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Development of C-20 Modified Betulinic Acid Derivatives as Antitumor Agents

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Abstract—Chemical modifications were performed on C-20 position of betulinic acid for a structure–activity relationship study. The evaluation of the compounds using human colon carcinoma HCT-116, human prostate adenocarcinoma PC3, and human melanoma cell lines M14-MEL, SK-MEL-2, and UACC-257 did not show any selective cytotoxicity towards melanoma cells. The results from both MTT reduction assay and SRB staining assay were comparable that no remarkable differences in cytotoxicity profile of the compounds were noticed. The C-20 position was found to be sensitive to the size and the electron density of the substituents in retaining the cytotoxicity of betulinic acid and was found to be undesirable position to derivatize.

For the past four decades, the incidence of melanoma has been increasing at a rate higher than that of any other type of cancer.[1,2] For patients with metastatic melanoma, DTIC [5-(3,3-dimethyl-1-triazenyl)-1*H*-imidazole-4-carboxamide] has been the most efficacious single chemotherapeutic agent, and combination therapy with other synthetic and recombinant agents has been used for treatment of human melanoma.[3] However, a durable complete response is uncommon and greater toxicity is often observed.[4,5] Thus, current treatment for patients with metastatic melanoma are unsatisfactory. During a drug discovery effort from natural resources for potential anticancer activity agents 3β-hydroxy-lup-20(29)-ene-28-oic acid, betulinic acid (**1**), has been isolated from *Ziziphus mauritiana* Lam. (Rhamnaceae) to show selective cytotoxicity against cultured human melanoma cells (MEL-2), as opposed to several other cell types including human epidermoid carcinoma of the mouth (KB).[6] The ability of betulinic acid to induce apoptosis in melanoma[6] and other cell types[7] and the favorable therapeutic index from the lack of toxicity towards normal cells[6] suggested betulinic acid to be an attractive and promising antitumor agent. The selective toxicity towards malignant melanoma as compared to normal melanocytes was underscored by a study reporting a lack of toxicity towards melanocytes in an in vivo model.[8] Other biological activities reported for betulinic acid include antiinflammatory activity[9] and inhibition of phorbol ester-induced epidermal ornithine decarboxylase (ODC) accumulation in the mouse ear model with subsequent inhibition of the carcinogenic response in the two-stage mouse skin model.[10] In addition, betulinic acid (**1**) was shown to inhibit human immunodeficiency virus (HIV) replication in H9 lymphocytes[11] and to block HIV-1 entry into cells.[12] Synthetic derivatives of betulinic acid have also been investigated as specific inhibitors of HIV-1.[13]

Recently, we reported the synthesis and biological evaluation of betulinic acid analogues against MEL-2 and KB cell lines.[14–16] Betulin (**2**), available in abundance up to 25% dry weight of bark from white birch bark,[17] has been synthetically converted to betulinic acid in a two-step process of high yield (Scheme 1).[14,15]

Betulinic acid has three positions (C-3, C-20, and C-28) where chemical modifications can be performed to yield derivatives for structure–activity relationship (SAR) study. We have performed modifications at the positions C-3 and C-28, and demonstrated that simple modifications of the parent structure of betulinic acid can produce potentially important derivatives, which may be developed as antitumor drugs.[14,15] In another study, we coupled betulinic acid with a series of amino acids at C-28 carboxylic acid position and demonstrated that this process can also produce potentially important

