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Synthesis and cytotoxic activity of 3-*O*-acyl/3-hydrazine/2-bromo/20,29-dibromo betulinic acid derivatives

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Abstract—A series of 3-*O*-acyl, 3-hydrazine, 2-bromo, and 20,29-dibromo betulinic acid derivatives (**1**–**27**) have been synthesized and screened for in vitro cytotoxic activity on human cancer cell lines MOLT-4, JurkatE6.1, CEM.CM3, BRISTOL8, U937, DU145, PA-1, A549, and L132. A number of compounds have shown $ED_{50} < 1$ μg/mL against the cancer cell lines tested and have shown better cytotoxicity than betulinic acid. Compounds **13**, **19**, **20**, **23**, and **27** were the best derivatives and were selected as lead molecules for further development. The structure–activity relationship has been described.

1. Introduction

Betulinic acid (3-β-hydroxy-lup-20(29)-en-28-oic acid) is a pentacyclic lupane-type triterpene and has been shown to possess several medicinal properties including anti-cancer[1,2] and anti-HIV[3] activities. Previous reports indicated that betulinic acid was a melanoma-specific cytotoxic compound,[1,2] however, more recent evidence indicates that betulinic acid possesses a broader spectrum of activity against other cancer cell types.[4–7] Betulinic acid was shown to act through induction of apoptosis[1,2] independent of the cell's p53 status.[7–9] A previous investigation had shown that betulinic acid inhibited the in vitro activity of aminopeptidase N,[10] an endogenous angiogenic factor and inhibited the mitochondrial function in endothelial cells.[11] Recently, the anti-angiogenic activity of some betulinic acid derivatives has been reported by us.[12,13] These findings have made betulinic acid a very attractive candidate for the clinical treatment of various forms of cancer. As a result, further studies have been performed to derive synthetic betulinic acid analogs in an effort to establish meaningful structure–activity relationships.

Here we report the synthesis of 3-*O*-acyl, 3-hydrazine, 2-bromo, and 20,29-dibromo betulinic acid derivatives (**1**–**27**) and their in vitro cytotoxicity against a number of human cancer cell lines. For the first time, the anti-leukemia and anti-lymphoma activity of betulinic acid and its derivatives (**1**–**27**) is being reported here.

2. Chemistry

Synthesis of betulinic acid derivatives **1**–**20**[2,12] has been described in Scheme 1. Betulinic acid was elaborated in four ways. In first, betulinic acid was treated, separately, with acetyl chloride, (*S*)-(−)-2-acetoxypropionyl chloride, trimethylacetyl chloride, and propionyl chloride in methylene chloride (DCM) to afford corresponding 3-*O*-acyl derivatives **1**, **2**, **5**, and **8**, respectively. Compounds **1** and **2** were reacted, separately, with methyl bromoacetate and sodium hydride in DMF to give 17-carboxymethyl carboxylate derivatives **3** and **7**, respectively. While in the second, betulinic acid was reacted with methyl bromoacetate, as for **3** and **7**, to yield 17-carboxymethyl carboxylate derivative **4** and which, upon hydrolysis with 10% sodium hydroxide afforded the respective acid derivative **6**. In the third, betulinic

