Betulinic acid induced tumor killing

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Publication date
2011

Chapter 3

Betulinic Acid Delivered in Liposomes Reduces Growth of Human Lung and Colon Cancers in Mice Without Causing Systemic Toxicity

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Anticancer Drugs 2011 Mar;22(3):223-33

Abstract
Betulinic Acid (BetA) is a plant-derived pentacyclic triterpenoid with a potent anti-cancer capacity that targets the mitochondrial pathway of apoptosis. BetA has a broad efficacy in vitro against prevalent cancer types, including lung-, colorectal-, prostate-, cervix- and breast-cancer, melanomas, neuroblastomas and leukemias. The cytotoxic effects of the compound against healthy cells are minimal, rendering BetA a promising potential anticancer drug. However, because of the weak hydrosolubility of BetA, it has been difficult to study its efficacy in vivo and a pharmaceutical formulation is not yet available.
We report the development of a liposome formulation of BetA and show its successful application in mice. Large liposomes, assembled without cholesterol to reduce their rigidity, efficiently incorporated BetA. Nude mice xenografted with human colon and lung cancer tumors were treated intravenously with the BetA-containing liposomes. Tumor growth was reduced to more than 50% compared with the control treatment, leading to an enhanced survival of the mice. Oral administration of the liposomal formulation of BetA also slowed tumor growth. Any signs of systemic toxicity caused by BetA-treatment were absent. Thus, liposomes are an efficient formulation vehicle for BetA, enabling its preclinical development as a non-toxic compound for the treatment of cancers.

Introduction

More than half of the common anticancer drugs are of natural origin. Examples are the taxanes and vinca alkaloids and their synthetic derivatives that target microtubules [1]. In current anti-cancer drug discovery, the development of small molecule inhibitors specifically targeting one enzyme in cancer cells has led to therapeutic progress. However, the plasticity and instability of the cancer genome often render these agents to be of modest clinical benefit [2]. Therefore, there is still a great need for broadly active multifunctional anticancer compounds to be used either alone or in a combined regime synergistically working with other anti-cancer chemotherapeutic drugs or treatments. Within the large group of plant-derived triterpenoids several compounds possess anti-tumor properties by exerting effects on multiple regulatory networks and on cellular metabolism [3]. One of the most promising members of this group is BetA, a lupane-type pentacyclic triterpenoid found in various plant sources [4,5]. BetA is easily synthesized in an oxidation process from its precursor betulin, which itself also has anti-cancer activity (although this is less than BetA) and is abundantly available from the bark of the white birch. In the 1990s, BetA was discovered as the most promising anti-cancer reagent in a screen of 2500 plant extracts and was selected for the Rapid Access to Intervention Development program of the National Cancer Institute [6]. The mode of action of BetA in inducing cytotoxicity in cancer cells has been investigated comprehensively [4,5]. Induction of mitochondrial damage and subsequently induced apoptosis were found among the prominent phenomena [7,8], but additional mechanisms like decreased expression of vascular endothelial growth factor and anti-apoptotic molecule surviving [9], suppression of STAT3 activation [10], inhibition of topoisomerases [5,11] and other mechanisms [5] can all contribute to the anti-tumor effect of BetA. Its capacity to induce tumor cell death has been demonstrated in vitro for a wide variety of cancer types, including melanoma, neuroblastoma, glioma, leukemia, and ovarian-, cervix-, prostate-, lung-breast and colorectal cancers [12-19]. The cytotoxicity of BetA against healthy cells *in vitro* was found to be only minimal [15,16,20], indicating a favorable therapeutic window.

On account of its highly lipophilic character, BetA cannot be dissolved and administered in aqueous solutions. Consequently, the study of the anti-cancer capacity of BetA *in vivo* has been difficult. The formulations of BetA that have been used so far *in vivo* are either not suitable for human application or are not precisely defined and thus cannot be standardized [6,9,16,21]. BetA has initially been discovered by showing its activity in athymic mice xenografted subcutaneously with human melanomas [6]. The inhibition of tumor growth was achieved by the intraperitoneal administration of BetA in a formulation with polyvinylpyrrolidone (PVP), which enabled its solubilization [6]. Subsequent studies have assessed the *in vivo* anti-cancer potential of BetA using different formulations. Nude mice subcutaneously grafted with human ovarian carcinoma IGROV-1 were shown to survive longer after treatment with BetA by the intraperitoneal route in a formulation of ethanol, Tween-80 and water

(10%/10%/80%) [16]. The oral application of BetA using corn oil as a vehicle was shown to inhibit the growth of human prostate cancer LNCaP tumors subcutaneously grafted in athymic mice [9]. Importantly, in these mice studies [6,9,16], and also in rats [22], systemic adverse effects of BetA-treatment were not observed, substantiating the potential of BetA as a non-toxic anti-cancer drug. To enhance the hydrosolubility of BetA and to broaden the formulation possibilities, research groups have synthesized derivatives of BetA that are less lipophilic [23-29]. However, as the lipophilic character of BetA is likely to be crucially involved in its pluripotent mechanism of action, which is responsible for its broad activity profile (manuscript in preparation), we sought a novel formulation of BetA itself. Thus, we had to address the lipophilicity of BetA in the development of a new formulation and for that reason embarked on liposomes as delivery system. Liposomes are small vesicles consisting of one or more concentric phospholipid bilayers with an aqueous core. Water-soluble drugs can be encapsulated in the aqueous phase, and hydrophobic drugs can be incorporated into the lipid bilayer membrane. Liposomes are attractive as a drug carrier because of their relatively high drug loading capacity, good biocompatibility, low toxicity, versatility and ease of preparation. Since the 1990s many clinical trials have been carried out with drugs in a liposome formulation and some are now standard therapies [30,31]. New generations of liposomes have been developed that target drugs to tumor cells or their supporting cells and protect drugs from metabolizing enzymes or enable prolonged action of the drug in the body by slow release from the liposomes [32,33]. Otherwise difficult to administer lipophilic drugs have been solubilized in liposomes [32]. Accordingly, we investigated the potential of liposomes to incorporate BetA with a payload sufficiently high to treat tumor-bearing mice.