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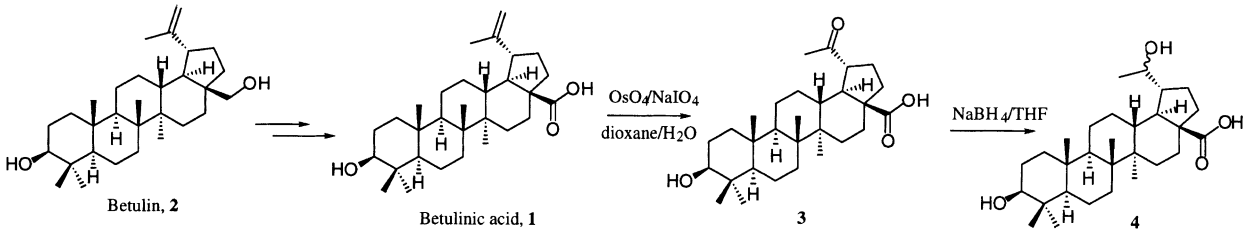


Scheme 1.

derivatives.[16] A number of amino acid conjugates of betulinic acid at C-28 position showed remarkably improved water solubility while retaining the selective cytotoxicity.

The above investigations demonstrated that simple modification of the parent structure of betulinic acid can produce a number of potentially important derivatives, which may improve the selective toxicity profile or introduce general toxic effects. However, results from a more extensive investigation using a greater number of derivatives is needed for SAR study for the design and ultimate synthesis of a more effective betulinic acid-derived antitumor agent. Here, we report the synthesis of betulinic acid derivatives modified at C-20 position and cytotoxicity evaluation of the derivatives using different tumor cell lines than previously tested. In this study, both 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT) reduction assay and sulphorhodamine (SRB) cellular protein staining assays were carried out and the results were compared.

Cell Lines, and Cytotoxicity Assay Using MTT

Human colon carcinoma line HCT-116, and human melanoma cell lines M14-MEL, SK-MEL-2, and UACC-257 were obtained from National Cancer Institute's Antineoplastic Drug Screen program, and maintained as described.[17] Cells were plated in a 96-well plate (5000–15,000 cells per well) 24 h prior to compound treatment. All test compounds were solubilized in dimethyl sulfoxide (DMSO) and were tested at five 5-fold dilutions (100, 20, 4, 0.8, and 0.16 μM). Each compound concentration was tested in quadruplicate wells. After 72 h incubation with test compounds, relative cell growth was assessed using MTT-based Cell Proliferation Kit I (Roche Molecular Biochemicals, Indianapolis, IN, USA), according to manufacturer's instructions. Briefly, at the end of treatment, cells were labeled with MTT (0.5 mg/mL), followed by solubilization with 10% SDS in 0.01 M HCl at 37 °C overnight. Absorbance at 570 nm was determined, and the percentage relative growth of cells (GI_{50}) in each compound treated well was determined on the basis of the cell growth in untreated control wells.[17]

Cell Lines and SRB Assay[15,16]

Human prostate adenocarcinoma cell line PC3 and human melanoma cell line SK-MEL-2 were obtained from ATCC, and maintained as described.[17] Cells were plated in a 96-well plate (5000–15,000 cells per well) 24 h prior to compound treatment. All test compounds were solubilized in DMSO and were tested at five 5-fold dilutions (100, 20, 4, 0.8, and 0.16 μg/mL). The final concentration of DMSO was less than 1% of the total volumn. Each compound concentration was tested in quadruplicate wells. After incubating the plates for 72 h at 37 °C (100% humidity with 5% CO_2 atmosphere), the cellular proteins were precipitated to the plates with trichloroacetic acid and stained with 0.4% SRB. Protein-bound SRB was solubilized with Tris base and read at 515 nm in an ELISA reader. The protein content of the compound-treated cells was compared to that of DMSO control and ED_{50} values were obtained.

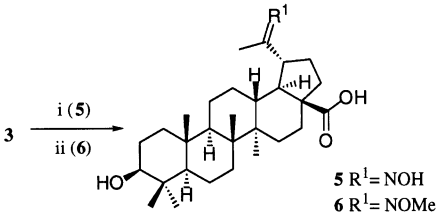
Chemical Modification at C20

The C-20 position of betulinic acid (**1**) has alkene functional group. The chemical modification at this position was initiated by converting the double bond to a ketone using $OsO_4/NaIO_4$ system (Scheme 1).[18,19] The ketone functionality was readily transformed to oximes and secondary alcohol (Scheme 2). The isopropenyl side chain was also brominated to afford a handle to put different substituents (Scheme 3). The products were purified using HPLC [gradient eluent system, CH_3CN/H_2O (80:20) to CH_3CN (100%)].