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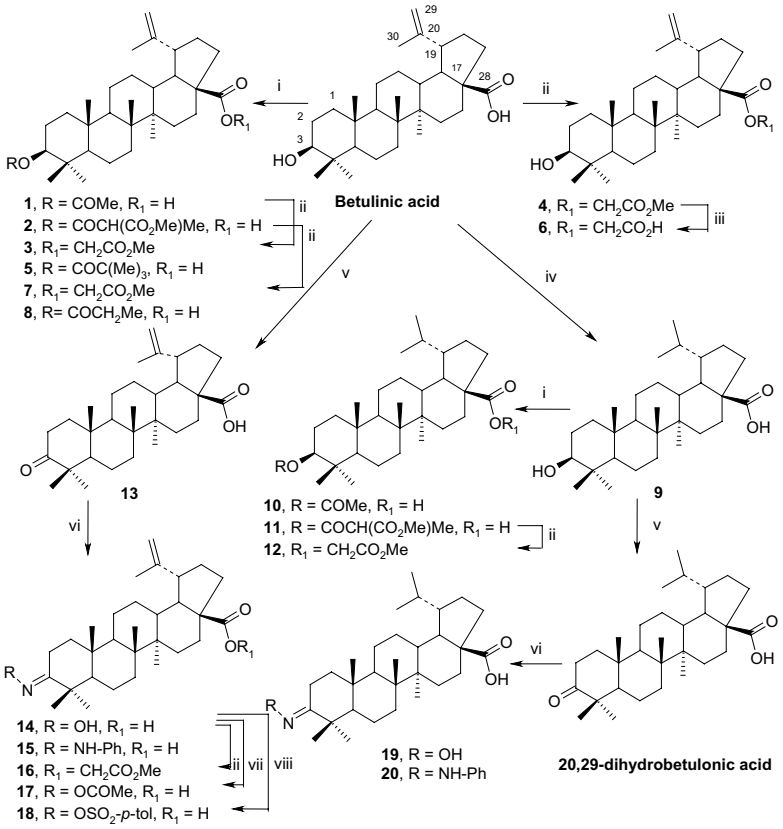


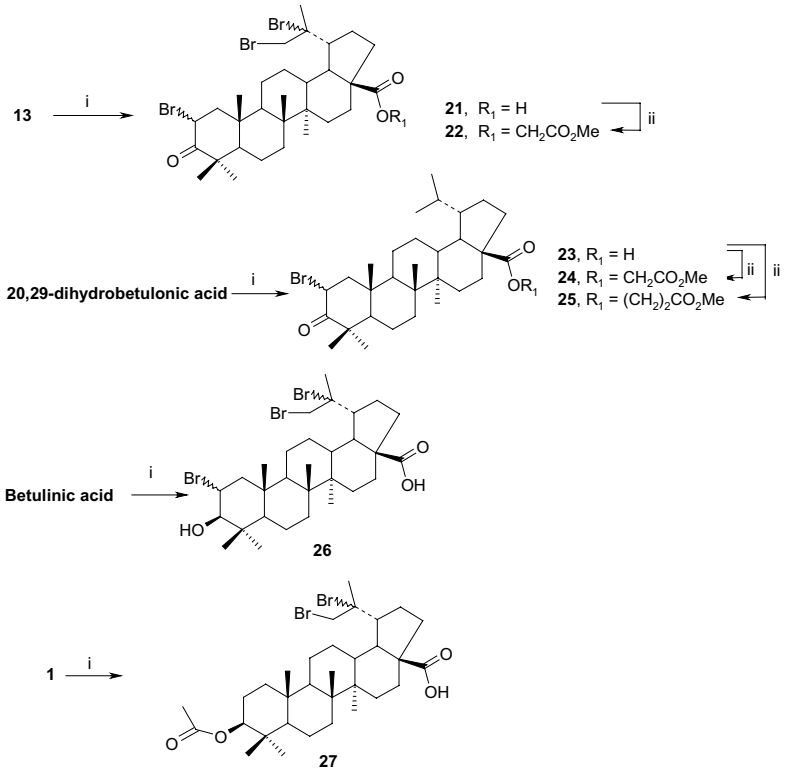
Scheme 1. Reagents and conditions: (i) R-Cl/DCM/pyridine; (ii) R_1-Br/NaH/DMF; (iii) 10% NaOH/MeOH; (iv) H_2/Pd–C/MeOH; (v) Jones' reagent; (vi) R-NH_2/NaOAc/EtOH; (vii) acetyl chloride/NaH/DMF; (viii) tosyl chloride/NaH/DMF.

