Boc-lysinated-betulonic acid: A potent, anti-prostate cancer agent

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Abstract—Betulonic acid, derived from betulinol, a pentacyclic styrene, has shown a highly specific anti-prostate cancer activity in vitro cell cultures. However, due to the lack of solubility of betulonic acid in aqueous medium, its potent anti-cancer activity in vivo has not been determined to the fullest extent. The present study describes the chemical synthesis of hydrophilic Boc-lysinated-betulonic acid, which has improved its solubility in an aqueous biocompatible solvent. Evaluation in cytotoxicity assays, Boc-lysinated-betulonic acid dissolved in phosphate-buffered saline (PBS) containing 22% ethanol and 4% human serum albumin, has shown 95.7% inhibition of LNCaP prostate cancer cells in culture after 72 h incubation at a concentration of 100 \( \mu \)M, but with little effect on normally proliferating fibroblast cells. In the in vivo assay, male athymic mice transplanted with human prostate LNCaP xenografts were injected with Boc-lysinated-betulonic acid intraperitoneally at a dose of 30 mg/kg daily for 17 days. The treated mice exhibited 92% inhibition of tumor growth as compared to controls. Histological sections of the tumors showed that Boc-lysinated-betulonic acid arrested mitosis and induced apoptosis, which was confirmed by TUNEL assay, Yo-Pro-1 staining, and the release of cleaved caspase-3 from the ex vivo in tumor culture. These studies, for the first time, demonstrate that a non-toxic hydrophilic lysinated derivative of betulonic acid and its solubility in a biocompatible aqueous medium has enhanced the bioavailability of the drug and has thus unleashed its full anti-prostate cancer activity.

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1. Introduction

Prostate cancer is the most frequently diagnosed cancer and is a leading cause of cancer morbidity and mortality in men in the United States with an estimated incidence of more than 916 new cases and 115 deaths per day.1 The use of chemotherapeutic agents in the treatment of cancer has become a widely used practice. However, the adverse effects of the cytotoxic drugs to the non-targeted normal cells have remained a major concern.2,3 Development of new drugs with high anti-prostate cancer activity and with low adverse effects is the most urgent demand to treat cancer.

The anti-cancer activity of terpenoids has been described earlier.4-6 Pentacyclic styrenes have shown anti-tumor activity against carcinosarcoma growth and Epstein-Barr virus in lymphoblastoid Raji cells7,8 as well as in nasopharynx carcinosarcoma.9 The in vivo anti-cancer activity of terpenoids was also shown against Walker-256 carcinosarcoma, as tested in mice and rats.10 It is noteworthy that betulinol, a pentacyclic alcohol of the group of styrenes as well as its other derivatives, has shown minimal cytotoxic effect on normally proliferating fibroblast cells and on non-target tissues.11

The structure of betulinol is based on a 30-carbon skeleton comprising of four six-membered rings and one five-membered ring, which has three sites available for chemical modification, at C-3, C-20, and C-28, to yield its derivatives for structure-function studies. Betulonic acid, a derivative of betulinol, has shown high cytotoxicity in vitro against LNCaP, PC-3, and DU-145 prostate cancer cells.12-15
Betulonic acid is soluble in organic solvents. However, due to the lack of solubility of betulonic acid in biocompatible aqueous solvents, the anti-cancer activity of betulonic acid in vivo has not been determined to the fullest extent. In the present study, Boc-lysinated-betulonic acid (hereafter, referred to as lysinated-betulonic acid) has been chemically synthesized as a hydrophilic derivative of betulonic acid and solubilized in a biocompatible aqueous solvent, namely, phosphate-buffered saline (PBS) containing 22% ethanol and 4% human serum albumin. The anti-cancer activity of both betulonic acid and its lysinated derivative was investigated in an in vitro MTT cytotoxicity assay as well as in vivo growth inhibition assay of human prostate LNCaP tumor xenografts in athymic mice. The mechanism of action of lysinated-betulonic acid was explored by histological examination, Yo-Pro-1 staining, TUNEL assay, and by the release of cleaved caspase-3 from the ex vivo tumor cultures, indicating that the lysinated-betulonic acid arrested mitosis and caused apoptosis of prostate LNCaP tumor cells.

