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New ionic derivatives of betulinic acid as highly potent anti-cancer agents

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Abstract

Betulinic acid is a natural compound with high *in vitro* cytotoxicity toward many cancer cells. However, the poor water solubility of this compound hampers an effective *in vivo* cancer study. We prepared new ionic derivatives of betulinic acid with higher water solubilities, without losing the structural integrity and functionality of this compound. As a result, these new ionic derivatives have shown much higher inhibitory effects against different cancer cell lines such as melanoma A375, neuroblastoma SH-SY5Y and breast adenocarcinoma MCF7. For A375 cell lines, the derivative **5** exhibited a low IC_{50} value of 36 μM (*vs* 154 μM for betulinic acid); for MCF7 cell lines, the derivative **5** also exhibited a low IC_{50} value of 25 μM (*vs* 112 μM for betulinic acid). The high cytotoxicity of these new derivatives can be linked to their greatly improved water solubility. Our assay method used little DMSO in aiding the dissolution of these derivatives to demonstrate the advantage of improved water solubility and to mimic the *in vivo* study conditions. The cell viability studies based on both MTT and LDH assay methods have confirmed the high inhibitory effect of our ionic derivatives of betulinic acid (particularly **4** and **5**) against different cancer cells.

Keywords

Betulinic acid; anti-cancer agent; melanoma cancer cell; ionic derivative; ionic liquid

Betulinic acid (3β-hydroxy-lup-20(29)-en-28-oic acid, **1**) is a natural pentacyclic lupane-type triterpene (Scheme 1) that can be found in various plants including birch trees. This compound and its derivatives possess many favorable biological properties such as anticancer, anti-HIV-1 (human immunodeficiency virus type-1), antibacterial, anti-malarial, anti-inflammatory, and anthelmintic activities.[1–6] Betulinic acid was initially known for its high cytotoxicity against human melanoma cancer cells,[7, 8] but later studies also suggest this

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Supplementary data
Supplementary data associated with this article can be found, in the online version, at XXX.

compound being a broad inhibitor of other cancerous tumors including aneuroectodermal tumors (such as neuroblastoma, medulloblastoma, glioblastoma and Ewing's sarcoma),[9–12] brain-tumors,[13] human gliomas,[14] leukemia,[15–17] human colon carcinoma and human prostate adenocarcinoma,[18–21] head and neck squamous carcinoma cells,[22] lung, colorectal, breast, and cervical cancer.[21]

The main mechanism of anti-cancer action of betulinic acid (**1**) is known as the induction of apoptosis in cells independent of their p53 status.[9–11, 20] The Debatin group[11, 23, 24] suggests that betulinic acid could induce mitochondria to undergo permeability transition (PT), which causes the release of cytochrome *c* into the cytosol, the activation of caspases (interleukin 1β-converting enzyme/Ced-3-like proteases), and the DNA fragmentation. Another mechanism indicates betulinic acid acting as the inhibitor of aminopeptidase N (APN); since APN is closely associated with extracellular matrix components, its inhibition is likely to prevent the melanoma invasion into basement membranes.[25] However, a later study suggested that betulinic acid showed no inhibition of this enzyme *in vivo* in endothelial cells or APN-positive tumor cells; alternatively, anti-angiogenic activity of betulinic acid is believed to occur through a modulation of mitochondrial function rather than APN activity in endothelial cells.[26] Other possible mechanisms include the protection of congenital melanocytic naevi (CMN) cells from UV-C (254 nm)-induced DNA strand breakage independent of p53 and p21,[27] the inhibition of topoisomerases I and IIα,[28] the inhibition of cholesterol acyltransferases (ACAT-1 and ACAT-2),[29] the activation of p38 and proapoptotic mitogen-activated protein kinases (MAPKs),[30] the inhibition of NF-κB expression,[31] and the decreased cyclin D1 expression and induced proteasome-dependent degradation of Sp proteins.[19] Therefore, the inhibition mechanism of betulinic acid on different cancerous cells is a rather complex mode of action.

