 3.06 (d, 1H, J=9.2 Hz, one of 23-CH₂), 3.41 (m, 2H, 3-CH and one of 23-CH₂), 3.65 (m, 2H, 28-CONHCH₂CONHCH₂(CH₂CH₂H₂)COOH), 4.12 (m, 2H, 23-ΟH and 28-CONHCH₂CONHCH₂(CH₂CH₂H₂)COOH), 4.32 (t, 1H, 3-ΟH), 4.53, 4.64 (d, 2H, J=32.7 Hz, 29=CH₂), 7.72 (m, 2H, 28-CONHCH₂CONHCH₂(CH₂CH₂H₂)COOH and 28-CONHCH₂CONHCH₂(CH₂CH₂H₂)COOH). MS (ESI): m/z [M-H]- 601.5. HR-MS (ESI, M+H) m/z: calcd for C₃₈H₄₅N₄O₆ 643.4686, found 643.4681.
N'-[β,23-dihydroxylup-20(29)-en-28-oyl]-2-amino-2-benzyl-ethanoyl]-L-leucine (7d): 6d (75.0mg, 0.10mmol). Yield: 58.0mg (79%) as a white solid, mp 213-215°C. IR (KBr, cm⁻¹) v 3417, 2951, 2868, 1715, 1639, 1519, 1467, 1383, 1200, 1153, 1045, 882, 699.¹ H-NMR (DMSO, 300MHz) δ 0.42, 0.52, 0.72, 0.78, 1.58 (s, 3H each, 24, 25, 26, 27and 30-CH₃), 2.88 (m, 4H, 19-CH and one of 23-CH₂, and 28-CONHCH(CH₂CH₃)CONHCH(CH₂CHCH₂CH₃)COOH), 3.40 (2H, m, 3-CH and one of 23-CH₂), 4.09 (d, 1H, 3-CH), 4.28 (m, 2H, 23-CH and 28-CONHCH(CH₂CH₃)CONHCH(CH₂CHCH₂CH₃)COOH), 4.49 (m, 1H, 28-CONHCH(CH₂CH₃)CONHCH(CH₂CHCH₂CH₃)COOH), 4.49, 4.58 (d, 2H, J=29.1 Hz, 29=CH₂), 6.70 (m, 5H, 28-CONHCH(CH₂CH₃)CONHCH(CH₂CHCH₂CH₃)COOH), 7.67 (d, 1H, J=7.8 Hz, 28-CONHCH(CH₂CH₃)CONHCH(CH₂CHCH₂CH₃)COOH). MS (EI): m/z [M-H]⁻ 731.5. HR-MS (ESI, M+H) m/z: calcd for C₃₆H₅₈N₂O₆ 733.5156, found 733.5159.

N'-[β,23-dihydroxylup-20(29)-en-28-oyl]-2-amino-2-benzyl-ethanoyl]-glucose (7e): 6e (69.0mg, 0.10mmol). Yield: 41.0mg (61%) as a white solid, mp 223-225°C. IR (KBr, cm⁻¹) v 3415, 2940, 2867, 1714, 1638, 1521, 1448, 1384, 1204, 1042, 886, 616.¹ H-NMR (DMSO, 300MHz) δ 0.41, 0.51, 0.75, 0.86, 1.58 (s, 3H each, 24, 25, 26, 27and 30-CH₃), 2.83 (m, 2H, 28-CONHCH(CH₂CH₃)CONHCH₂COOH), 3.03 (m, 2H, 19-H and one of 23-CH₃), 3.36 (m, 2H, 3-CH and one of 23-CH₂), 3.79 (m, 2H, 28-CONHCH(CH₂CH₃)CONHCH₂COOH), 4.09 (d, 1H, J=4.9 Hz, 3-CH), 4.32 (t, 1H, 23-CH), 4.48 (m, 1H, 28-CONHCH(CH₂CH₃)CONHCH₂COOH), 4.48, 4.59 (d, 2H, J=32.4 Hz, 29=CH₂), 7.20 (m, 5H, 28-CONHCH(CH₂CH₃)CONHCH₂COOH), 7.67 (d, 1H, J=8.6 Hz, 28-CONHCH(CH₂CH₃)CONHCH₂COOH), 7.92 (t, 1H, 28-CONHCH(CH₂CH₃)CONHCH₂COOH), MS (EI): m/z [M-H]⁻ 675.5. HR-MS (ESI, M+H) m/z: calcd for C₃₅H₆₀N₂O₆ 677.4530, found 677.4535.