2. Results

2.1. Synthesis and characterization of betulonic acid and lysinated-betulonic acid

The synthesis of betulonic acid from betulinol using hydrogen peroxide as an oxidizing agent yielded an 88% recovery and 99% purity as compared to only 30% recovery and 80% purity using Jones reaction.16–18 The purity of betulonic acid and lysinated-betulonic acid was substantiated by a single peak with retention times of 11.044 and 10.936 min, respectively, as determined by gas chromatography (GC) (Suppl. Fig. 1a and b). The confirmation of the chemical structure of betulonic acid and lysinated-betulonic acid as determined by NMR spectroscopy confirmed their pentacyclic styrene nature. The molecular mass of betulonic acid and lysinated-betulonic acid was determined by a Quattro II triple quadrupole instrument with electrospray ionization mass spectrometric analysis (MALDI) in the positive mode. The [M+H]+ and [M+NH4]+ ions m/z 455 and m/z 472 were determined for betulonic acid (Suppl. Fig. 2a and b). The MSMS spectra showed an m/z of 544, 471, and 455 for betulonic acid. The lysinated ester of betulonic acid appeared to be a single compound, with m/z 697 singly protonated ion (Suppl. Fig. 3a-c). At a higher resolution, more slowly recorded ESI-MS scan, the monoisotopic molecular mass of the neutral compound was computed to be 696.5 ± 0.2 Da. The average molecular weight of betulonic acid and of the lysinated derivative was estimated to be approximately 469 and 663, respectively. The NMR and mass data validated that, within the acceptable limits of experimental error, one lysine residue was conjugated to one molecule of betulonic acid. The NMR spectroscopy and mass spectroscopy characterization of betulonic acid and its lysinated derivative is presented in the supplementary data.

2.2. In vitro studies: inhibition of growth of human prostate cancer cells in culture by betulonic acid dissolved in DMSO

As shown in Table 1A, in the MTT assay betulonic acid, at a concentration of 10 μM, inhibited the growth of LNCaP, DU-145, and PC-3 prostate cancer cell lines in culture with little cytotoxicity to normally proliferating fibroblast cells. Of the three human prostate cancer cell lines, the effects of betulonic acid on LNCaP cells at different concentrations as well as at different incubation time periods (Table 1B) showed that betulonic acid inhibited the growth of LNCaP cells in a dose-dependent manner at 24, 48, and 72 h of incubation. Betulonic acid showed the highest growth inhibition at 88.4% of LNCaP cells at a concentration of 10 μM after 72 h incubation.

As shown in Figure 1, in the AIG assay, betulonic acid exhibited significant inhibition in cellular growth and tumorigenicity of all three prostate cancer cell lines at a concentration of 10 μM in DMSO after 14 days of incubation. The colony formation rate in the soft agar significantly decreased to 42% (P < 0.01) for LNCaP (Fig. 1B), 40% (P < 0.05) for DU145 (Fig. 1B), and 74% for PC-3 cells (P < 0.05) (Fig. 1B), as compared with their corresponding controls. Interestingly, the untreated LNCaP cells grew as three-dimensional tubular structures representing a high metastatic nature; whereas the betulonic acid treated cells completely lacked this activity. It is noteworthy that betulonic acid at the same concentration displayed little effect on normal fibroblast cells (Fig. 1A).