Materials and Methods

Cancer cell lines

Human lung cancer cell line A549 and human colon cancer cell line SW480 were cultured under standard conditions in Iscove's modified Dulbecco's medium supplemented with 8% fetal calf serum, L-glutamine (2 mmol/l), penicillin (100 U/ml) and streptomycin (100 µg/ml). The cells were maintained in a logarithmic growth phase and were ~60% confluent when harvested for tumor challenge.

Animals

Female athymic nude Foxn1 mice were used for all the experiments. At the start of each experiment the mice were 5 weeks old. Experiments were performed with groups of six mice each.

Preparation of BetA-containing liposomes, Rhodamine B-PE-containing liposomes and empty liposomes

BetA-containing liposomes and empty liposomes were prepared by the film method [34]. In brief, a lipid solution was prepared in chloroform, containing egg-phosphatidylcholine (EPC, from Lipoid GmbH, Ludwigshafen, Germany) and egg-phosphatidylglycerol (EPG, Lipoid) in a molar ratio of 10/2. For BetA-containing liposomes, BetA was added to the lipid solution and was also dissolved in chloroform. Rhodamine- phosphatidylethanolamine (headgroup labeled PE with lissamine rhodamine B; Avanti Polar lipids, Alabaster, Al, USA) was used as a fluorescent marker in the lipid bilayer of

some liposome preparations. Rhodamine-PE was added to the lipid solution at 0.1 mol% of total lipids. In all cases, a lipid film was created by rotary evaporation of the lipid solution and the film was hydrated in phosphate buffered 0.8% saline. The resulting liposomes were filtrated once through a polycarbonate filter membrane of 8.0 µm to remove un-encapsulated BetA. Liposome particle size distributions were measured using dynamic light scattering, detected at an angle of 90^0 to the laser beam on a Malvern 4700 System (Malvern Instruments Ltd., Malvern, UK) and were shown to be on average between 1 um and 1.5 um. The polydispersity indexes were between 0.5 and 0.7 indicating large variations in size distribution. Phosphate concentration of the liposomes was determined with a phosphate assay according to Rouser [35]. To determine BetA concentration, BetA-liposomes were dissolved in ethanol and, using BetA as standard, BetA concentration was determined by HPLC (15 cm LiChrospher RP-18, 8 µm column) using a mobile phase of acetonitril/water in a ratio of 80/20 (V/V) with pH 3. Detection was performed with a UV-detector at 210 nm. The final liposome preparation contained approximately 70 µmol phospholipid per ml and approximately 6 mg BetA per ml. Thus, the liposomes contained approximately 85 µg BetA/µmol phospholipid. Empty liposomes also contained an average of 70 µmol/ml of phospholipids. The liposomes were diluted to a concentration of 5 mg/ml BetA, which enabled us to carry out an in vivo treatment with batches of BetA-liposomes containing an equal amount of BetA. Liposomes were stored at 4°C until use.

Tumor cell injection, calculation of tumor volumes and analysis of tumor growth

Per mouse, 10^6 tumor cells (resuspended in 100 µl PBS/0.5% bovine serum albumin) were subcutaneously injected into one flank of each mouse. The tumor size was measured twice a week during the course of the experiment. For calculation of the tumor size, two sides of the tumor (length L and width W) were measured, tumor volume was calculated as $L \times W^2 \times ½$ [36]. The effects of BetA-treatment on tumor growth at specific time points were analyzed, using GraphPad Prism software (La Jolla, California, USA), by two-way ANOVA with Bonferroni posttests for statistical analyses or using the (average) area under the curve (AUC) per group of mice (treated versus control treated) over the full treatment period [37] and an independent *t*-test was carried out on these AUC data for statistical analysis. The statistical analysis of the survival curves was carried out with the Log-rank test.

Treatment of mice

Mice injected with tumor cells were divided into two groups consisting of six mice each and injected three times per week with empty liposomes (200 µl control group) or BetA-containing liposomes (200 µl) containing 5 mg/ml of BetA (BetA group). Liposomes were injected intravenously (i.v.) into the tail vein or were applied via oral injection using a gavage needle.

Determination of in vitro stability of BetA-liposomes in serum

In brief, 1 ml of BetA-containing liposomes was incubated with 2 ml of either mouse serum, foetal calf serum or human serum for 1 h at 37^0C. The tube containing the liposome suspension in serum was centrifuged to sediment the liposomes. The pelleted liposomes were separated from the supernatant (containing disintegrated liposomes) and from both fractions BetA was extracted using ethyl acetate. The BetA content in both fractions was determined by HPLC according to a standard procedure.