acid was hydrogenated with Pd/C in methanol resulted 20,29-dihydrobetulinic acid (**9**). Compound **9** was reacted in two ways; on one side, it was treated, separately, with acetyl chloride and (*S*)-(−)-2-acetoxypropionyl chloride, as for **1** and **2**, to provide 3-*O*-acyl 20,29-dihydrobetulinic acid derivatives **10** and **11**, respectively. Compound **11** was reacted with methyl bromoacetate, as for **4**, to provide compound **12**. On the other hand, compound **9** was converted into 20,29-dihydrobetulonic acid by Jones' oxidation and which, upon treatment, separately, with hydroxylamine and phenylhydrazine afforded 3-hydroxyloxime 20,29-dihydrobetulonic acid (**19**) and 3-phenylhydrazone 20,29-dihydrobetulonic acid (**20**), respectively. In the last, betulinic acid was converted, as for 20,29-dihydrobetulonic acid, into betulonic acid (**13**). Reaction of **13** with hydroxylamine and phenylhydrazine, separately, afforded 3-hydroxyloxime betulonic acid (**14**) and 3-phenylhydrazone betulonic acid (**15**), respectively. Treatment of **14** with methyl bromoacetate, as for **4**, yielded compound **16**. Compound **14** was reacted, separately, with acetyl chloride and *p*-toluenesulfonyl chloride to afford compounds **17** and **18**, respectively.

Bromination[2,12] of betulinic acid and its derivatives has been described in Scheme 2. The betulonic acid (**13**) was brominated by liquid bromine in DCM to give 2,20,29-tribromobetulonic acid (**21**) and later, upon its treatment with methyl bromoacetate and sodium hydride in DMF yielded its 17-carboxymethyl carboxylate derivative **22**. While bromination of 20,29-dihydrobetulonic acid, as for **21**, resulted 2-bromo 20,29-dihydro-betulonic acid (**23**). Reaction of **23**, separately, with methyl bromoacetate and 2-bromo ethylacetate, as for **22**, yielded ester derivatives **24** and **25**, respectively. Bromination of betulinic acid and 3-*O*-acetyl betulinic acid (**1**), as for **21**, resulted in 2,20,29-tribromobetulinic acid (**26**) and 3-*O*-acetyl 20,29-dibromobetulinic acid (**27**), respectively. All the compounds were characterized by spectroscopic tools.

3. Results and discussion

In vitro cytotoxic activity of betulinic acid and its derivatives (**1**–**27**) was determined by performing the MTT cytotoxicity assay and the cytotoxicity data is summarized in Table 1. Here we report for the first time the in vitro anti-leukemia/lymphoma activity of betulinic acid and its derivatives. In order to establish this activity profile, we have enlarged the panel using different cell lines of leukemia/lymphoma: MOLT-4 (human lymphoblastic leukemia), JurkatE6.1 (human lymphoblastic leukemia), CEM.CM3 (human lymphoblastic leukemia), BRISTOL8 (human B-cell lymphoma), U937 (human histiocytic lymphoma). Subsequently, we tested the activity of betulinic acid and its derivatives (**1**–**27**) in other cell lines and found activity in DU145 (human prostate), PA-1 (human ovary), A549 (human lung), and L132 (human lung).



Scheme 2. Reagents and conditions: (i) liquid Br_2/DCM; (ii) R_1-Br/NaH/DMF.

Table 1. The cytotoxicity data of betulinic acid and its derivatives (**1**–**27**) [ED_{50} (μg/mL) ± SD]