2.3. Comparison of growth inhibition of human prostate LNCaP cells by lysinated-betulonic acid dissolved in DMSO and PBS: 22% ethanol/4% albumin in the MTT assay

As shown in Table 2A, the lysinated-betulonic acid dissolved in DMSO inhibited the growth of LNCaP cells to, approximately, 40% at a concentration of 10 μM
after 48 as well as 72 h of incubation. However, at a concentration of 100 \(\mu\)M, the inhibition of growth increased to 80% at all incubation times. It is noteworthy that lysinated-betulonic acid dissolved in PBS/ethanol/albumin solvent showed similar cytotoxicity on LNCaP cells at the concentration of 10 \(\mu\)M, however, it increased to 95.7% at 100 \(\mu\)M after 72 h of incubation (Table 2B). Lysinated-betulonic acid at a concentration of 100 \(\mu\)M also caused significant inhibition of growth of DU-145 and PC-3 prostate cancer cells in MTT assay at 24, 48, and 72 h of incubation with little cytotoxicity to normally proliferating fibroblast cells (Table 2C), indicating that the improved solubility of lysinated-betulonic acid in the PBS/ethanol/albumin solvent provided higher availability of the drug.

### Table 2. Cytotoxicity of lysinated-betulonic acid determined by MTT assay

<table>
<thead>
<tr>
<th>Concentration ((\mu)M)</th>
<th>Incubation time (h)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2A</td>
<td>24</td>
</tr>
<tr>
<td>10</td>
<td>19.0 ± 14.4</td>
</tr>
<tr>
<td>100</td>
<td>76.5 ± 22.6</td>
</tr>
<tr>
<td>2B</td>
<td>16.33 ± 5.51</td>
</tr>
<tr>
<td>100</td>
<td>50.33 ± 13.65</td>
</tr>
<tr>
<td>Cells</td>
<td>24</td>
</tr>
<tr>
<td>DU-145</td>
<td>71.0 ± 1.61</td>
</tr>
<tr>
<td>PC-3</td>
<td>53.0 ± 1.68</td>
</tr>
<tr>
<td>Fibroblasts</td>
<td>0</td>
</tr>
</tbody>
</table>

The data are shown as means ± SD of three independent experiments. Percent growth inhibition of DU-145, PC-3 and fibroblast by lysinated-betulonic acid in PBS/ethanol/albumin at a concentration of 100 \(\mu\)M. Percent growth inhibition of LNCaP cells by lysinated-betulonic acid in medium/DMSO. Percent growth inhibition of LNCaP cells by lysinated-betulonic acid in PBS/ethanol/albumin.

Figure 1. Effect of betulonic acid at a concentration of \(10^{-3}\) M on colony formation rate of prostate cancer cell lines and fibroblast as determined in the anchorage-independent growth (AIG) assay: (A) growth of cells after incubation with betulonic acid for 14 days, (B) colony formation percent compared to the control.

2.4. In vivo effect of lysinated-betulonic acid dissolved in PBS/EtOH/albumin on LNCaP prostate cancer cell xenografts in male athymic mice

Visible tumors developed by day 12 post-subcutaneous implantation of approximately 7 million LNCaP cells in matrigel in male athymic mice. As shown in Figure 2A, the average tumor volume in control mice increased continuously from the day 1 until day 17 of injections with a net increase of \(433 ± 177\) mm\(^3\). In contrast, the average tumor volume in mice injected with lysinated-betulonic acid decreased on day 3 and then remained almost constant at, approximately, \(40 ± 2\) mm\(^3\) until day 17. The inhibition of tumor growth in the treated mice was approximately 92% as compared to the tumor volume in untreated control mice on day 17 (Fig. 2A). The difference in the tumor volumes between the control group and the lysinated-betulonic acid treated mice is clearly visible in the photographs of mice in Figure 2B and C, respectively. These results were reproduced in a repeat experiment under the same conditions. There...
was an 89% inhibition of tumor growth in the lysinated-betulonic acid injected mice as compared to the control group.

2.5. Immunohistochemical and histological studies of tumor tissues

Frozen sections of the tumors identified specific antigens PSA and PSMA in tumor cells of both lysinated-betulonic acid treated and control groups of mice. The hematoxylin–eosin stained section of the tumor tissue from control mice revealed mitotic cancer cells indicating growth with large vesicular nuclei and prominent nucleoli, both characteristics of cell viability (Fig. 3A). In contrast, the tumor cells of the lysinated-betulonic acid treated mice were devoid of mitotic division and metabolically less active (Fig. 3B), exhibiting dark chromatin and inapparent nucleoli. The TUNEL assay detected apoptotic cells in the hematoxylin–eosin stained sections of the tumor tissue. The appearance of apoptotic cells in the sections of the tumor tissue of the drug treated mice produced a brown stain indicative of apoptosis (Fig. 3D). The cells in the control group did not show any brown stain, revealing lack of apoptotic cells (Fig. 3C).