Immunohistochemistry and measurement of Rhodamine-PE in organ and tumor sections

Organs were collected in formalin and embedded in paraffin according to standard protocols. After deparaffinization and an endogenous peroxigenase quenching step (30 min at room temperature in 1.5% H_2O_2 in PBS) antigen retrieval was undertaken by cooking samples for 10 minutes in Natrium-Citrate, pH 6. Immunostaining was performed using an anti-mouse proliferating cell nuclear antigen (PCNA) antibody (SC-56; Santa Cruz, CA, USA). After incubation with a secondary, biotinylated antibody, an AB-complex reagent (=streptavidin-biotin-horseraddish peroxidase; K0355, DAKO,

Denmark) was applied for one hour before DAB (di-aminebenzamine; Sigma, St Louis, MO, USA) coloring. For counterstaining Eosin-hematoxyline (Fluka, Buchs, Switzerland) was used.
Measurement of Rhodamine-PE fluorescence in organ and tumor sections was taken on deparaffinized slides in mounting solution (vector shield) containing 4',6-diamidino-2-phenylindole (DAPI).

Results

Development of a liposome formulation incorporating BetA with high efficiency

In the foregoing experiments we tested the activity of BetA in nude mice bearing human cancers that we have previously found sensitive *in vitro* [19], using published formulations of BetA. Mice were treated either intraperitoneally with the PVP formulation of BetA [6] or orally with BetA dissolved in corn oil [9], being the most successful formulations in the literature. However, any treatment effect on tumor growth was absent (data not shown). After the mice had been killed, we inspected the abdominal cavity of mice treated with BetA-PVP and observed large deposits of precipitate on the liver (Fig. 1), which were absent in the mice treated with the PVP vehicle only, and therefore, suggested a shortcoming in the bioavailability of BetA using this formulation. Therefore, we embarked on liposomes as the drug vehicle, aiming to generate liposomes with a high payload of BetA. The efficiency of drug loading into liposomes depends primarily on liposome size and (lipid) composition and the physiochemical characteristics, for example, hydrophobicity, of the drug molecule. We first tested small liposomes with a size of 0.1 – 0.2 μm, also referred to as long-circulating liposomes, which did incorporate not more than 1 mg/ml BetA. Such liposomes, because of their small size and prolonged circulatory half-life, could potentially extravasate into the tumor tissue by virtue of the locally enhanced capillary permeability thereby delivering BetA to the tumor tissue [38]. However, when athymic mice xenografted with human lung cancer A549, which is sensitive for BetA *in vitro* [19], were treated i.v. with these BetA-containing small liposomes using a feasible scheme of injections (200 μl), three times per week, tumor growth was not impeded (data not shown). The encapsulated BetA concentration of approximately 1 mg/ml resulted in an *in vivo* BetA dose of approximately 10 mg/kg of body weight (BW) per injection (200 ul). Such a dose, injected (i.v.) three times per week, is possibly too low to reach an anti-tumor effect, as is known from the literature (see refs. 6,9,16,21 and table 2). Therefore, we pursued the assembly of liposomes containing a higher BetA payload. Incorporation of BetA in large unsized liposomes was much more efficient, reaching a BetA incorporation of approximately 6 mg/ml BetA. Initially, we assembled the BetA-containing large liposomes with cholesterol; however this resulted in a very rigid bilayer and liposome filtration was hard to accomplish. Cholesterol is known to improve liposome stability and to provide membrane rigidity [39,40]. Apparently, the incorporation of both cholesterol and BetA in the bilayer worked together to render the bilayer extremely rigid, a phenomenon of cooperative membrane rigidification also observed for cholesterol together with carotenoids [41]. Large liposomes without cholesterol incorporated equal levels of approximately 6 mg/ml BetA, but

these bilayers were more flexible and allowed filtration. The liposome particle size was on average between 1 µm and 1.5 µm with a broad size distribution. The higher BetA payload would enable – with an injection volume of 200 µl and after dilution of the liposomes to 5 mg BetA per ml - an *in vivo* BetA concentration of 50 mg/kg of BW per dose, shown before to be efficacious in xenograft tumor models (see refs. 6,9,16,21 and table 2). We tested the BetA-containing large liposomes without cholesterol (further designated 'BetA-containing liposomes') for their *in vivo* effect against prevalent human cancer types xenografted in athymic mice. As size and lipid composition are crucial parameters in determining the behaviour of liposomes after systemic administration, we also studied *in vitro* stability of the BetA-liposomes in serum and their fate *in vivo*.

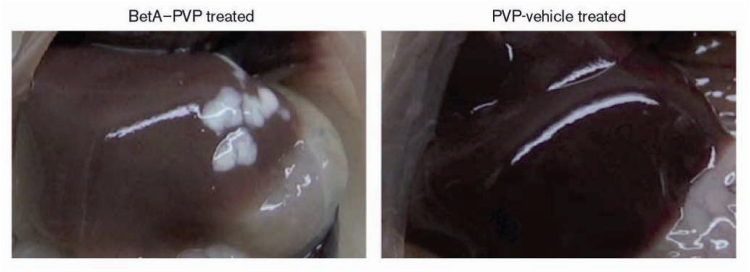

Figure 1: Formation of intra-abdominal deposits of BetA-PVP in treated mice.
White deposits of BetA-PVP complexes were observed on the liver of a representative mouse that received multiple injections of BetA-PVP (left), whereas mice that were injected with PVP as control vehicle only did not show deposits (right). BetA was co-precipitated with PVP as described previously [6,60]. Briefly, BetA and PVP were dissolved in methanol and mixed (ratio BetA to PVP was 1:4). Subsequently, the mixture was dried in a speed-vacuum system and dissolved in phosphate buffer solution.