Compound	Cell line								
	MOLT-4	JurkatE6.1	CEM.CM3	BRISTOL8	U937	DU145	PA-1	A549	L132
1	3.1 ± 0.2	1.8 ± 0.4	—	4.8 ± 1.2	1.6 ± 0.3	2.6 ± 0.5	4.1 ± 0.9	>10.0	>10.0
2	1.2 ± 0.4	0.9 ± 0.1	1.6 ± 0.5	1.7 ± 0.3	1.1 ± 0.1	1.6 ± 0.7	3.6 ± 0.1	>10.0	>10.0
3	4.0 ± 2.2	1.4 ± 0.1	3.2 ± 0.1	5.3 ± 0.5	>10.0	>10.0	5.9 ± 1.6	>10.0	>10.0
4	2.9 ± 0.6	3.1 ± 0.4	5.9 ± 0.3	7.4 ± 0.5	5.4 ± 0.1	1.9 ± 0.1	3.1 ± 0.1	>10.0	>10.0
5	2.4 ± 0.6	>10.0	>10.0	>10.0	>10.0	1.0 ± 0.1	0.7 ± 0.0	>10.0	>10.0
6	3.1 ± 0.0	>10.0	>10.0	6.1 ± 0.0	>10.0	>10.0	1.3 ± 1.2	>10.0	>10.0
7	3.9 ± 0.0	7.4 ± 0.7	6.4 ± 0.4	4.5 ± 0.6	>10.0	2.9 ± 0.1	7.1 ± 1.2	>10.0	>10.0
8	3.3 ± 0.7	2.1 ± 0.6	5.5 ± 0.1	2.1 ± 0.2	6.8 ± 0.5	4.2 ± 0.8	3.7 ± 0.8	>10.0	>10.0
9	0.6 ± 0.4	4.9 ± 3.9	1.3 ± 0.1	4.4 ± 0.3	0.9 ± 0.1	0.7 ± 0.1	0.7 ± 0.1	2.2 ± 0.2	2.4 ± 0.1
10	1.1 ± 0.9	1.6 ± 0.1	1.0 ± 0.1	3.4 ± 0.1	1.8 ± 0.1	0.9 ± 0.3	0.8 ± 0.1	2.3 ± 0.7	2.9 ± 0.7
11	2.8 ± 0.0	3.8 ± 0.1	3.1 ± 0.0	2.1 ± 0.2	4.0 ± 0.3	1.0 ± 0.3	1.3 ± 0.1	2.1 ± 0.9	7.7 ± 2.1
12	2.8 ± 0.8	5.5 ± 1.7	2.5 ± 0.1	3.4 ± 2.0	>10.0	>10.0	0.5 ± 0.1	>10.0	>10.0
13	1.2 ± 0.1	0.4 ± 0.3	4.4 ± 0.2	2.6 ± 0.1	2.8 ± 0.6	3.1 ± 1.3	1.2 ± 0.4	>10.0	>10.0
14	3.4 ± 0.7	1.9 ± 1.6	3.7 ± 0.4	5.1 ± 1.7	2.0 ± 1.1	2.8 ± 1.2	8.1 ± 0.1	>10.0	7.3 ± 3.0
15	3.4 ± 0.7	2.6 ± 0.6	3.1 ± 0.1	3.4 ± 1.3	2.4 ± 1.3	2.4 ± 0.8	5.3 ± 0.3	>10.0	4.3 ± 1.4
16	1.7 ± 1.0	0.5 ± 0.3	1.4 ± 0.1	1.4 ± 0.7	1.7 ± 0.3	5.5 ± 0.8	6.5 ± 0.0	>10.0	>10.0
17	2.2 ± 0.7	2.9 ± 0.1	4.2 ± 0.1	3.4 ± 0.1	5.5 ± 0.8	2.9 ± 2.5	3.7 ± 2.5	>10.0	5.3 ± 2.5
18	2.7 ± 0.2	4.9 ± 0.4	1.5 ± 0.1	3.3 ± 0.4	4.8 ± 0.4	2.8 ± 0.1	1.8 ± 1.2	>10.0	>10.0
19	2.1 ± 0.2	1.8 ± 0.0	2.6 ± 0.1	1.6 ± 0.1	2.4 ± 0.3	1.1 ± 0.4	0.7 ± 0.1	1.8 ± 0.3	1.5 ± 0.6
20	1.4 ± 0.0	6.3 ± 0.0	2.9 ± 0.6	3.7 ± 0.1	2.2 ± 1.1	0.6 ± 0.1	0.4 ± 0.0	>10.0	7.4 ± 2.9
21	5.9 ± 1.6	2.1 ± 0.5	2.8 ± 1.6	3.8 ± 3.3	2.1 ± 0.2	3.6 ± 1.8	5.1 ± 2.5	>10.0	>10.0
22	1.3 ± 0.0	1.1 ± 0.2	1.2 ± 0.1	1.9 ± 0.3	0.7 ± 0.1	4.5 ± 2.3	2.5 ± 0.7	>10.0	>10.0
23	0.4 ± 0.1	0.7 ± 0.2	0.3 ± 0.1	2.9 ± 0.1	1.3 ± 0.8	3.0 ± 0.1	0.5 ± 0.0	>10.0	>10.0
24	1.7 ± 0.4	2.8 ± 0.0	2.1 ± 0.1	3.4 ± 0.1	1.1 ± 0.1	6.8 ± 1.3	3.2 ± 0.1	>10.0	>10.0
25	1.2 ± 0.0	1.9 ± 0.3	1.9 ± 0.1	2.3 ± 0.2	1.2 ± 0.1	6.6 ± 3.1	4.8 ± 1.9	>10.0	>10.0
26	3.6 ± 0.0	>10.0	6.8 ± 0.1	7.0 ± 0.1	>10.0	>10.0	6.0 ± 2.7	>10.0	>10.0
27	2.8 ± 0.0	6.9 ± 1.7	2.7 ± 0.1	1.4 ± 0.2	0.3 ± 0.0	2.5 ± 1.6	2.4 ± 2.4	>10.0	>10.0
Betulinic acid	1.23 ± 0.7	0.65 ± 0.04	0.98 ± 0.03	0.84 ± 0.05	0.69 ± 0.01	1.13 ± 0.35	>10.0	>10.0	1.3 ± 0.55