2.6. Effects of lysinated-betulonic acid on the ex vivo growth of LNCaP tumor tissue in culture

Portions of the tumor tissue harvested from control as well as from lysinated-betulonic acid injected mice were grown in culture medium for 12 days. The tumor tissue from the control mice grew as shown in Figure 4A for days 0, 5, and 9. As shown in Figure 4B, the tumor from one of the drug treated tumor also showed growth, however, after the addition of the drug daily from day 5 in the culture medium, the tumor tissue decreased in size up to day 12, indicating a direct inhibitory effect of the lysinated-betulonic acid on the growth of the tumor. Whereas, as shown in Figure 4C, the tumor tissue from the other drug treated mice did not show any growth presumably because of the absence of any viable cells.

The profile of tumor growth for 12 days is shown in Figure 4D. The tumor tissue of the control mice grew continuously from day 1 to day 12 with a 215.23% increase in the tumor size. The tissue from one treated mouse showed 68.3% growth on day 5, however, after the addition of the drug in the culture medium, the tumor size decreased to 13.6% on day 12. The tumor tissue from the other treated mice did not grow at all (Fig. 4D).

On day 12, both control and drug treated tumor cells were double stained with Hoechst 33342 and Yo-Pro-1, and representative images of tumor tissues were obtained by confocal laser scanning microscopy (Fig. 4E–G). In the tissue from the control mice, nuclei of the cells did not stain green, indicating the absence of apoptosis, whereas proliferating viable cells on the periphery of the tumor tissue stained blue with Hoechst 33342 (Fig. 4E). On the other hand, the images in Figure 4F and G showed that the nuclei in the drug treated tumor cells were stained green by the Yo-Pro-1, indicating apoptosis.
2.7. Detection of cleaved caspase-3 in tumor tissue

Tumor tissues from both drug treated and control samples were subjected to SDS–PAGE, and the cleaved caspase-3 was detected in Western blots using cleaved caspase-3 monoclonal antibody. As shown in Figure 4H, the cleaved caspase-3 was visible in drug treated tumor sample, whereas no cleaved caspase-3 release was detected in the control tissue.

3. Discussion

Metastatic prostate carcinoma is associated with a high morbidity and mortality rate with a medium survival of, approximately, 12–15 months.19 Available treatment alternatives include, radiotherapy after radical retropubic prostatectomy, radical prostatectomy, external beam radiation, prostate brachytherapy, and androgen ablation of the prostate.20 Until recently, despite androgen suppression, no cytotoxic agent has been able to change the progression of metastatic prostate cancer. Androgen ablation therapy remains the main course of treatment with advanced disease. However, it has no effect on hormone-independent cancer cells.21,22 Chemotherapeutic agents result in less than a 10% response in advanced prostate carcinoma, in part due to increased resistance of androgen-independent cells to apoptosis.23 However, the severe side effects of chemotherapy have remained a major problem.

Betulonic acid showed the highest cytotoxic effect on LNCaP human prostate cancer cells in cell culture as compared to other betulinol derivatives. Betulonic acid was synthesized by a modified method using H2O2 as the oxidizing agent, resulting in higher yield and purity, compared with the previous method using Jones’ reagent.17 Betulonic acid showed high cytotoxicity in vitro in MTT assay and significant inhibition of colony and tumor growth in AIG assay on LNCaP, DU-145, and PC-3 human prostate cells, with little effect on normally proliferating fibroblast cells.

