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Synthesis of 3-*O*-acyl/3-benzylidene/3-hydrazone/3-hydrazine/17-carboxyacryloyl ester derivatives of betulinic acid as anti-angiogenic agents

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Abstract—New 3-*O*-acyl, 3-benzylidene, 3-hydrazone, 3-hydrazine, 17-carboxyacryloyl ester derivatives of betulinic acid (**2**–**6**, **8**–**11**, **13**, **17**, **18**, **21**, and **22**) were synthesized and evaluated in vitro for anti-angiogenic activity on endothelial cell cytotoxicity, specificity, and tube-formation ability. All derivatives reported here showed $IC_{50} < 4\,\mu g/mL$. Compounds **3**, **9**, **10**, **17**, **21**, and **22** have shown better cytotoxicity ($IC_{50} < 1.2\,\mu g/mL$) than betulinic acid (**1**) and improved endothelial cell specificity (ECS > 10) in some cases. Compounds **10**, **17**, and **18** have shown 20%, 32%, and 48% reduction in TLS, respectively, and were found better than betulinic acid (**1**). We have shown that 20,29-dihydrobetulinic acid derivatives have better anti-angiogenic activity as compared to betulinic acid or its other derivatives.

1. Introduction

Betulinic acid (**1**), a pentacyclic triterpene has been reported to be a selective inducer of apoptosis in various human cancers.[1–3] Recent studies have revealed that betulinic acid (**1**) is cytotoxic to endothelial cells[4] and inhibits invasion and tube formation of endothelial cells at noncytotoxic concentrations through a modulation of mitochondrial function.[4,5] It also inhibits in vitro enzymatic activity of aminopeptidase N, which is known to play an important role in angiogenesis.[6]

Previously, we reported the in vitro anti-cancer activity of derivatives of betulinic acid on leukemia, lymphoma, prostate, lung, and ovarian cancer cell lines.[7] Recently, the anti-angiogenic activity of some betulinic acid derivatives has been reported by us.[4,8] In order to better understand the structure–activity relationships, we have synthesized several derivatives of betulinic acid and studied their effect on endothelial cells in vitro. Here we report the synthesis of 3-*O*-acyl, 3-benzylidene, 3-hydrazone, 3-hydrazine, 17-carboxyacryloyl ester derivatives of betulinic acid (**2**–**6**, **8**–**11**, **13**, **17**, **18**, **21**, and **22**) with $IC_{50} < 4\,\mu g/mL$ on ECV304 cell line, their endothelial cell specificity (ECS) and percentage inhibition of tube-like structure (TLS) formation of ECV304 cells.

2. Chemistry

Synthesis of 3-*O*-acyl, 3-phenylhydrazone, and 17-carboxyacryloyl ester derivatives of betulinic acid (**2**–**6**, **8**–**11**, and **13**)[4] has been described in Scheme 1. Betulinic acid (**1**) on treatment with acryloyl chloride in presence of sodium hydride and solvent DMF afforded to 17-carboxyacryloyl ester derivative **2**. Betulinic acid (**1**), upon reaction separately with 3-(trifluoromethyl)benzoyl chloride, 4-pentylbenzoyl chloride and 4-heptylbenzoyl chloride, yielded corresponding 3-*O*-acyl derivatives **3**, **4**, and **6**, respectively. Upon treatment of **1** with (2,5-dimethoxyphenyl)acetyl chloride afforded compound **5**. Hydrogenation of betulinic acid (**1**) with Pd/C gave 20,29-dihydro betulinic acid (**7**) and which was later converted, using the similar method as