To further examine the structural features responsible for biological activities of betulinic acid and optimize its pharmacological effects, a number of derivatives of betulinic acid have been prepared and evaluated, mainly targeting on the modifications of C-3 hydroxyl, C-20 alkene, and C-28 carboxylic acid groups.[1, 2, 4, 6, 32] Despite the promising results on *in vitro* examination of betulinic acid and its derivatives over different cancer cell lines, there is only limited *in vivo* research on mice.[7, 19, 33, 34] A main reason is likely to be the high lipophilic characteristics and the poor water-solubility of betulinic acid and its derivatives although some formulations have been used during *in vivo* studies (such as co-precipitating with polyvinylpyrrolidone,[7] mixing with corn oil and 1% DMSO,[19] or dissolving in a mixture of 10% ethanol, 10% Tween-80 and 80% water[33]). The solubility of betulinic acid in water is only about 0.02 μg mL^{-1} at room temperature.[35] Its solubility in common organic solvents at 25 °C is also fairly low; e.g., 1% (w/v) in ethanol and 5% (w/v) in DMSO.[36] A limited number of derivatives of betulinic acid were prepared with improved water solubility and biological activity comparing with unmodified betulinic acid.[1, 4, 32] Building on the early progress and our experience with ionic liquids,[36, 37] we anticipate that novel ionic derivatives of betulinic acid may exhibit a further improved water solubility and anti-cancer activity. Our recent study[38] suggests new ionic derivatives of betulinic acid have showed improved water solubility and better inhibition against HIV-1 protease. In this study, we hypothesize new ionic derivatives will also be better inhibitors against cancer cells than betulinic acid itself.

The idea of preparing ionic derivatives of betulinic acid originated from the common concept that many ionic compounds are water-soluble. Recently, there is a rising interest in converting crystalline medicinal molecules into so-called "ionic liquid active pharmaceutical ingredients" (IL-APIs)[39–41] by making them ionic and pairing them with counter-ions; the purpose is to eliminate the problematic polymorphism of pharmaceutical drugs and to add a second biological functionality complementary to the API. For example, sodium ibuprofen

(an anti-inflammatory) can be paired with didecyldimethylammonium bromide (antibacterial and anti-inflammatory) to produce an IL-API (didecyldimethylammonium ibuprofenate) which retains dual biological roles.[42]

Motivated by the molecular flexibility, we recently designed ionic derivatives of betulinic acid with improved aqueous solubility and found they have a high inhibitory effect against HIV-1 protease.[38] As shown in Scheme 2, derivatives **2** and **3** contain anions of betulinic acid conjugated with glycine at C-28 carboxylic acid positions; **4** and **5** simply contain anions of betulinate, and cations of benzalkonium and cholinium respectively. These new compounds represent minimum modifications of betulinic acid structure and minimum disruption of its biological functions, and have introduced the second functionality (benzalkonium compounds are known for their antibacterial properties;[39] cholinium compounds are usually non-toxic, and choline chloride is even an essential micronutrient for human health[43]).

In this study, we examined the inhibitory effect of betulinic acid (**1**) and its four derivatives (**2–5**) against four cancer cell lines (i.e. melanoma A375, neuroblastoma SH-SY5Y, breast adenocarcinoma MCF7 and epidermoid carcinoma A431) (see experimental procedures in Supplementary Data). As shown in Fig 1, IC_{50} values (*in vitro* cytotoxicity activity) of betulinic acid (**1**) are in the range of 112–353 μM for these cell lines. In previous studies, IC_{50} values of betulinic acid typically fall in the rage of 0.5–17 μg/mL depending on the type of cell lines and assay methods,[1, 4, 6, 32] but could also fall in a higher range such as 23–108 μM for mesenchymal (CEM, K562, K562-tax), epithelial (HT29, PC-3), and neuroektodermal (SK-MEL2) tumors.[44] The discrepancy between our high IC_{50} values and literatures' low values can be explained by the difference in DMSO concentrations during *in vitro* assay studies. Conventionally, betulinic acid is dissolved in DMSO and further diluted by DMSO before being added to the cell culture; the final DMSO concentration in the cell culture is usually in the range of 0.5–2% (such as 0.5%,[8, 21] up to 0.75%,[33] 1%,[18] and 2%[44]). The presence of DMSO (even at the low concentration) is to assist the solubilization of betulinic acid since this compound is poorly soluble in water. Although a low concentration of DMSO is not inhibitory to most cell growth, this does not reflect the *in vivo* study where DMSO is not present in animal cells at this concentration (0.5–2%). The low water solubility has been a major challenge for *in vivo* investigation of betulinic acid. In our study, all compounds (**1–5**) were dissolved in DMSO initially at 5 mg/mL as stock solutions, but they were further diluted by cell culture media (*vs* by DMSO in previous studies) to appropriate concentrations. The final DMSO concentration in cell culture was kept at only 0.1% (v/v). Although some compounds (such as **1**) began to precipitate out upon dilution by aqueous media, this delivery mode mimics the *in vivo* scenario better than the previous approach. Thus, our higher IC_{50} values are likely to match closely to *in vivo* experiments. Similarly, Yazan et al[45] used this method to dilute the stock solution of betulinic acid in DMSO (10 mg/mL) by the cell culture medium to final concentrations of 0.1–0.7%, and reported relatively high IC_{50} values of betulinic acid toward human mammary carcinoma cell line MDA-MB-231 and human promyelocytic leukemia cell line HL-60 as 58 μg/mL and 134 μg/mL respectively [*vs* lower IC_{50} values of 12.5 μg/mL (for K562 leukemia cell line)[17] and ~40 μg/mL (for L1210 leukemia cell line)[15]].