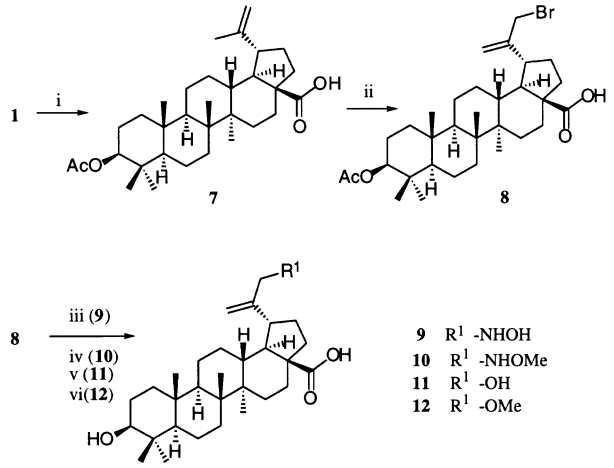
Experimental

Ozonolysis of betulinic acid using $OsO_4/NaIO_4$ system[18,19]

Double bond in betulinic acid (**1**) was converted to ketone moiety using $OsO_4/NaIO_4$ involved ozonolysis. To a dioxane/H_2O (35 mL/5 mL) solution containing betulinic acid (**1**, 1.0 g) was added OsO_4 (50 mg) and stirred until the solution became dark grey. $NaIO_4$ (5 g)



Scheme 2. (i) $HONH_3Cl/NaOAc/MeOH$; (ii) $MeONH_3Cl/NaOAc/MeOH$.



Scheme 3. (i) Acetic anhydride/pyridine; (ii) NBS/CCl_4; (iii) $HONH_3Cl/NaOAc/i$PrOH; (iv) $MeONH_3Cl/NaOAc/i$PrOH; (v) $LiOH/DMF/H_2O$; (vi) NaOAc/MeOH.

was added in small portions over 3 h and stirred for 3 days at room temperature. The reaction mixture was poured into separatory funnel containing ethyl acetate (200 mL) and washed with water (200 mL×3). The water layer was back extracted with ethyl acetate (100 mL×2). The organic layer was combined, dried ($MgSO_4$), filtered, and the solvent was removed under vacuum to afford a residue. The residue was chromatographed over silica gel (230–400 mesh) using 1:1 ethyl acetate/petroleum ether eluent system to afford 75% yield of ketone **3**. The resulting ketone **3** was reduced ($NaBH_4$ in refluxing THF)[15] to afford the secondary alcohol **4** (80%).

Preparation of oximes 5 and 6

The ketone **3** (50 mg each) was subjected to methoxylammonium chloride and hydroxylammonium chloride condensation in the presence of NaOAc in ethanol[15,20] to afford the corresponding oximes **5** and **6** in quantitative yield, respectively (Scheme 2).

Preparation of brominated betulinic acid-derived analogues

Hydroxyl group at C-3 of betulinic acid (**1**) was acetylated (acetic anhydride/pyridine) to the corresponding **7**, followed by bromination using NBS/CCl_4 to afford **8** in quantitative yield (Scheme 3).[13] Brominated betulinic acid **8** was converted to the corresponding hydroxyl amine **9** (85%) using $HONH_3Cl/NaOAc/i$PrOH system and methoxyl amine **10** (74%) using $CH_3ONH_3Cl/NaOAc/i$PrOH system, respectively. Treatment of **8** with $LiOH/DMF/H_2O$ and NaOMe/MeOH afforded corresponding alcohol **11** and ether **12** in quantitative yield, respectively.