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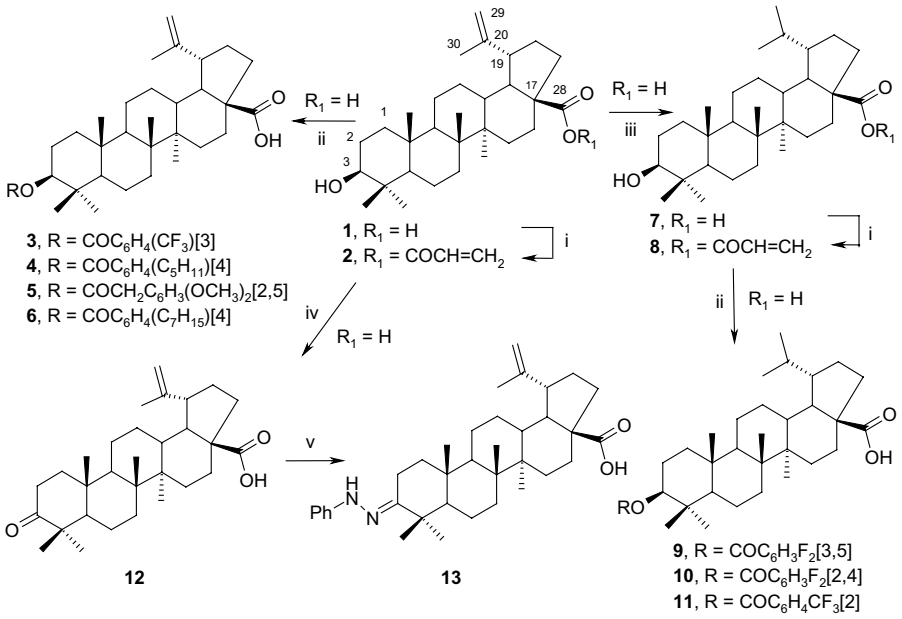
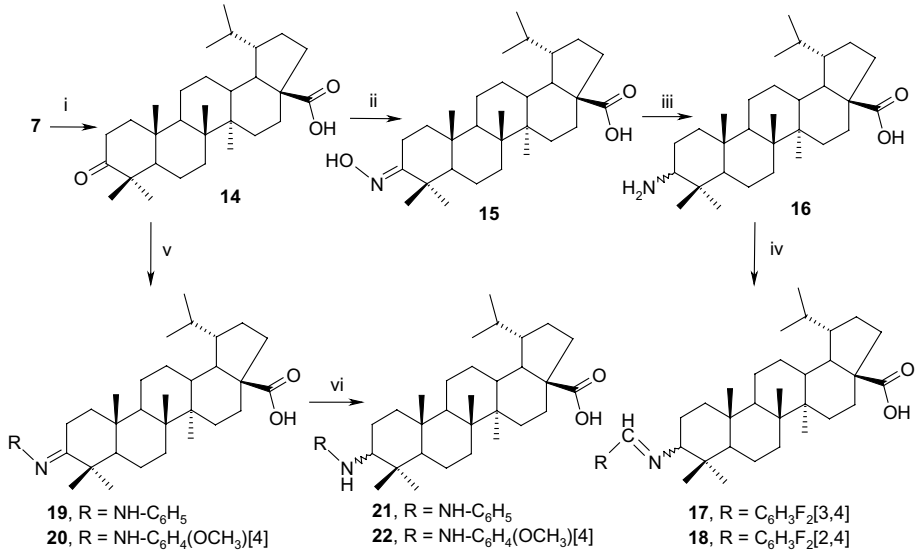


Scheme 1. Reagents and conditions: (i) R_1-Cl/NaH/DMF; (ii) R-Cl/DCM/Py; (iii) H_2/Pd–C/MeOH; (iv) Jones' reagent; (v) $PhNHNH_2$/NaOAc/MeOH.

discussed for the synthesis of **2**, to its 17-carboxyacryloyl ester derivative **8**. Similarly as discussed for the synthesis of **3**–**6**, 20,29-dihydro betulinic acid (**7**) was converted to 3-*O*-acyl-20,29-dihydro betulinic acid derivatives (**9**–**11**). On the other hand, upon Jones' oxidation of betulinic acid (**1**), betulonic acid (**12**) was formed and which, was further reacted with phenyl hydrazine and sodium acetate to provide 3-phenylhydrazone betulonic acid (**13**).