More importantly, our ionic derivatives (**2–5**) have consistently showed much lower IC_{50} values against four cancer cell lines (Fig 1), indicating their higher cytotoxicities than **1**. In particular, IC_{50} values of **4** and **5** are at least 3–4 folds lower than **1** against melanoma A375, neuroblastoma SH-SY5Y, and breast adenocarcinoma MCF7 (Fig 1a, b and c respectively). Betulinic acid (**1**) was less effective in inhibiting the growth of epidermoid carcinoma A431 (IC_{50} = 353.2 μM), which is consistent with the literature's finding.[8] However, our ionic derivatives still showed much improved cytotoxicity, especially **3** and **4**

with half of the IC_{50} value of **1**. The overall cytotoxicities of these compounds decrease in the order of **5**, **4** > **3** > **2** >**1**. A similar inhibition trend for these compounds was observed for HIV-1 protease in our early study.[38] The greatly improved anti-cancer properties of these new ionic derivatives can be loosely linked to their higher water solubilities. As suggested by our earlier study,[38] **2** and **3** are about three- and two-fold more water-soluble than **1** whilst **5** is at least 100 times more water soluble than **1**. In this study, we found **4** is about two times more water soluble than **1**.

To compare the concentration-dependence of cancer cell inhibition by different derivatives, we illustrate the cell viability of those four cancer cell lines at different concentrations of compounds (**1–5**) based on the MTT assay method. A typical comparison is shown in Fig 2 for melanoma A375 (others are provided in Supplementary Data as Fig S1–S5). After 24 h of cell incubation following the addition of compound **1** or **5**, melanoma cell viability decreases as the compound concentration increases, but **5** requires much lower molar concentration to reach 50% inhibition than **1**. A longer incubation time (48 h) of cells with each compound further decreases the cell viability, but not significantly. Same trends can be observed for other cell lines in the presence of betulinic acid derivatives (Fig S1–S5). These comparisons further confirm that ionic derivatives are more cytotoxic to cancer cells, particularly **4** and **5**.

Lactate dehydrogenase (LDH) is a soluble enzyme located in the cytosol, and is released into the surrounding culture medium upon cell damage or lysis, processes that occur during both apoptosis and necrosis. Therefore, the level of LDH in the cell lysate is a reliable indicator for cell membrane integrity, and thus a measurement of cytotoxicity. Using the LDH assay (see experimental procedures in Supplementary Data), we measured the optical density (at 490 nm) of LDH released from cancer cells upon the addition of different concentrations of **1–5**. A typical illustration is shown in Fig 3 (others are provided in Supplementary Data as Fig S6–S10). Once again, we can see the higher cytotoxicity at a higher concentration of **1** or **5**; in particular, **5** requires a much lower concentration than **1** to have 50% inhibition (Fig 3), implying the higher cytotoxicity of **5**. Overall, based on the LDH assay, the cytotoxicities of these compounds decrease in the order of **5**, **4** > **3** > **2** > **1**, which is consistent with our conclusion from MTT assay.

Since many tumors produce acidic extracellular environments,[46] compounds **4** and **5** can easily gain protons (H^+) to turn back to betulinic acid (however, the ionic forms of **4** and **5** are essential to assist the dissolution and transport of these compounds to cancerous cells), and it is also known betulinic acid exhibits a greater inhibition against melanoma cell growth at acidic conditions (≤ 6.8).[15, 47] In particular, **5** is not expected to have toxic effects on healthy cells because betulinic acid has low toxic effects against healthy human cells *in vitro*,[14, 21, 33, 48, 49] and no *in vivo* toxicity was observed in mice even at a dose of 500 mg/kg;[7] cholinium salts are typically nontoxic (for example, choline chloride is an essential micronutrient and human nutrient,[43] and cholinium alkanoates are environmentally benign and biodegradable[50]). However, in our future study, the toxicity of these derivatives on healthy cells will be further investigated.