The hydrophobic nature of betulonic acid and consequent lack of solubility in aqueous solvents had remained a limitation in achieving full expression of its anti-cancer activity in vivo. Hence, a lysinated derivative of betulonic acid was synthesized to yield a hydrophilic compound. The chemical conjugation of lysine to betulonic acid was facilitated by the use of selectively protected amino groups of the lysine to avoid the intramolecular interference and permit the formation of a
covalent bond exclusively between the C-28 of betulonic acid and the free amine group at C-6 of the lysine. Release of the ester group at the C-terminal of the lysine yielded the hydrophilic lysinated-betulonic acid with enhanced solubility in aqueous medium. The solubility of lysinated-betulonic acid was only 0.7 mg/mL in 20% DMSO, while the solubility of betulonic acid was 0.5 mg/mL. However, the solubility of the lysinated-betulonic acid increased to 5.0 mg/mL in the PBS/ethanol/albumin solvent compared to 1.8 mg/mL of betulonic acid in the same solvent system.

Anti-prostate cancer activity of lysinated-betulonic acid was determined using LNCaP prostate cancer cells, which are diagnostic of human prostate cancer. Lysinated-betulonic acid dissolved in the PBS/ethanol/albumin solvent showed higher inhibition of LNCaP cell growth at a concentration of 100 μM after 72 h incubation as compared to lysinated-betulonic acid dissolved in DMSO (Table 2A and B). It may be mentioned that lysinated-betulonic acid at 100 μM concentration also causes significant inhibition of growth of hormone-independent DU-145 and PC-3 cells in the MTT assay with little cytotoxicity to normally proliferating fibroblast cells (Table 2C).

The higher activity of lysinated-betulonic acid against prostate cancer was further confirmed by using xenograft tumors in vivo. Lysinated-betulonic acid, at a dose of 30 mg/kg body weight, showed up to 92% growth inhibition of the LNCaP xenograft tumors transplanted in athymic male mice (Fig. 2A). The mice had been treated for 17 days. However, it is tempting to speculate that if the experimental mice were continued to be treated further, the tumors might have been further suppressed. The mice injected with lysinated-betulonic acid showed little change in body weight or vital signs or visible toxic effect during the entire period of treatment. The results of two independent in vivo experiments in a total of 16 male athymic mice confirm and validate the anti-cancer efficacy of lysinated-betulonic acid, solubilized in the PBS/ethanol/albumin, in the inhibition of LNCaP xenograft tumor growth.

Anti-cancer effect of the lysinated-betulonic acid was further demonstrated by histological studies of the tumor, showing the absence of dividing cells in the tumors of treated mice, whereas the tumor tissues from control mice showed abundant cell division with a mitotic index of 3–12 (Fig. 3A and B). From our preliminary studies, the mechanism of action of lysinated-betulonic
acid was demonstrated to be predominantly apoptosis as shown by specific Yo-Pro-1 staining of the ex vivo tumor tissues (Fig. 4E–G) and the detection of caspase-3 enzyme from the tumors treated with the drug (Fig. 4H). The TUNEL assay, which detects the breakdown of DNA strands of the tumor cells, further supported that lysinated-betulonic acid caused the apoptotic death of LNCaP cancer cells (Fig. 3C and D).

4. Conclusion

The results of the present study demonstrate that the newly synthesized hydrophilic lysinated derivative of betulonic acid soluble in PBS containing 22% ethanol and 4% albumin showed significant inhibition of growth of human prostate cancer cells in vitro as well as dramatic inhibition of growth of LNCaP xenograft tumors in male athymic mice in vivo. The enhanced anti-prostate cancer activity of lysinated-betulonic acid may be attributed to a higher solubility and consequent-ly bioavailability of the drug in vivo. These studies have provided a much needed breakthrough to undertake dose–response, toxicity, and drug delivery studies of lysinated-betulonic acid as an anti-prostate cancer drug and have indicated that lysinated-betulonic acid has a promise alone or in conjunction with other drugs, radiation, and surgical treatment modalities to provide an improved therapy of prostate cancer.