Table 1 In-vitro stability of betulinic acid-containing liposomes in serum

	Mouse serum	Human serum	Fetal calf serum
BetA stable in liposome[a]	68.8% (1.2%)	68.4% (14.7%)	73.2%
BetA released[b]	31.1% (1.8%)	31.5% (8.3%)	26.7%

[a]BetA (betulinic acid; %) in stable liposomes after 1-h incubation in serum at 37°C. The mean standard error of the mean is indicated; incubation in fetal calf serum was performed once.
[b]BetA (%) released in serum after 1-h incubation at 37°C.

Intravenously applied BetA-liposomes effectively reduce outgrowth of lung and colon tumors

BetA-liposomes were compared with empty control liposomes in groups of six athymic nude mice subcutaneously grafted with human lung cancer cell line A549 or human colon cancer cell line SW480. These tumors were sensitive to BetA treatment *in vitro* in our previous study [19]. Treatment was started two days after the tumor challenge. Mice were injected i.v. three times per week with 200 µl BetA-liposomes containing 5 mg/ml BetA. BetA treatment of mice grafted with lung cancer A549 and mice with colon cancer SW480 resulted in significantly slowed tumor growth compared with the growth in control-treated mice (Fig. 2A). The reduction in average tumor volume was 55% for A549 tumors at day 95, and

59% for SW480 tumors at day 33, indicating that the tumor volumes in the BetA-treated mice were less than half of the volumes in the control mice for both cancer types. When tumor growth was analyzed over the full treatment period using the average area under the curve (AUC) per group of mice, the average AUC for BetA-treated mice was reduced with 49% (P=0.025) compared with control treated mice for A549 tumors and was reduced by 51,5% (P=0.011) for SW480 tumors.

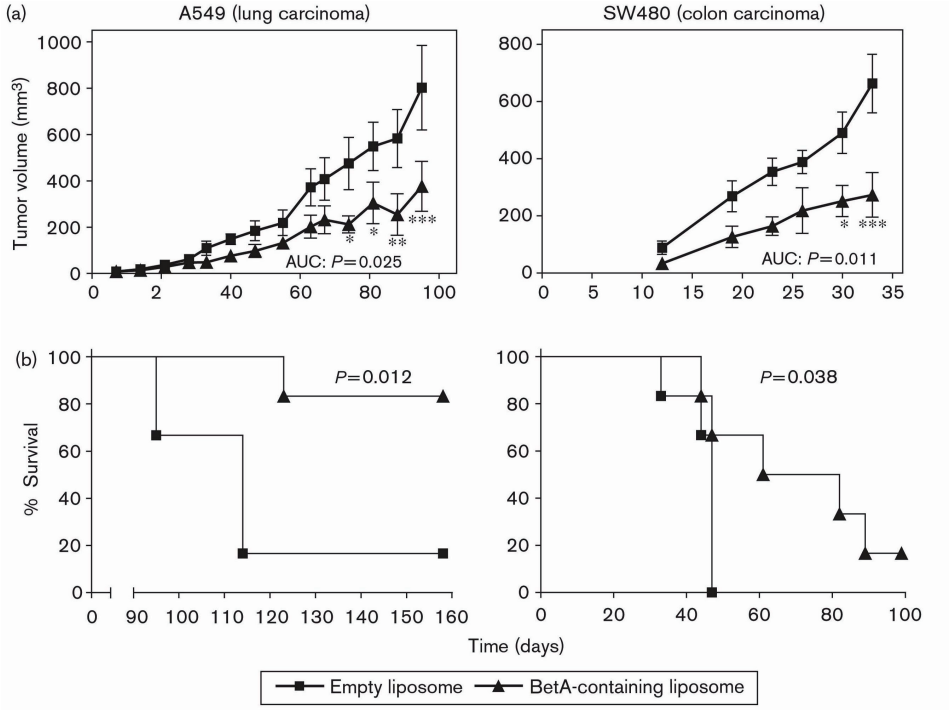

Figure 2: Intravenous administration of BetA-liposomes reduces tumor growth and prolongs survival of tumor-bearing mice.

*Nude mice (six per group) were injected subcutaneously with either A549 lung cancer cells (two groups) or SW480 colon cancer cells (two groups) and treated three times per week i.v. with either 200 µl BetA-liposomes or empty liposomes starting two days after tumor cell injection. The BetA concentration reached per injection was 50 mg/kg body weight. (200 µl of 5 mg BetA/ml liposomes was injected). The treatment was continued for three months for A549 tumors and two months for SW480 tumors. During the course of the experiment tumor volumes (Panel A) and bodyweight was monitored (see Fig. 3). Mice were killedd when tumor size was more than 1000 mm^3. In panel B, survival times of mice injected with either A549 (left) or SW480 tumors (right) are shown. *P < 0.05; **P < 0.01; ***P < 0.001.*

The two most important goals in cancer treatment are prolonged survival without reduction in the quality of life.
In accordance with regulations in The Netherlands, the mice were killed when tumors were more than 1000 mm^3. Tumor-bearing mice treated with BetA-liposomes showed a clear survival advantage compared with the control treated mice (Fig. 2B). In particular, mice with A549 tumors showed greatly enhanced survival upon BetA-treatment (Fig. 2B). Importantly, in line with the literature, no signs of systemic toxicity were observed by monitoring general behavior, appetite and mice body weight (Fig. 3). In addition, the white blood cell count in the mice, as an indication of hematopoietic toxicity, was not affected after 2 months of i.v. BetA-treatment (data not shown). Together, these results indicate that BetA-liposomes have the potential to slow the outgrowth of tumors from lung and colon carcinomas, thereby prolonging life, without inducing systemic adverse effects.