Discussion

The isopropenyl group was successfully converted to ketone **3** and subsequent chemical manipulation yielded C-20 modified derivatives **4**–**6**. Furthermore, the isopropenyl group was converted to brominated betulinic acid derivative **8** that provided a handle to carry out chemical modifications to afford compounds **9**–**12**.

In addition to the above synthesized compounds, a number of other betulinic acid derivatives (**13**–**16**) were also tested (Fig. 1). The cytotoxicity evaluation of these compounds showed that betulonic acid (**13**) was the most active compound. When the double bond was oxidized to a ketone **3**, loss of the cytotoxicity was observed, suggesting that the presence of highly electronegative oxygen atom may be changing the electrostatic property of betulinic acid, rendering it less toxic.

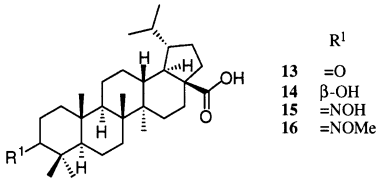


Figure 1.

Table 1. Cytotoxicity profile of betulinic acid derivatives against selected human cancer cell lines

Compound	GI50 (μM) MTT assay				ED50 (μg/mL) SRB assay	
	HCT-116	M14-MEL	SK-MEL-2	UACC-257	PC3	SK-MEL-2
1	2.7	2.7	3.2	1.5	1.9	2.2
3	>50	>50	>50	>50	>50	>50
4	>50	>50	>50	>50	>50	>50
5	>50	>50	>50	>50	>50	>50
6	>50	>50	>50	>50	>50	>50
7	11.3	6.4	3.9	4.5	7.2	6.9
9	—[a]	—	—	—	>50	>50
10	—	—	—	—	>50	>50
11	9.8	16.2	9.3	>50	8.4	9.0
12	8.6	9.0	>50	12.7	9.4	10.1
13	—	—	—	—	1.3	1.6
14	—	—	—	—	2.2	2.0
15	—	—	—	—	3.0	3.4
16	—	—	—	—	16.4	21.2

The assay was performed twice in quadruplicate.
[a]Not tested.

Converting the ketone functionality to the corresponding oximes (**5** and **6**) also appears to result in the loss of cytotoxicity, probably due to the same reason described above. These results suggest that the cytotoxicity profile of betulinic acid dervatives may be sensitive to the size of the substituents at the C-20 position in addition to the electrostatic sensitivity. The hydroxyl amine **9**, methoxyl amine **10**, primary alcohol **11**, and the methoxyl ether **12** also showed the loss of cytotoxity. Dihydrobetulinic acid (**14**) retained the cytotoxicity of the parent compound, betulinic acid (**1**). Based on these results, we conclude that C-20 position of betulinic acid is not a favorable place to derivatize to improve cytotoxicity.

During this investigation highly unexpected results were observed (Table 1). In the previous studies,[15,16] human melanoma cell line MEL-2 (obtained from UIC medical school) was routinely used to represent human melanoma model, and human epidermoid carcinoma of the mouth KB cell lines was routinely used to represent human non-melanoma model to evaluate the selective cytotoxicity of betulinic acid and its derivatives. In this present study, human colon carcinoma cell line HCT-116 and human prostate adenocarcinoma cell line PC3 were used in place of KB cells as the non-melanoma models, while human melanoma cell lines M14-MEL, SK-MEL-2, and UACC-257 were used as the melanoma models to evaluate the selective cytotoxicity. Interestingly, the evaluation of these compounds using the different tumor cell lines did not show any selective cytotoxicity. The results from both MTT reduction assay and SRB staining assay were comparable that no remarkable differences in cytotoxicity profile of the compounds were noticed. Because of these unexpected results, we are currently in a process of testing selected betulinic acid derivatives, including the parent compound, using other non-melanoma cell lines to evaluate whether betulinic acid indeed possess selective cytotoxicity against human melanoma cells or not.

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