5. Materials and methods

5.1. Chemicals

Prostate cancer cells, namely LNCaP, DU145, and PC-3, and corresponding culture media were purchased from American Type Culture Collection (ATCC, Manassas, VA). The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrasodium bromide (MTT) kits were also obtained from ATCC. Matrigel was purchased from BD Biosciences (Bedford, MA). Yo-Pro-1 and Hoechst 33342 stains were purchased from Molecular Probes (Carlsbad, CA). Rabbit polyclonal antibody to caspase-3 was obtained from Cell Signaling (Beverly, MA). Streptavidin-HRP and diaminobenzidine (DAB) kits were obtained from Ventana Medical Systems (Tucson, AZ). Terminal deoxynucleotidyl Transferase (TdT) and Biotin-16-dUTP were purchased from Roche (Indianapolis, IN). ECL protein detection kit and
Betulinol was first treated with pyridinium chloride by a procedure described in Figure 5, Reaction A.

The BCA protein assay kit and RIPA nitrocellulose membranes were obtained from Pierce (Rockford, IL). N$_2$-Butyl-oxycarbonyl-N$_{a}$-benzoxycarbonyl-lysine and all other chemicals were obtained from Sigma–Aldrich (St. Louis, MO). Injectable grade (98%) ethanol and 4% pure human serum albumin were obtained from American Regent and Biocell, respectively.

5.2. Mice

Athymic mice, 13 weeks of age, were purchased from The National Cancer Institute (NCI) and were housed in a pathogen-free environment under controlled conditions of light and humidity with free access to food and water. All mice studies were performed according to the guidelines of the Institutional Animal Care and Use Committee and Research Animal Resource Center of Weill Medical College of Cornell University, New York (protocol #0402-209A).

5.3. Synthesis of lysinated-betulonic acid

Betulinol was isolated from the non-saponifiable fraction of crude sulfate soap prepared by boiling the outer bark of the white birch tree in sodium hydroxide (NaOH), sodium sulfate (Na$_2$SO$_4$), sodium sulfite (Na$_2$SO$_3$), and sodium thiosulfate (Na$_2$S$_2$O$_3$) at 110–120 °C. Betulinol was then crystallized in acetone. Betulonic acid was synthesized from betulinol by a procedure described in Figure 5, Reaction A where betulinol was first treated with pyridinium chlorochromate (PCC) in dichloromethane (CH$_2$Cl$_2$). Betulonic acid was obtained by further oxidation on betulone aldehyde using 30% aqueous hydrogen peroxide (H$_2$O$_2$). In order to increase the hydrophilicity, lysine, a biocompatible amino acid, was conjugated to betulonic acid. Lysine was chosen due to its ability to form two amino bonds as well as for its long steric flexibility. Additionally, lysine is known to be fairly stable in most synthetic reactions.

Esterification of Boc-Lys(Cbz-OH) generated a methyl ester. Selective deprotection of benzoxycarbonyl (Cbz) was achieved by hydrogenolysis to release the primary amine (Fig. 5, Reaction B). The conjugation reaction of the deprotected lysine derivative to betulonic acid was performed in CH$_2$Cl$_2$ using 1-hydroxybenzotriazole hydrate (HOBT) to activate the C-28 of betulonic acid using 1,3-dicyclohexylcarbodiimide (DCC) as the catalyst (Fig. 5, Reaction C). The reaction occurred selectively between the amine and carboxyl group, whereas the ketone and alkenes on betulonic acid were unaffected and yielded lysinated-betulonic acid ester (Boc-Lys-Ome-betulonic acid). The methyl ester on the C-terminus of lysine was then hydrolyzed by treatment with lithium hydroxide monohydrate (LiOH·H$_2$O) to obtain lysinated-betulonic acid as a white powder (Fig. 5, Reaction C).

Betulonic acid and lysinated-betulonic acid were dissolved in DMSO at concentrations of 2.22 and 3.41 mg/mL, respectively, and diluted with culture medium containing 10% fetal bovine serum (FBS) to yield a final concentration of 1.0 mM, in 20% DMSO. Lysinated-betulonic acid was also solubilized in ethanol with a concentration of 5.0 mg/mL and diluted with phosphate-buffered saline (PBS) containing 4% human serum albumin to yield a concentration of 1.0 mM containing 14% ethanol for in vitro cytotoxicity assays in cell culture, and to 2.2 mM concentration in PBS containing 4% albumin and 22% ethanol for the in vivo assay in athymic male mice.