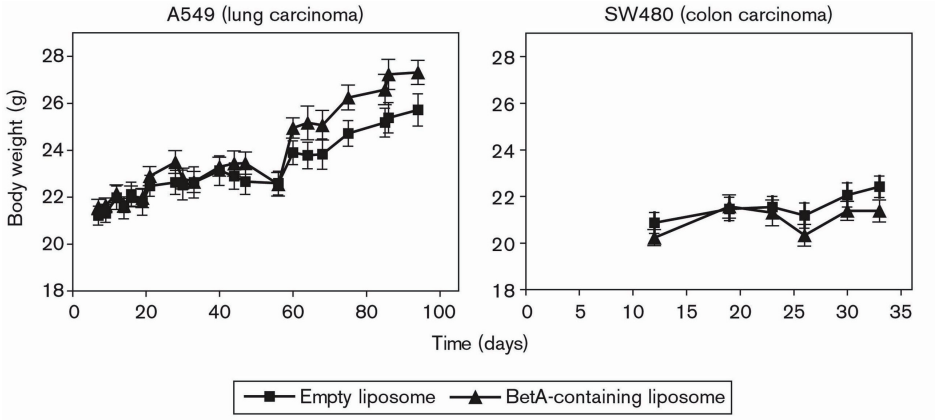

Figure 3: Body weight of tumor-bearing mice treated i.v with BetA-liposomes or control liposomes. *The average body weight per group of six mice bearing either A549 lung cancer tumors (left) or SW480 colon cancer tumors, monitored during the course of the experiment is shown.*

BetA-liposomes are relatively stable in serum and serve as stable drug vehicle

Large liposomes can not be passively targeted to the tumor. Only small liposomes (size ~100 nm) are small enough to passively infiltrate tumor endothelium, due to the neovasculature of tumors being hyperpermeable [38], whereas they are excluded from normal endothelium. Thus, we reasoned that the BetA-containing liposomes function merely as formulation vehicle. For assessing their drug delivery capacity, we studied the fate of these liposomes in the body. The behavior of the liposomes after systemic administration is, to a great extent, determined by the size and (lipid) composition of the liposomes. Cholesterol is mostly included in liposomes to increase their stability [39,40]. Size is important as large liposomes are rapidly recognized by the mononuclear phagocyte system and show, in general, less stability than small liposomes [42]. We studied the stability of the BetA-

containing liposomes, which were assembled without cholesterol, by measuring *in vitro* BetA-release in serum. As shown in Table 1, BetA-containing liposomes are relatively stable, because after one hour incubation in serum still approximately 70% of BetA is still incorporated in the liposomes, whereas empty liposomes (either with or without cholesterol) fall readily apart in serum (data not shown). This result indicates that incorporation of BetA in the liposomes drastically improved their stability. It is likely that the increased stability of the BetA-containing liposomes is reflected in their *in vivo* tissue distribution. To study this, BetA-containing liposomes and control empty liposomes were assembled which contained rhodamine-phosphatidylethanolamine (Rho-PE) and these liposomes were injected. One hour after injection the mice were killed and the organs and tumors were isolated. The Rho-PE label was only detected in the liver when incorporated in BetA-containing liposomes and not when Rho-PE-labeled empty liposomes were injected (Fig. 4). This indicates that also *in vivo* the BetA-liposomes are relatively stable, because of the incorporation of BetA, and are delivered in the liver, most likely to the Kupffer cells.