Scheme 2 describes the synthesis of 3-benzylidene and 3-hydrazine 20,29-dihydrobetulinic acid derivatives (**17**, **18**, **21**, and **22**).[4] The 20,29-dihydrobetulinic acid (**7**) was oxidized to 20,29-dihydrobetulonic acid (**14**) using Jones' reagent and which, was then elaborated in two ways. In the first, compound **14** was treated with hydroxylamine hydrochloride in pyridine to afford 3-hydroxyloxime 20,29-dihydrobetulonic acid (**15**) and which, upon treatment with platinum oxide in acetic acid, yielded 3-amino 20,29-dihydrobetulinic acid (**16**). The 3-benzylidene 20,29-dihydrobetulinic acid derivatives **17** and **18** were prepared from the reaction of **16** with 3,4-difluorobenzaldehyde and 2,4-difluorobenzaldehyde, respectively. In the second, compound **14** was treated separately with phenylhydrazine and 4-methoxyphenyl hydrazine to afford 3-hydrazone derivatives **19** and **20**, respectively. Upon hydrogenation of **19** and



Scheme 2. Reagents and conditions: (i) Jones' reagent; (ii) $H_2NOH{\cdot}HCl$/Py; (iii) H_2/PtO_2/AcOH; (iv) RCHO/MeOH; (v) RNH_2/NaOAc/EtOH; (vi) H_2/Pt/AcOH.

20 in the presence of platinum sponge catalyst in glacial acetic acid afforded 3-hydrazine 20,29-dihydrobetulinic acid derivatives **21** and **22**, respectively. All the compounds were characterized by spectroscopic and analytical tools.

3. Results and discussion

Betulinic acid derivatives (**2**–**6**, **8**–**11**, **13**, **17**, **18**, **21**, and **22**) were screened for cytotoxicity on human endothelial cells (ECV304) and tumor cell lines DU145 (Prostate), L132 (Lung), PA-1 (Ovary) and HT-29 (Colon) and the endothelial cell specificity (ECS) was calculated as shown in Table 1. All betulinic acid derivatives reported here showed $IC_{50} < 4\,\mu g/mL$ on ECV304 cell line and compounds (**3**, **9**, **10**, **17**, **21**, and **22**) have shown better cytotoxicity ($IC_{50} < 1.2\,\mu g/mL$) than betulinic acid (**1**) and improved endothelial cell specificity (ECS > 10) in some cases. Compound **17** exhibited the best cytotoxicity ($IC_{50} = 0.35\,\mu g/mL$) and compounds **21** and **22** have shown the best ECS, particularly, against DU145 cell line.

All the betulinic acid derivatives were screened for inhibition of tube-like structure (TLS) formation of ECV304 cells in a Matrigel™ tube formation assay. Compounds **10**, **17**, and **18** have shown 20%, 32%, and 48% reduction in TLS, respectively, and were found better than betulinic acid (**1**) as shown in Figure 1. Figure 2 shows the formation of TLS by ECV304 cells on matrigel (left panel) and its inhibition by incubation for 48 h with compound **18**.

Structure–activity relationship indicated that upon protection of C-28 carboxylic acid in betulinic acid or its 20,29-dihydro derivative (compounds **2** and **8**), cytotoxicity was lowered. In the 3-*O*-acyl betulinic acid derivatives (**3**–**6**), compound **3**, having an electron withdrawing group in aromatic ring, was the most cytotoxic with low to moderate ECS while substitution