5.4. In vitro cytotoxicity of betulonic acid and lysinated-betulonic acid on human prostate cancer cells by MTT assay

Human prostate cancer cell lines LNCaP, PC-3, and DU-145 as well as human fibroblast cell lines were obtained from ATCC and were, respectively, maintained in RPMI-1640, F-12K, MEM, and DMEM supplemented with 10% fetal bovine serum at 37 °C under 5% CO$_2$ in a humidified atmosphere in an incubator. The wells of a 96-well culture plate were precoated with 3% lysine to achieve optimal cell adhesion. Approximately, 60,000–100,000 cells in 0.1 mL culture medium per well were plated. The cells were incubated with 10 μL of the drug solutions with a final concentration of 10 and 100 μM for 24, 48, and 72 h. Ten microliters of 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide was added into each well at the end of each incubation period and placed in the incubator. The cells were periodically examined under an inverted microscope for the appearance of an intracellular punctate purple precipitate, formazan, which indicated the presence of live cells. When the purple precipitate was clearly visible under the microscope, 100 μL of the detergent was added to all wells to dissolve the formazan. The absorbance of each well was determined at a wavelength of 540 nm using an automatic ELISA reader coupled with a Titerteck Multiskan Ascent (Thermo Labsystems, Finland).

5.5. Anchorage-independence growth (AIG) assay

AIG assay was performed to determine the ability of betulonic acid to inhibit tumor cell growth by forming three-dimensional colonies in soft agar in 6-well culture plates. Prostate cancer cells, LNCaP, PC-3, and DU-145, as well as normal proliferating fibroblasts were suspended in 2 mL of RPMI, F12K and DMEM containing 0.33% agar, respectively. A 2 mL matrix made up of 0.6% agar was overlaid on the agar in the absence or presence of betulonic acid at a final concentration of 10 μM. The cultures were incubated at 37 °C in a humidified environment under 5% CO$_2$ for 14 days without medium change. The cultures were fixed in Cornoys’s fixative (acetic acid/100% ethanol 1:3 v/v), the size as well as the numbers of anchorage-independent tridimensional colonies (≥ 40 μm diameter) formed were determined under 10-fold magnification.
5.6. In vivo effect of lysinated-betulonic acid on LNCaP prostate cancer cell xenografts in athymic mice

Human prostate LNCaP cells were cultured in T-75 culture flasks in RPMI-1640 cell culture medium with 10% FBS until 80–90% confluent. Cells were scraped from the bottom of the flask into phosphate-buffered saline (PBS) and collected by centrifugation. In a pilot study, five athymic male mice were transplanted on the left flank of the abdomen with approximately 7 million LNCaP prostate cancer cells in matrigel28 to determine the time required to grow visible tumors. In the following study with 12 athymic male mice, 7 million LNCaP cells in matrigel were implanted in each mouse on the left flank area. Visible tumors grew in 12 days following implantation. The mice were randomly divided into two groups with four mice each. Each mouse in the experimental group was injected intraperitoneally with lysinated-betulonic acid at a dose of 30 mg/kg body weight daily for 17 days. Mice in the control group were injected with the solvent alone. The experiment was repeated under the same conditions.

Mice were weighed daily and observed for vital signs and visible adverse effect. The change in the tumor volume was determined daily, by the aid of caliper and calculated according to the formula \( \pi/6(l \times w \times h) \).29 Mice were sacrificed by CO\(_2\) asphyxiation at the end of the experiment on day 17. The tumor tissue was dissected out from each mouse under sterile conditions and subjected to further studies.