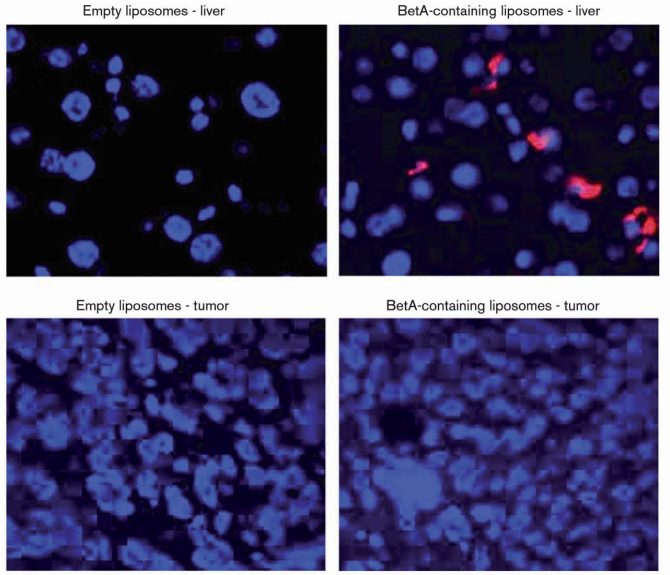

Figure 4: Fate of Rhodamine-PE labeled BetA-liposomes and empty liposomes in vivo.
Mice were injected with 200 µl Rho-PE-labeled BetA-liposomes or empty liposomes and killed after one hour. Organs (liver and kidney) and tumor were isolated and slides were prepared. Slides were stained with 4',6-diamidino-2-phenylindole (DAPI) as fluorescent nuclear stain (blue fluorescence). The Rhodamine B fluorescence was monitored at an excitation of 540 nm and emission of 625 nm. Only in the liver slide of the mice that received labeled BetA-liposomes the Rhodamine is detected (red fluorescence) (upper right panel), indicating that liposomes reached the liver dependent on BetA incorporation. No Rho-PE signal was found in tumors (lower panels) or kidney (not shown) after injection of either Rho-PE labeled empty- or BetA-liposomes.

Orally applied BetA-liposomes slow the growth of SW480 colon cancer tumors

The usual administration route of liposomes is by i.v. injection; however oral application is possible. For instance, oral application of liposomes containing a derivative of cytosine arabinoside [43] or gemcitabine [44] has been shown to exert potent anti-tumor effects. It is expected that the liposomes will disintegrate in the digestive tract, after which BetA is released. Using corn oil as solubilization agent and vehicle, oral application of BetA has been shown previously to be effective in mice against xenografted prostate cancer LNCaP tumors [9]. Therefore, we were interested in knowing whether BetA-containing liposomes are also effective after oral administration. To compare the effects of oral versus i.v. application of BetA we again used the SW480 colon cancer model with identical experimental parameters. Athymic nude mice were injected subcutaneously with SW480 tumor cells and oral treatment (200 µl, three doses per week) of BetA-containing liposomes (containing 5 mg/ml of BetA) or empty control liposomes was started two days after tumor challenge. Tumor size and body weight (as an indication of general health) were monitored during the course of the experiment. The SW480 tumors in mice that orally received BetA-containing liposomes were smaller at all time points, up to an average reduction in tumor volume of 51% on day 33, indicating a slowed tumor outgrowth (Fig. 5A). The reduction in tumor volume in mice orally treated with BetA-containing liposomes over the full treatment period expressed as the average AUC, was 42% ($P = 0.18$). Consequently, these mice, on average, survived longer (mice were killed when tumors were >1000 mm^3; Fig. 5B), although the treatment effect of orally applied BetA-containing liposomes was somewhat less effective than after i.v. administration. Any signs of systemic toxicity were absent and average body weight was similar in BetA-treated and control mice (Fig. 5C). To verify that the oral treatment had no toxic effects, specifically in the tractus digestivus, we analyzed immunohistological sections of the small intestines. The histological structure was normal in the BetA-treated mice and no decrease in proliferation of cells localized in the crypts was observed using proliferating cell nuclear antigen (PCNA) as a marker (Fig. 5D).

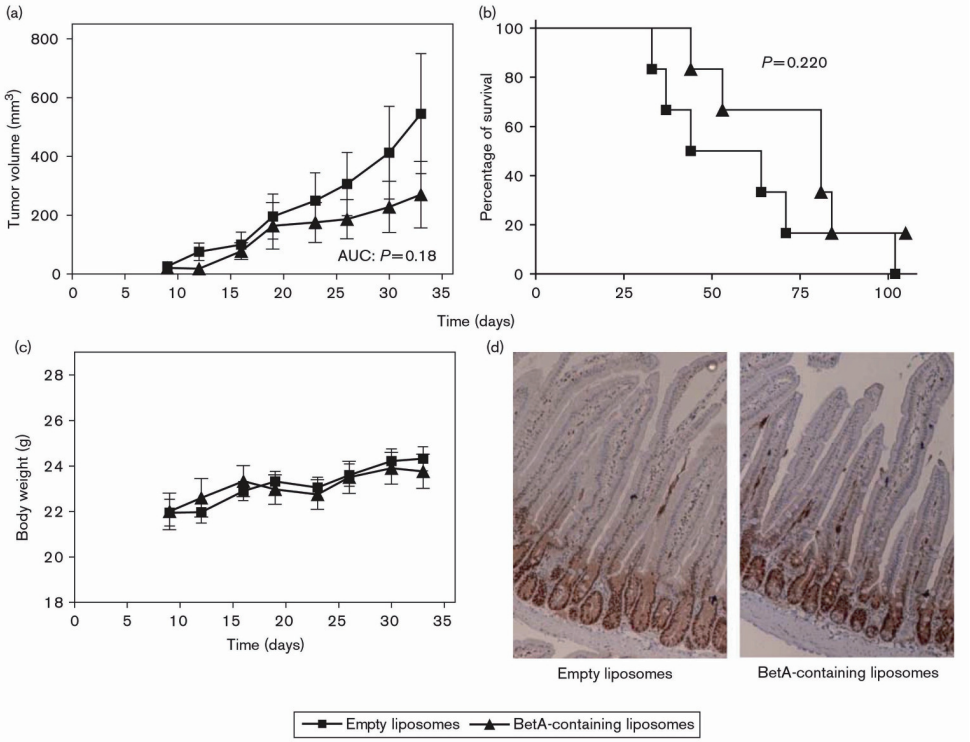

Figure 5: Oral administration of BetA-containing liposomes reduces tumor growth and prolongs survival of SW480 tumor-bearing mice.