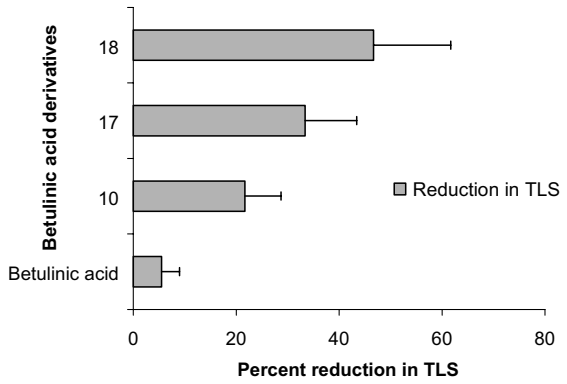


Figure 1. Inhibition of TLS by betulinic acid (**1**) and derivatives **10**, **17**, and **18** at noncytotoxic concentration.

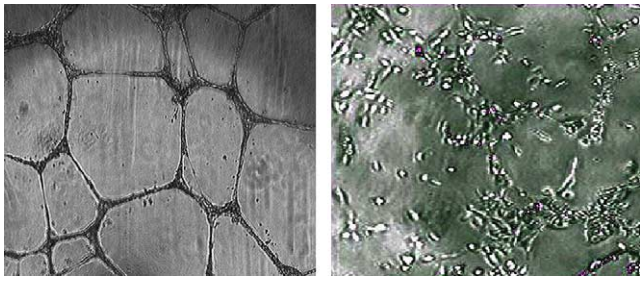


Figure 2. Representative image analysis photograph of formation of TLS by ECV304 cells on Matrigel (left panel) and inhibition of TLS by incubation with **18** after 48 h (right panel).

by a bulky group like pentyl or heptyl (compounds **4** and **6**) or electron donating group in the aromatic ring in **5**, lowered the cytotoxicity as well as ECS. Unlike compounds **3**–**6**, in the 3-*O*-acyl 20,29-dihydrobetulinic acid series (**9**–**11**), compounds **9** and **10**, possessing fluoro group, exhibited better cytotoxicity and ECS while compound **11**, bearing an electron withdrawing group, lowered the cytotoxicity and ECS. Introduction of a phenylhydrazone function at position-3 (**13**) in betulinic acid also lowered the cytotoxicity. It indicated that

Table 1. IC_{50} and ECS ratios of betulinic acid (**1**) and its derivatives (**2**–**6**, **8**–**11**, **13**, **17**, **18**, **21**, and **22**) on ECV304 cells

Compound	IC_{50} (μg/mL)	ECS ratio			
	ECV304 (endothelial)	DU145 (prostate)	L132 (lung)	PA-1 (ovary)	HT-29 (colon)
2	4.0 ± 0.74	>2.5	0.43	>1	2.5
3	0.9 ± 0.10	>11.1	3.0	>4.4	>11.1
4	1.9 ± 0.33	1.05	3.1	>2.1	1.84
5	1.8 ± 0.40	0.55	2.5	—	>5.6
6	1.7 ± 0.49	>5.8	4.1	>2.3	>5.89
8	2.0 ± 0.61	>2.0	2.0	0.75	5.0
9	0.7 ± 0.34	4.5	1.7	—	>14.3
10	0.7 ± 0.55	3.5	1.5	2.3	2.4
11	2.6 ± 0.80	>1.5	1.5	>1.53	3.84
13	2.4 ± 0.75	2.7	1.9	2.08	4.2
17	0.35 ± 0.14	6.2	7.1	3.7	11.4
18	2.4 ± 0.68	1.04	1.4	0.54	2.04
21	0.5 ± 0.31	19.8	1.6	7.0	3.5
22	0.4 ± 0.27	21.1	—	—	0.87
Betulinic acid (1)	1.2 ± 0.44	2.2	1.0	3.2	—

'—' Not done; '>' IC_{50} in tumor cells greater than highest concentration tested.

electron withdrawing group in betulinic acid derivatives (**3**–**6**) is playing a crucial role for eliciting better activity while it did not improve the activity in 20,29-dihydrobetulinic acid derivatives (**9**–**11**).