5.7. Immunohistochemical study of the tumor tissues

A portion of the dissected tumors from mice was frozen immediately at −80°C in optimal cutting gel. Frozen sections of the tumors were treated with monoclonal antibodies to localize prostate specific membrane bound antigen (PSMBA) and prostate specific antigen (PSA) as markers to identify prostate cancer cells.30

5.8. Histological studies

A portion of the tumor tissue was fixed in 4% formalin for 24 h and transferred to 70% ethanol. Eight micrometer thick sections of the fixed tissue were stained with hematoxylin–eosin for histological examination for cell types and structure.

5.9. Ex vivo growth of tumor tissues in culture

A portion of the tumors excised from control and lysinated-betulonic acid treated mice was used for in vitro growth in RPMI culture medium. The tumor was cut into small pieces of approximately 4 mm\(^2\) and immersed into wells of a 12-well culture plate containing a mixture of matrigel and RPMI culture medium in a 1:1 ratio. After the matrigel solution solidified, culture medium was added to each well and changed daily. On day 5 of the culture, tumor tissues from drug treated mice were incubated with lysinated-betulonic acid at final concentration of 5.5 \times 10^{-4} M. Solvent alone was added to the wells with tissue from control mice. The tissues were cultured for 12 days. Diameter measurements and photographs of the tumors were taken daily using an Olympus IX 70 inverted microscope and DP11 image analyzer.

5.10. Detection of apoptosis by Yo-Pro-1 immunofluorescence staining

On day 12, tumor tissues cultured in a 1:1 ratio of matrigel and RPMI culture medium supplemented with 10% fetal bovine serum (FBS) were incubated with 80 µg/mL of Hoechst-33342 stain for 1 h at 37°C under 5% CO\(_2\) and 95% air. The tissues were then incubated with 4 µM Yo-Pro-1 stain for an additional hour. Confocal laser scanning fluorescence microscopy was used to measure viable and apoptotic tumor cells.31

5.11. Western blot

On day 12, the ex vivo tumor tissues were lysed in 100 µL RIPA buffer (0.1% sodium dodesylsulfate, 0.5% sodium deoxycholate, 1% NP-40, 150 mM NaCl, 50 mM Tris–HCl, 2.5 mM EDTA, 25 mM NaF, 10 mM sodium pyrophosphate, 1 mM sodium orthovanadate, and protease inhibitors such as 100 mM PMSF, 1 µg/mL aprotinin, 1 µg/mL leupeptin, and 1 µg/mL pepstatin). The samples were centrifuged at 16,000 g for 30 min at 4°C. The protein concentration in the supernatant was determined using Pierce’s BCA assay kit. Aliquots of 20 µg protein samples were subjected to 10% SDS–polyacrylamide gel electrophoresis (PAGE). The protein bands were transferred onto a nitrocellulose membrane using a semi-dry blot device. The membrane was blocked with 5% dried milk in Tris-buffered saline and blotted with 1:100 rabbit polyclonal caspase-3 and mouse monoclonal vinculin antibodies overnight. The membrane was then blotted with a second antibody conjugated with horseradish peroxidase (HRP) and images were developed using an ECL protein detection kit with a chemiluminescence device.

5.12. Detection of apoptotic cells by terminal deoxy-nucleotidyl transferase biotin-dUTP nick end labeling (TUNEL) assay

The tumor tissues from mice were examined by the TUNEL assay to detect 3′ free hydroxyl ends (3-OH) of DNA strands created by nucleases in apoptotic cells.32 Sections of tumor tissues were treated for 20 min with Proteinase K (20 µg/mL in PBS), equilibrated for 10 min with TdT buffer, and incubated for 2 h in the TdT mix containing 100 U TdT and 0.5 µL Biotin-16-dUTP. Biotin-16-dUTP labeled in 3-OH of DNA was detected by streptavidin–HRP and visualized using diaminobenzidine (DAB) for color reaction according to the manufacturer’s instructions. The in situ molecular detection was performed using 8 µm thick paraffin sections with an automated staining processor (Discovery XT).
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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmc.2006.05.048.

References and notes

2. Perry, C. M.; McTavish, D. Drugs Aging 1995, 7, 49–74.