Two groups of nude mice (6 per group) were injected subcutaneously with SW480 colon cancer cells and treated three times per week orally with either 200 µl BetA-containing liposomes or empty liposomes starting two days after tumor cell injection. The BetA concentration reached per injection was 50 mg/kg body weight. (200 µl liposomes containing 5 mg/ml of BetA was injected). The treatment was continued until mice had to be killed (tumors >1000 mm^3). During the course of the experiment tumor volumes (Panel A) and bodyweight were monitored (Panel C). In panel B, survival times of the SW480 tumor bearing mice are shown. At the time point that mice had to be killed (tumor volume > 1000 mm^3) the small intestines were isolated from mice of each group, slices were prepared for immunohistochemistry, stained with proliferation marker PCNA (see text) and analyzed by microscopy (Panel D). A representative result of one mouse from each group is shown. AUC, area under the curve.

Discussion

Cancer is a leading cause of death worldwide with lung and colorectal cancer having the highest incidence and mortality [45]. Novel effective treatments for these cancer types in particular but also other malignancies are still urgently needed. BetA has been proven very efficacious *in vitro* against many prevalent cancer types including breast, prostate, lung and colorectal carcinomas [4,5]. However, the promise that BetA showed *in vitro* has not yet been translated into many successful preclinical *in vivo* studies (summarized in Table 2). Although impressive reduction in growth and even regression of human melanomas has been reached by intraperitoneal treatment with BetA administered in PVP [6], this BetA

formulation has not yet been effectively applied for the treatment of other tumors. Experiments in our hands using this BetA-PVP formulation were not successful, likely because of the observed emergence of BetA-PVP deposits on the liver (Fig. 1). Other *in vivo* studies showed either limited anti-cancer effects [16,21] and/or used a BetA formulation that is either not approved for human application or not pharmaceutically acceptable [9,16]. The biggest hurdle to overcome using the anticancer potential of BetA *in vivo* is its highly lipophilic character. Therefore, we decided to investigate the potential of liposomes, which are approved for usage in humans and are especially suited for incorporation of hydrophobic compounds, as a drug carrier of BetA.

The potential of liposomes to solubilize BetA has been reported in the literature [46]. Another study showed incorporation of BetA in phospholipid nanosomes (small liposomes with an average diameter approximately 190 nm, created by supercritical fluid technology) with a maximally achieved BetA content of 87 µg/ml [47]. However, neither of these liposome formulations of BetA was tested *in vivo* [46,47]. Our results showed a maximal BetA incorporation in small liposomes (size 100 – 200 nm) of 1 mg/ml, which translates to 10 mg/kg body weight per dose (200 µl) in a mouse. However, when tested *in vivo* in our A549 xenograft model, the small BetA-containing liposomes failed to slow tumor growth (data not shown). Although small liposomes will advantageously target drugs to tumors in a passive manner, we reasoned that their BetA payload is too small, and therefore tested large liposomes for their capacity to entrap BetA.

Large liposomes, assembled without cholesterol, contained a fivefold-enhanced BetA incorporation (approximately 6 mg/ml). This allowed us to successfully treat mice carrying implanted A549 and SW480 tumors i.v. with the large BetA-liposomes (Fig. 1). Instead of functioning as a targeted drug carrier, which would be the case for small liposomes, the large BetA-liposomes serve merely as a biocompatible solubilizing vehicle for BetA. The *in vitro* stability of the large liposomes (lacking cholesterol) was strongly improved after incorporation of BetA as shown by their serum stability (Table 1). Indeed, when administered in tumor-bearing mice, the Rho-PE labeled BetA-liposomes were found in the liver, and, as expected, not in the tumor (Fig. 3). From the liver BetA may redistribute in the body to ultimately reach the tumor. Metabolism of BetA in the liver, which likely occurs after administration of BetA-liposomes because BetA is known to be metabolized by liver microsomes [21], may lead to several metabolites with anti-cancer activity [48]. Metabolism of BetA by various microorganisms, which resembles mammalian metabolism [49,50], gave rise to metabolites of which some exerted a more potent anti-melanoma effect than BetA itself [51,52].

As summarized in table 2, for the treatment of human melanoma engrafted in nude mice, BetA doses ranging from 5 - 500 mg/kg bodyweight were shown to be effective (melanoma is among the cancers most sensitive to BetA) [6]. Growth inhibition of prostate cancer LNCaP tumors was achieved using BetA doses of 30 or 60 mg/kg of bodyweight administered per week [9] and treatment with BetA at a dose of 200 mg/kg of bodyweight per week resulted in prolonged survival of mice

engrafted with human ovarian cancer IGROV-1[16]. Although these studies differ greatly in various parameters, taken together with our results, the data suggest that for BetA treatment to show an effect the amount of BetA administered per week in nude mice should be at least approximately 30 mg/kg of body weight.