Between the 3-benzylideno 20,29-dihydrobetulinic acid derivatives **17** and **18**, compound **17** showed high cytotoxicity and low to moderate ECS along with good anti-TLS while compound **18** showed lowered cytotoxicity and ECS with high anti-TLS activity. It seemed that the position of fluoro group in the aromatic ring has a vital role in eliciting both cytotoxicity and anti-TLS activity. Compounds **21** and **22** of 20,29-dihydro-3-hydrazine series, exhibited high cytotoxicity and high ECS indicating that substitution of an electron donating group in the aromatic ring has slightly improved the activity. It clearly pointed that the electron donating group in 20,29-dihydrobetulinic acid was required for eliciting better activity than betulinic acid.

4. Conclusion

In the present study we have shown that 20,29-dihydrobetulinic acid derivatives have better anti-angiogenic acivity as compared to the other derivatives of betulinic acid. In our earlier work, we had speculated that the double bond between the position-20 and 29 in betulinic acid was playing a crucial role in eliciting high endothelial cell cytotoxicity, the endothelial cell specificity, and inhibition of tube-like structures.[8] The present studies confirmed our earlier results. We predict that the 'high' and 'moderate' ECS compounds specifically target endothelial cells and can be grouped under potent anti-angiogenic compounds while 'low' ECS compounds would supplement their already reported cytotoxic activity against tumor cells. Further studies would be done to test the best derivatives in vivo to ascertain the effect on in vivo experimental angiogenesis as well as determine the mechanism of action of these compounds.

5. Materials and methods

5.1. Chemicals

MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide, Sigma, USA], Matrigel™ (Becton Dickinson, USA), DMEM (Dulbeccos modified Eagles medium), and fetal bovine serum, FBS (Gibco BRL, USA), DMSO (Merck, India). Chemicals used in synthesis were purchased from Sigma, USA.

5.2. Cell culture

ECV304 cell line was generously gifted by Dr. Takahashi (Tokyo University, Tokyo, Japan). Human tumor cell lines DU145 (prostate), L132 (lung), HT-29 (colon), and PA-1 (ovary) cell lines have been procured from NCCS, Pune, India. Cell lines were grown in DMEM, containing L-glutamine and 25 mM HEPES and supplemented with 10% fetal bovine serum, penicillin (100 units/mL), streptomycin (100 lg/mL), and amphotericin B (0.25 lg/mL) at 37 °C, 5% CO_2, 100% humidity.

5.3. Cytotoxicity assay

Cells (1.5×10^4) were incubated with the compounds dissolved in DMSO (final DMSO concn <0.1%), in triplicate wells to obtain drug concentration of 0.5–4 μg/mL. Cytotoxicity was measured after 72 h using MTT assay as described by Mosmann.[9] Each experiment was repeated thrice and mean IC_{50} values (half-maximal cytotoxicity) have been reported. ECS ratios were calculated using the formula: IC_{50} (tumor cell)/IC_{50} (endothelial cell). ECS < 10 was designated low, between 10 and 20 as moderate and >20 as high endothelial specificity.

5.4. Tube-like structure (TLS) formation assay

The method as described by Shinji et al. was followed.[10] Briefly, 10^4 ECV304 cells in growth medium (DMEM containing 10% FBS) were seeded on Matrigel™ (70 μL). Compounds were solubilized in DMSO and were added in duplicate wells at noncytotoxic concentration and incubated for 18 h following which the control cells start to form an intense network of tube-like structures. The absence of cytotoxicity of betulinic acid and its derivatives on ECV304 cells at the above time point was confirmed by suitable controls. The total tube length was measured by image analysis and percentage inhibition of tube formation was calculated compared to controls. A qualitative assessment was performed by viewing the tube-like structures under the microscope and scoring for inhibition of endothelial sprouting, capillary network formation, and intussusception.

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