Data is mean of three experiments; '>10' ED_{50} not obtained even at 10 μg/mL; '—' not done.

The structure–activity relationship was studied keeping in mind the activity on different cancer sub-types that is leukemia/lymphoma, prostate, ovary, and lung cancer. The activity of derivatives was compared with betulinic acid in each panel.

Amongst leukemia/lymphoma cancer cell lines, 2-bromo 20,29-dihydrobetulinic acid (**23**) was threefold more potent than betulinic acid on MOLT-4 and CEM.CM3 cell lines and had similar activity as betulinic acid on JurkatE6.1 cell line. The 20,29-dihydrobetulinic acid derivative (**9**) was twofold more potent than betulinic acid on MOLT-4 and had similar activity as betulinic acid on CEM.CM3 and U937 cell lines. Betulonic acid (**13**) was 1.5-fold more potent than betulinic acid on JurkatE6.1 and had similar activity as betulinic acid on MOLT-4 cell line. Compounds **2** and **10**, having 2-acetoxypropionyl and acetyl substituent at position-3, respectively, have shown similar activity as betulinic acid on MOLT-4 cell line. Compound **10** also had similar activity as betulinic acid on CEM.CM3 cell line. The 20,29-dihydrobetulinic acid derivative **20** and 17-carboxyalkyl carboxylate derivatives (**22** and **25**) of 2-bromo betulinic acid had similar activity as betulinic acid on MOLT-4 cell line. Compound **22** also had similar activity as betulinic acid on CEM.CM3 cell line. This indicated that although *O*-acyl, oxime and hydrazone substituents at position-3 had some effect on the cytotoxicity but it did not significantly improve the activity of betulinic acid. In this cancer subtype, it seemed that the bromo group at position-2 in betulinic acid derivative, was the most suitable substituent for eliciting better anti-leukemia/lymphoma activity.

In prostate cancer cell line (DU145), the best compound was the 3-phenylhydrazone derivative (**20**) of 20,29-dihydrobetulinic acid, which has shown around two-fold more activity than betulinic acid, while its 3-hydroxyloxime derivative **19** had similar activity as betulinic acid. The 20,29-dihydrobetulinic acid (**9**) and its 3-acetyl and 3-(2-acetoxy) propionyl derivatives (**10** and **11**) were slightly better than betulinic acid. Compound **5**, 3-*O*-trimethylacetyl derivative of betulinic acid, had similar activity as betulinic acid. It indicated that the hydrazone functionality at position-3 in 20,29-dihydrobetulinic acid was the best substituent for anti-prostate activity.