Table 2 Anticancer effects of betulinic acid treatment *in vivo*, reported in published studies and in this study

BetA formulation	References	BetA concentration *in vivo* per treatment dose	Treatment route and schedule	Average dose/ week	Tumor model[a] (all models s.c.)	Start treatment[b] (day/volume)	Effect on tumor growth
PVP	[6]	5, 50, 250, 500 mg/kg BW	i.p.-6 × every third or fourth day	10–1000 mg/kg BW	Melanoma	1 day or approximately 600 mm^3	Complete inhibition[c]
Ethanol, Tween-80, H_2O	[16]	100 mg/kg BW	i.p.-6 × every third or fouth day	200 mg/kg BW	Ovarian carcinoma	1 day	Longer survival[c]
1% DMSO in corn oil	[9]	10 and 20 mg/kg BW	oral-7 × every second day	30–60 mg/kg BW	Prostate carcinoma	10 days	Complete inhibition
Vehicle[d]	[21]	10 mg/kg BW	i.v.-14 × each day	70 mg/kg BW	Primary colon carcinoma	Approximately 1400 mm^3	Inhibition
Large liposomes	Current	50 mg/kg BW	i.v./oral-3 × per week	150 mg/kg BW	Lung, colon carcinoma	2 days	Inhibition

BetA, betulinic acid; BW, body weight; DMSO, dimethyl sulfoxide; i.p., intraperitoneally; i.v., intravenously; PVP, polyvinylpyrrolidone; s.c, subcutaneously.
[a]Tumors were from melanoma MEL-1 and MEL-2 cells (Ref. [6]), ovarian carcinoma IGROV-1 (Ref. [16]), prostate carcinoma LNCaP (Ref. [9]), and primary colon adenocarcinoma cells (Ref. [21]).
[b]Start of treatment indicated in days after tumor injection or indicated as tumor volume at the first treatment, as specified in the respective reference.
[c]In Ref. [6], also regressions are shown of 600 mm^3 tumors (after six treatment doses of 50 mg/kg of BW during 18 days). In Ref. [16], survival is shown; tumor growth is not detailed.
[d]The vehicle used is not specified in Ref. [21]. In this study a derivative of BetA was shown to exert improved cytotoxicity.

Interestingly, the oral administration of BetA-containing liposomes also resulted in a reduction in SW480 tumor volumes (Fig. 4). This result is in concordance with the reported complete growth inhibition of LNCaP tumors by oral application of BetA in corn oil [9] and confirms that the liposomes serve as drug carrier without providing a tumor-targeting effect. Using a similar treatment scheme (three times per week, a dose of 200 µl) the oral route of administration in our hands was suggested to be somewhat less efficacious than i.v. treatment of BetA-containing liposomes (Fig. 2B and Fig. 5B). This can be attributed, likely, to digestive processes in the tractus digestivus causing a possibly limited absorption of BetA, and consequently, the BetA concentration in the circulation of the orally-treated mice being lower than in mice receiving BetA-containing liposomes i.v.

Our study indicates that BetA treatment, provided that sufficiently high *in vivo* concentrations are reached, can strongly reduce tumor growth. Whether higher *in vivo* BetA concentrations can be reached by the optimization of liposomal composition and/or treatment schemes, and whether that may halt *in vivo* tumor growth still more efficiently are important questions that need to be addressed now. An advantage of oral treatment in future experiments is the possibility to treat mice with higher doses that are applied more frequently, which is hardly possible for i.v. application. Under such a treatment scheme, oral treatment may be more effective than i.v. application. Besides its disadvantageous lipophilic character, we confirmed the complete absence of systemic toxicity after BetA treatment. This is the most advantageous feature of BetA as potential anti-cancer drug. The large BetA-containing liposomes are not feasible and approved for human i.v. application, but oral administration of these liposomes is obviously allowed in humans. The prospect of BetA, which needs a relatively high *in vivo* concentration

compared with other chemotherapeutic drugs, may be especially in its synergizing therapeutic effect when applied together with other anti-cancer drugs. Several *in vitro* studies have suggested this role for BetA [20,53-56]. For instance, BetA was shown to synergize with vincristine [54], to cooperate with TRAIL therapy [53], and to work additive together with 5-fluorouracil [56] and irradiation [20]. BetA was also found to be active against chemoresistant colon cancer cell lines [55]. Being a non-toxic and inexpensive compound, BetA is a favorable adjuvant drug provided that effective concentrations can be reached in humans. Such drugs are greatly needed for the treatment of, among others, colorectal cancer for which the current combined treatment protocols cause serious systemic toxicity and, for many patients, are not successful [57]. We provide a first efficacious vehicle for the potential clinical application of BetA that can be standardized. Liposomes, and possibly other carriers of lipophilic drugs, such as polymeric micelles [58] or self-emulsifying drug delivery systems [59], hold promise for clinical drug delivery of BetA and can be used in its further preclinical development.

In conclusion, we showed that BetA can be efficiently incorporated in large liposomes enabling the efficacious treatment of tumor-bearing mice. The liposomes were stabilized through BetA-incorporation as shown by their tissue distribution in the liver and *in vitro* stability in serum. The liposomal formulation of BetA, administered three times per week i.v. with a dose of 50 mg/kg body weight, efficiently reduced the growth of human colon and lung tumors in nude mice, leading to extended mice survival. Oral application similarly resulted in slowed colon tumor growth and enhanced survival. Monitoring of behavior, body weight and histology of small intestines of BetA-treated mice did not show any adverse systemic toxicity. The development of this effective BetA-liposome formulation encourages the preclinical study of BetA as a broadly applicable non-toxic anti-cancer agent.

Acknowledgements: We thank Liesbeth van Bostelen and Marco Guadagnoli who have skilfully helped with the *in vivo* experiments.

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