N'-[β,23-dihydroxylup-20(29)-en-28-oyl]-2-amino-2-(2'-isobutyl)-ethanoyl]-glucose (7f): 6f (66.0mg, 0.10mmol). Yield: 46.0mg (72%) as a white solid, mp 188-190°C. IR (KBr, cm⁻¹) v 3429, 2949, 2866, 1708, 1636, 1530, 1447, 1384, 1226, 1042, 886, 620.¹ H-NMR (DMSO, 500MHz) δ 0.50, 0.77, 0.83, 0.87, 1.62 (s, 3H each, 24, 25, 26, 27and 30-CH₃), 3.02 (m, 2H, 19-CH and one of 23-CH₂), 3.40 (2H, m, 3-CH and one of 23-CH₂), 3.73 (m, 2H, 28-CONHCH(CH₂CHCH₂CH₃)CONHCH₂COOH), 4.09 (d, 1H, J=4.8 Hz, 3-CH), 4.34 (m, 2H, 23-CH and 28-CONHCH(CH₂CHCH₂CH₃)CONHCH₂COOH), 4.52, 4.64 (d, 2H, J=58.8 Hz, 29=CH₂), 7.54 (d, 1H, J=8.5 Hz, 28-CONHCH(CH₂CHCH₂CH₃)CONHCH₂COOH), 7.80 (t, 1H, 28-CONHCH(CH₂CHCH₂CH₃)CONHCH₂COOH), MS (EI): m/z [M+H]⁺ 643.5, [M+Na]⁺ 665.4, [M-H]⁺ 641.4. HR-MS (ESI, M+H) m/z: calcd for C₃₄H₆₂O₆ 643.4686, found 643.4684.
2.3 Cytotoxic activity in vitro[11]

The cytotoxic activity in vitro was measured using the MTT assay. The tumor cell line panel consisted of A549, BEL-7402, SF763, B16 and HL-60 (final concentration in the growth medium was $2\sim 4\times 10^5$/mL). MTT solution (20µL/well) was added after cells were treated with drug for 48 h, and cells were incubated for a further 4 h at 37°C. The purple formazan crystals were dissolved in 150µl DMSO. After 5 min, the plates were read on an automated microplate spectrophotometer at 570 nm. Assays were performed in triplicate in three independent experiments. The concentration required for 50% inhibition of cell viability (IC$_{50}$) was calculated. In all of these experiments, three replicate wells were used to determine each point.

3. Results and Discussion

As shown in Figure 2, treatment of 1 with Ac$_2$O and then (COCl)$_2$ in CH$_2$Cl$_2$ and then H$_2$NCHR’COOCH$_3$·HCl in the presence of DMAP produced compounds 4a-f in 51-67% yields. Hydrolysis of 4a-f with 4N NaOH in THF and CH$_3$OH furnished derivatives 5a-f (71-84%), among which, 5a, 5c and 5d were then treated with H$_2$NCHR$_2$COOCH$_3$·HCl in the presence of EDC and HOBT to give corresponding amides 6a-g in the yields of 63-80%. Finally, alkaline catalyzed hydrolysis of the resulted amides gave C-17 amide derivatives 7a-g in the yields of 61-85%.[13-14]

The cytotoxic activity of 23-hydroxy betulinic acid, betulinic acid and all derivatives in vitro was determined by the MTT cytotoxicity assay, and the result is summarized in Table 1. Many different cell lines were used: A549 (human lung carcinoma), BEL-7402 (human hepatoma), SF-763 (human cerebroma), B16 (mice melanoma), HL-60 (human leukaemia). The MTT assay results showed that most of the 23-hydroxy betulinic acid derivatives had better cytotoxic activities against the tested cells than betulinic acid and 23-hydroxy betulinic acid.

Compounds 4a-f displayed moderate cytotoxic activities against all cell lines expect 4a and 4b. Compounds 5a-f showed no cytotoxicity, despite the carboxylic acid substitution at the end of C-28 side chain. The reason maybe that the polarity of the compounds is too strong and affect the penetration of them into cells.

In the series of 6a-g, only 6g revealed potent cytotoxicity against all cell lines with IC50 values ranging from 22.65 to 31.97 µM. In A549 and HL-60 cell lines, 6a-f had better activity than betulinic acid and 23-hydroxy betulinic acid. In SF-763, compounds 6b, 6e, 6f exhibited better activity than betulinic acid and 23-hydroxy betulinic acid. Compounds 6a, 6c, 6d showed better activity than 23-hydroxy betulinic acid but less potent than betulinic acid. In BEL-7402 and B-16, 6a-f had better activity than 23-hydroxy betulinic acid but weaker than betulinic acid.

Compounds 7a-g showed almost the same cytotoxic activity as 23-hydroxy betulinic acid. The end of the C-28 side chain in 7a-g was carboxylic acid, which maybe affect the activity of derivatives due to the large polarity.

4. Conclusions

In summary, a series of novel C-28 amide derivatives modified on 17-carboxylic acid of 23-hydroxy betulinic acid were obtained and tested for their cytotoxic activities against five human tumor cell lines in vitro. Most of the amide derivatives showed moderate potent cytotoxic activities on all the tested cells except for compounds 5a-f. The compounds 4a, 4b and 6g have the most potent cytotoxic activity. The terminal group and branched chains on the C-28 side chain maybe have a major impact on their antitumor activity.

The further structure modification and SAR studies of 23-hydroxy betulinic acid derivatives are in progress in our laboratory and the results will be reported in due course[15].
Table 1. The cytotoxicity data of 23-hydroxy betulinic acid and its derivatives [IC50 (µmol/L) ±SD]

<table>
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<th>Compound</th>
<th>Cell line</th>
<th>A549</th>
<th>BEL-7402</th>
<th>SF-763</th>
<th>B16</th>
<th>HL-60</th>
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<td>81.36±3.54</td>
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<td>45.13±7.07</td>
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BA: betulinic acid, HBA: 23-hydroxy betulinic acid
Data is mean of three experiments.

Acknowledgments

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References