In conclusion, our new ionic derivatives of betulinic acid have exhibited high cytotoxicities toward several cancer cell lines based on MTT and LDH assay methods. The IC_{50} values of **4** and **5** are 3–4 folds lower than that of betulinic acid. The improvement is mainly due to the preservation of betulinic acid structure and the increase in water solubility. This study also confirms the inhibitory effect of betulinic acid derivatives against different cancer cells, not just melanoma cells.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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References and notes

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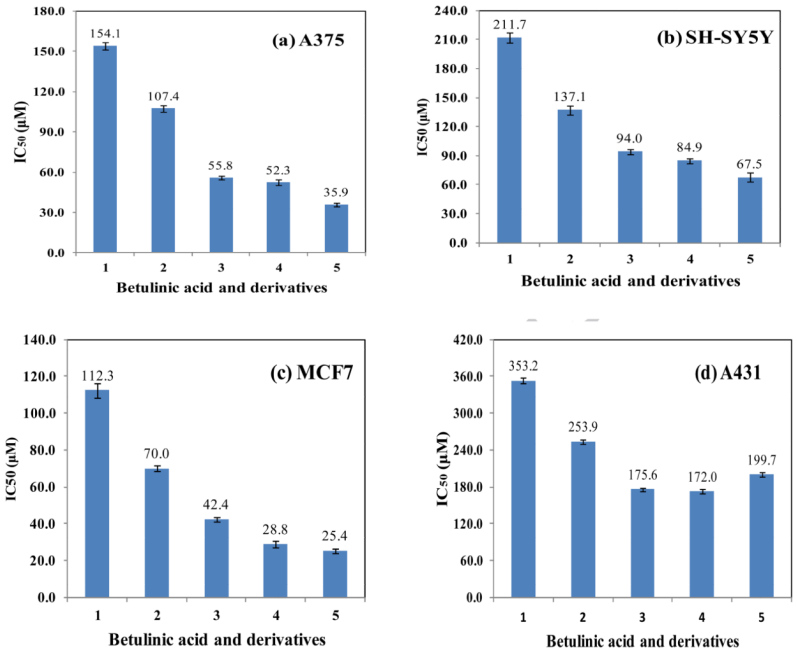


Fig. 1.

IC_{50} values of betulinic acid and derivatives against four cancer cell lines: (a) melanoma A375, (b) neuroblastoma SH-SY5Y, (c) breast adenocarcinoma MCF7, and (d) epidermoid carcinoma A431.

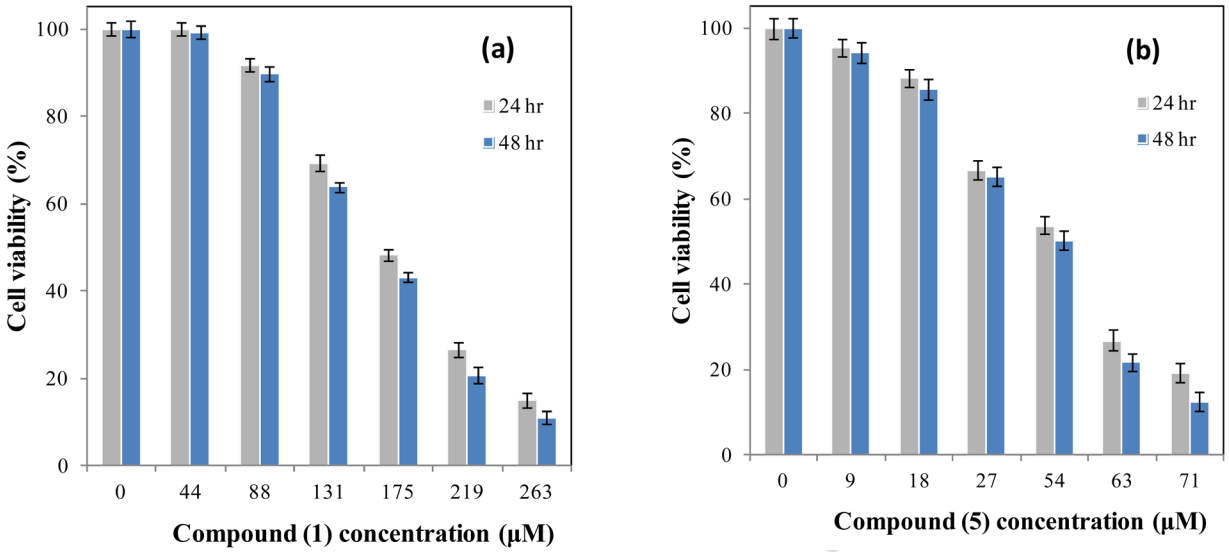


Fig 2.