In ovarian cancer cell line, all the betulinic acid derivatives (**1**–**27**) have shown several fold better cytotoxicity than betulinic acid. In particular, the 20,29-dihydrobetulinic acid derivatives **9**–**12**, **19**, **20**, and **23** were relatively highly potent. As in the case of anti-prostate agents, here also, compound **20** was the most potent. It pointed that hydrazone substituent at position-3 in 20,29-dihydrobetulinic acid plays a key role in eliciting anti-ovarian as well as anti-prostate activity.

In lung cancer cell lines (A549 and L132), the cytotoxicity against A549 cell line, was several fold increased after converting betulinic acid into 20,29-dihydrobetulinic acid derivatives **9**–**11** and **19**. In L132 cell line, though, none of the derivatives (**1**–**27**) were found better than betulinic acid. However, 3-hydroxyloxime 20,29-dihydrobetulinic acid (**19**) was the most potent on L132 cell line. It clearly showed that oxime group in 20,29-dihydrobetulinic acid plays a crucial role in eliciting anti-lung cancer activity.

4. Conclusion

In the present study, several derivatives have shown better cytotoxicity than betulinic acid. The 2-bromo 20,29-dihydrobetulonic acid derivative (**23**) had shown broad spectrum cytotoxicity. It had better cytotoxic activity than betulinic acid in two out of five cancer cell lines of leukemia/lymphoma and similar activity as betulinic acid on JurkatE6.1 cell line. The betulonic acid (**13**) and 3-*O*-acyl 20,29-dibromobetulinic acid (**27**) were more active than betulinic acid in one out of five cell lines of leukemia/lymphoma. The 3-hydroxyloxime 20,29-dihydrobetulonic acid (**19**) had shown better activity than betulinic acid in one of the two lung cancer cell lines. The 3-phenylhydrazone 20,29-dihydrobetulonic acid (**20**) had several fold better anti-prostate as well as anti-ovarian cancer activity than betulinic acid. The present study pointed that bromo group in betulinic acid is vital for anti-leukemia/lymphoma activity. The oxime group at position-3 is essential for anti-lung activity while the hydrazone substituent is responsible for eliciting anti-prostate and anti-ovarian activities. And in general, C-28 carboxylic acid group in betulinic acid and its derivatives was found essential for providing cytotoxic activity. Earlier studies have shown that C-20 side chain is not good for structural modifications.[14] On the contrary, it was interesting to note that in the present study, C-20 double bond in betulinic acid was not critical for the cytotoxic activity in the cell lines tested since upon its hydrogenation, the cytotoxicity of the derivatives was affected significantly. Based on these studies, compounds **13**, **19**, **20**, **23**, and **27** have been selected as 'LEAD' molecules and further studies are under progress to determine the ADME characteristics and in vivo activity in animal models.

5. Materials and methods

5.1. Chemicals

MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) was obtained from Sigma, USA, DMEM (Dulbecco's modified Eagles medium) from Gibco BRL, USA, fetal bovine serum (FBS) from Gibco BRL, USA, dimethyl sulfoxide (DMSO) from Merck, India, antibiotic solution (containing penicillin and streptomycin) from Hyclone, USA. Chemicals used in synthesis were purchased from Sigma, USA.

5.2. Cell culture

Human tumor cell lines MOLT-4 (human lymphoblastic leukemia), JurkatE6.1 (human lymphoblastic leukemia), CEM.CM3 (human lymphoblastic leukemia), BRISTOL8 (human B-cell lymphoma), U937 (human histio-

cytic lymphoma), DU145 (prostate), PA-1 (human ovary), A549 (lung), and L132 (lung) 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 μg/mL), and amphotericin B (0.25 mg/mL) at 37 °C, 5% CO_2, 100% humidity.

5.3. Cytotoxicity assay

Cells (1.5×10^4) were incubated with the compounds (**1**–**27**) dissolved in DMSO (final DMSO concn < 0.1%), in triplicate wells to obtain drug concentration of 0.1–10 μg/mL. Cytotoxicity was measured after 72 h using MTT assay as described by Mosmann.[15] Each experiment was repeated thrice and mean ED_{50} values (half-maximal cytotoxicity) have been reported.

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