Cell viability of melanoma A375 at different concentrations of (a) compound **1** and (b) compound **5** (based on MTT cell assay, cell incubation time after adding the compound was 24 or 48 h).

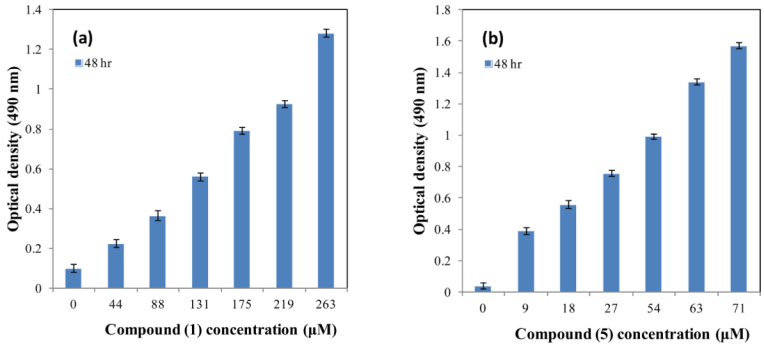
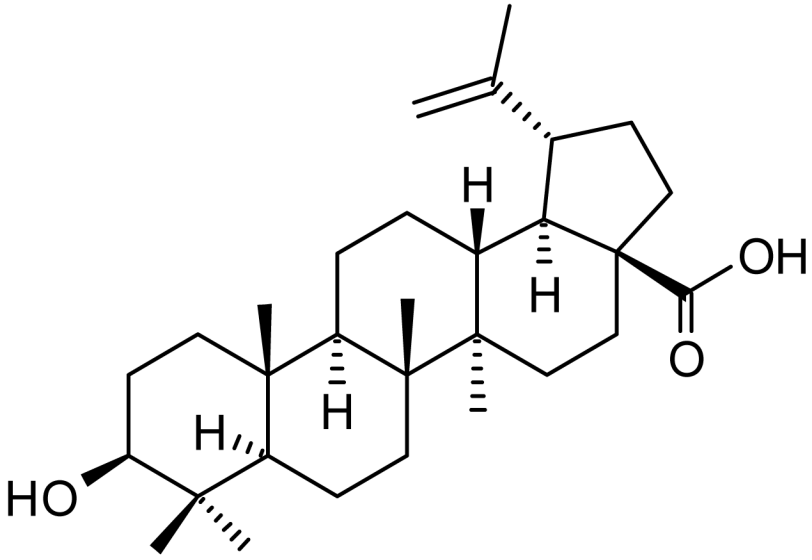
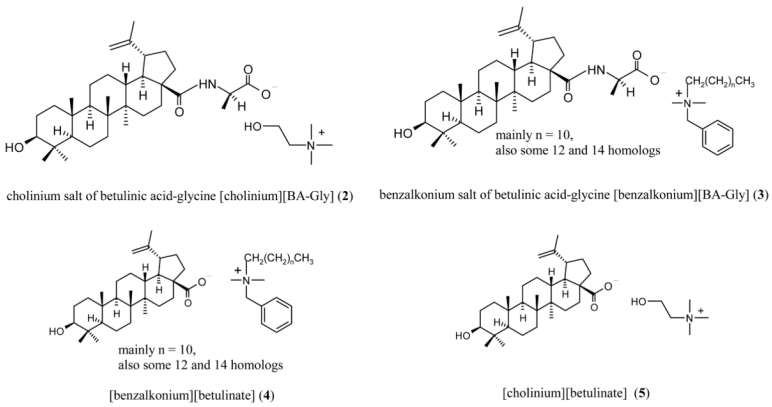


Fig 3.

Optical density of LDH released from melanoma A375 at different concentrations of (a) compound **1** and (b) compound **5** (based on LDH cell assay, cell incubation time after adding the compound was 48 h).



Scheme 1.
Structure of betulinic acid (**1**).



Scheme 2.
Ionic derivatives (**2**–**5**) of betulinic acid.